

1 **Spliced peptides and cytokine driven changes in the**
2 **immuno-peptidome of melanoma**

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5 **Running title: Spliced and linear HLA-I peptides in melanoma.**

6

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9 HLA, Immunotherapy.

10

11 **Summary**

12 Antigen-recognition by CD8⁺ T cells is governed by the pool of peptide antigens
13 presented on the cell surface in the context of HLA class I complexes. Recent studies
14 have shown not only a high degree of plasticity in the immunopeptidome, but also that
15 a considerable fraction of all presented peptides is generated through proteasome-
16 mediated splicing of non-contiguous regions of proteins to form novel peptide
17 antigens. Here we used high-resolution mass-spectrometry combined with new
18 bioinformatic approaches to characterize the immunopeptidome of melanoma cells in
19 the presence or absence of interferon- γ . In total, we identified more than 60,000
20 peptides from a single patient derived cell line (LM-MEL-44) and demonstrated that
21 interferon- γ induced marked changes in the peptidome with an overlap of only ~50%
22 between basal and treated cells. Around 6-8% of the peptides were identified as *cis*-
23 spliced peptides, and 2213 peptides (1827 linear, 386 *cis*-spliced peptides) were
24 derived from known melanoma-associated antigens. These peptide antigens were
25 equally distributed between the constitutive and interferon- γ induced peptidome. We

1 next examined additional HLA-matched patient derived cell lines to investigate how
2 frequently these peptides were identified and found that a high proportion of both
3 linear and spliced peptides were conserved between individual patient tumors,
4 drawing on data amassing to over 100,000 peptide sequences from these extended
5 data sets. Moreover, several of these peptides showed *in vitro* immunogenicity across
6 multiple melanoma patients. These observations highlight the breadth and complexity
7 of the repertoire of immunogenic peptides that can be exploited therapeutically and
8 suggest that spliced peptides are a major new class of tumor antigens.

9

10

1 **Introduction:**

2 Antigen recognition by cytotoxic T cells and subsequent tumor 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 tumor mutations as an important source of
7 immunogenic antigens in the context of melanoma and other cancers(2). Nonetheless,
8 tumors with low mutational burdens can respond to checkpoint inhibitor therapy, and
9 the presence of a high tumor-mutational load does not necessarily correspond to the
10 efficacy of treatment(3). The human leukocyte antigen (HLA) class I-bound peptides
11 (*p*-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 neoantigens to the overall tumor
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 tumor 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 is expressed under steady state conditions. The
25 expression of an immunoproteasome, the subtype expressed by dendritic cells and

1 other cells of the immune system, may be induced in tumor 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
4 immunoproteasome in a range of human melanoma cell lines in the presence of the
5 inflammatory cytokine IFN γ *in vitro*, and in melanoma patient inflamed tumors
6 (characterized by presence of tumor infiltrating lymphocytes (TILs)) *ex vivo*(12).
7 Dependant on the proteasome subtype expressed by the cell, we have shown that a
8 single melanoma antigen (NY-ESO-1) can be processed into several different
9 epitopes. These differences in antigen processing led to concomitant change in the
10 ability of antigen specific T cells to target the tumor cell. Thus, the potential for a
11 tumor cell to ‘look’ substantially different to CD8⁺ T cells, depending on
12 inflammation at the tumor site, arises. Moreover, recent studies by ourselves and
13 others(13,14) have shown that a significant proportion of *p*-HLA-I are not
14 genomically templated and result from post-translational proteasome splicing (ligation
15 of non-contiguous small polypeptide segments from the same or different proteins).
16 To date, these peptides have been missed in most neoantigen discovery studies due to
17 the lack of appropriate bioinformatics tools(15,16).
18 In this study we have used high resolution mass spectrometry approaches combined
19 with a novel bioinformatics workflow to identify linear and spliced *p*-HLA-I
20 presented in the melanoma immunopeptidome in the presence or absence of the
21 cytokine IFN γ . These included a number of linear and *cis*-spliced peptides derived
22 from melanoma-associated antigens. A series of identified linear and *cis*-spliced
23 peptides were tested for *in vitro* immunogenicity across multiple melanoma patients
24 and healthy donors. While the peptide repertoire changed significantly,
25 immunogenicity of selected peptide pools from different treatment conditions (+/-

1 IFN γ) *in vitro* did not change across melanoma patients. However, T lymphocyte
2 responses to pools of IFN γ upregulated peptides were not seen in healthy donors. We
3 also demonstrate that *cis*-spliced peptides were widely presented by melanoma cells
4 and immunogenic in multiple donors. These findings have significant implications for
5 cancer immunotherapy as well as for fundamental questions such as induction of
6 immune-tolerance, T cell repertoires and immune recognition.

7

1 **Results:**

2 *The melanoma immunopeptidome is composed of linear and spliced peptides*

3 We have established a comprehensive repository of HLA class I peptide ligands
4 presented by a patient derived melanoma cell line (LM-MEL-44) utilizing either the
5 constitutive proteasome (-IFN- γ) or the immunoproteasome (+IFN- γ). Using three
6 biological replicates for each condition, we identified around 60,000 peptides
7 presented across all HLA class I allotypes expressed by LM-MEL-44 cells (Table S1).
8 Approximately 6-8% of the peptides in each sample were conservatively assigned as
9 *cis*-spliced in origin (Fig. 1A, Table S1), being derived from non-contiguous
10 sequences of the same protein. This proportion of peptides of *cis*-spliced origin is in
11 agreement with previous studies (14,17,18). As expected for HLA class I epitopes, the
12 majority of peptides were 9 amino acids in length with no apparent difference
13 between linear and *cis*-spliced sequences (Fig.1B).

14 Using NetMHC 4.0 binding prediction algorithm, more than 80% of the linear
15 peptides were assigned to at least one of the HLA class I alleles expressed on the
16 surface of the LM-MEL-44 cells (HLA-A*02:01, B*40:01/*44:02,
17 C*03:04/*05:01)(19) suggesting that the majority of the identified peptide sequences
18 can be considered genuine HLA class I ligands (Fig. 1C, Table S2). The percentage of
19 *cis*-spliced peptides predicted to bind to HLA-A or HLA-C molecules was found to be
20 comparable to linear epitopes, but intriguingly substantially fewer *cis*-spliced peptides
21 were predicted to bind to HLA-B*40:01 and HLA-B*44:02, suggesting that LM-
22 MEL-44 cells generate a lower number of *cis*-spliced peptides that conform to the
23 consensus-binding motif of these HLA-B allotypes (Fig. 1C). Moreover, a
24 significantly higher percentage of unassigned sequences was observed amongst the
25 *cis*-spliced epitopes, which is in agreement with previous reports (13,14) and which

1 can be attributed to the fact that binding algorithms such as NetMHC 4.0 are
2 exclusively trained on linear peptide sequences.

3 In addition, it should be noted that we did not identify in our entire dataset any of the
4 mutational neoantigens that have been described for the LM-MEL-44 cell line based
5 on exome sequencing data (20). However, this is not surprising as this cell line has a
6 relatively low mutational load (Table S3).

7

8 *The generation of spliced peptides is not a random process*

9 To address whether spliced peptides are randomly generated, we comparatively
10 analyzed the overlap of both linear and spliced peptides across our three biological
11 replicates. A total of 1399 and 1795 *cis*-spliced peptides were identified in at least two
12 of the three biological replicates for the IFN γ -untreated and treated samples, which
13 corresponds to 47.6% and 52.2%, respectively (Fig. 2A). Importantly, a similar
14 overlap was observed for the linear epitopes (48.8% and 53.9%, respectively), which
15 suggests that the generation of *cis*-spliced peptides is not a random process. Of note,
16 the comparatively low overlap between the replicates can be rather attributed to the
17 stochastic nature of data-dependent acquisition mass spectrometry (DDA-MS), which
18 is particularly pronounced when acquiring highly complex samples that contain
19 individual analytes of low abundance (such as HLA peptide samples).

20 To investigate whether the identified *cis*-spliced peptides are also expressed on other
21 cell lines with a similar HLA signature, we analysed the cell lines LM-MEL-53 and
22 LM-MEL-33 by DDA-MS. LM-MEL-53 cells are derived from the same patient as
23 LM-MEL-44 cells, but from another metastasis at a different point in time (21). In
24 contrast, LM-MEL-33 (HLA-A*02:01/A*03:01, B*40:02/*47, C*03:04/*06:02) cells
25 have been isolated from a different patient that shares three HLA alleles with LM-

1 MEL-44 (21). 47% and 28% of the identified *cis*-spliced peptides from LM-MEL-44
2 were also identified on LM-MEL-53 and LM-MEL-33 cells, respectively (Fig. 2B,
3 Table S1), which further confirms that the generation of *cis*-spliced peptides is not a
4 random process, but more importantly, that *cis*-spliced peptides have a significant
5 potential for cancer immunotherapy.

6

7 *IFN γ -treatment alters melanoma HLA class I immunopeptidome*

8 Considering the well-described clinical relevance of so called “hot” versus “cold”
9 tumor microenvironments and previous work demonstrating the influence of cytokine
10 exposure on antigen-presentation pathways (10), we wanted to examine the impact of
11 IFN γ exposure on the immunopeptidome. IFN γ treatment led to the identification of a
12 considerably higher number of HLA epitopes than that from untreated cells,
13 consistent with the upregulation of HLA molecules at the cell surface (Fig. 3A, Fig.
14 S1) (10). Moreover, for common peptides we observed, on average, an increase in
15 peptide abundance in the cytokine treated samples. Of note, only 44.7% of the linear
16 and 52.5% of the spliced peptides were identified under both conditions suggesting
17 that the addition of IFN γ significantly impacts the composition of the
18 immunopeptidome.

19 To understand whether IFN γ exposure changes the abundance of individual epitopes
20 independent of the HLA expression levels, we identified epitopes that were present
21 across all replicates (a total of 4942 peptides) and calculated their log₂ fold change
22 between IFN γ treated and untreated samples after median normalization of their ms1
23 intensities to remove any bias introduced through varying levels of IFN γ -induced
24 HLA expression (Fig. 3B, Table S1). A considerable number of epitopes changed in
25 abundance by a factor of at least 2 (both up- and down), confirming that the addition

1 of IFN γ substantially alters HLA class I presentation, while not affecting the
2 proportion of presented *cis*-spliced epitopes on LM-MEL-44 melanoma cells.
3 Interestingly and despite median-normalized ms1 intensities, most of the peptides
4 predicted to bind to HLA-B*40:01 and HLA-B*44:02 were still upregulated upon
5 IFN γ exposure, which correlates to the enhanced upregulation of HLA-B molecules
6 in response to IFN γ compared to HLA-A and HLA-C molecules (10).

7

8 *Identification of novel cancer specific peptides in the melanoma immunopeptidome*

9 Next we screened a panel of identified peptides including sequences from
10 melanoma/cancer-associated antigens (MAA) (22,23) and tumor antigens with
11 demonstrated immunogenicity(24-26). We identified a total of 2213 peptides in our
12 dataset (1827 linear, 386 spliced peptides) derived from 142 different MAAs (Fig. 4A,
13 Table S4). A large proportion (~45%) of linear peptides have not been previously
14 reported (Fig. 4B) (24). Furthermore, almost all of those peptides generated by
15 splicing constitute potentially novel epitopes. Of the previously reported epitopes, the
16 majority were detected in both the presence and absence of IFN γ , whereas >58% of
17 novel peptides were exclusive to IFN γ treated samples, demonstrating the importance
18 of carefully considering experimental conditions for epitope discovery (Fig. 4C).

19

20 *Melanoma patients expressing immunoproteasome genes have a survival advantage*

21 Tumor recognition *in vivo* relies on the processing and generation of cognate peptides
22 within the tumor cells. Using OncoLnc(27), we mined gene-expression data generated
23 by the TCGA Research Network (<http://cancergenome.nih.gov/>) for correlation of
24 both immuno- and constitutive- proteasome-specific genes with survival in melanoma
25 patients. We found that expression of all three immunoproteasome-specific subunits

1 was significantly associated with survival in melanoma patients. Conversely,
2 constitutive proteasome-specific subunits were associated with decreased melanoma
3 patient survival (Fig. S2).

4 Since immunoproteasome subunits are also expressed by immune cells, including
5 intra-tumoral T cells that are themselves associated with better prognosis, we removed
6 the top quartile of samples with the highest CD3 expression. Following removal of
7 these samples, we found that a significant survival benefit, associated with 2/3
8 immunoproteasome subunits, was maintained (Fig. 5). Furthermore, presence of the 3
9 constitutive proteasome subunits was associated with a trend towards decreased
10 survival. This indicates that patients whose tumors express an immunoproteasome
11 have survival benefit which is specifically associated with this proteasome type. This
12 effect persists, albeit to a lesser extent, when we removed to tumors that showed the
13 highest CD45 infiltration, thus including non T cell lineage immune cells and APCs
14 (Fig. 5). As immunoproteasome expression in tumors is largely driven by cytokine
15 exposure it remains unclear if this is merely a footprint of a (previous) successful
16 immune recognition or if it is part of the pre-conditions to allow for such an immune
17 response.

18

19 *CD8⁺ T lymphocytes frequently recognized novel linear melanoma-specific epitopes*

20 In this study we identified several novel linear melanoma-specific peptides predicted
21 to be bind the HLA-allotypes presented by a tumor-derived melanoma cell line (HLA-
22 A*02:01, B*40:01/*44:02, C*03:04/*05:01). Importantly, this included HLA-
23 A*02:01, one of the most prevalent HLA-types, and therefore a common target for
24 peptide identification and therapeutic focus. We addressed functional immunogenicity
25 of a selection of these peptides, by using them to stimulate CD8⁺ T lymphocytes in

1 PBMC derived from healthy donors or melanoma patients (Fig.6, Table S5). In doing
2 these studies, selected donors were matched for at least two HLA-allotypes, (across
3 HLA-A/B/C) and 3 melanoma patients were matched across all three. Of note, the
4 LM-MEL-44 cell line was derived from melanoma patient 2 and, melanoma patient 6
5 shares none of the HLA alleles from this cell line, serving as a negative control.
6 Alongside these assays using melanoma antigen-derived peptides, we also assessed
7 differences in functional immunogenicity of immuno- or constitutive- proteasome
8 processed epitopes. This was done by pooling a selection of those peptides which
9 were most strongly up- or down- regulated following IFN γ treatment (Fig. 6A and B,
10 Table S5). We found that though also observed in healthy donors, CD8⁺ T
11 lymphocyte responses to the tested peptides were more frequently seen in melanoma
12 patients (Fig. 6A (individual) and 6B (combined donors)). Novel peptides derived
13 from 15 of the melanoma antigens identified in our screen stimulated specific CD8⁺ T
14 lymphocyte responses (over 2% TNF α ⁺ cells) in three or more donors, demonstrating
15 novel, functional, melanoma T cell epitopes (Fig. 6A, Table S5, S6, representative
16 examples, Fig. S3A). Of those where a clear HLA-binding prediction could be
17 determined, 54.5% (n=6) were predicted to bind to HLA-A*02:01, 36.4% (n=4) to
18 HLA-B*44:02, and 9.1% (n=1) to HLA-C*05:01. The strongest responses were
19 induced by the peptides derived from SART1 (U4/U6.U5 tri-snRNP-associated
20 protein 1) and PGK1 (Phosphoglycerate kinase 1), both of which stimulated responses
21 in 2-7% of T lymphocytes from 4 melanoma patients. Both of these peptides were
22 predicted to bind to HLA-A*02:01. When a selection of peptides were pooled in
23 groups of those up/down regulated or unchanged following IFN γ treatment, no
24 appreciable difference in functional immunogenicity in melanoma patients was
25 observed between groups. One pool in each group was made on the basis of higher *in*

1 *silico*-predicted immunogenicity (www.iedb.org(28), Fig. 6A,B, asterisks). However,
2 these groups did not display enhanced ability to activate CD8⁺ T lymphocytes in
3 either melanoma patients or healthy donors.

4
5

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

8 The potential implications of the presence of spliced peptides for all facets of
9 immunity have sparked intense discussions in the last 4 years(14,29-31). In cancer,
10 their presence would dramatically widen the repertoire of potentially targetable
11 epitopes and may allow for many more tumor-specific antigens (including mutational
12 derived neoantigens) being presented in various HLA-contexts(16). So far only 6
13 immunogenic *cis*-spliced HLA-I bound peptides derived from 4 different proteins(29)
14 have been described and most of them have been discovered by T cell assays rather
15 than by mass spectrometry(32-37). To test some of the identified spliced peptides for
16 their ability to activate CD8⁺ T cells *in-vitro* we synthesized 26 *cis*-spliced peptides
17 based on (i) their *de novo* sequencing confidence score, (ii) their binding prediction
18 score for HLA alleles expressed on LM-MEL-44 cells (HLA-A*02:01, HLA-
19 B*40:01, HLA-B*44:02 or HLA-C*05:01) and (iii) the quality of their peptide
20 spectrum matches (PSMs). When employed as pools of 8-9 individual peptides, all 3
21 pools evoked immune-responses as measured by intracellular TNF α production in
22 CD8⁺ T cells (Fig. 7A and peptide sequences listed in Fig. 7B) in multiple melanoma
23 patient and healthy donor derived PBMCs (example shown in Fig. S3B).
24 Given the differences in the potential to stimulate HLA-A2 positive vs. negative
25 patient and healthy donor samples, most of the immunogenic peptides derived from

1 Pools I and III in our assays seem to be HLA-A2 associated. To identify specific
2 immunogenic peptides, PBMCs were stimulated with the listed peptide pools (Fig.
3 7B) for 10-12 days followed by single peptide re-stimulation. Six out of 26 peptides
4 induced a TNF- α response above DMSO background (Fig. 7C) in more than one
5 patient sample. Of note, the peptide demonstrating the highest immunogenicity (1832,
6 shown as example in Fig. S3C) based on these assays is a spliced peptide derived
7 from the cancer-testis antigen MAGE-C2 (LILGLLTKV) and showed CD8⁺ T cell
8 activation across all 4 patients. Matched mixed effect analysis showed significant
9 differences across peptides and peptide 1832 and FEC represented the treatments with
10 significant differences to DMSO. However, the other shown peptides displayed higher
11 immunogenic potential when compared to their respective DMSO control, but with
12 very high patient-to-patient variability, as expected in these types of data,
13 Interestingly, peptide 1832 was identified across all replicates of LM-MEL-44, -33
14 and -53 (Table S6). All spliced peptides that tested positive for immunogenicity in our
15 assays were subjected to T2 peptide binding assays to examine HLA-A2 binding. As
16 shown in Fig. S4, these peptides all stabilize HLA-A2, albeit to a lesser extent than
17 the well described modified ELAGIGILTV HLA-A2 peptide (aa26-35) from the
18 melanoma antigen Melan-A(38) with some just showing minor stabilization.
19 Of note, we did not find any particular pattern in the length of the *N*- and *C*-terminal
20 segments of these spliced peptides nor in the distance between these segments on the
21 protein level (Fig. S5). Taken together, these data show that these spliced peptides can
22 serve as *bona fide* anti-cancer targets and provide a large number of additional targets
23 would have not been considered using previous MS-based epitope discovery
24 strategies.
25

1 **Discussion:**

2 In this study we have described a detailed and in-depth immunopeptidome presented
3 on a patient-derived melanoma cell line (LM-MEL-44) generated from a lymph node
4 metastasis. Our qualitative assessment of the immunopeptidome yielded around
5 60,000 high confidence peptide identifications that encompassed two culture
6 conditions (+/- IFN γ) to gain insights into the influence of differences in the
7 microenvironment of the cells on the global immunopeptidome. Furthermore, we
8 demonstrated consistence of over 50% of these peptides with a temporally distinct
9 autologous tumor sample, and 37% with a tumor from a different donor. The well-
10 described effect of IFN α in mediating changes to the composition of the antigen
11 processing machinery, coupled with reports of differences in antigen processing
12 between the constitutive and the immunoproteasome, led us to expect a degree of
13 difference between the two immunopeptidomes. Nevertheless, our observation that
14 ~55% of linear and 47% of spliced HLA class I epitopes were exclusive to either
15 IFN γ treated or untreated conditions, was striking. Our observations are also
16 consistent with recent studies in ovarian and lung cancer(10,11) that demonstrated
17 profound changes between cytokine treatment conditions.

18 To have a closer look at the “tumor-specific” immunopeptidome landscape, we
19 focussed on MAA-derived peptides. More than 50% of novel peptides that we
20 identified were exclusively presented in the presence of IFN α . Interestingly, of the
21 MAA epitopes that have been previously described in other studies, only 27.7% were
22 present uniquely in IFN γ treated conditions (Fig. 4C). This observation suggests that
23 many immunoproteasome processed epitopes may be as yet undescribed, since
24 traditional approaches to identify tumor associated antigens have largely been
25 undertaken using cells lines under steady state conditions (*i.e.* which express only the

1 constitutive form of the proteasome). It is evident from our study that the steady state
2 immunopeptidome may vary dramatically from the *in vivo* tumor scenario depending
3 on the tumor microenvironment at any given time. Though our functional studies did
4 not reveal a difference in the immunogenicity of peptides derived from either
5 IFN γ treated or untreated conditions, in the *in vivo* setting a T cell response to IFN γ
6 related epitopes is likely to be aided by correlative IFN γ influences, such as
7 upregulation of surface HLA(39). The potential for tumor escape from CD8⁺ T
8 lymphocyte killing due to whole scale change to the immunopeptidome upon
9 initiation of an anti-tumor responses, and corresponding induction of IFN γ , is clear
10 from our studies. These data become particularly significant in the context of recent
11 studies demonstrating that tumors with an IFN γ -inflamed, or ‘hot’ microenvironment
12 are associated with better prognosis, and are more likely to be amenable to treatment
13 with immune checkpoint inhibitors(40). It seems conceivable that *in vivo* the
14 difference between immunopeptidomes is indeed of immunological relevance to
15 disease progression and overall patient prognosis. Taken together, it is tempting to
16 speculate that antigens processed *via* the immunoproteasome may represent an
17 untapped resource of “IFN γ -associated neo-epitopes”.

18 This remarkable plasticity in the peptide landscape of melanoma is further increased
19 by the presence of spliced peptides. The identification of spliced peptides as tumor
20 antigens in cancer was first described in 2004 in both the FGF5 protein in renal
21 cancer(32) and the gp100 protein in melanoma(33), and since then only a further 4
22 *cis*-spliced peptides have been described in cancer(29). Of these 6 spliced peptides, 3
23 have been shown to be processed exclusively by the constitutive proteasome, and 2 by
24 both the constitutive and immunoproteasome (and 1 undetermined)(29).

1 Several bioinformatics tools are now available to reliably identify spliced
2 peptides(13,14,17,18,41). Nevertheless, the contribution of spliced peptides to the
3 overall immunopeptidome has been reported in a range from 2 to 40% and is still
4 heavily debated. Recent studies have identified *cis*-spliced peptides in the cancer and
5 viral infection context (16,17,41), but few have provided experimental evidence of
6 their immunogenicity (41). In this study we identified 386 *cis*-spliced peptides that
7 were potentially derived from MAA and therefore considered as potential candidates
8 to induce therapeutic immune responses. We demonstrated that generation and
9 presentation of spliced peptides is not a random process since within three biological
10 replicates we found comparable reproducibility of both linear and spliced peptides.
11 Interestingly, more than 50% of spliced peptides identified in the LM-MEL-44 cell
12 line were present on at least one of the other two distinct cell lines (LM-MEL-53 and
13 33). In addition, we demonstrated the immunogenicity of 6 *cis*-spliced epitopes tested
14 across multiple patients, strengthening the argument that *cis*-splicing is a random,
15 functional process leading to diversification of the antigenic pool of peptides. In how
16 far increased potential to evoke CD8+ T cell activation reflects meaningful and
17 translatable anti-tumour effects remains to be tested in much larger patient cohorts
18 with additional clinical data and in a more formal setting.

19 This study and other recent publications which focused on the identification of spliced
20 peptides (16,42,43) and their impact on the plasticity of the immunopeptidome, will
21 beyond doubt open up new questions and opportunities in the field. These will range
22 from the basic understanding of immune-tolerance, autoimmunity and thymic
23 selection to opportunities for development of novel peptide-based therapeutics. This
24 includes vaccines in an infectious and cancer setting where predictability and HLA-

1 binding characteristics of linear and constitutive proteasome-derived peptides were
2 potentially limiting factors.

3

4 **Materials and Methods**

5 *Human ethics approval*

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

9

10 *Melanoma cell line culture*

11 Establishment and characterization of the melanoma cell lines used has been
12 previously described(44,45). Cells were cultured in RF10 consisting of RPMI 1640, 2
13 mM Glutamax, 100 IU/ml Penicillin, 100 µg/ml Streptomycin and 10% heat-
14 inactivated fetal calf serum (all Invitrogen). For induction of immunoproteasome
15 catalytic subunits, cells were incubated with 100 ng/ml IFN γ (Peprotech) for 72 h
16 prior to experiments.

17

18 *Melanoma cell line sequencing*

19 Whole exome sequencing of the LM-MEL-44 cell line was performed using the
20 NimbleGen EZ Exome Library v2.0 kit and run on a Illumina Hiseq2000 instrument
21 as previously described(46). Sequence reads were aligned to the human genome (hg19
22 assembly) using the Burrows–Wheeler Aligner (BWA) program(47). Single
23 nucleotide variants (SNVs) and indels were identified using the GATK Unified
24 Genotyper(48), Somatic Indel Detector(49) and MuTect (Broad Institute)(50).

25

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

2 HLA class I peptides were eluted from LM-MEL-44, 33 and 53 cells (prior to or after
3 treatment with IFN γ) as described previously(51-54). In brief, for replicate one of
4 LM-MEL-44, 3×10^9 cells were lysed in 0.5 % IGEPAL, 50 mM Tris-HCl pH 8.0,
5 150 mM NaCl supplemented with protease inhibitors (CompleteProtease Inhibitor
6 Cocktail Tablet; Roche Molecular Biochemicals) for 45 min at 4 °C. Lysates were
7 cleared by ultracentrifugation at 40,000 g and HLA class I complexes were
8 immunoaffinity purified using DT9 (anti HLA-C) and W6/32 (pan anti-HLA-I)
9 monoclonal antibodies. For replicate two and three of LM-MEL-44 and also LM-
10 MEL-33 and LM-MEL-53, 5×10^8 cells (for each sample) were lysed in 0.5 %
11 IGEPAL, 50 mM Tris-HCl pH 8.0, 150 mM NaCl supplemented with protease
12 inhibitors for 45 min at 4 °C. Lysates were cleared by ultracentrifugation at 40,000 g
13 and HLA class I complexes were immunoaffinity purified using W6/32 (pan anti-
14 HLA-I) monoclonal antibody.

15

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

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

1 reconstituted to 12 μ l with 0.1 % formic acid (FA). Indexed retention time (iRT)
2 peptides(55) were spiked in for retention time alignment.

3

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

5 For the first replicate of LM-MEL-44 we used a Dionex UltiMate 3000 RSLCnano
6 system equipped with a Dionex UltiMate 3000 RS autosampler, the samples were
7 loaded via an Acclaim PepMap 100 trap column (100 μ m x 2 cm, nanoViper, C18, 5
8 μ m, 100 Å; Thermo Scientific) onto an Acclaim PepMap RSLC analytical column (75
9 μ m x 50 cm, nanoViper, C18, 2 μ m, 100 Å; Thermo Scientific). The peptides were
10 separated by increasing concentrations of 80 % ACN / 0.1 % FA at a flow of 250
11 nl/min for 65 min and analyzed with a QExactive Plus mass spectrometer (Thermo
12 Scientific). In each cycle, a full ms1 scan (resolution: 70.000; AGC target: 3e6;
13 maximum IT: 120 ms; scan range: 375-1800 m/z) preceded up to 12 subsequent ms2
14 scans (resolution: 17.500; AGC target: 1e5; maximum IT: 120 ms; isolation window:
15 1.8 m/z; scan range: 200-2000 m/z; NCE: 27). To minimize repeated sequencing of
16 the same peptides, dynamic exclusion was set to 15 s and the ‘exclude isotopes’
17 option was activated.

18 For second and third replicates of LM-MEL-44, LM-MEL-33 and LM-MEL53 we
19 used a Dionex, Sunnyvale, CA, UltiMate 3000 RSLCnano system equipped with a
20 Dionex UltiMate 3000 RS auto sampler, the samples were loaded via an Acclaim
21 PepMap 100 trap column (100 μ m x 2 cm, nanoViper, C18, 5 μ m, 100Å; Thermo-
22 Fisher Scientific, Waltham, MA) onto an Acclaim PepMap RSLC analytical column
23 (75 μ m x 50 cm, nanoViper, C18, 2 μ m, 100 Å; ThermoFisher Scientific). The
24 peptides were separated by increasing concentrations of 80 % ACN/0.1 % FA at a
25 flow of 250 nL/min for 158 min and analyzed with an Orbitrap Fusion™ Tribrid™

1 mass spectrometer (ThermoFisher Scientific). 6 μ L of each sample fraction was
2 loaded onto the trap column at a flow rate of 15 μ L/min.
3 Orbitrap Fusion™ Tribrid™ mass spectrometer (ThermoFisher Scientific) was set to
4 data-dependent acquisition mode with the following settings: All MS spectra (MS1)
5 profiles were recorded from full ion scan mode 375-1800 m/z in the Orbitrap
6 at 120,000 resolution with automatic gain control (AGC) target of 400,000 and
7 dynamic exclusion of 15 s. The top 12 precursor ions were selected using top speed
8 mode at a cycle time of 2 s. For MS/MS, a decision tree was made which helped in
9 selecting peptides of charge state 1 and 2-6 separately. For single charged analytes
10 only ions falling within the range of m/z 800-1800 were selected. For +2 to +6 m/z s
11 no such parameter was set. The c-trap was loaded with a target of 200,000 ions with
12 an accumulation time of 120 ms and isolation width of 1.2 amu. Normalized
13 collision energy was set to 32 (high energy collisional dissociation (HCD)) and
14 fragments were analyzed in the Orbitrap at 30,000 resolution.

15

16 *DDA data analysis*

17 Linear and *cis*-spliced peptide sequences were identified as described previously(14).
18 In brief, the acquired .raw files from six LM-MEL-44 line were searched with
19 PEAKSStudio X (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) against
20 the human UniProtKB/SwissProt (v2017_10) database, which was manually corrected
21 for the single nucleotide variants characteristic of the LM-MEL-44 cell line as
22 identified by whole exome sequencing. The parent mass error tolerance was set to 10
23 ppm for *de novo* sequencing and database search and the fragment mass error
24 tolerance to 0.02 Da for both searches. Oxidation of M and deamidation of N & Q
25 were set as variable modifications and a FDR cutoff of 1% was applied. High

1 confidence *de novo* peptide sequences without any linear peptide match in the
2 provided database were further interrogated with the “Hybrid finder” algorithm and
3 the identified *cis*-spliced candidate sequences from all 6 samples combined together
4 and added back to the original UniProtKB/SwissProt database and all data researched
5 using PEAKS DB. Linear and *cis*-spliced peptides in this search were extracted at 5%
6 FDR to create the final list of identified peptides. For identification of linear and *cis*
7 spliced peptides from LM-MEL-33 and 53, we used this combined database and the
8 same setting on PEAKS studio.

9

10 *Binding prediction*

11 We used the NetMHC4 (56,57) algorithms for binding predictions for both spliced
12 and linear peptides. A default rank cut-off of 2 was implemented as a binder peptide.

13

14 *Immunogenicity prediction.*

15 We used the Immune Epitope Database and Analysis Resource (IEDB:
16 www.iedb.org), Class I Immunogenicity algorithm for immunogenicity predictions of
17 linear peptides(58). The peptides used in functional assays indicated as “predicted
18 immunogenic” had an immunogenicity score of between 0.37 and 0.55 (Table S5).

19

20 *T cell stimulation assay*

21 To assess T cell responses selected peptides were synthesized (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, 2 mM 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 of 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. Statistics were performed using GraphPad Prism. For peptide responses in
7 Figure 6a a repeated-measures 2-way ANOVA was used, and for *cis*-spliced peptide
8 assays in Figure 7 a matched mixed effect analysis with Dunnett's multiple
9 comparison test was used to compare each column to DMSO.

10

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

12 To assess antigen responses, T-lymphocytes were restimulated (following 10 - 12
13 days incubation as outlined above) with peptide pools for 4-8 h in TCRPMI in
14 presence of 10 μ g/ml brefeldin A (BFA, Golgi plug). Cells were washed with PBS
15 (Invitrogen) labeled with live/dead fixable violet stain (Invitrogen), then incubated
16 with antibodies against CD3 and CD8 (BD biosciences) for 15 min at 4°C. Samples
17 were washed and fixed for 20 min at 4°C. Cells were permeabilized and stained with
18 anti-TNF α (eBiosciences) for 25 min at 4°C. The gating strategy was: SSC/FSC;
19 Singlets; SSC/LD⁻; CD3⁺/CD8⁺; CD8⁺/TNF α ⁺(Fig. S6). Data were acquired on a
20 FACSCanto (BD biosciences, VIC Australia) and analyzed with FlowJo software
21 (Version 10, FlowJo, Ashland OR, USA). To account for the large variation in DMSO
22 background CD8⁺ T cell activity across multiple donors, signals were normalized by
23 subtracting the background from DMSO control treated samples in each case.

24

1 *HLA-A2 stabilization assays*

2 The binding activity of the peptides was assayed by measuring peptide-induced
3 stabilization of HLA-A2 on TAP-deficient T2 cells by flow cytometry. T2 cells were
4 cultured in RF-10 (RPMI with 10% serum, 5% Glutamine, 5% Pen/Strep) in T25
5 flasks for 2 - 3 days before the assay. T2 cells (2×10^5 cells/well) were cultured for 16
6 h at 37 °C in 5 % CO₂ in 200 µL RF10 in 96-well U-bottomed plates in presence or
7 absence of 10 µg/ml of synthetic peptides. Peptides from Melan-A (modified aa26-35
8 ELAGIGILTV and aa 60-72) served as positive or negative controls respectively. All
9 peptides were tested in triplicate.

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

15

16 *Validation spliced peptides using retrospectively synthesized peptides and retention*
17 *time prediction*

18 We validated the identity of a panel of spliced peptides (including peptides that were
19 tested for immunogenicity), using 28 synthetic peptides (Mimotopes, Melbourne,
20 Australia) by comparing chromatographic retention and MS/MS spectra with the
21 original p-HLA (Fig. S7). The PKL files of the synthetic peptide and corresponded
22 eluted peptides were exported from PEAKS X studio software. For evaluating the
23 similarity between two spectra, we predicted all b- and y-ions for each sequence and
24 then extracted the corresponding intensity for each ion (with a fragment mass error
25 tolerance of 0.1 Da). The Pearson correlation coefficient and the corresponding p-

1 value (Prism version 8.01, GraphPad) between the log₁₀ intensities of identified b-
2 and y-ions in the synthetic and sample derived spectra were calculated (Fig. S7 and
3 S8) (59). The closer a correlation coefficient to 1, the more identical the spectra. All
4 tested peptides were found to have a *p*-value of less than 0.05.

5 We calculated the iRT index of each synthetic peptide and corresponding eluted
6 peptide using the retention time of a standard set of reference peptides (iRT kit,
7 Biognosys) that were spiked into all samples (Fig. S8) (55).

8 We also used GPTIME tool (59) to compare the predicted versus actual
9 chromatographic retention time of both the identified linear and spliced peptides. For
10 each data set generated from LM-MEL-44 cell lines (6 replicates in total) we sorted
11 peptides (8-12 mer peptides without modification) based on the $-\log P$ score (high to
12 low) from Peaks Studio software. We used the first 1000 peptides for training the
13 algorithm and then used the trained algorithm to predict the retention times of all
14 linear and spliced peptides in the corresponding dataset (Fig. S9). We also calculated
15 Grand Average of Hydropathy (GRAVY) Score of all identified 8-12 mer peptides
16 (Table S1) (60).

17

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17

18

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8

1 **Figure legends:**

2 **Figure 1. The melanoma immunopeptidome consists of linear and spliced**
3 **peptides in the presence and absence of IFN γ .** **A**, 6-8 % of peptides presented by
4 the LM-MEL-44 cell line were *cis*-spliced peptides across 3 replicates. **B**, Length
5 distribution analysis showed that majority of linear and *cis*-spliced peptides were 9
6 mers in the presence and absence of IFN γ . **C**, Using the NetMHC4.0 algorithm,
7 binding of identified 8-12 mer peptides to all HLA-I haplotypes presented by LM-
8 MEL-44 was predicted.

9

10 **Figure 2. Generation and presentation of spliced peptides is not random process.**

11 **A**, Reproducibility of linear and *cis*-spliced peptides across biological replicates with
12 around half of the of linear *cis*-spliced of peptides identified in at least two out of
13 three replicates. **B**, A high proportion of linear and spliced peptides presented on LM-
14 MEL-44 cell were also identified on other melanoma cell lines. The yellow circle
15 (defined as 100%) represents all peptides that were identified across all six LM-MEL-
16 44 samples. The green and orange circles represent the proportion of those peptides
17 that were also identified to be presented on LM-Mel-53 and LM-Mel-33, respectively.

18

19 **Figure 3. The melanoma immunopeptidome is substantially altered following**

20 **exposure to IFN γ .** **A**, Around 20% of linear and 25% of spliced peptides presented by
21 HLA-I were lost following treatment with IFN γ , alongside a concurrent increase in
22 novel peptides. **B**, Among peptides identified in 3 replicates both with and without
23 IFN γ , an elevation in the number of peptides that bound to HLA-B, compared to
24 HLA-A and C, was observed.

25

1 **Figure 4. Novel and previously described linear and spliced peptides from**
2 **melanoma specific antigens were identified.** A, Both linear and *cis*-spliced peptides
3 derived from MAA were identified in the LM-MEL-44 immunopeptidome. B, A
4 substantial proportion of identified MAA have not been previously described. C,
5 Proportion of MAA-associated epitopes in the presence and absence of IFN γ .
6 Almost 70% of novel identified peptides were solely present in the presence of IFN γ
7 or generated through post translational splicing. IEDB website (www.iedb.org) (28),
8 SysteMHC (<https://systemhcatlas.org/>)(62) and (63) were used as resources for known
9 peptides.

10

11 **Figure 5. Immunoproteasome expression is associated with survival benefit in**
12 **melanoma patients.** Using the TCGA-SKCM and FM-AD datasets looking at nevi
13 and melanomas, we selected patients whose tumors had highest (top quartile) or
14 lowest (bottom quartile) expression of each immunoproteasome (IP) or constitutive
15 proteasome (cP) subunits as indicated in the figure. To correct for immune infiltration,
16 the top quartile of CD3g expressing samples were removed. The remainder of samples
17 with high or low expression of a given proteasome subunit were plotted on the basis
18 of patient survival over time using a Kaplan Meier survival curve
19 (astatsa.com/LogRankTest/).

20

21 **Figure 6: Immunogenicity of identified melanoma-associated epitopes.**

22 Selected MAA peptides were pooled and incubated at 10 μ M final concentration with
23 PBMC from healthy donors (n=4) or melanoma patients (n=6) for 10 days in presence
24 of IL-2. Melanoma patient 2 is the patient from which the LM-MEL-44 cell line was
25 derived. Melanoma patient 6 was a HLA-unmatched negative control. All other

1 donors were HLA-matched over at least two allotypes. On day 10, CD8⁺ T
2 lymphocytes were re-stimulated with individual peptides. Additionally cells were
3 stimulated with pools of selected peptides whose HLA-presentation was found to be
4 up/down regulated or unchanged in presence of IFN γ as indicated. TNF α expression
5 measured by ICS. Data show the percentage of TNF α ⁺ CD8⁺ T lymphocytes in
6 response to each peptide as individual values (A) or combined (B). * denotes peptides
7 with the highest *in silico* predicted immunogenicity. In (A) all responses shown were
8 significantly greater (p<0.05) than their respective matched DMSO control and the
9 line denotes a conservative, arbitrary, cut-off for peptides inducing an immune
10 response. Peptide pools outline in Table S5.

11

12 **Figure 7. Immunogenicity of cis-spliced peptide pools.** **A**, PBMCs from melanoma
13 patients and healthy donors (HD) were stimulated with pooled peptides (n=8-9) for 10
14 days in the presence of IL-2. Cells were re-stimulated with the same pools for 8 h in
15 the presence of BFA and TNF α expression measured by ICS. DMSO and FEC served
16 as negative or positive control respectively. **B**, Amino acid sequence of peptide pools
17 used in A. **C**, PBMCs from melanoma patients pre-stimulated with the pooled
18 peptides as in A were re-stimulated with single peptides from the same pool after 12
19 days for 8 h in the presence of BFA and TNF α expression measured by ICS. DMSO
20 and FEC served as negative or positive control respectively. % of TNF α ⁺ CD8⁺ cells
21 of peptides that gave a signal above background (DMSO) are shown. Statistics was
22 performed using Graphpad Prism as described in Material and Methods, ****
23 p<0.0001 and ** p<0.001 after Dunnett's multiple comparison test.

24

1 **Supplementary Figures:**

2 **Figure S1. IFN γ treatment increase the expression of HLA-I molecules.** The
3 melanoma cell lines LM-MEL-33, -44, and -53 were cultured in presence/absence of
4 100 ng/ml IFN γ for 72 h. Cells were labelled with anti-pan HLA-I and the expression
5 of HLA-I on the cell surface was determined by flow cytometry. *=p<0.05,
6 **=p<0.001.

7

8 **Figure S2.** Using the TCGA-SKCM and FM-AD datasets looking at nevi and
9 melanomas, we selected patients whose tumors had highest (top quartile) or lowest
10 (bottom quartile) expression of each immunoproteasome (IP) or constitutive
11 proteasome (cP) subunits as indicated in the figure. Samples with high or low
12 expression of a given proteasome subunit were plotted on the basis of patient survival
13 over time using a Kaplan Meier survival curve (astatsa.com/LogRankTest/).

14

15 **Figure S3. The FACS plot examples for the peptide stimulations.** **A**, FACS plot
16 showing TNF α vs CD8 in selected patients re-stimulated with either linear peptides.
17 **B**, pools of spliced peptides. **C**, or single spliced peptides. DMSO stimulations served
18 as negative and background control and FEC as positive control. Labelling denotes
19 first stimulation and re-stimulation.

20

21

22 **Figure S4.** *Cis*-spliced peptides that stimulated CD8⁺ T cells as measured by TNF α so
23 a higher degree than DMSO in at least 1 patient plus some randomly picked *cis*-
24 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 **Figure S5.** A, Length distribution of the region separating the N- and C-terminal
5 segment of a cis-spliced peptide on the protein level (in amino acids). B-F, Length
6 distribution of N- and C-terminal segments of *cis*-spliced peptides, shown for 8-12
7 mers (in amino acids).

8

9 **Figure S6.** Flow cytometry gating strategy. **A**, Cells were gated based on size (FSC-
10 A/SSC-A), doublets and dead cells were excluded, and this strategy was used for all
11 samples. The DMSO-treated negative control. **B**, was used to set the TNF α ⁺/CD8⁺
12 gate after CD3⁺/CD8⁺ gating. An example of a peptide-specific CD8⁺ T cell response
13 is shown (C).

14

15 **Figure S7.** Pearson r value correlation score of b and y ions in spectra from 28 eluted
16 *cis*-spliced peptides from LM-MEL-44 cell line compared with their synthetic
17 versions. This analysis approved the authenticity of 27 sequences and disapproved
18 one sequence.

19

20 **Figure S8.** Comparison of MS2 spectra and iRT value of 28 *cis*-spliced peptides
21 versus their corresponding synthetic version.

22

23 **Figure S9.** Comparison of predicted vs real retention time of linear and *cis*-spliced
24 peptides by using GPTIME tool (55) . There was not a difference between linear and
25 spliced peptide in this analysis in any of datasets.

1 **Supplementary Tables:**

2 **Table S1.** Identified Linear and *cis*-spliced peptides. Sheet 1; all 8-12 mer identified
3 peptides and their presence in other cell lines or reported before (note that in spliced
4 peptides, L stands for both leucine and isoleucine.). Sheet2; Shared peptides between
5 six LM-MEL-44 samples. Sheet 3. List of LM-MEL-44 peptides presented in each
6 condition. Sheet 4-13 raw export of PEAKS X software for each dataset.

7 **Table S2.** Binding prediction for identified linear and *cis*-spliced peptides from LM-
8 MEL-44 cell line

9 **Table S3.** Predicted mutated peptides based on LM-MEL-44 sequencing data

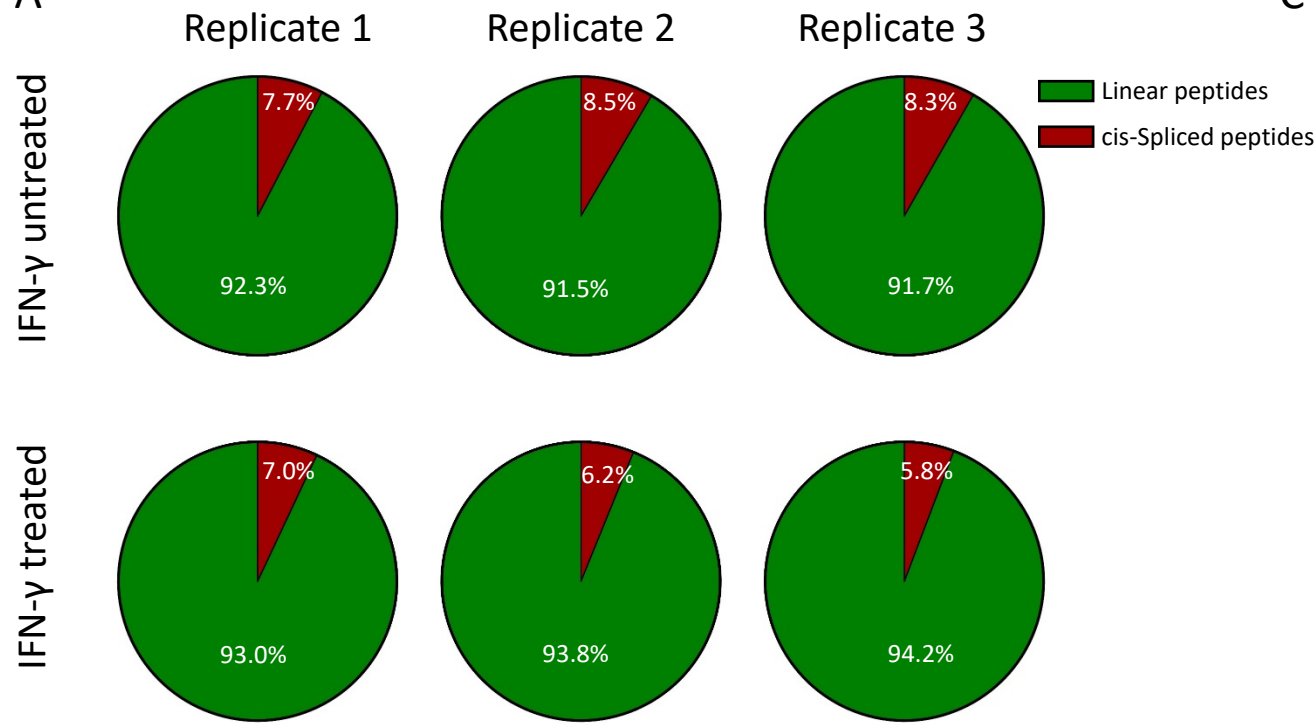
10 **Table S4.** Melanoma associated antigens derived linear and *cis*-spliced peptides

11 **Table S5.** Selected linear peptides used for immunogenicity assay

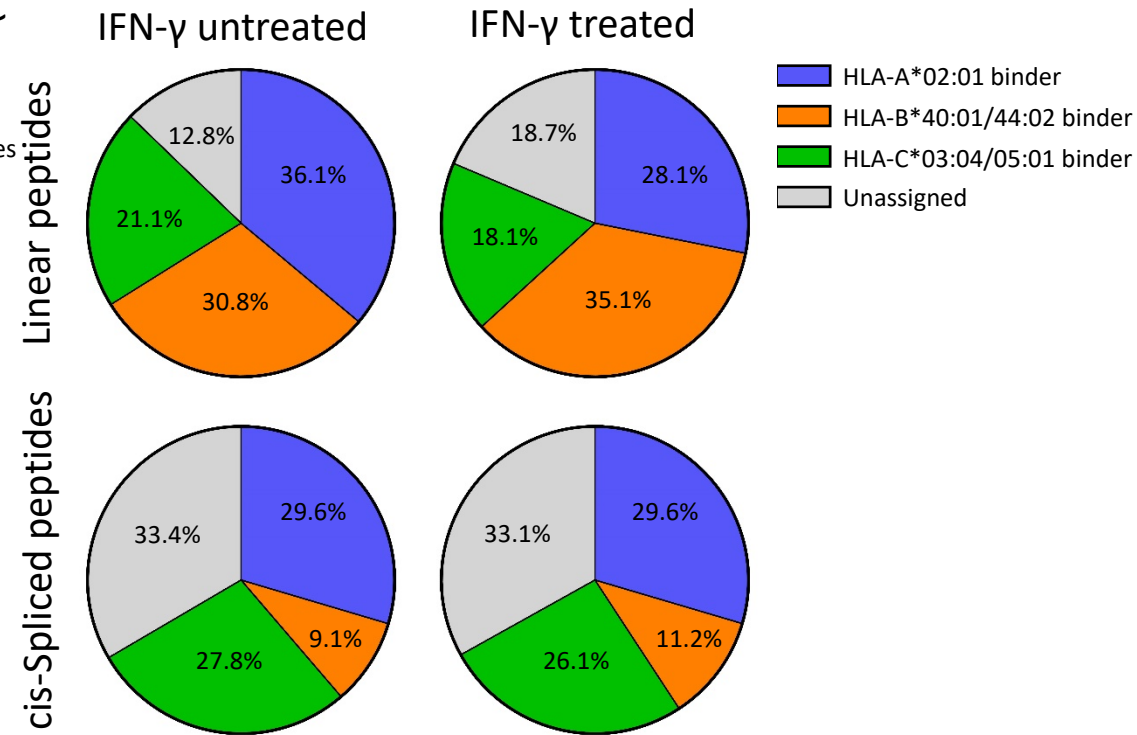
12 **Table S6:** Presence of immunogenic linear and spliced sample across different cell
13 lines

14

Fig.1 A



C



B

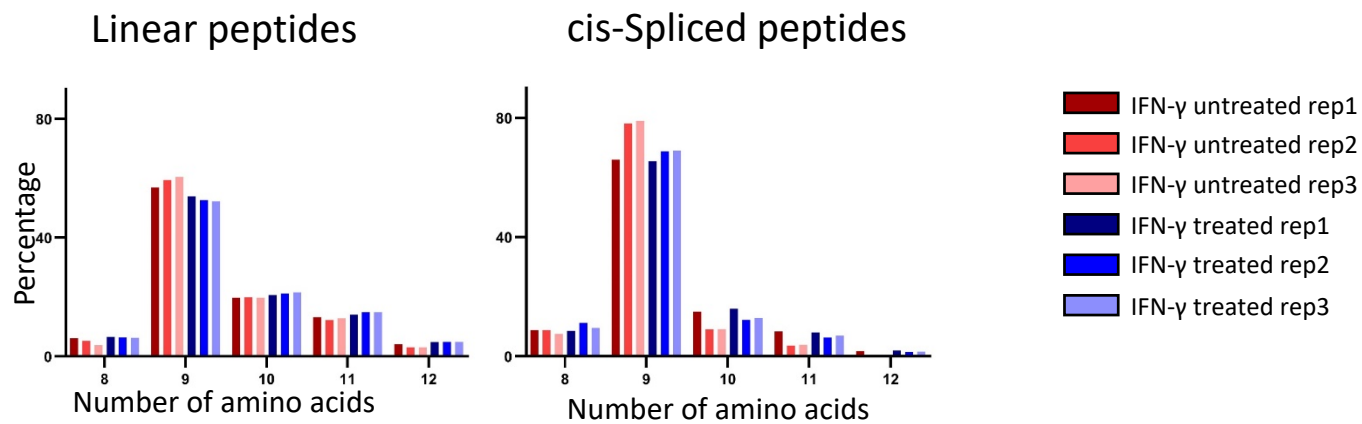
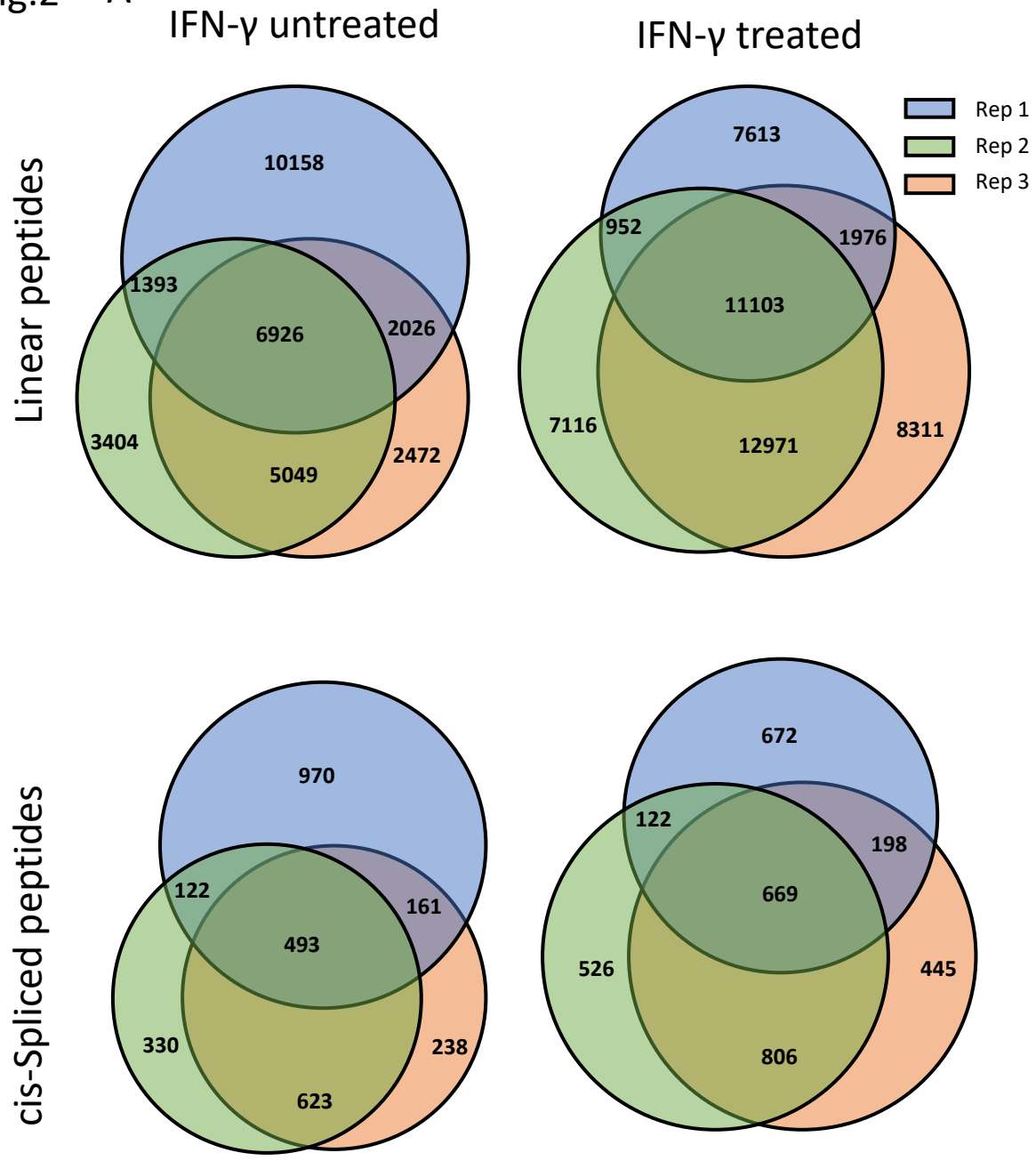


Fig.2

A



B

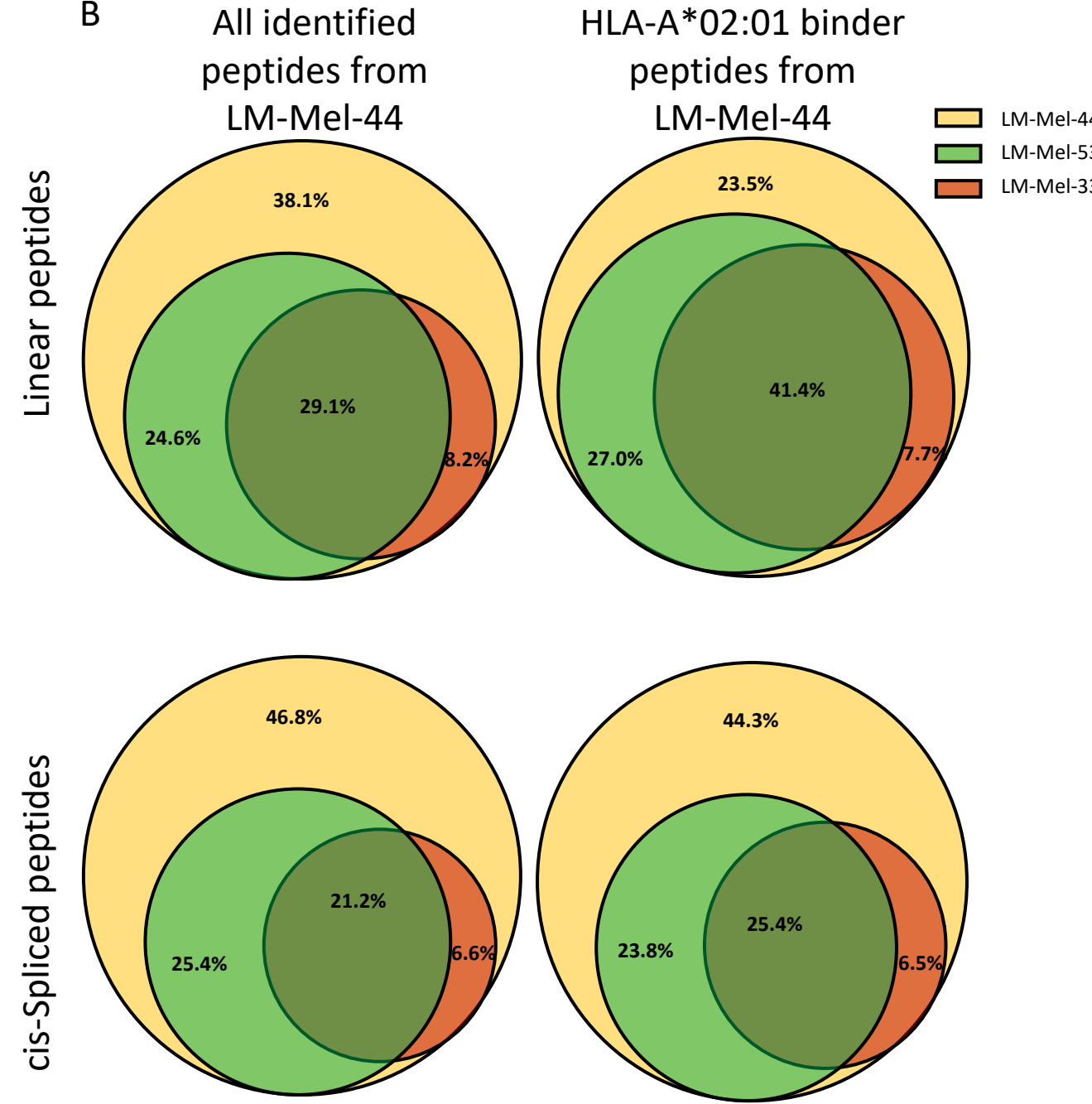
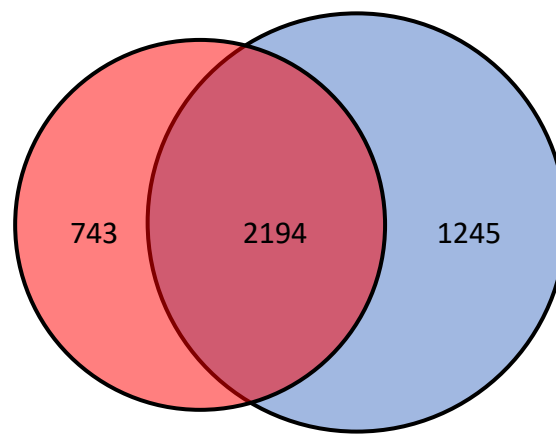
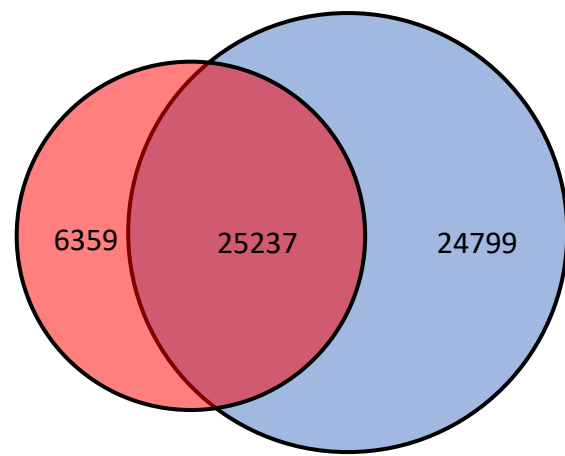


Fig.3

A

Linear peptides

cis-Spliced peptides



IFN- γ untreated
IFN- γ treated

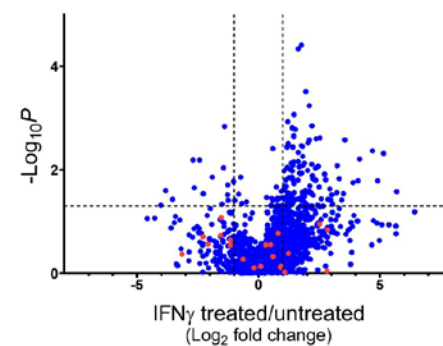
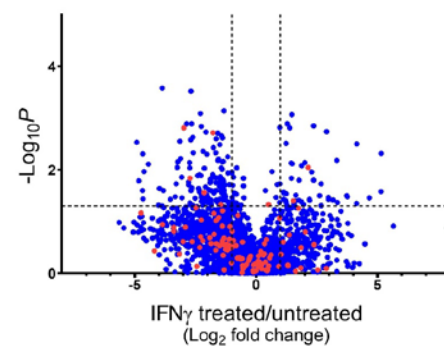
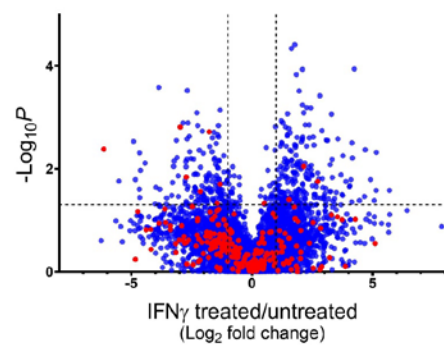
B

All peptides

HLA-A*02:01

HLA-B*40:01

Linear peptides
Cis-Spliced peptides



HLA-B*44:02

HLA-C*03:04

HLA-C*05:01

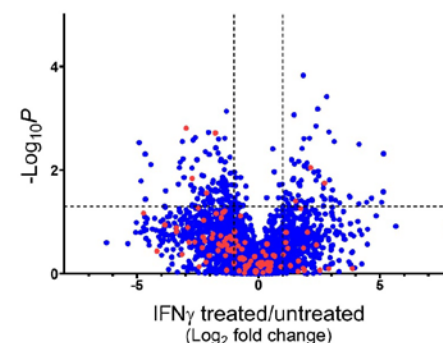
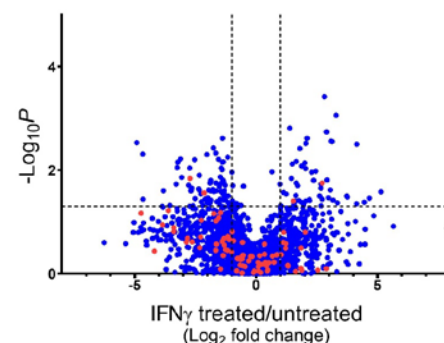
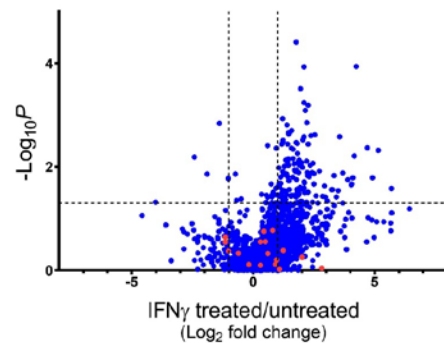
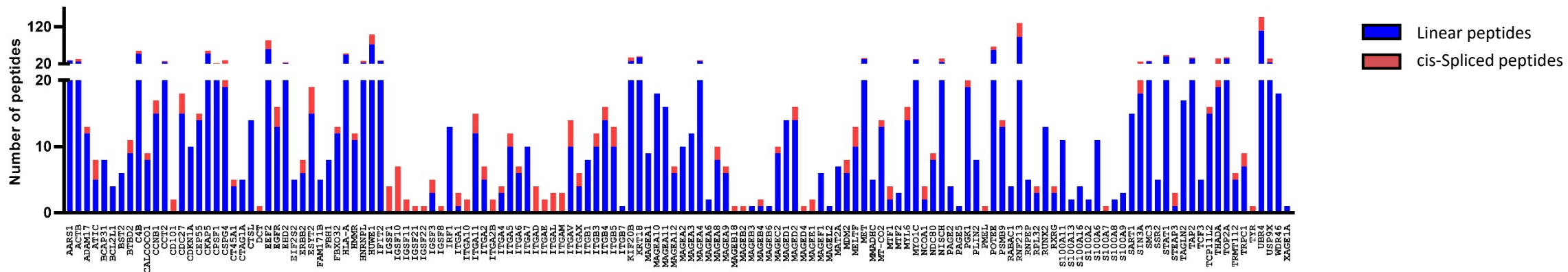
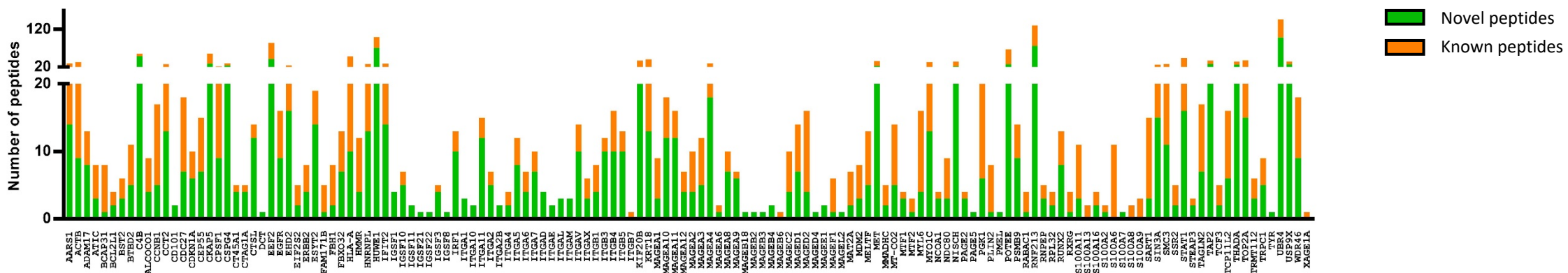


Fig.4

A



B



C

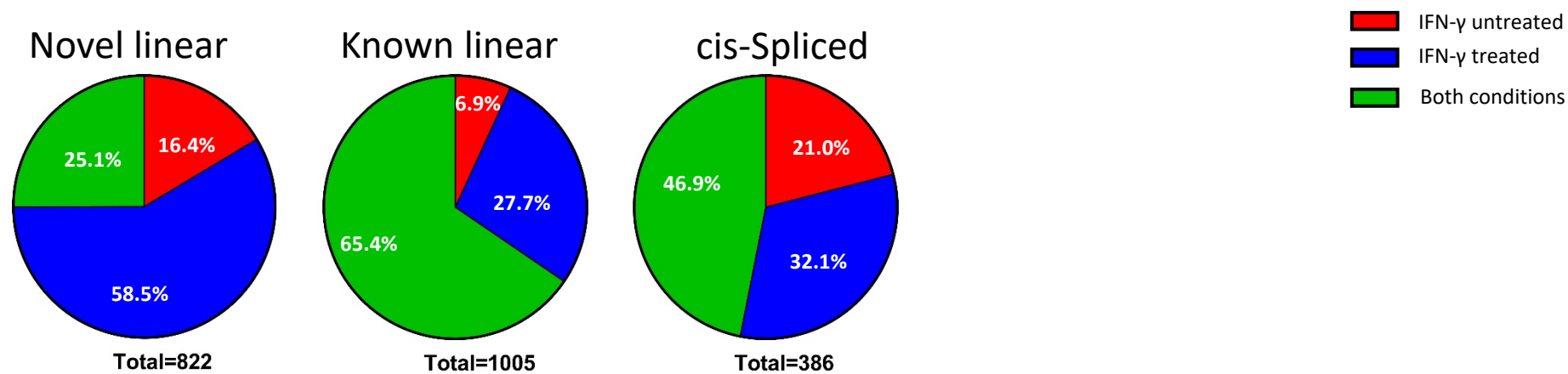
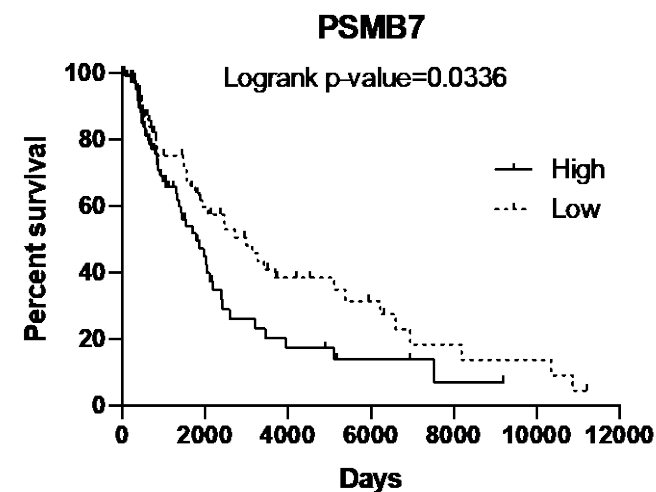
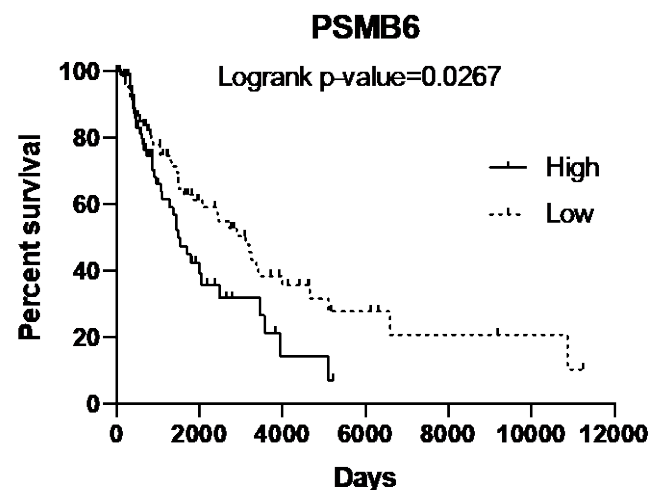
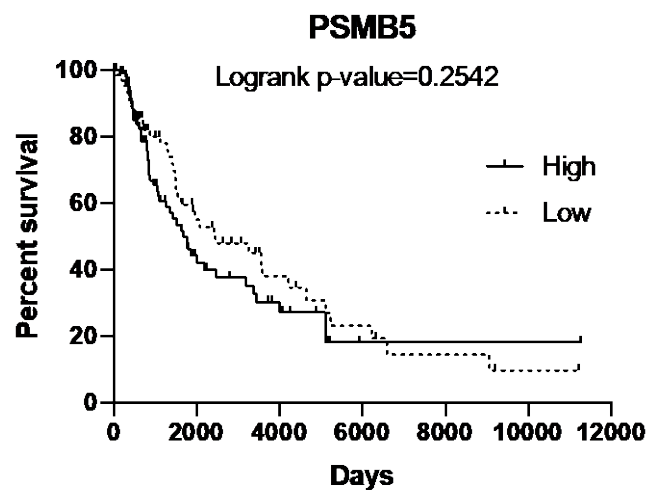


Fig.5

Constitutive proteasome genes – top quartile of CD3 expressing samples removed



Immunoproteasome genes – top quartile of CD3 expressing samples removed

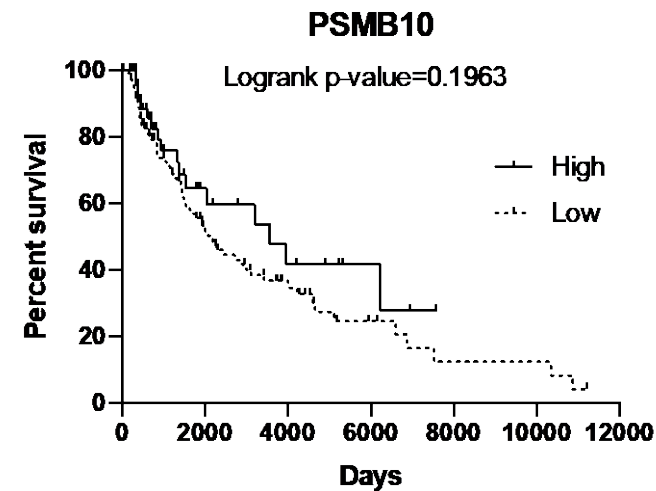
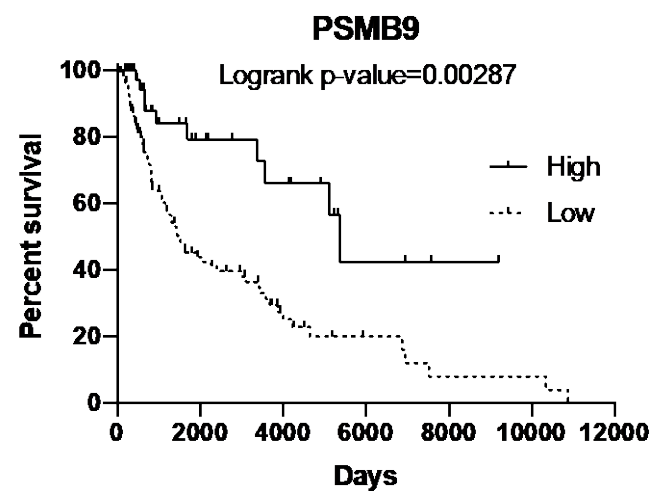
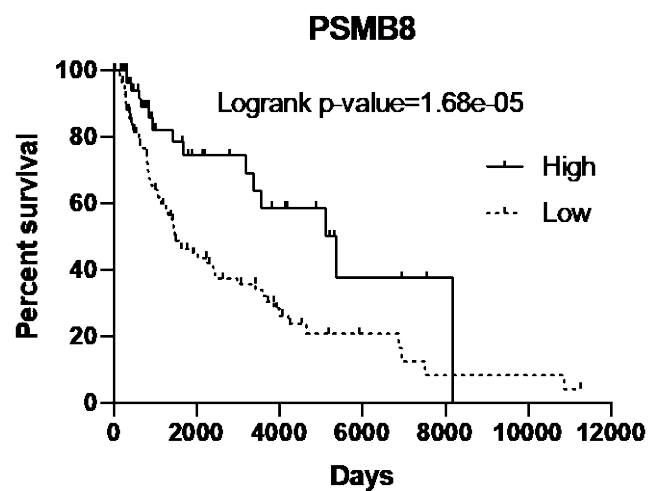
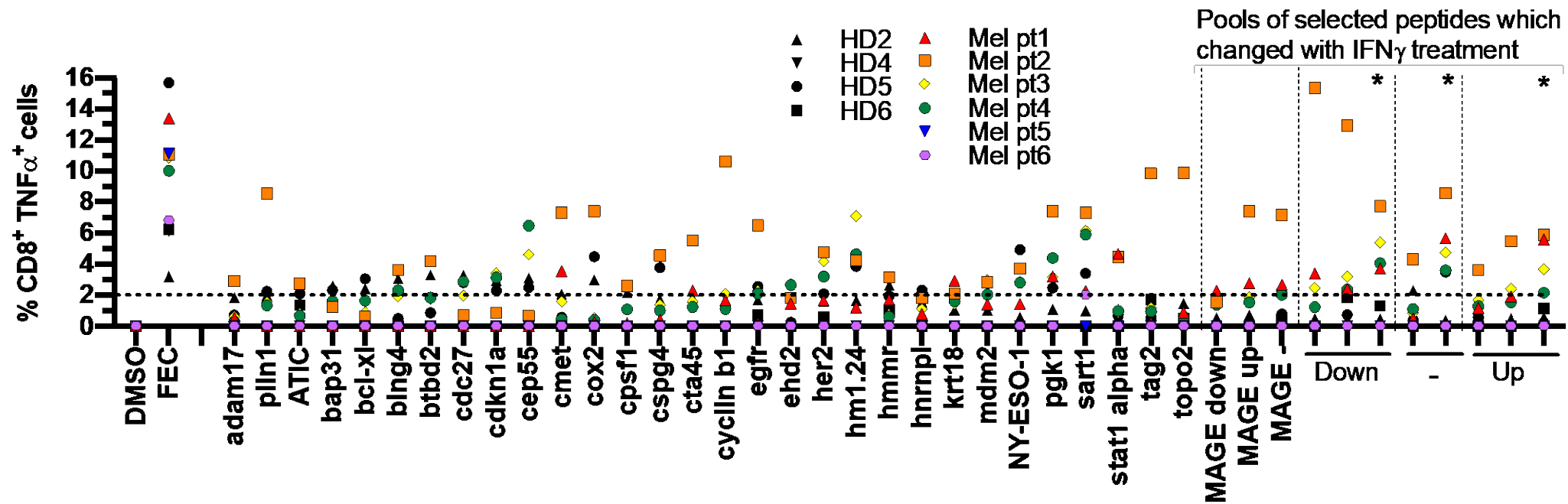


Fig.6

A



B

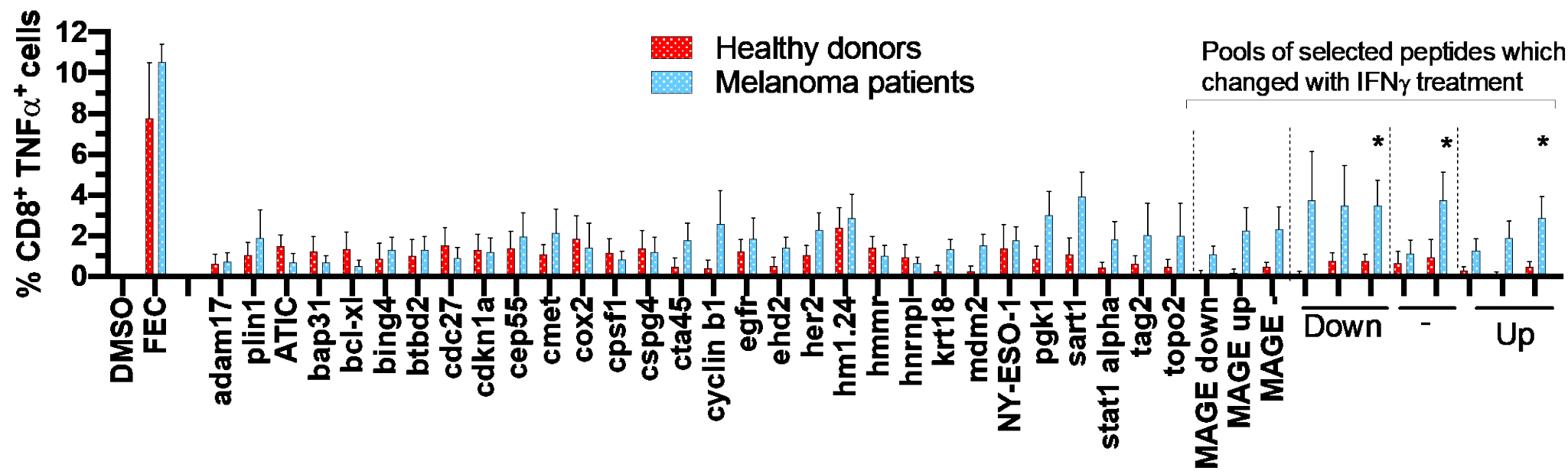
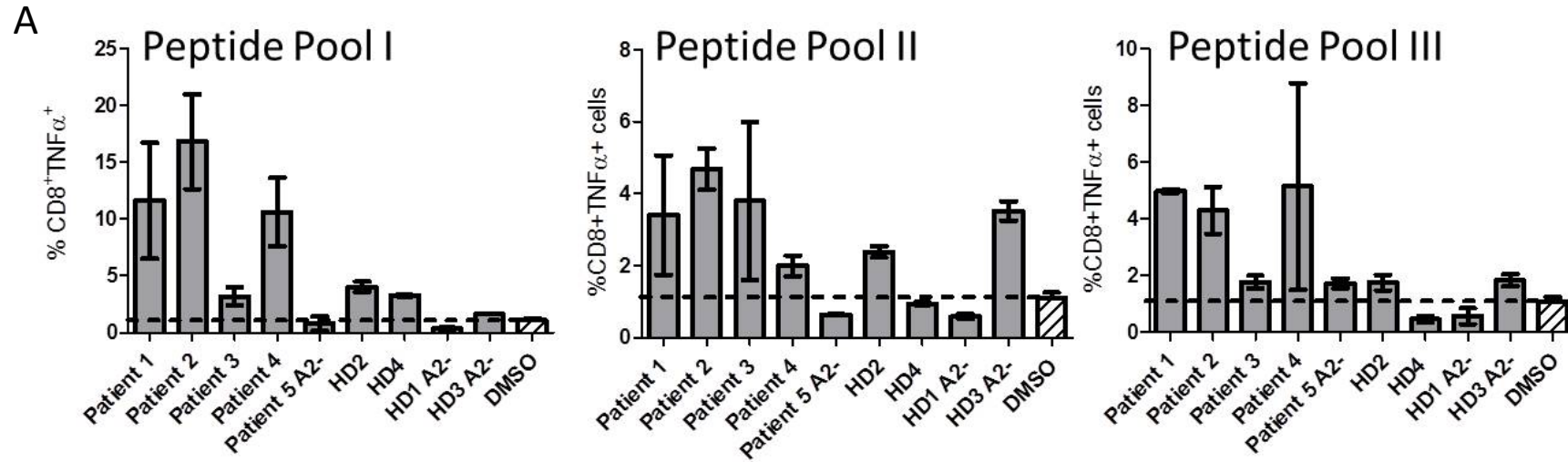


Fig.7



B

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

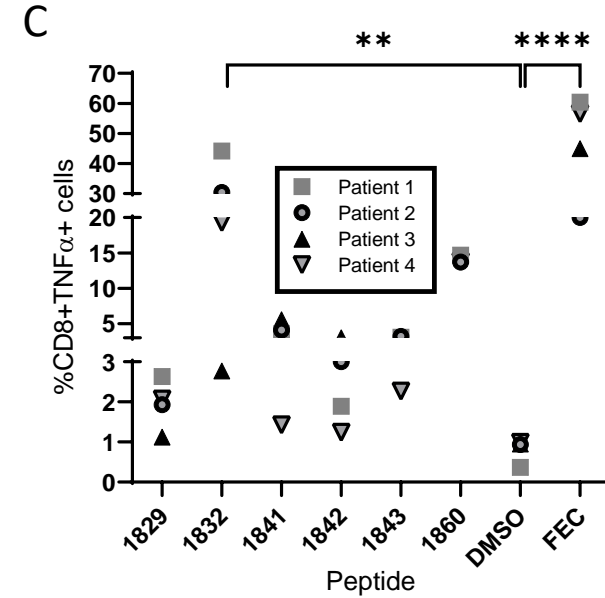


Fig.S1

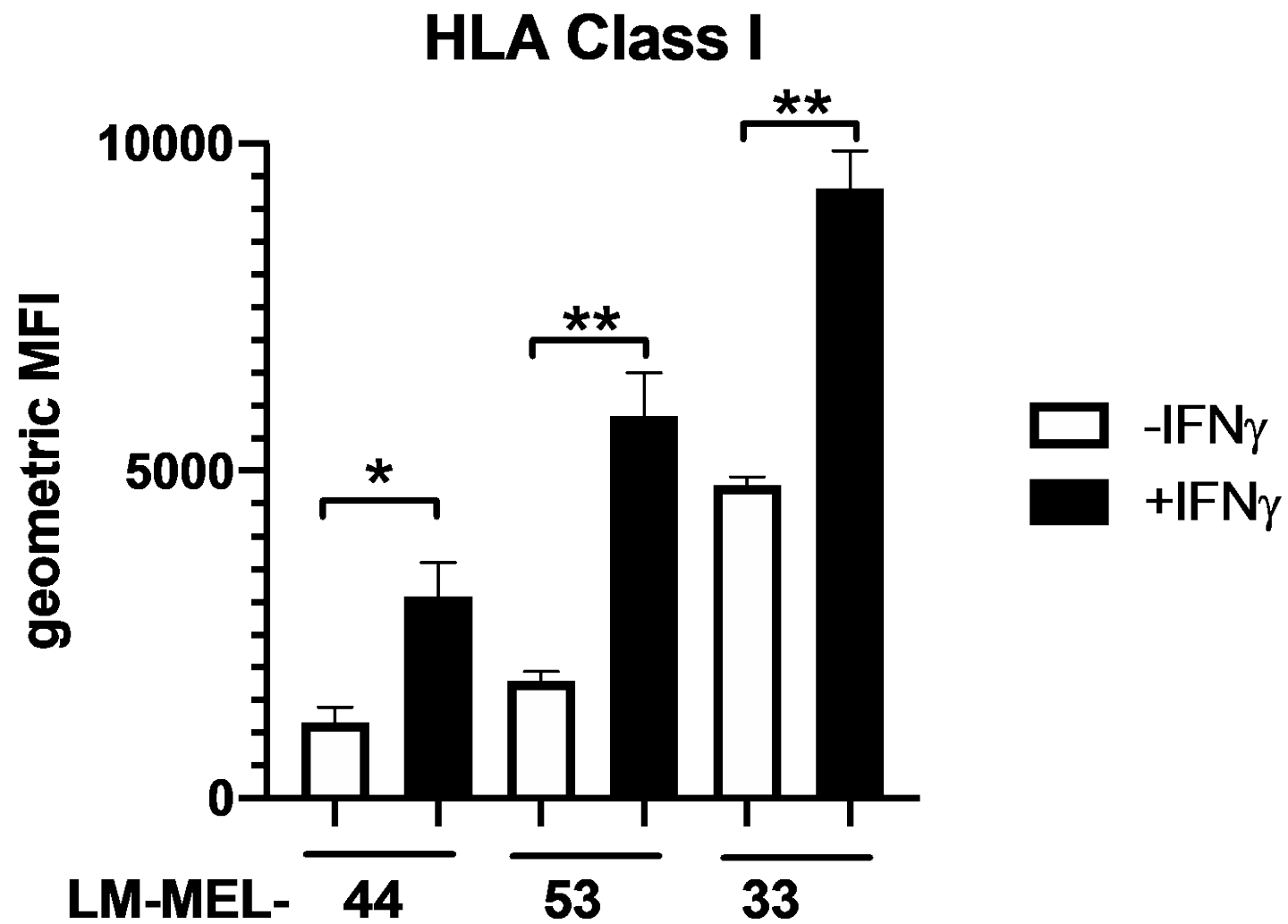
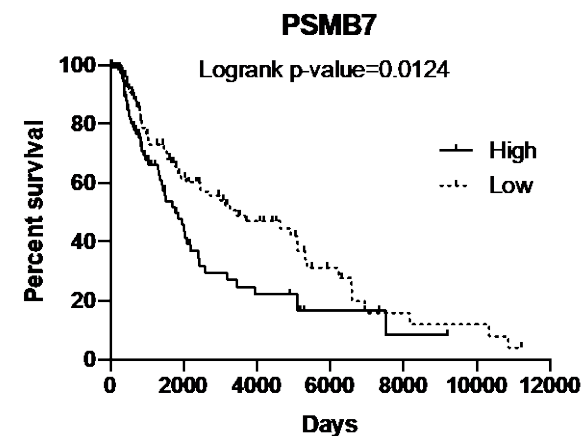
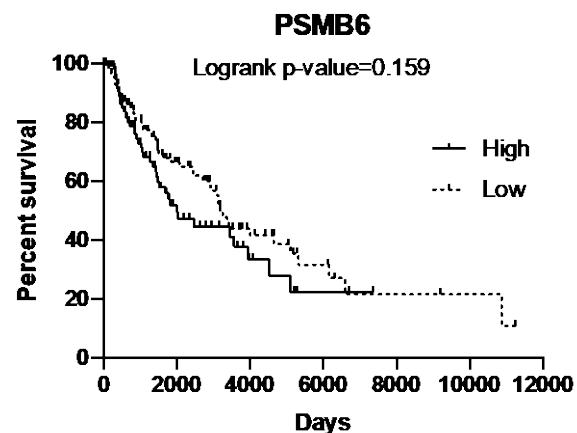
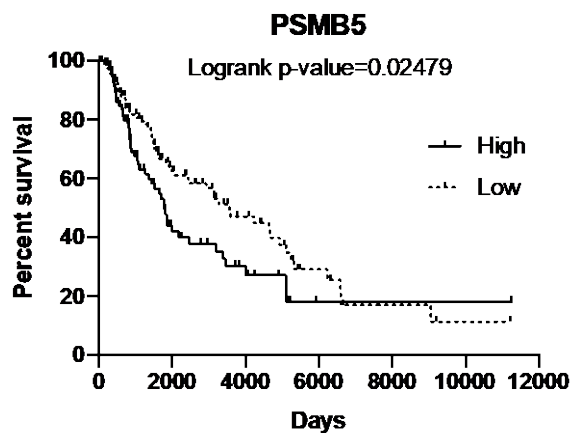


Fig.S2

Constitutive proteasome genes



Immunoproteasome genes

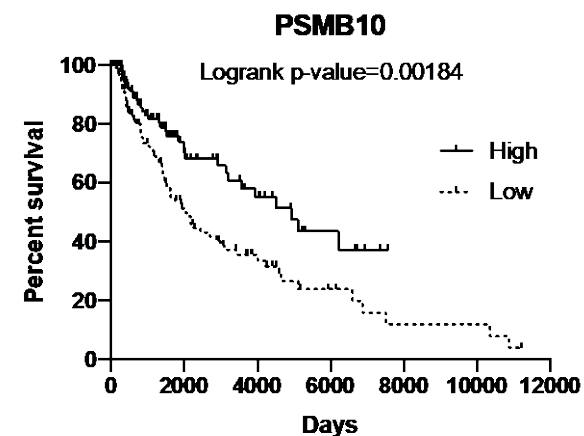
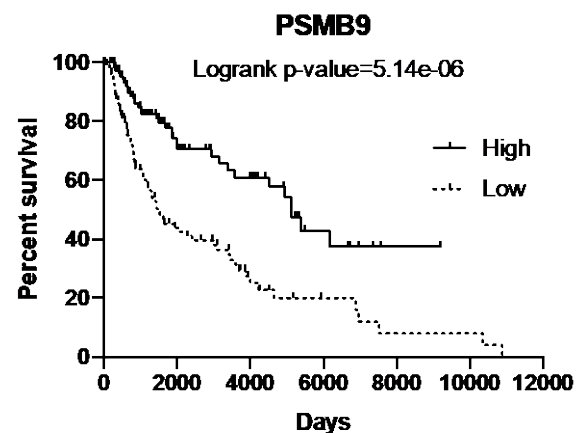
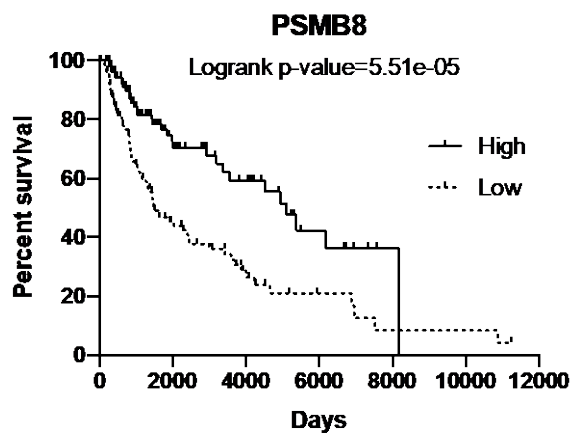


Fig.S3

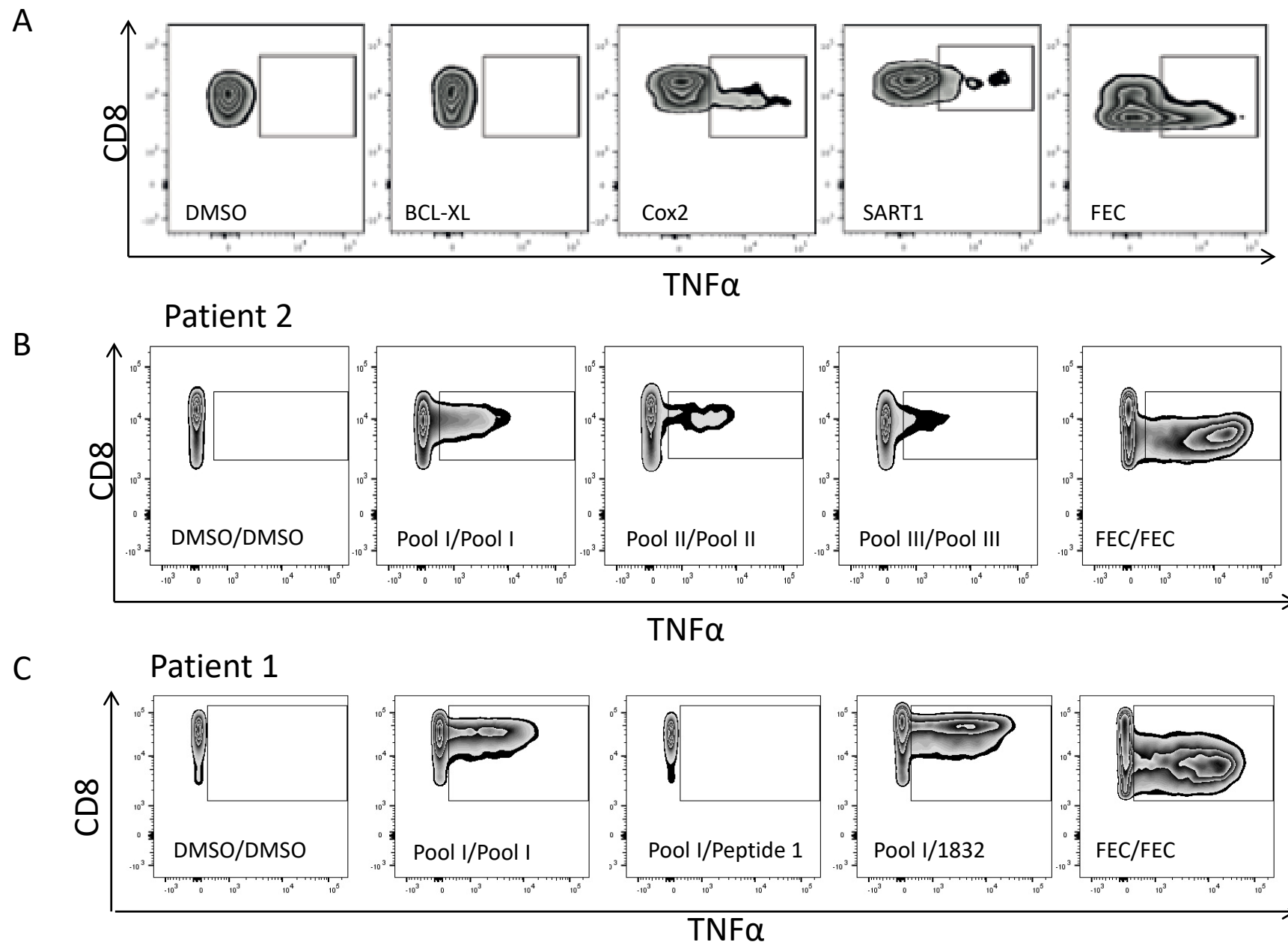


Fig.S4

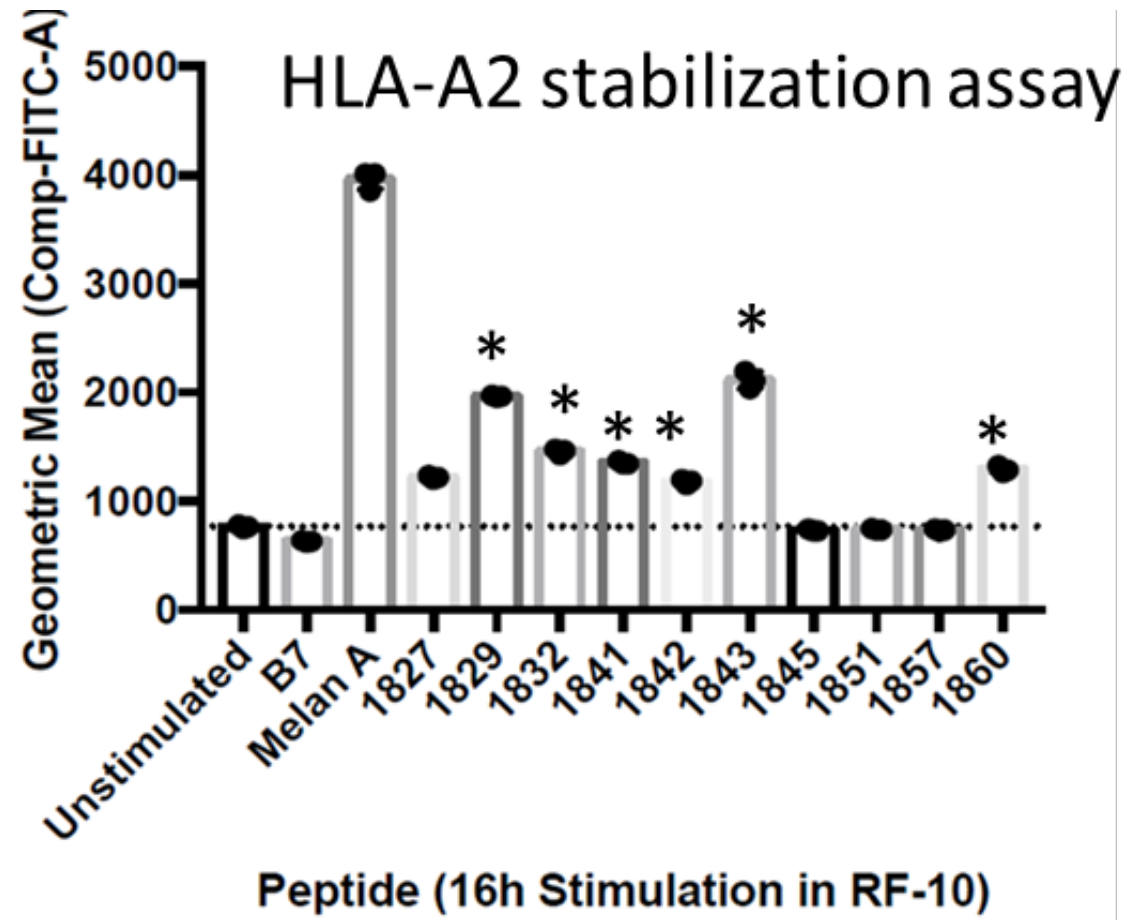


Fig.S5

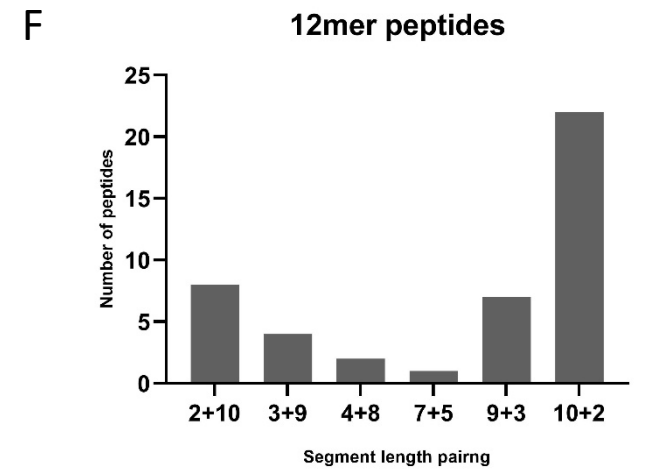
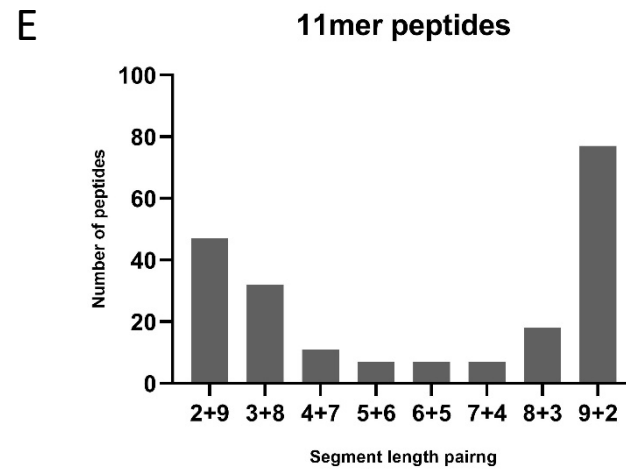
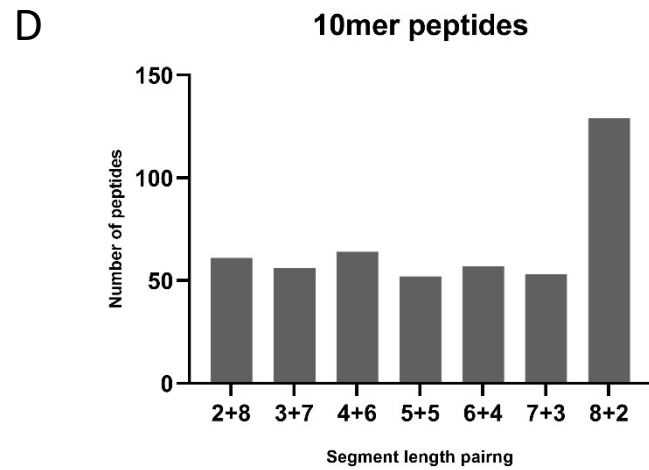
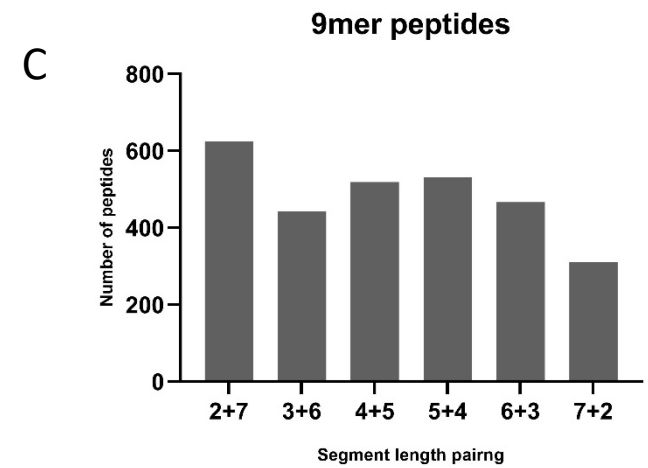
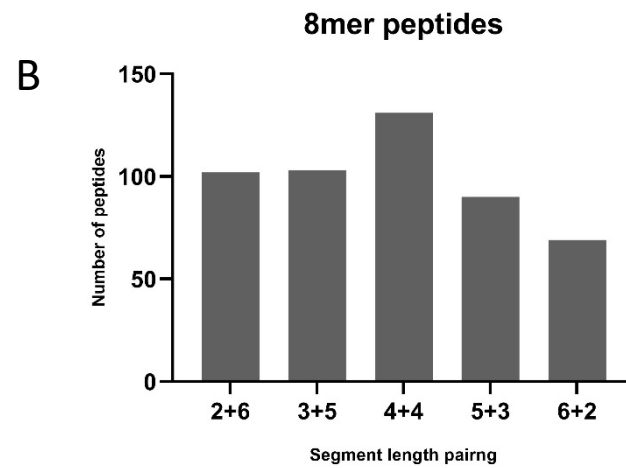
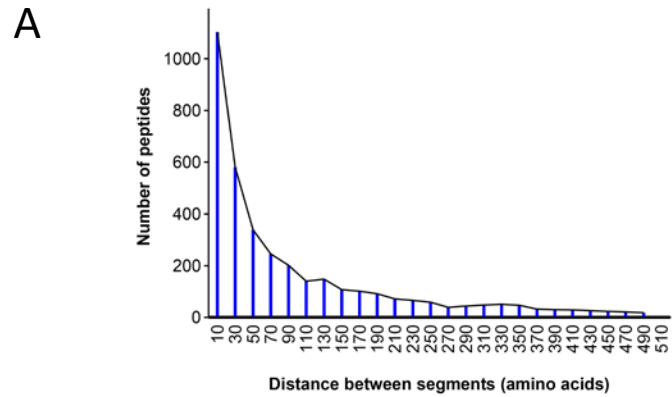
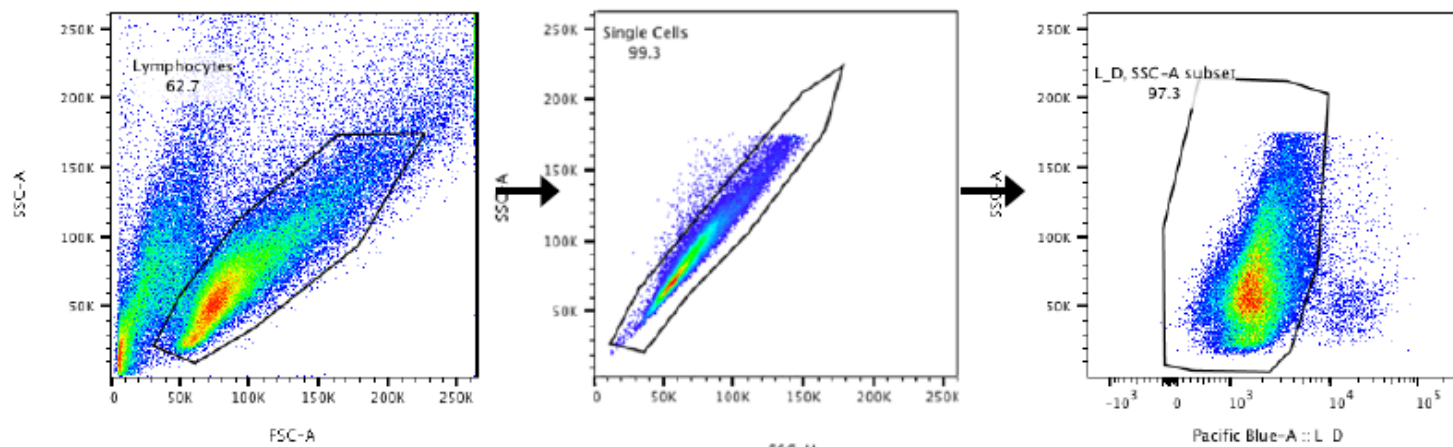
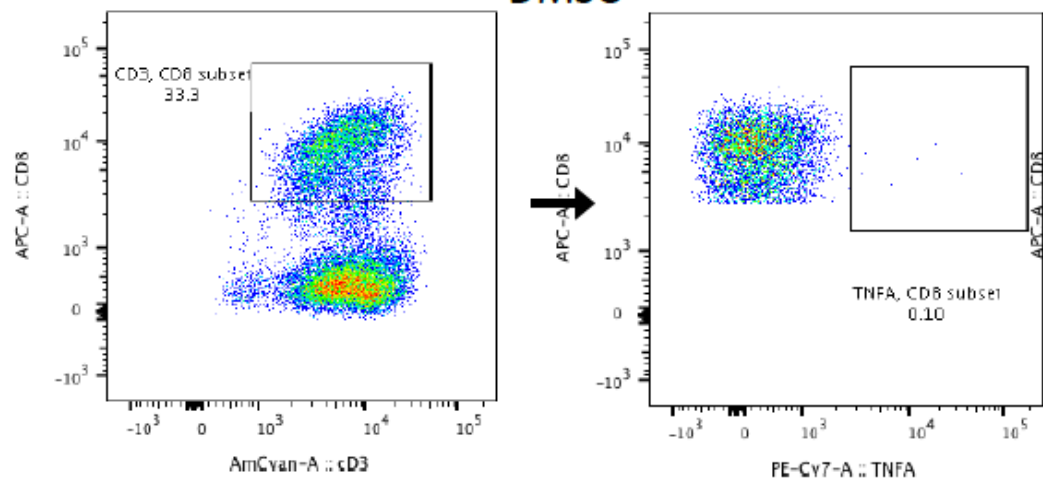


Fig.S6

A



B



C

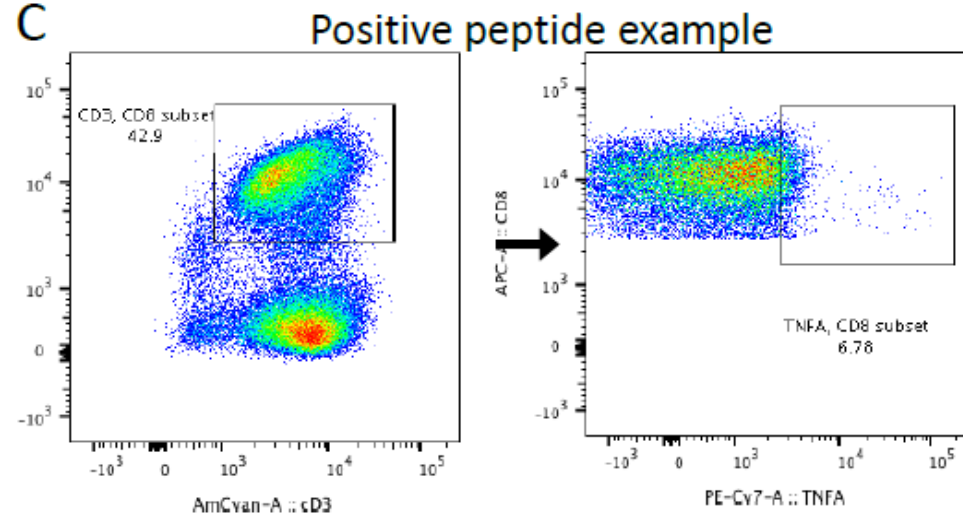
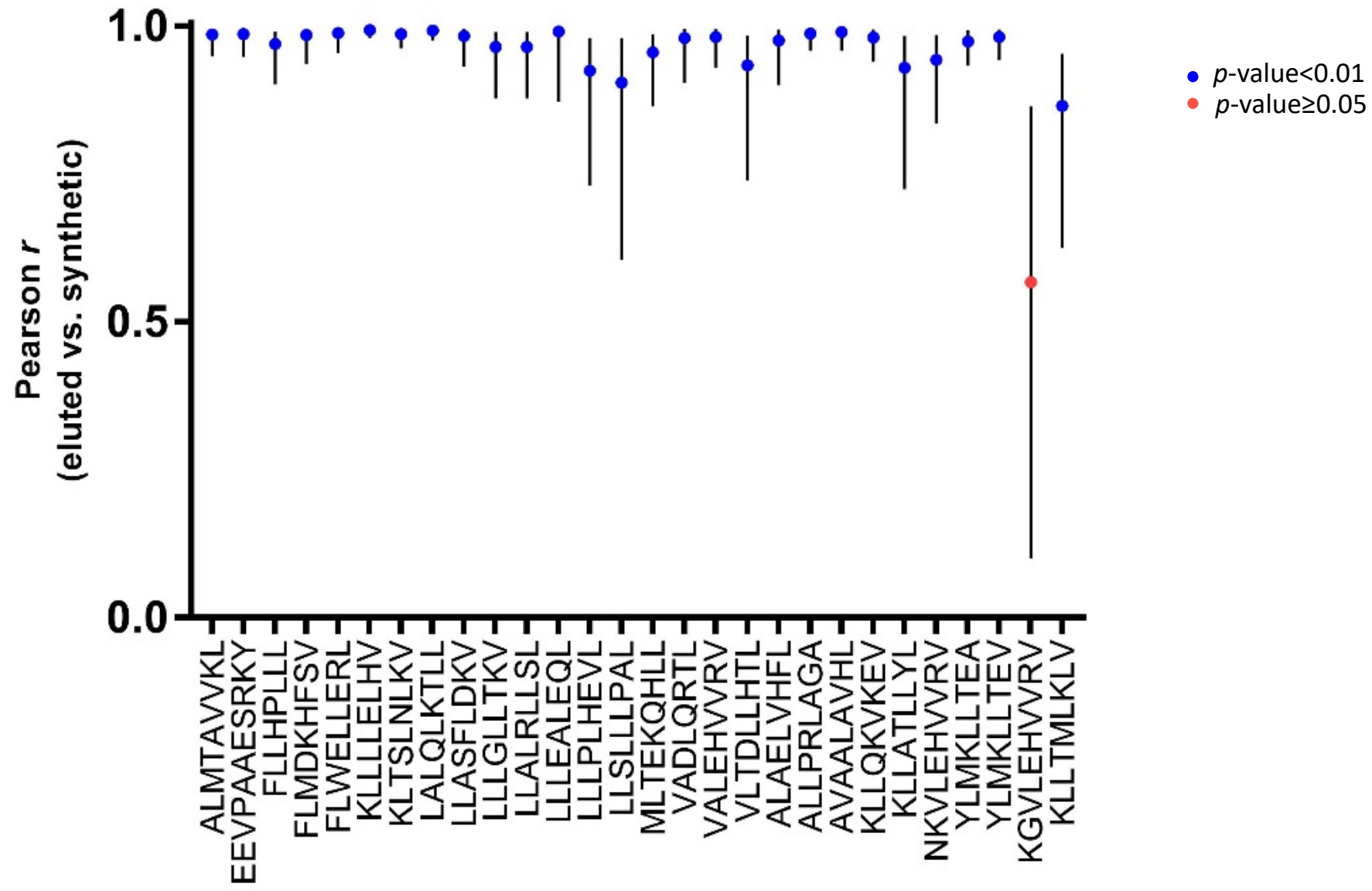


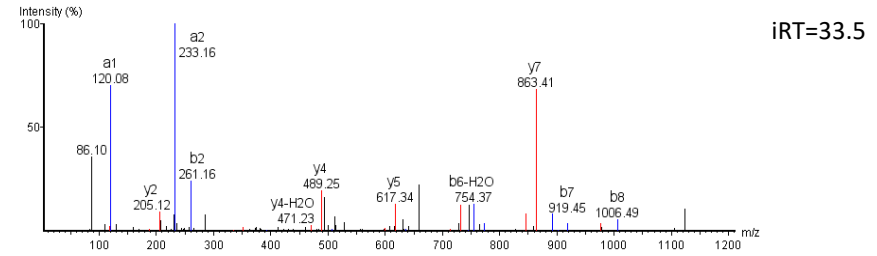
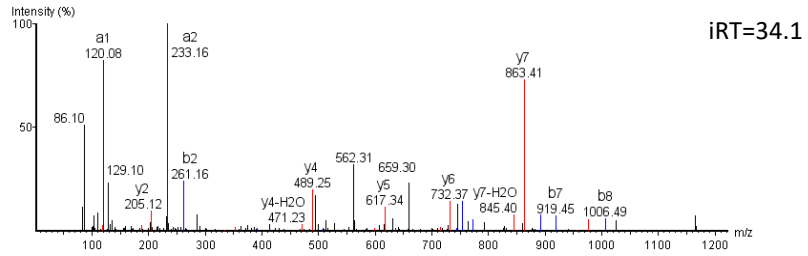
Fig.S7



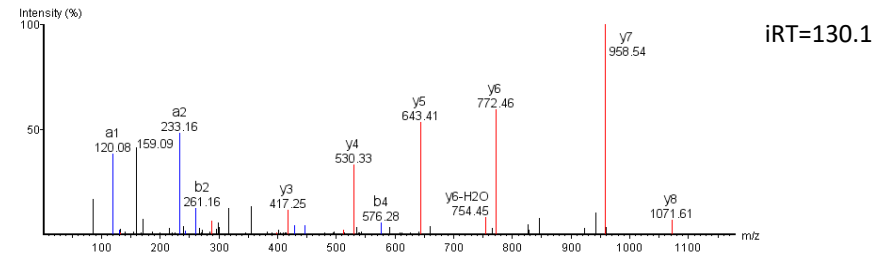
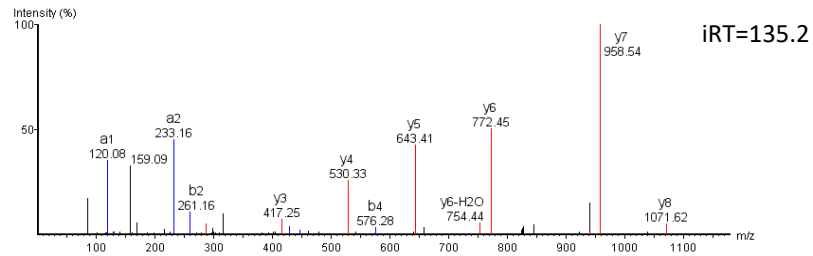
Eluted peptide

Synthetic peptide

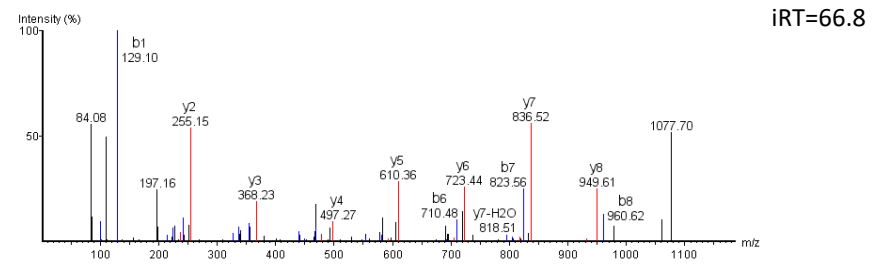
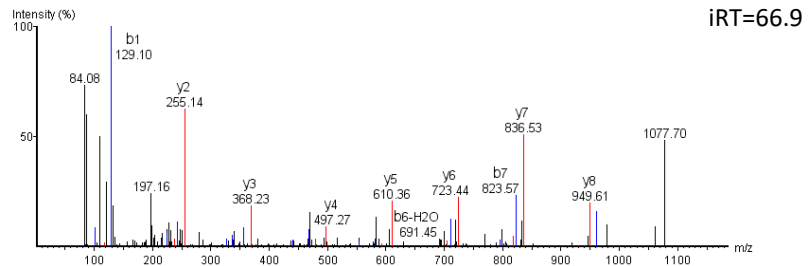
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FLWELLERL



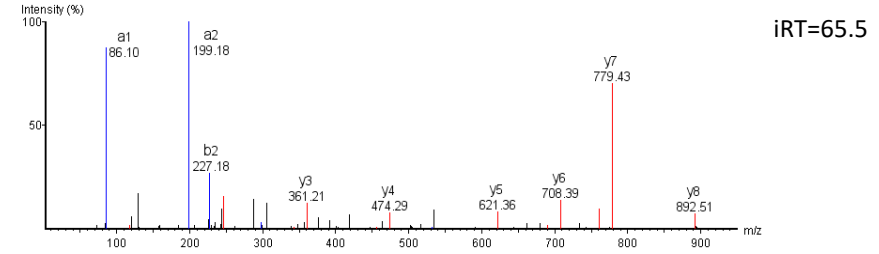
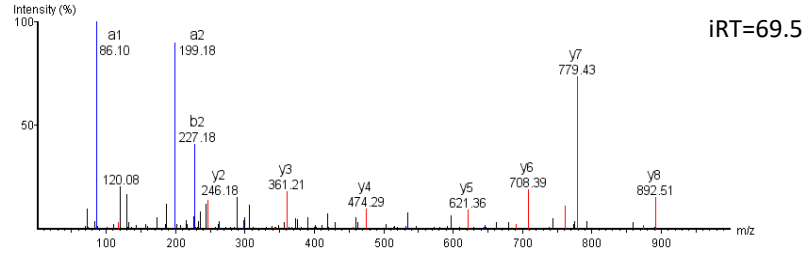
KLLLLLHV



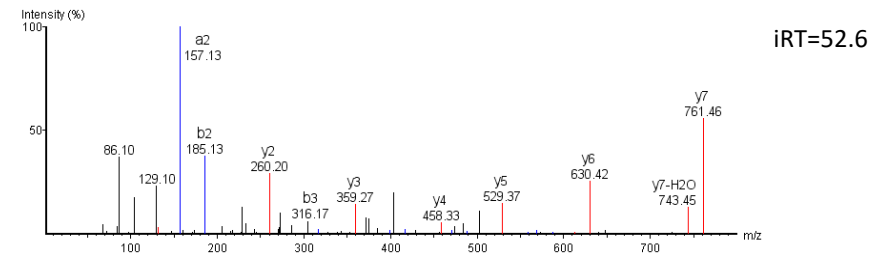
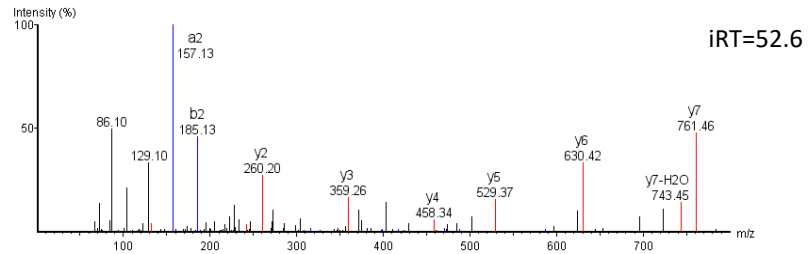
Eluted peptide

Synthetic peptide

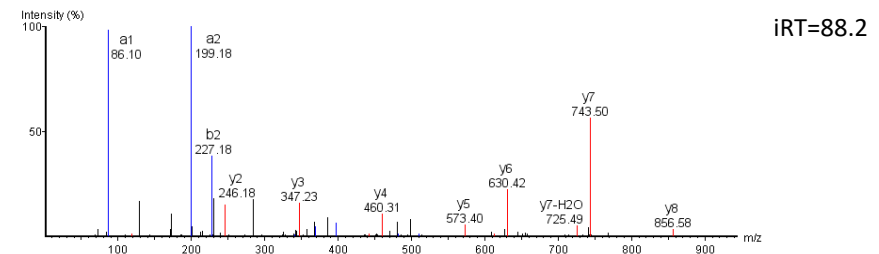
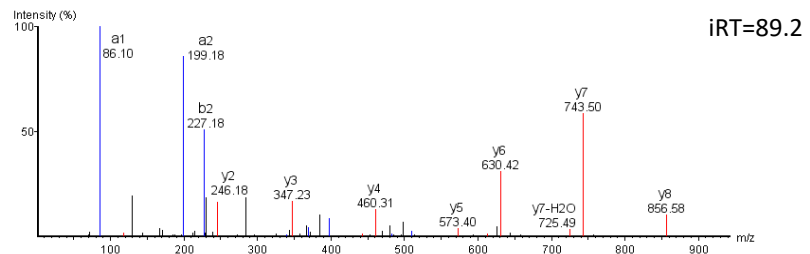
LLASFLDKV



ALMTAVVKL



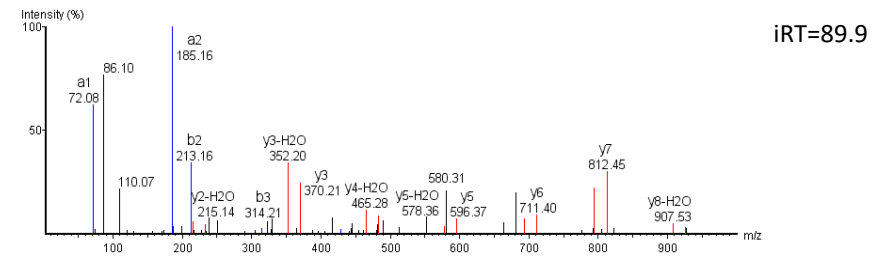
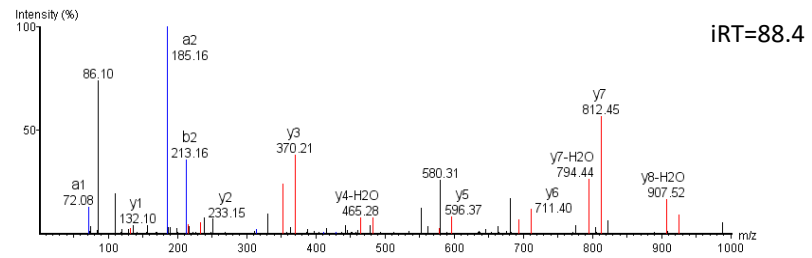
LLLGLLTKV



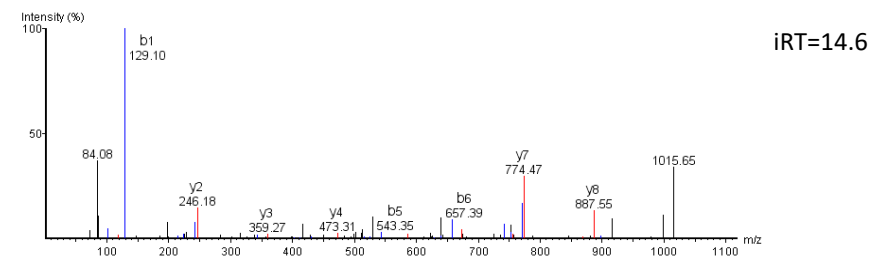
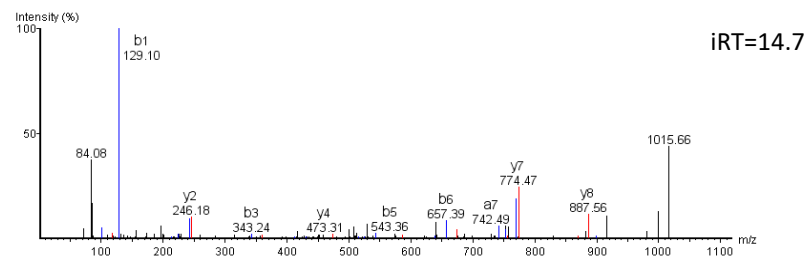
Eluted peptide

Synthetic peptide

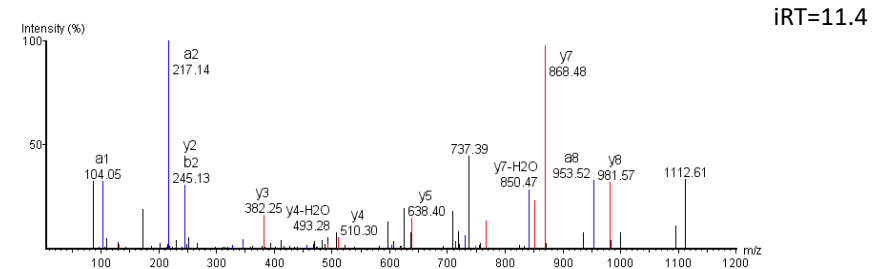
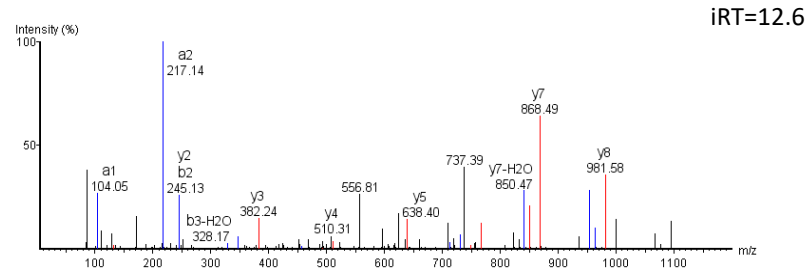
VLTDLLHTL



KLTSNLKV



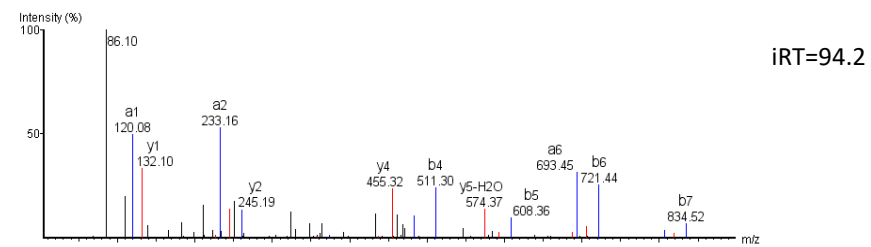
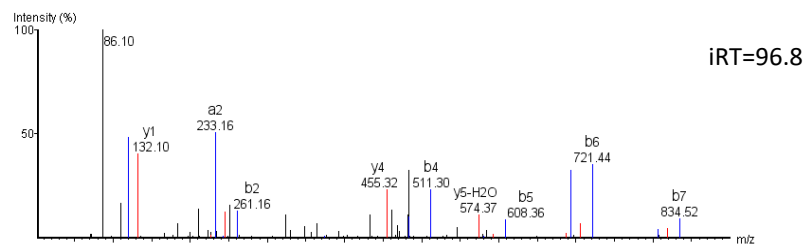
MLTEKQHLL



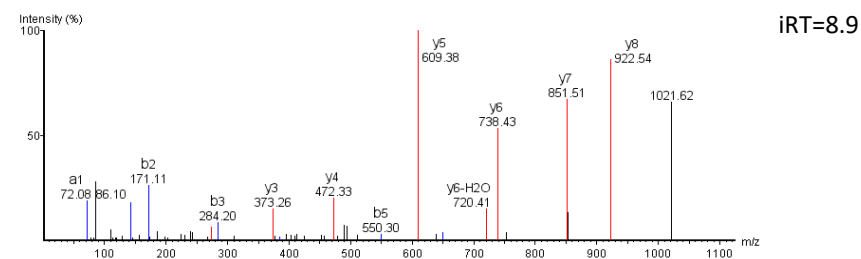
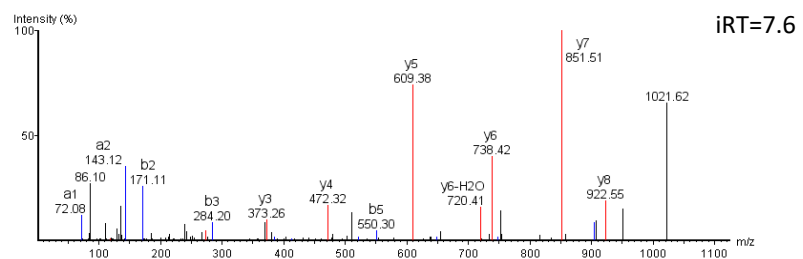
Eluted peptide

Synthetic peptide

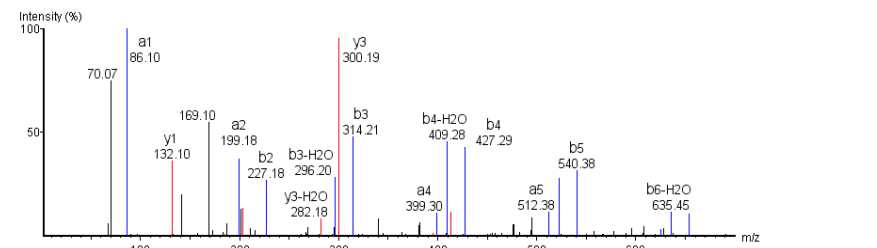
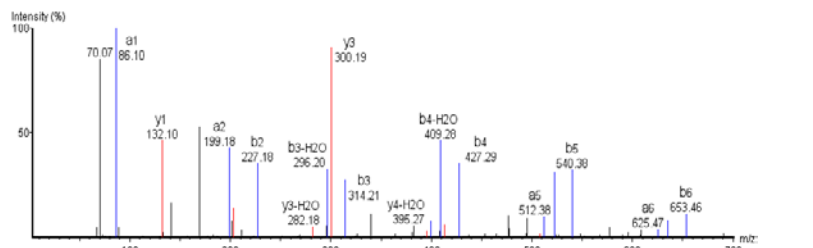
FLLHPLLL



VALEHVVRV



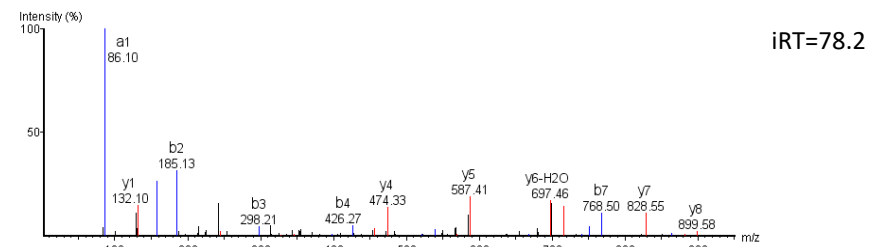
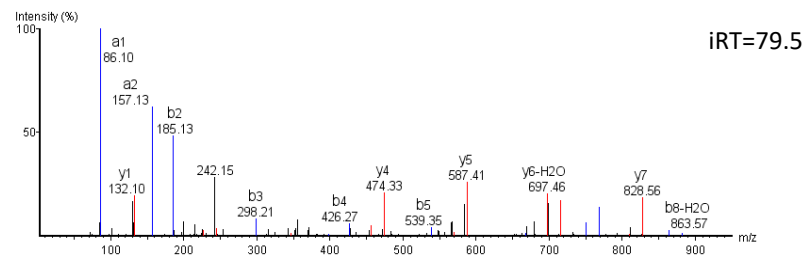
LLSLLLPA



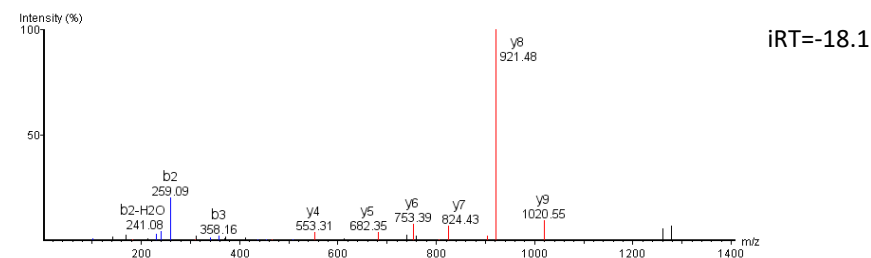
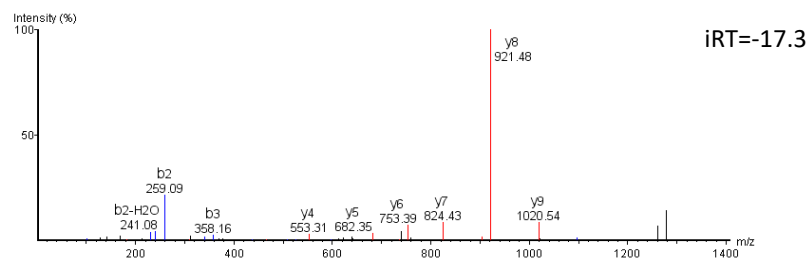
Eluted peptide

Synthetic peptide

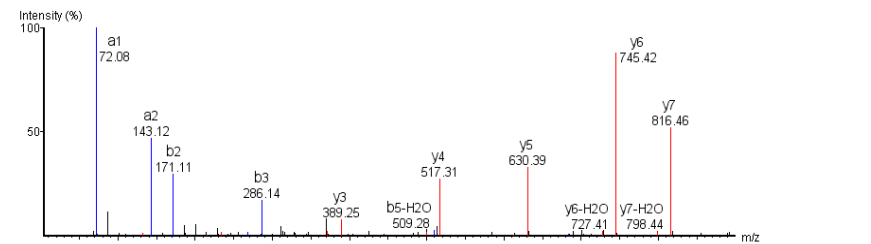
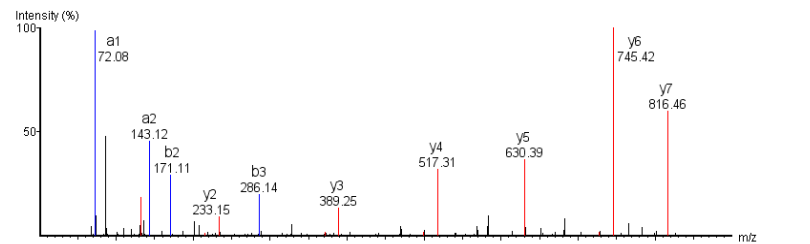
LALQLKTL



EEVPAESRKY



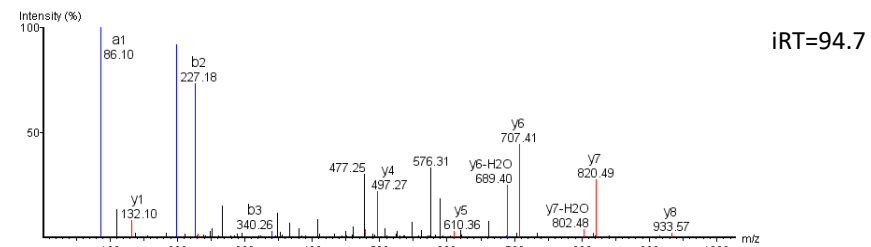
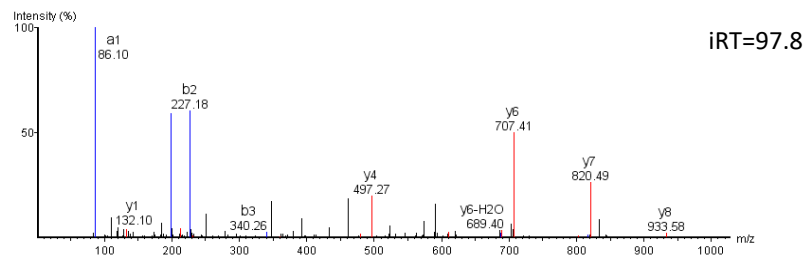
VADLQRTL



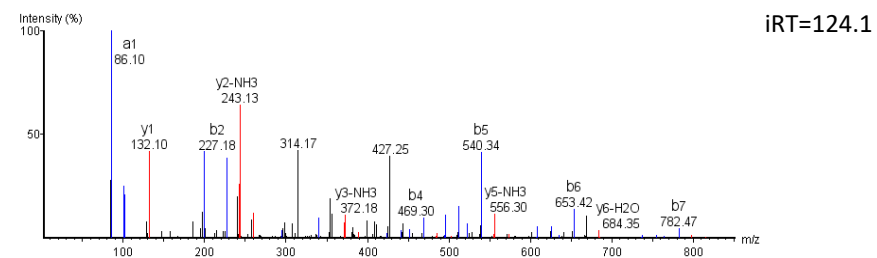
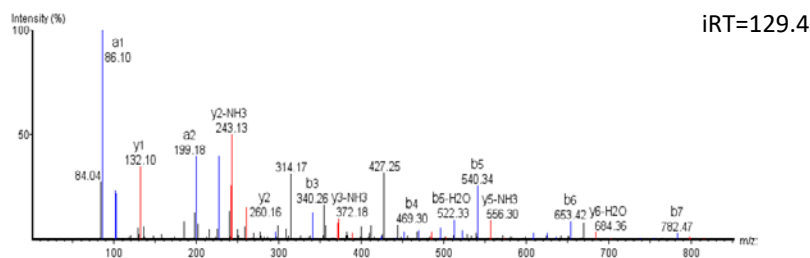
Eluted peptide

Synthetic peptide

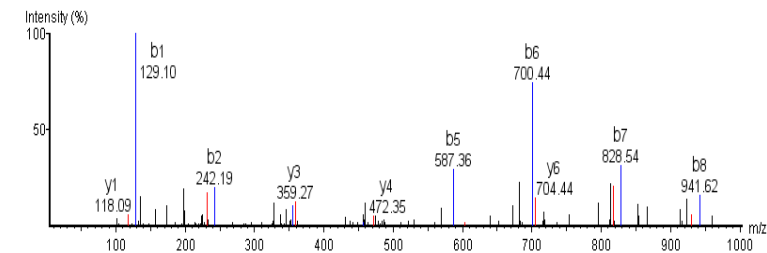
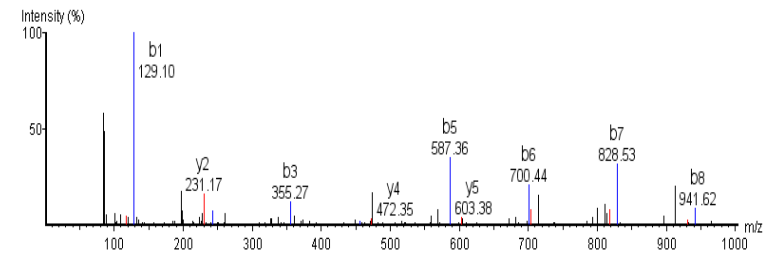
LLLPLHEVL



LLLEALEQL

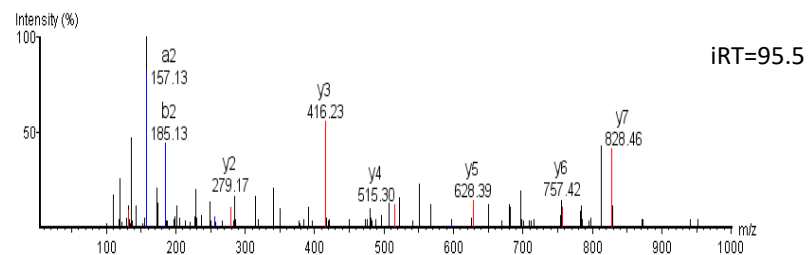


KLLTMLKLV

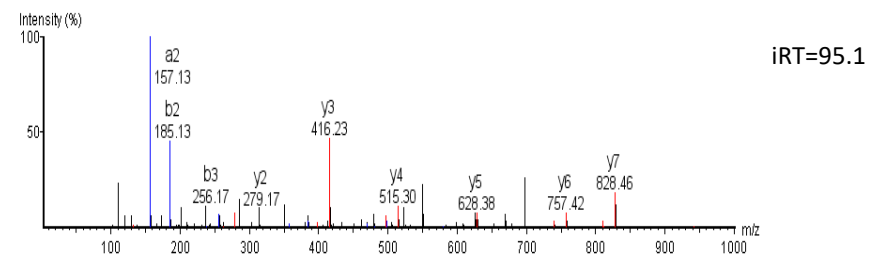


Eluted peptide

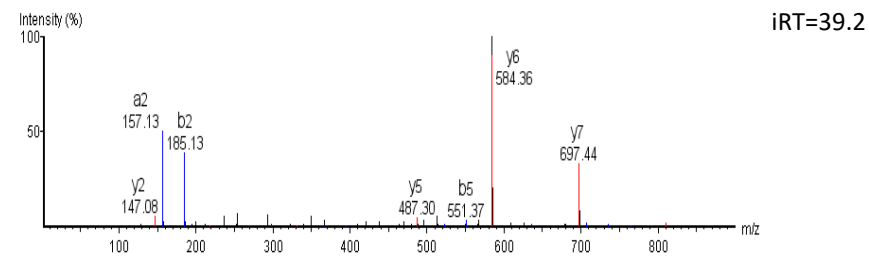
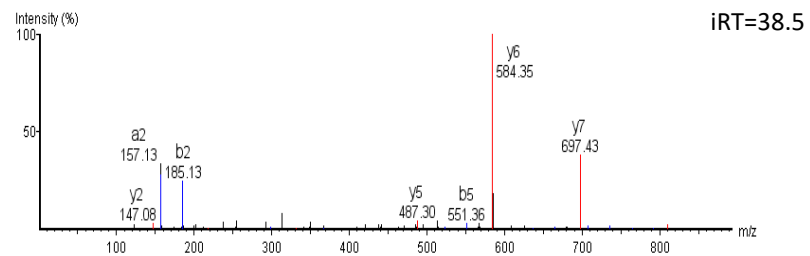
ALAELVHFL



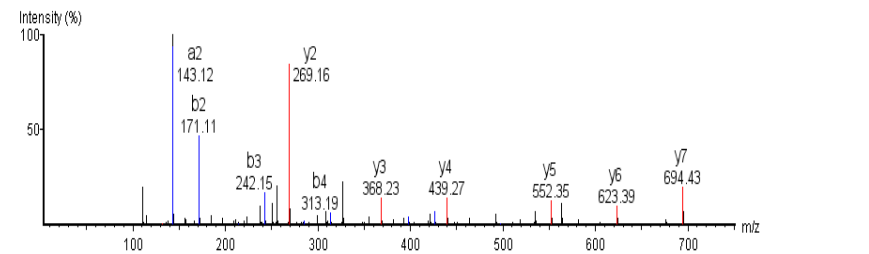
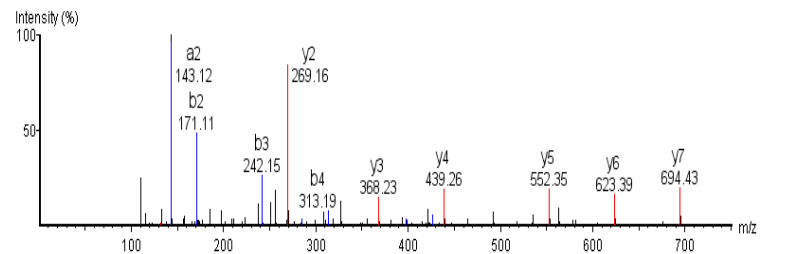
Synthetic peptide



ALLPRLAGA



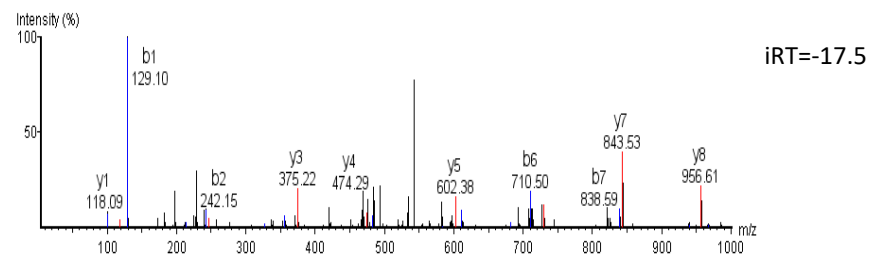
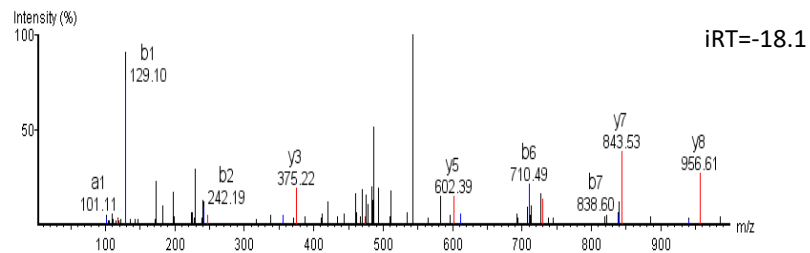
AVAALAVHL



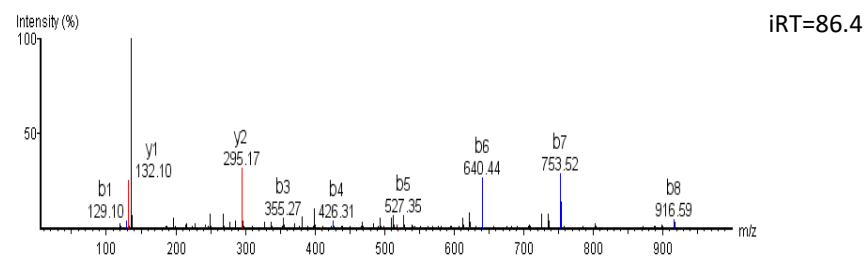
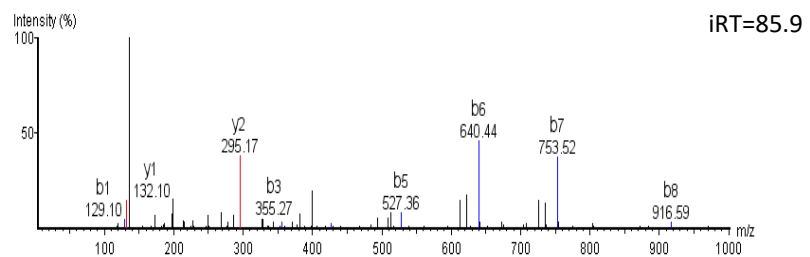
Eluted peptide

Synthetic peptide

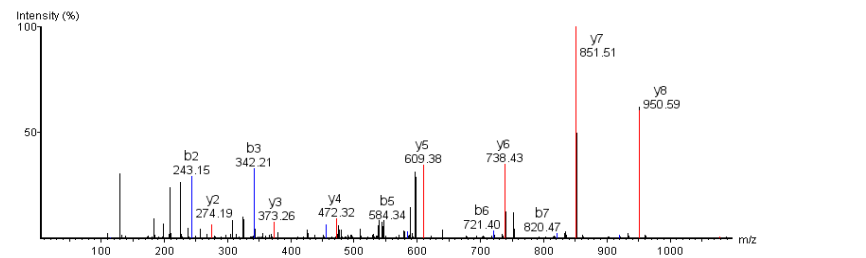
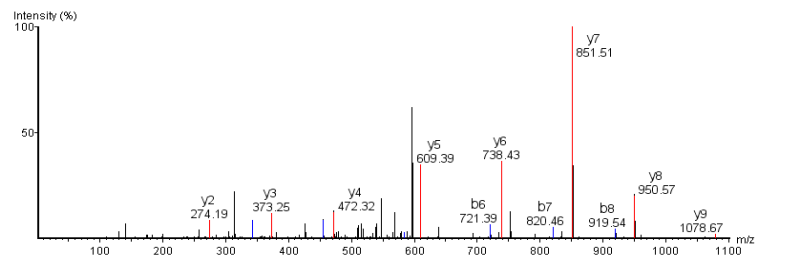
KLLQKVKEV



KLLATLLYL



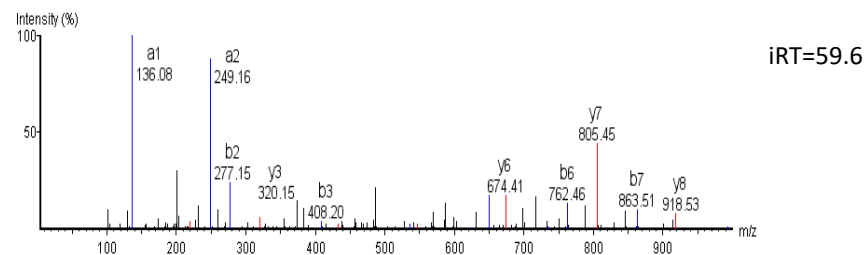
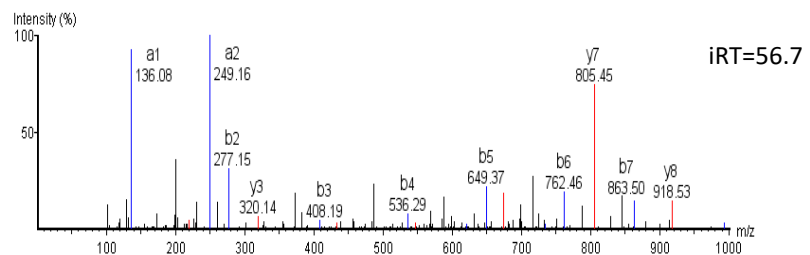
NKLVLEHVV RV



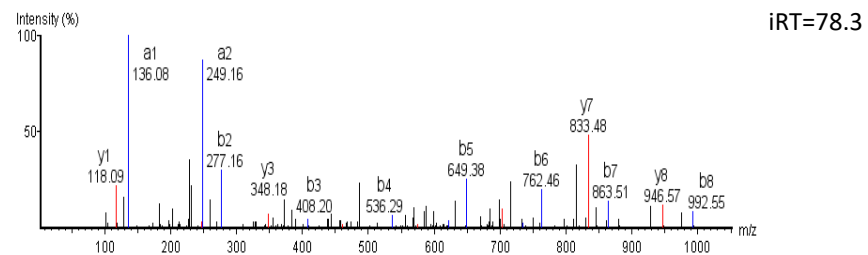
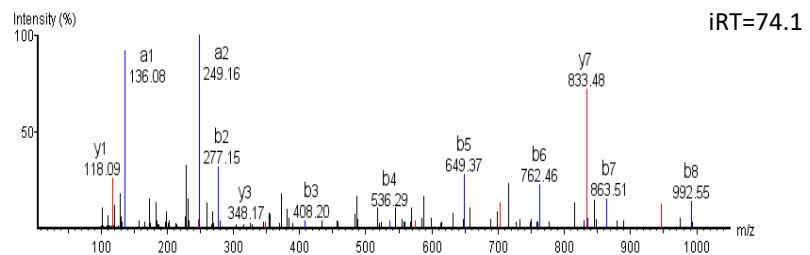
Eluted peptide

Synthetic peptide

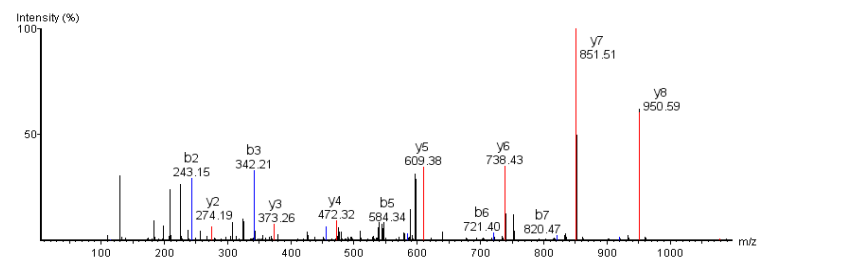
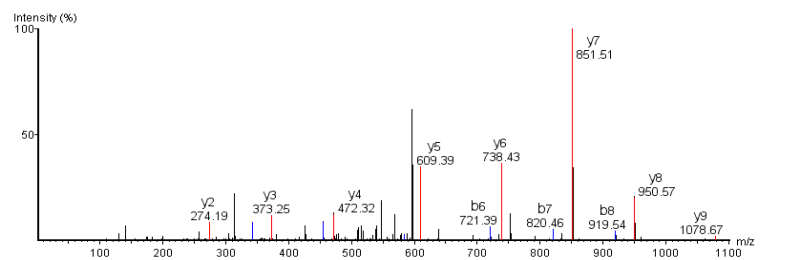
YLMKLLTEA



YLMKLLTEV

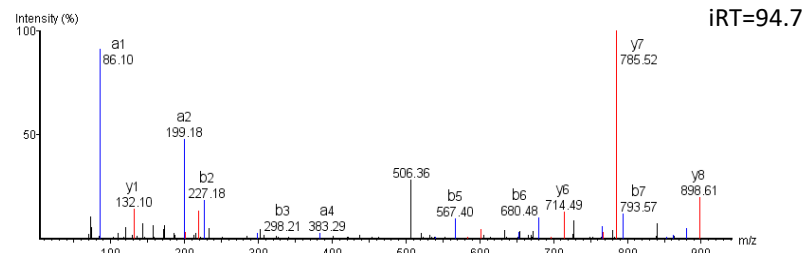


KGVLEHVVRV



Eluted peptide

LLALRLLSL



Synthetic peptide

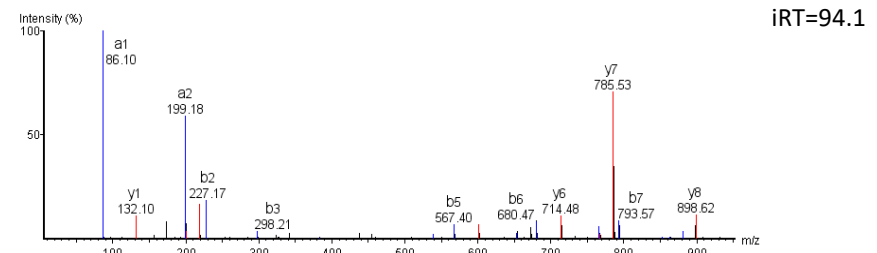


Fig.S9

