

Nicola Ternette¹ and Anthony W. Purcell²

¹ The Jenner Institute, Target Discovery Institute Mass Spectrometry Laboratory, University of Oxford, OX3 7FZ, United Kingdom

² Infection and Immunity Program & Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, Victoria 3800, Australia.

The use of peptides to infer protein expression is very familiar to all of those who undertake bottom-up proteomics studies of cells, tissues and fluids. Remarkably an analogous process is used by the immune system to report on the presence of infectious micro-organisms, malignancy and other abnormalities within the body. This process known as antigen processing and presentation relies on the selection and presentation of peptides on the surface of cells as a bound complex with Major Histocompatibility Complex (MHC) molecules. The isolation and characterisation of these MHC-bound peptides, predominantly through the use of mass spectrometry, has been coined immunopeptidomics and is the subject of this special issue.

Immunopeptidomics as a field has rapidly matured in the past decade and has moved from the domain of just a handful of specialised laboratories to now being more generally applied by the proteomics and immunology communities. The development of immunoproteomics was pioneered by the groups of Don Hunt with Victor Englehard and Alessandro Sette [1-5] and Hans-Georg Rammensee [6-9]. Over the years the protocols developed by these groups have been refined but the basic premise of isolating peptide-MHC complexes under near native conditions and the analysis of the bound peptide cargo primarily by LC-MS/MS remains unchanged. What has changed is the instrumentation and sensitivity of the measurements which have allowed analysis of thousands of peptides using this technique. With such information rich and deep datasets reporting on peptide epitope display by a variety of different cell types and tissues have come profound insights into fundamental mechanisms of antigen presentation (e.g. [2, 4, 10-17]) and how this may be perturbed by various environmental or infectious agents (e.g. [18-20]). The maturation of immunopeptidomics has also facilitated the identification of T cell epitopes in a variety of diseases including autoimmunity (e.g. [21-24]), cancer (e.g. [25-31]) and infectious disease (e.g. [32-35]). It has revealed the critical role that post-translational modification (e.g. [24, 30, 31, 36-40]) has in the immune response and allowed enumeration of specific epitopes with great accuracy and precision (e.g. [41-43]).

In this special issue, we have collected contributions that apply immunopeptidomics in the context of infectious disease [44, 45], cancer [46], autoimmune disease [47] and graft versus host disease [48]. Furthermore, the development of HLA binding prediction and the use of high quality mass spectrometric data is discussed in two review articles [49, 50]. Enrichment strategies prior to MS analysis are compared for suitability of antigen discovery [51] and alternative instrument acquisition strategies to the standard data-dependent acquisition (MS3 [45], PRM [48], EThcD [52] and DIA [53]) are discussed. An important outcome of a recent meeting that focussed on immunopeptidomics and the establishment of the Human Immunopeptidome project [54] are a series of guidelines to support publication of immunopeptidomics data sets. In this issue Lill et al. [55] publish these community driven guidelines on the Minimal Information About an Immuno-Peptidomics Experiment (MIAIPE) which emphasise the requirement for authors to not only report the data and

peptide identities but also the LC conditions and MS acquisition parameters with sufficient accuracy to allow reproduction of studies and understand the quality of deposited data sets. There are two main strategies to obtain high quality HLA ligand data. The most common approach is the enrichment of HLA-ligand complexes using MHC pan- or allele-specific antibodies prior to acid elution of ligands (immunoprecipitation, IP). This approach has been shown to produce highly specific datasets and is further advantageous due to its suitability for the isolation of HLA-peptide complexes from both cells and tissue samples. IP workflows have been applied to both membrane-bound and soluble HLA. For example in this issue, Komov and colleagues [56] examine both membranal and soluble HLA-A*02:01 peptide cargoes and address a fundamental question in antigen processing surrounding the rate limiting steps in the surface expression of HLA-peptide complexes. Here they show that, if soluble HLA-A*02:01 is overexpressed in cells with endogenous HLA-A*02:01 expression, no quantitative alteration was observed on the membrane-bound HLA-A*02:01 peptidome. This suggests that peptide supply is not a limiting factor in antigen presentation and that the peptide pool available for loading of these molecules in the endoplasmic reticulum is supplied in excess.

Direct elution of HLA ligands with acidic buffers from intact cells is also possible to enrich HLA (mild acid elution, MAE) and has the advantage of not requiring the use of large amounts of antibodies. This approach is further valued as it can distinguish membrane-presented from ER-resident HLA ligand presentation, which cannot always be discriminated in IP enriched ligand data. However, these experiments have to be carefully controlled for viability of the cells during the acid stripping of MHC-peptide complexes from the cell surface. Lanoix *et al.* [51] compare IP and MAE workflows for their sensitivity to detect HLA ligand sequences using B-lymphoblastic cell lines B-LCL and B-ALL cells that differ in MHC surface expression density (3E6 vs 5E5 molecules/cell, respectively). The authors demonstrate that up to 6.4-fold more sequences could be identified using IP and conclude that IP is the superior methodology for analysis.

Lanoix *et al.* [51] further report that 0.4% of HLA-presented peptides in the B-lymphoblastic cell lines contain amino acid variants caused by specific nonsynonymous single nucleotide variants (SNV) in these lines. Such peptides are termed minor histocompatibility antigen (MiHAs) in the context of transplantation due to their potential to cause T cell reactivity in a MiHA negative donor (graft versus host disease (GVHD)). The absence of host cross-reactive responses to the allelic counterpart (AC) of the respective MiHA had previously been attributed to the absence of presentation of the AC in the donor. Here, Bijen *et al.* address the question of presentation of MiHA and AC using a targeted MS approach combining SILAC and PRM methodologies [48] for increased sensitivity of MiHA detection. Using three known MiHA antigens, the authors first show that T cell clones that recognize the MiHA antigens are specifically recognizing the variant and not the AC. They further show that the MiHA antigens can bind both host and AC HLA with similar affinities, and that both peptides are presented with similar abundance on the cell surface.

Targeted methods are also used in the report by Blatnik *et al.* [45], who show that the use of prediction methods in combination with a targeted MS3 strategy can support identification of low abundance HLA peptides on the cell surface. The authors chose to analyse a HLA-A2-positive cervical cancer cell line (CaSki) for presentation of peptides originating from two viral oncogenic driver proteins, Human papillomavirus (HPV) E6 and E7. HLA binding prediction for HLA-A*02:01 of both proteins was used to establish a peptide screening library, and this library was further refined using *in vitro* binding assays. The authors report here supporting data for 11 out of the 17 candidate peptides, all originating from E7, to be presented by HLA-A*0201 on CaSki cells.

Such targeted approaches can be extended by DIA acquisition and the use of peptide libraries. This methodology has first been applied to HLA class I immunopeptidomics data by the Purcell and Aebersold laboratories [57, 58]. A first attempt to apply a non-targeted, data independent acquisition approach to HLA class II peptidomes is reported here by Ritz et al. [53] and is applied to studying the peptidomes of both lymphoma cell lines and also of soluble HLA class II molecules found in human serum.

Data for class II ligand analysis is still underrepresented in published datasets and online repositories and is valuable for the training of class II prediction engines. Performance and potential of using MS data for training of predictors is summarised by Alvares *et al.* [50]. Such targeted MS² and MS³ methods, in addition to DIA strategies increase the sensitivity of detection, but cannot perform as discovery experiments, in particular for modified or cryptic antigenic sequences. Ramarathinam et al. identify a naturally presented Kynurenine peptide originating from HIV-1 env that is presented by HLA and suggest that the modified counterpart can elicit specific T cell responses, while the unmodified form is not immunogenic [44].

An important limitation of immunopeptidomics to date is the amount of starting material needed for sufficient enrichment of MHC-peptide complexes for subsequent analysis. In this issue, Olsson et al. [52] compare the immunopeptidome of T cell sub-populations (conventional, regulatory and activated T cell populations) from single donors, advancing the field towards single cell population analyses. Here, spectra are acquired in HCD and EThcD and phosphorylated and cysteinylated peptides were identified from proteins that are central to T cell activation and migration.

Ternette et al. [46] describe common tumour-associated antigens in triple-negative breast cancer cohort that share HLA-A2 expression, as determined by immunohistochemistry. Authors further refine their analysis by determining the average tumour-associated presentation cohort coverage (aTaCC) of proteins, that reflects the coverage of protein antigens presented by HLA in a given analysed cohort and which therefore shortlist the most relevant tumour-associated antigens presented by all patients. The reported proteins with highest aTaCC values are known tumour markers, but are also expressed in healthy tissues and will have to be carefully evaluated for their potential to be used as targets in immunotherapeutic approaches.

Yair-Sabag et al report the important finding that the HLA B27:05 allotype (associated with the autoimmune disease Ankylosing spondylitis (AS)) and HLA B27:09 (not associated with AS development) allow lysine as an anchor residue at position P2, which is most often restricted to Arginine. This is important for the consideration of ligands important in the development of AS [47].

Finally, Faridi et al. [59] suggest approaches to refine shotgun data in order to improve the sensitivity of shotgun experiments by application of hybrid fragmentation to increase coverage, refinement of protein databases by libraries generated from RNAseq data, incorporation of MHC peptide characteristics to database scores, de novo sequences approaches or synthetic peptide libraries. Immunopeptidomics is experiencing exponential growth both in terms of data in online repositories and publications. In the coming years this methodology promises to answer a variety of unknowns in the field of antigen recognition and response.

References:

[1] Hunt, D. F., Michel, H., Dickinson, T. A., Shabanowitz, J., *et al.*, Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-A^d. *Science* 1992, 256, 1817-1820.

- [2] Henderson, R. A. M., H. Sakaguchi, K. Shabaniwitz, J. Appela, E. Hunt, D. F. Engelhard, V. H., HLA-A2.1-Associated Peptides from a Mutant Cell Line: A Second Pathway of Antigen Presentation. *Science* 1992, 1264-1266.
- [3] Hunt, D. F., Henderson, R. A., Shabanowitz, J., Sakaguchi, K., *et al.*, Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* 1992, 255, 1261-1263.
- [4] Sette, A., Ceman, S., Kubo, R. T., Sakaguchi, K., *et al.*, Invariant chain peptides in most HLA-DR molecules of an antigen-processing mutant. *Science* 1992, 258, 1801-1804.
- [5] Henderson, R. A., Cox, A. L., Sakaguchi, K., Appella, E., *et al.*, Direct identification of an endogenous peptide recognized by multiple HLA-A2.1-specific cytotoxic T cells. *Proc Natl Acad Sci U S A* 1993, 90, 10275-10279.
- [6] Rotzschke, O., Falk, K., Deres, K., Schild, H., *et al.*, Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* 1990, 348, 252-254.
- [7] Falk, K., Rötzschke, O., Deres, K., Metzger, J., *et al.*, Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. *The Journal of Experimental Medicine* 1991, 174, 425-434.
- [8] Falk, K., Rötzschke, O., Stevanovic, S., Jung, G., Rammensee, H.-G., Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 1991, 351, 290-296.
- [9] Rammensee, H. G., Falk, K., Rotzschke, O., Peptides naturally presented by MHC class I molecules. *Annu Rev Immunol* 1993, 11, 213-244.
- [10] Delong, T., Wiles, T. A., Baker, R. L., Bradley, B., *et al.*, Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. *Science* 2016, 351, 711-714.
- [11] Liepe, J., Marino, F., Sidney, J., Jeko, A., *et al.*, A large fraction of HLA class I ligands are proteasome-generated spliced peptides. *Science* 2016, 354, 354-358.
- [12] Seamons, A., Sutton, J., Bai, D., Baird, E., *et al.*, Competition between two MHC binding registers in a single peptide processed from myelin basic protein influences tolerance and susceptibility to autoimmunity. *J Exp Med* 2003, 197, 1391-1397.
- [13] Zarlign, A. L., Luckey, C. J., Marto, J. A., White, F. M., *et al.*, Tapasin is a facilitator, not an editor, of class I MHC peptide binding. *J Immunol* 2003, 171, 5287-5295.
- [14] Remesh, S. G., Andreatta, M., Ying, G., Kaever, T., *et al.*, Unconventional Peptide Presentation by Major Histocompatibility Complex (MHC) Class I Allele HLA-A*02:01: BREAKING CONFINEMENT. *The Journal of biological chemistry* 2017, 292, 5262-5270.
- [15] McMurtrey, C., Trolle, T., Sansom, T., Remesh, S. G., *et al.*, Toxoplasma gondii peptide ligands open the gate of the HLA class I binding groove. *eLife* 2016, 5.
- [16] Pymm, P., Illing, P. T., Ramarathnam, S. H., O'Connor, G. M., *et al.*, MHC-I peptides get out of the groove and enable a novel mechanism of HIV-1 escape. *Nature structural & molecular biology* 2017, 24, 387-394.
- [17] Martin-Esteban, A., Guasp, P., Barnea, E., Admon, A., Lopez de Castro, J. A., Functional Interaction of the Ankylosing Spondylitis-Associated Endoplasmic Reticulum Aminopeptidase 2 With the HLA-B*27 Peptidome in Human Cells. *Arthritis & rheumatology (Hoboken, N.J.)* 2016, 68, 2466-2475.
- [18] Illing, P. T., Vivian, J. P., Dudek, N. L., Kostenko, L., *et al.*, Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature* 2012, 486, 554-558.
- [19] Ostrov, D. A., Grant, B. J., Pompeu, Y. A., Sidney, J., *et al.*, Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire. *Proc Natl Acad Sci U S A*. 2012, 109, 9959-9964. doi: 9910.1073/pnas.1207934109. Epub 1207932012 May 1207934129.
- [20] Norcross, M. A., Luo, S., Lu, L., Boyne, M. T., *et al.*, Abacavir induces loading of novel self-peptides into HLA-B*57: 01: an autoimmune model for HLA-associated drug hypersensitivity. *AIDS (London, England)* 2012, 26, F21-29.
- [21] Ooi, J. D., Petersen, J., Tan, Y. H., Huynh, M., *et al.*, Dominant protection from HLA-linked autoimmunity by antigen-specific regulatory T cells. *Nature* 2017, 545, 243-247.
- [22] Wiles, T. A., Delong, T., Baker, R. L., Bradley, B., *et al.*, An insulin-IAPP hybrid peptide is an endogenous antigen for CD4 T cells in the non-obese diabetic mouse. *Journal of autoimmunity* 2017, 78, 11-18.
- [23] Fissolo, N., Haag, S., de Graaf, K. L., Drews, O., *et al.*, Naturally presented peptides on major histocompatibility complex I and II molecules eluted from central nervous system of multiple sclerosis patients. *Molecular & cellular proteomics : MCP* 2009, 8, 2090-2101.
- [24] Molberg, Ø., McAdam, S. N., Körner, R., Quarsten, H., *et al.*, Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nature Medicine* 1998, 4, 713.
- [25] Neidert, M. C., Kowalewski, D. J., Silginer, M., Kapolou, K., *et al.*, The natural HLA ligandome of glioblastoma stem-like cells: antigen discovery for T cell-based immunotherapy. *Acta neuropathologica* 2018, 135, 923-938.

- [26] Loffler, M. W., Kowalewski, D. J., Backert, L., Bernhardt, J., *et al.*, Mapping the HLA ligandome of Colorectal Cancer Reveals an Imprint of Malignant Cell Transformation. *Cancer research* 2018.
- [27] Schuster, H., Peper, J. K., Bosmuller, H. C., Rohle, K., *et al.*, The immunopeptidomic landscape of ovarian carcinomas. *Proc Natl Acad Sci U S A* 2017, *114*, E9942-e9951.
- [28] Bassani-Sternberg, M., Braunlein, E., Klar, R., Engleitner, T., *et al.*, Direct identification of clinically relevant neopeptides presented on native human melanoma tissue by mass spectrometry. *Nature communications* 2016, *7*, 13404.
- [29] Depontieu, F. R., Qian, J., Zarling, A. L., McMiller, T. L., *et al.*, Identification of tumor-associated, MHC class II-restricted phosphopeptides as targets for immunotherapy. *Proc Natl Acad Sci U S A* 2009, *106*, 12073-12078.
- [30] Zarling, A. L., Polefrone, J. M., Evans, A. M., Mikesch, L. M., *et al.*, Identification of class I MHC-associated phosphopeptides as targets for cancer immunotherapy. *Proc Natl Acad Sci U S A* 2006, *103*, 14889-14894.
- [31] Cobbold, M., De La Peña, H., Norris, A., Polefrone, J. M., *et al.*, MHC Class I-Associated Phosphopeptides Are the Targets of Memory-like Immunity in Leukemia. *Science Translational Medicine* 2013, *5*, 203ra125.
- [32] Ternette, N., Yang, H., Partridge, T., Llano, A., *et al.*, Defining the HLA class I-associated viral antigen repertoire from HIV-1-infected human cells. *European journal of immunology* 2016, *46*, 60-69.
- [33] van Els, C. A., Herberts, C. A., van der Heeft, E., Poelen, M. C., *et al.*, A single naturally processed measles virus peptide fully dominates the HLA-A*0201-associated peptide display and is mutated at its anchor position in persistent viral strains. *European journal of immunology* 2000, *30*, 1172-1181.
- [34] Alvarez-Navarro, C., Cragolini, J. J., Dos Santos, H. G., Barnea, E., *et al.*, Novel HLA-B27-restricted epitopes from Chlamydia trachomatis generated upon endogenous processing of bacterial proteins suggest a role of molecular mimicry in reactive arthritis. *The Journal of biological chemistry* 2013, *288*, 25810-25825.
- [35] Meiring, H. D., Soethout, E. C., Poelen, M. C., Mooibroek, D., *et al.*, Stable isotope tagging of epitopes: a highly selective strategy for the identification of major histocompatibility complex class I-associated peptides induced upon viral infection. *Molecular & cellular proteomics : MCP* 2006, *5*, 902-913.
- [36] van Lummel, M., Duinkerken, G., van Veelen, P. A., de Ru, A., *et al.*, Posttranslational modification of HLA-DQ binding islet autoantigens in type 1 diabetes. *Diabetes* 2014, *63*, 237-247.
- [37] Petersen, J., Purcell, A. W., Rossjohn, J., Post-translationally modified T cell epitopes: immune recognition and immunotherapy. *J Mol Med (Berl)* 2009, *87*, 1045-1051.
- [38] Alpizar, A., Marino, F., Ramos-Fernández, A., Lombardía, M., *et al.*, A Molecular Basis for the Presentation of Phosphorylated Peptides by HLA-B Antigens. *Molecular & Cellular Proteomics* 2017, *16*, 181-193.
- [39] Marino, F., Mommen, G. P. M., Jeko, A., Meiring, H. D., *et al.*, Arginine (Di)methylated Human Leukocyte Antigen Class I Peptides Are Favorably Presented by HLA-B*07. *Journal of Proteome Research* 2017, *16*, 34-44.
- [40] Zarling, A. L., Ficarro, S. B., White, F. M., Shabanowitz, J., *et al.*, Phosphorylated peptides are naturally processed and presented by major histocompatibility complex class I molecules in vivo. *J Exp Med* 2000, *192*, 1755-1762.
- [41] Purcell, A. W., Croft, N. P., Tschärke, D. C., Immunology by numbers: quantitation of antigen presentation completes the quantitative milieu of systems immunology! *Current opinion in immunology* 2016, *40*, 88-95.
- [42] Croft, N. P., Smith, S. A., Wong, Y. C., Tan, C. T., *et al.*, Kinetics of Antigen Expression and Epitope Presentation during Virus Infection. *PLoS pathogens* 2013, *9*, e1003129.
- [43] Dudek, N. L., Tan, C. T., Gorasia, D. G., Croft, N. P., *et al.*, Constitutive and inflammatory immunopeptidome of pancreatic beta-cells. *Diabetes* 2012, *61*, 3018-3025.
- [44] Ramarathnam, S. H., Gras, S., Alcantara, S., Yeung, A. W. S., *et al.*, Identification of Native and Posttranslationally Modified HLA-B*57:01-Restricted HIV Envelope Derived Epitopes Using Immunoproteomics. *Proteomics* 2018, e1700253.
- [45] Blatnik, R., Mohan, N., Bonsack, M., Falkenby, L. G., *et al.*, A Targeted LC-MS Strategy for Low-Abundant HLA Class-I-Presented Peptide Detection Identifies Novel Human Papillomavirus T-Cell Epitopes. *Proteomics* 2018, e1700390.
- [46] Ternette, N., Olde Nordkamp, M. J. M., Muller, J., Anderson, A. P., *et al.*, Immunopeptidomic Profiling of HLA-A2-Positive Triple Negative Breast Cancer Identifies Potential Immunotherapy Target Antigens. *Proteomics* 2018, e1700465.
- [47] Yair-Sabag, S., Tedeschi, V., Vitulano, C., Barnea, E., *et al.*, The Peptide Repertoire of HLA-B27 may include Ligands with Lysine at P2 Anchor Position. *Proteomics* 2018, *18*, e1700249.
- [48] Bijen, H. M., Hassan, C., Kester, M. G. D., Janssen, G. M. C., *et al.*, Specific T Cell Responses against Minor Histocompatibility Antigens Cannot Generally Be Explained by Absence of Their Allelic Counterparts on the Cell Surface. *Proteomics* 2017.

- [49] Creech, A. L., Ting, Y. S., Goulding, S. P., Sauld, J. F. K., *et al.*, The Role of Mass Spectrometry and Proteogenomics in the Advancement of HLA Epitope Prediction. *Proteomics* 2018.
- [50] Alvarez, B., Barra, C., Nielsen, M., Andreatta, M., Computational Tools for the Identification and Interpretation of Sequence Motifs in Immuno-peptidomes. *Proteomics* 2018.
- [51] Lanoix, J., Durette, C., Courcelles, M., Cossette, E., *et al.*, Comparison of the MHC I Immuno-peptidome Repertoire of B-Cell Lymphoblasts Using Two Isolation Methods. *Proteomics* 2018, e1700251.
- [52] Olsson, N., Schultz, L. M., Zhang, L., Khodadoust, M. S., *et al.*, T-Cell Immuno-peptidomes Reveal Cell Subtype Surface Markers Derived From Intracellular Proteins. *Proteomics* 2018, e1700410.
- [53] Ritz, D., Sani, E., Debiec, H., Ronco, P., *et al.*, Membranal and Blood-Soluble HLA Class II Peptidome Analyses Using Data-Dependent and Independent Acquisition. *Proteomics* 2018.
- [54] Caron, E., Aebersold, R., Banaei-Esfahani, A., Chong, C., Bassani-Sternberg, M., A Case for a Human Immuno-Peptidome Project Consortium. *Immunity* 2017, 47, 203-208.
- [55] Lill, J. R., van Veelen, P. A., Tenzer, S., Admon, A., *et al.*, Minimal Information About an Immuno-Peptidomics Experiment (MIAIPE). *Proteomics* 2018, e1800110.
- [56] Komov, L., Kadosh, D. M., Barnea, E., Milner, E., *et al.*, Cell Surface MHC Class I Expression is Limited by the Availability of Peptide-Receptive 'Empty' Molecules Rather than by the Supply of Peptide Ligands. *Proteomics* 2018, e1700248.
- [57] Caron, E., Espona, L., Kowalewski, D. J., Schuster, H., *et al.*, An open-source computational and data resource to analyze digital maps of immuno-peptidomes. *eLife* 2015, 4.
- [58] Schittenhelm, R. B., Sivanewaran, S., Lim Kam Sian, T. C., Croft, N. P., Purcell, A. W., Human Leukocyte Antigen (HLA) B27 Allotype-Specific Binding and Candidate Arthritogenic Peptides Revealed through Heuristic Clustering of Data-independent Acquisition Mass Spectrometry (DIA-MS) Data. *Molecular & cellular proteomics : MCP* 2016, 15, 1867-1876.
- [59] Faridi, P., Wayne Purcell, A., Croft, N. P., In Immuno-peptidomics We Need a Sniper Instead of a Shotgun. *Proteomics* 2018.