Mass Spectrometric Characterization of Peptides Associated with Molecules of the Major Histocompatibility Complex

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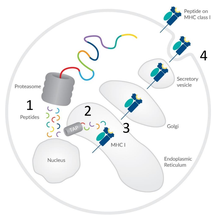
1. Introduction

The major histocompatibility complex (MHC) is a region of highly polymorphic genes encoding for glycoproteins (MHC molecules) that form part of the cell-mediated branch of the acquired immune system. In the cytosol, cellular self and foreign (non-self) proteins are constantly being degraded; it is the peptides generated that are presented, non-covalently bound to MHC molecules, on the surface of cells for inspection by T-lymphocytes. CD8+ cytotoxic T lymphocytes (CTL) recognize peptides presented by MHC Class I, and CD4+ helper T lymphocytes recognize peptides presented by MHC Class II. The recognition of peptide presented by MHC Class I results in the destruction of the presenting cell by the CTL.

Characterizing the factors associated with T cell recognition of peptide/MHC complexes is an attractive proposition for anyone interested in generating tools for targeted cell destruction. In the field of oncology the obvious application is the targeted destruction of cancerous cells. The molecular level characterization of peptides associated with molecules of the major histocompatibility complex requires a targeted protein complex enrichment, an unbiased peptide elution and finally a peptide analysis method. Most frequently immunoprecipitation is used to isolate the target complex followed by peptide elution performed under conditions minimizing protein contamination and finally peptide analysis is accomplished by mass spectrometry.

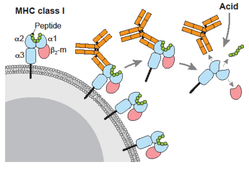
Here we present a case study of our recent work optimizing and performing a workflow for the analysis of peptides associated with Class I MHC molecules. The goal of the assay optimization was to minimize the amount of antibody required for the assay, to minimize the amount of biological material needed from which the complex is isolated and to achieve the optimum sensitivity towards the hitherto unknown target peptides.

2. MHC Peptide Presentation

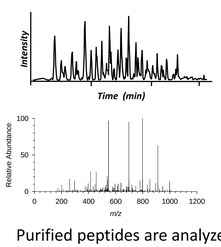


The processing of an antigen within a cell is prerequisite for antigen recognition by T lymphocytes via their T cell receptors (TCRs). Processing describes the sequence of events that takes an intra- or extra-cellular protein in it native state and leads to presentation of proteolytically derived fragments on the cell surface bound to an MHC molecule. The cartoon shows 1) Protein Processing, 2) Peptide transport into the endoplasmic reticulum (ER), 3) Peptide assembly with class I molecules and 4) Peptide presentation on the cell surface.

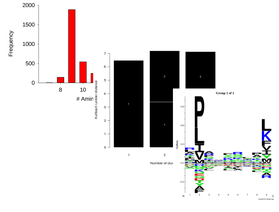
3. Workflow



Cells are lysed and resulting preparations are subjected to immunoaffinity capture. Following multiple washes peptides are eluted from the MHC cleft with acid.



Purified peptides are analyzed by LC-MS/MS using nano-scale chromatography combined with a Fusion Lumos mass spectrometer using electron-transfer/higher-energy collision dissociation (EThcD)[1] fragmentation. MaxQuant[2] is used for peptide identification and quantitation.



Processed data can be further interrogated using bioinformatics tools. For example NetMHC[3,4] or GibbsCluster[5] allow the elucidation of binding strengths and motifs.

4. Materials and Methods

Cell Culture
HCT-116 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1x penicillin/streptocymin. Once the cells reach 80% confluent, they were further passaged 1:5 in Hyperflasks or harvested by Accutase treatment. After washing twice in PBS, harvested cell pellets with defined density were stored at 80°C.

HLA-I Complexes Enrichment
HCT-116 cells were lysed by homogenization with Polytron PT2100 benchtop homogenizer at 4°C with 0.25% Deoxycholic Acid, 1% Octylthioglucoside, 1mM EDTA, 0.2mM Iodoacetamide, Roche HALT in PBS. The lysate was cleared by centrifugation at 20,000 ×*g* for 20 minutes at 4°C. The HLA-I complex was isolated with W6/32 antibody (Cayman, Ann Arbor, MI) bound to Protein A-Sepharose 4B beads (Invitrogen, Carlsbad, CA). SDS-PAGE was employed to confirm crosslinking. The antibody/bead matrix was washed thoroughly prior to use. The HLA-I complex was isolated from the cleared lysate by overnight incubation at 4°C.

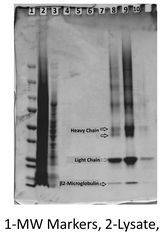
HLA-I Peptides Enrichment
The capture matrix was washed thoroughly with 20mM Tris.HCl, 150mM NaCl and the MHCI complex eluted with multiple additions of 5% acetic acid. HLA-I complex and peptides are visualized by SDS-PAGE following elution. The eluent was transferred to a 3kDa spin filter pre-blocked with angiotensin, and centrifuged at 14,000 x g for 20 minutes. HLA-I complex presented peptides pass through the spin filter membrane while the HLA-I complex is trapped above the membrane.

Liquid Chromatography and Mass Spectrometry
Isolated peptides were desalted by Stage-Tip and analyzed by nano LC-MS/MS with a ThermoFisher nLC-1200 HPLC system interfaced to a ThermoFisher Fusion Lumos operating in EThCD mode. Peptides were loaded on a trapping column and eluted over a 75µm x 50cm analytical column (Thermo Fisher P/N ES-803) at 300nL/min using a 2hr reverse phase gradient; both columns were packed with PepMap RSLC C18, 2 µm resin (Thermo Scientific). The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 60,000 FWHM and 17,500 FWHM for MS and MS/MS respectively. The instrument was run with a 3s cycle for MS and MS/MS.

MS Data Analysis
Data were processed with MaxQuant version 1.5.3.17 (Max-Plank Institute for Biochemistry) operating on a dedicated 64 core server with 128GB of RAM. MaxQuant employs the Andromeda search engine. Data were searched against the UniProt Human reference proteome with no-specific enzyme specificity, and N-terminal acetylation and methionine oxidation as variable modifications. The second peptide identification option in Andromeda was enabled. A false discovery rate of 0.01 was required for peptides, no protein false discovery rate was set.

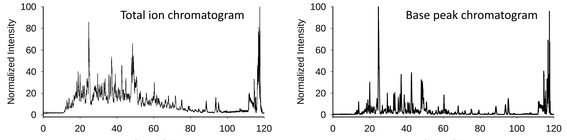
5. Results I

SDS-PAGE



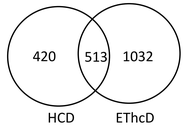
1-MW Markers, 2-Lysate, 3-Flow Thru, 4-7-Washes, 8-Eluate, 9-3kDa MWCO Retentate, 10-3kDa MWCO Filtrate

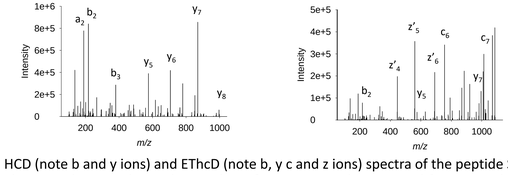
LC-MS/MS



Representative LC-MS/MS chromatograms from 50% load of 500M cell enrichment

HCD versus EThcD

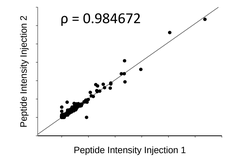


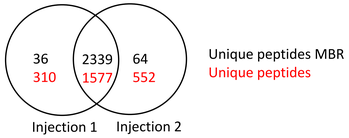


HCD (note b and y ions) and EThcD (note b, y c and z ions) spectra of the peptide SEYMNNKEA
The venn diagram shows the number of unique peptides acquired from a single sample using both approaches.

6. Results II

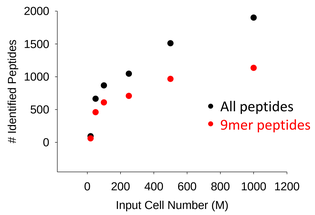
Analytical Replicates

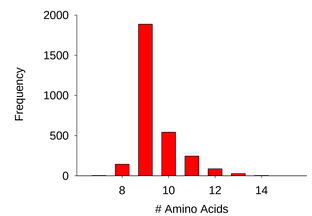




A single sample (1Billion cell input) was injected twice. The data were processed with and without the MaxQuant Match Between Runs (MBR) feature enabled. The Pearson correlation was calculated with the MBR data.

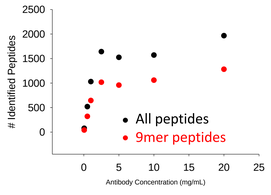
Varying Input Cell Number with a Fixed Antibody Concentration





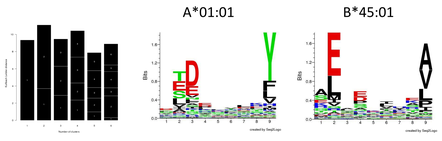
In an effort to better understand the HLA-I peptide yield and to improve practical application of this assay we varied the input amount while holding the antibody concentration constant (20mg/mL). The histogram shows the distribution on peptide lengths in the combined data. The scatter plot includes data for all the observed peptides and exclusively the 9 mers.

Varying Antibody Concentration with a Fixed Input Cell Number



Here we considered the antibody concentration and its impact on the HLA-I peptide yield. The input was 500M cells. Reducing the high antibody concentration associated has the potential to reduce costs and again improve the practical application of this assay. The scatter plot includes data for all the observed peptides and exclusively the 9 mers.

Motif Analysis



Once identified the putative MHC peptide list can be processed to assess binding strength and to extract motif information. These data can be used to confirm or predict HLA allele information. Here data specific to HCT116 cells are presented.

7. References

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3. http://www.cbs.dtu.dk/services/NetMHC/ Andreatta M. *et al.,* Bioinformatics 2016, 32, 511-7 "Gapped sequence alignment using artificial neural networks: application to the MHC class I system."
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5. http://www.cbs.dtu.dk/services/GibbsCluster/ Andreatta M. *et al., Bioinformatics* (2012) doi: 10.1093/bioinformatics/bts621 "Simultaneous alignment and clustering of peptide data using a Gibbs sampling approach"