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A detailed quantification of antigen processing by endosomal compartments provides important information on the pattern of protein fragmentation. Here, we describe a protocol that combines gradient purified endosomes, incubated with antigens, followed by hot spot analysis of MS/MS-sequenced peptides. The analysis identifies differences in endosomal antigen processing by dendritic cells under diverse experimental conditions.
Protocol

A protocol for qualitative and quantitative measurement of endosomal processing using hot spot analysis

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SUMMARY

A detailed quantification of antigen processing by endosomal compartments provides important information on the pattern of protein fragmentation. Here, we describe a protocol that combines gradient purified endosomes, incubated with antigens, followed by hot spot analysis of MS/MS-sequenced peptides. The analysis identifies differences in endosomal antigen processing by dendritic cells under diverse experimental conditions. For complete details on the use and execution of this protocol, please refer to Clement et al. (2021).

BEFORE YOU BEGIN

1. Make sure you have enough stock solutions (described in materials and equipment) for all procedures.
2. Ensure that the ultracentrifuge and water bath are available, and at the right temperature, since once the gradient purification and endosomal digestion start they cannot be paused.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Bovine Serum Albumin solution | Sigma-Aldrich | Catalog # A9576 |
| Protease Inhibitor Cocktail | Roche | Catalog # 04693116001 |
| Sodium Acetate 3 M pH 5.5 | Thermo Fisher | Catalog # AM9740 |
| Micro BCA Protein Assay Kit | Thermo Fisher | Catalog # 23235 |
| HEPES Buffer | Fisher Scientific | Catalog # BP299-100 |
| Acetonitrile Optima™ LC/MS | Fisher Scientific | Catalog # A955-4 |
| Formic Acid Optima™ LC/MS | Fisher Scientific | Catalog # A11710X1-AMP |
| Percoll | Millipore Sigma | Catalog # P1644-100ML |

(Continued on next page)
## MATERIALS AND EQUIPMENT

### Materials

#### 1. Homogenization buffer

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 2.5 M sucrose in HEPES, pH 7.4 | 1× | 5 mL |
| Complete protease inhibitors (Roche) (10×) | 1× | 5 mL |
| EDTA (0.5 M) | 1 mM | 0.1 mL |
| PBS | n/a | 39.9 mL |
| Total | n/a | 50 mL |

**Storage conditions:** on ice for maximum 4 h.
**STEP-BY-STEP METHOD DETAILS**

△ CRITICAL: All solutions, materials, rotors, and equipment are precooled and operated at 0°C–4°C.

Concentrations of substrate protein(s) and late endosomes are titrated to determine the optimal protein concentration to enable more than 90% sequence coverage.

Time of incubation for antigen processing is titrated to determine processing time when the highest number of epitopes are retrieved.

**Mice injection with FLT3-L and dendritic cell preparation**

This methodology has been previously published (Clement et al., 2016, Clement et al. 2021) (Figure 1)
Homogenization of dendritic cells and late endosomal preparation

- **Timing:** 2 h

1. *In vivo* Flt3L-induced splenic murine DC (1–3 × 10⁸), (Clement et al., 2016) are washed three times in 20 mL PBS and pelleted for 10 min at 500 × g.

   The cellular pellet is resuspended in 1 mL PBS containing 0.25 M sucrose, 20 mM HEPES (pH 7.4) supplemented with complete protease inhibitor cocktail (containing 1 mM EDTA).

2. Cells are homogenized in a Dounce homogenizer twice, each time for 10 min, on ice. The homogenate is spun at 150 × g for 10 min, at 4°C in a desktop microcentrifuge after each homogenization step.

3. The pellet contains cellular debris and nuclei: the homogenate is examined under a microscope to ensure complete cellular lysis.

4. The combined supernatants from two rounds of homogenization steps (2 mL) are loaded above 9 mL of 27% Percoll, which is laid over 1 mL of 2.5M sucrose in 20 mM HEPES (pH 7.4) cushion and centrifuged for 1 h at 34,000 × g, in open-top ultracentrifuge polycarbonate tubes at 4°C in a SW Ti41 rotor using a Beckman Optima XPN 100K Ultracentrifuge.

5. The band above the sucrose cushion corresponds to the total lysosomal fraction (Figure 2).

6. The band at the interface, enriched in late and early endosomes, is removed with a P1000 pipette.

7. The collected interface is further separated on a gradient made of 9 mL 10% Percoll laid on top of 1 mL of 2.5 m sucrose in 20 mM HEPES (pH 7.4) cushion and centrifuged for 1 h at 34,000 × g, at 4°C in a SW Ti41 rotor using a Beckman Optima XPN 100k Ultracentrifuge.

8. The late endosomal fraction is collected at the interface between the Percoll and the sucrose layer (Figure 2).

9. The late endosomal fraction is washed three times with 30 mL of PBS containing 0.25 M sucrose and 20 mM HEPES (pH 7.4) for 10 min each time at 3000 × g and 4°C.

10. A small aliquot from the late endosomal fraction is used to determine the total protein concentration using the BCA assay.

The endosomal preparation was originally published by Castellino and Germain (Castellino and Germain, 1995).

In vitro processing of HEL and OVA proteins using purified late endosomes

- **Timing:** 7–13 h

11. Native OVA and HEL (3–5 µg from a 2 mg/mL stock solution) are incubated with late endosomes purified from control C57BL/6J or Ob/Ob mice (0.5–1 µg of total endosomal protein) in 50 mM
sodium acetate buffer pH 5.5 (with 2.5 mm EDTA and 1 mm DTT) for 6 and 12 h at 37°C. The total assay volume is 200 µL.

12. The proteolysis reaction is quenched with 0.5% acetonitrile and 5% formic acid.

13. Processed peptides are extracted through a 10 kDa MWCO (molecular weight cut-off), using 10 kDa centrifugal filter units, by centrifugation in a desktop microcentrifuge at 8000 g for 10 min.

14. Peptides are purified using C18 zip tips and lyophilized using a Speed-Vac microcentrifuge (Clement et al., 2016; Clement et al., 2021)

15. Peptide extracts are reconstituted in 25 µL 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid and separated on a nano-ACQUITY (Waters Corporation, Milford, MA) UPLC with technical triplicate injections.

16. A 3.0 µL injection is loaded in 5% acetonitrile containing 0.1% formic acid at 4.0 µL/min for 4.0 min onto a 100 µm I.D. fused-silica precolumn packed with 2 cm of 5 µm (200 Å) Magic C18AQ (Bruker-Michrom, Auburn, CA).

17. The used solvents are A) water (0.1% formic acid); and B) acetonitrile (0.1% formic acid). A linear gradient is developed from 5% solvent A to 35% solvent B in 90 min.

18. Ions are introduced by positive electrospray ionization via liquid junction into a Q Exactive hybrid mass spectrometer (Thermo12 Fisher Scientific).

19. Mass spectra are acquired over m/z 300–1750 at 70,000 resolution (m/z-200), and data-dependent acquisition (DDA) selected the top 10 most abundant precursor ions in each scan for tandem mass spectrometry by HCD fragmentation using an isolation width of 1.6 Da, collision energy of 27, and a resolution of 17,500.

20. DDA raw files are filtered, de novo sequenced and assigned with protein ID using Peaks 8.5/X/X+ software (Bioinformatics Solutions, Waterloo, Canada), by searching against the Swiss-Prot FASTA database (https://www.uniprot.org), using selected species corresponding to the processed antigens.

21. The following settings are applied for peptide search: “no enzyme”, parent mass tolerance of 15 ppm or lower (10–12 ppm) using monoisotopic mass, and fragment ion mass tolerance of 0.05–0.1 Da (depending on the mass spectrometer used to collect the raw files). Methionine, lysine, proline, arginine, cysteine and asparagine oxidations (+15.99 on CKMNPR), deamidation of asparagine and glutamine (NQ-0.98) and pyro-Glu from glutamine (Q-18.01 N-term) are set as variable modifications at the first round of search.

22. The estimation of false discovery rate (FDR) using decoy-fusion algorithm is enabled during the PEAKS DB search in PEAKS (Figure 3).

23. Data are validated using the false discovery rate (FDR) method built in PEAKS 8.5/X/X+ and protein identifications are further accepted if they can be identified with a confidence score (−10logP)>20 for peptides and (−10logP)>20 for proteins (corresponding to p<0.01); a minimum
of 1 peptide per protein after data are filtered for less than 1.0% FDR for peptides and less than 1.5% FDR for proteins identifications.

24. In addition, selected peptides epitopes with $15 < \log_{10}(C) < 20$ are included in the data set after manual inspection of their tandem mass spectrometry (MS/MS) spectra.

25. An independent validation of the MS/MS-based peptides identifications is performed with Scaffold (version Scaffold_4.6.2 and higher, Proteome Software Inc., USA) using the compatible ‘’mzid’’ files of all samples exported from PEAKS.

26. Peptide identifications are accepted if they can be established at greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction. Protein identifications can be accepted if they can be established at greater than 95.0% probability and contained at least 1 identified peptide (Figure 4).

27. Once the peptide epitopes derived from each antigen processing are identified and validated using the described FDR and statistics tests implemented in PEAKS and/or Scaffold, the built-in label-free quantitation (LFQ) PEAKS Q module is further employed to compare the sequence space and the quantity for the peptidomes derived from different late endosomal processing conditions.

28. A heat map generated in PEAKS’ label-free method is used to contrast the hot spots for antigen processing. The LFQ heat map is based on the relative abundance of all peptide features detected in multiple samples (including the m/z, RT (retention time) and peptide precursor’s MS1 area) (Figures 5A and 5B).

EXPECTED OUTCOMES

Hot spot analysis will allow to visualize the sequence coverage and quantify the copy number for each processed epitope. The technique is very valuable for:

Comparing antigen processing efficiency between/among endosomes purified from different types of antigen presenting cells (APC). For example, comparison between dendritic cells and B cells or macrophages, or comparison with non-professional APC such as fibroblasts and endothelial cells.
Comparing antigen processing efficiency between/among endosomes purified from the same APC under different experimental conditions, to determine whether a particular epitope is generated more or less efficiently.

Quantitative analysis of epitope processing in endosomes harvested from wild type and enzyme knockout mice (for example mice deficient in cathepsin S, F, L, to name few) to determine which enzyme(s) is involved in generating a particular epitope.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The peptides epitopes, generated from late endosomal processing, are quantified using the integrated MS1 areas of the corresponding precursor ions using LFQ analysis in PEAKS. The PEAKS implemented Benjamin–Hochberg method is used to adjust the p value to the false discovery rate (FDR) for all protein that have already passed the other filters related to the quality of MS2 fragmentation and peptide sequence coverage which is extracted from MS/MS sequencing data. A p value of <0.05 is considered statistically significant. Only protein groups with significance scores passing the calculated FDR will be exported from the “Protein” view in PEAKS. When there are replicates in each group of protein (and corresponding processed peptides), ANOVA is implemented in PEAKS to determine the statistical significance for the calculated log10 (peptides abundance) which is direct proportional with the log 10 (MS1 area) of each peptide epitope.

Figure 4. Chromatogram of the endosomal processed peptidomes and related OVA sequence coverage
Base peak intensities and extracted chromatograms for OVA, as processed by B6 (upper panel) and Ob/Ob (lower panel) endosomes. MS/MS derived OVA peptide-sequence coverage is highlighted in yellow and amino acids with identified post-translational modifications are shown in green.
LIMITATIONS

Multiple titration experiments of both LE and protein antigen must be employed to determine the optimum time for antigen incubation with LE at 37°C so that under- or over-digestion processes can be avoided. Such situations would limit the hot spot analysis due to either limited number of generated peptides and thus lower sequence coverage, or to the lack of quantitative data on overlapped processed peptides, respectively.

TROUBLESHOOTING

Problem 1 (step 9)

Antigen over-digestion, which would cleave epitopes into single amino acids. We encountered this problem with low molecular weight proteins with a non-globular structure, such as insulin, which can be easily processed in a very short amount of time.

Potential solution

To avoid epitope overdigestion an experimental time course of processing efficiency is required to determine the optimal incubation time to retrieve the highest number of peptides.

Figure 5. Hot spot analysis of HEL digested peptides

One representative (out of three biological replicates) hot spot analysis of peptide epitopes, and HEL sequence coverage, following processing by late endosomal organelles (LE) purified from (A) B6 and (B) Ob/Ob mice. Highlighted in red are the epitopes which were identified only in the HEL processed by B6 LE and missing from the HEL processed by ObOb LE. One representative Ven diagram reporting unique peptide epitopes from B6 and Ob/Ob LE HEL-processing.
Problem 2 (step 12)
Aggregation of peptides prior to mass spectrometry and MS/MS sequencing. This aggregation and poor resolution of peptides elution on the reversed-phase chromatographic column during nano LC/MS/MS could interfere with the MS/MS during fragmentation using the selected collision energy and fragmentation mode (CID and/or HCD).

Potential solution
Testing of different v/v ratios of formic acid and acetonitrile in the final solubilization solutions before injection into the mass spectrometer. Testing of different amount of peptides (ng to low ug) to be injected in the mass spectrometer since overloading the chromatographic column with peptides could result in poor resolution of the eluted peptides which in turn, could complicate the final MS/MS fragmentation and sequencing.

Problem 3 (steps 9–14)
Presence of residual Sucrose or Percoll solutions that would interfere with both antigen processing and MS/MS analysis of the generated peptides.

Potential solution
If residual Sucrose and Percoll are noted an additional wash can be added following endosomal harvesting.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Laura Santambrogio: las4011@med.cornell.edu

Materials availability
This study did not generate new unique reagents

Data and code availability
The datasets generated during this study are available at Clement et al., 2021.

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AUTHOR CONTRIBUTIONS
C.C.C. and P.P.N. performed the experiments and analyzed the data; L.J.S. and L.S. analyzed the data and wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests

REFERENCES
Castellino, F., and Germain, R.N. (1995). Extensive trafficking of MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. Immunity 2, 73–88.
Clement, C.C., Becerra, A., Yin, L., Zolla, V., Huang, L., Merlin, S., Follenzi, A., Shaffer, S.A., Stern, L.J., and Santambrogio, L. (2016). The Dendritic Cell Major Histocompatibility Complex II (MHC II) Peptidome Derives from a Variety of Processing Pathways and Includes Peptides with a Broad Spectrum of HLA-DM Sensitivity. J. Biol. Chem. 291, 5576–5595.
Clement, C.C., Nanaware, P.P., Yamazaki, T., Negróni, M.P., Ramesh, K., Morozova, K., Thangasswamy, S., Graves, A., Kim, H.J., Li, T.W., et al. (2021). Pleiotropic consequences of metabolic stress on the major histocompatibility complex class II molecule antigen processing and presentation machinery. Immunity 54, 721–736.