Improved identification of allele-specific MHC class II immunopeptidome

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KEY FINDING

In cellulo **biotinylation can be utilized to enrich MHC class II for immunopeptidomic studies.**

INTRODUCTION

Professional antigen presenting cells (APCs) such as dendritic cells, macrophages, and B cells present extracellular antigens to activate CD4$^+$ T cells to elicit an immune response. Distinction of self from non-self begins with uptake of extracellular material by phagocytosis. Phagocytosed antigens derived from pathogens, self proteins, or biologic drugs are digested in the lysosome and the resulting peptides are loaded onto MHC class II molecules. These peptide-MHC complexes (pMHC) are then probed by CD4$^+$ helper T cells, which are activated if they detect their cognate antigen. Activated CD4$^+$ T cells coordinate responses to the "foreign" antigen, including cytokine production, help for B cell antibody production, and stimulation of other immune cells.

In humans, there are three main MHC class II alleles that present peptides, namely HLA-DR, HLA-DQ, and HLA-DP, each of which is composed of two proteins with many potential haplotypes. These MHC molecules present peptides of length around 15 amino acids, although the core of the peptides which bind to the MHC cleft is around 9 amino acids. Each peptide contains two to four anchor residues that form most of the interactions with MHC and dictate which MHC that peptide will bind. These patterns form canonical motifs and can help in the prediction of binding affinity of any given amino acid sequence. However, these prediction tools rely on the availability of large quantities of high-quality experimental data. While peptides presented by HLA-DR have been reasonably well characterized due to the availability of a high-quality immunoprecipitation (IP) antibody to this allele (L243), peptides presented by HLA-DQ and HLA-DP are less well characterized.

Detailed characterization of peptides which bind to MHC class II haplotypes has the potential to aid in the development of therapies for autoimmune disorders in two ways.

1. Better understanding of the antigens driving disease could lead to better therapies.
2. Extending the long-term efficacy of treatments which are administered over a lifetime requires ensuring that biologic drugs used to treat these conditions do not themselves elicit immune responses.

STUDY DESIGN

In this study, we have developed a workflow to improve the experimental identification of allele-specific MHC class II-associated peptides by biotinylating the α chain of HLA-DR in live cells with intact MHC class II machinery. This was accomplished using the HLA-DR-null HL-60 cell line, cotransfecting with BirA, and Avi-tagged MHC expression constructs. Supply of exogenous biotin ensured the specific biotinylation of MHC, which was then enriched from lysates using either streptavidin or HLA-DR-specific antibody. Peptides were eluted from these complexes and identified by mass spectrometry.

This procedure successfully captured all HLA-DR expressed by the cells and peptides identified by the traditional immunoaffinity approach were compared with the biotinylation approach. Use of this workflow will aid in the characterization of peptides presented by specific class II alleles, and further the treatment of class II-associated disease and the development of more successful biologic drugs.

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STUDY DESIGN *(CONTINUED)*

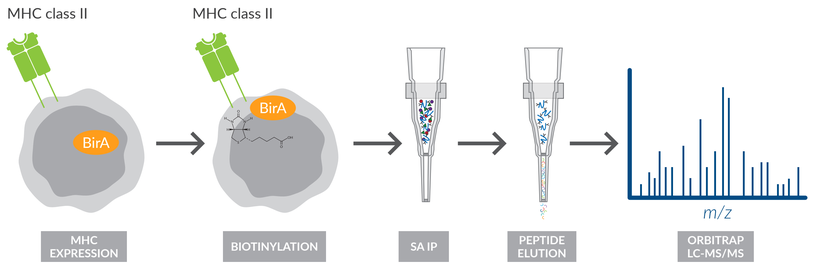

FIGURE 1 – Workflow
HL-60 cells (HLA-DR null) were transfected with HLA-DRA*01:01, DRB1*04:01 with an AviTag peptide, and BirA. Prior to harvest, exogenous biotin was provided for labeling of the target. Cells were lysed and HLA-DR was enriched by either streptavidin or antibody pulldown. Peptides were eluted and sequenced by mass spectrometry.

RESULTS

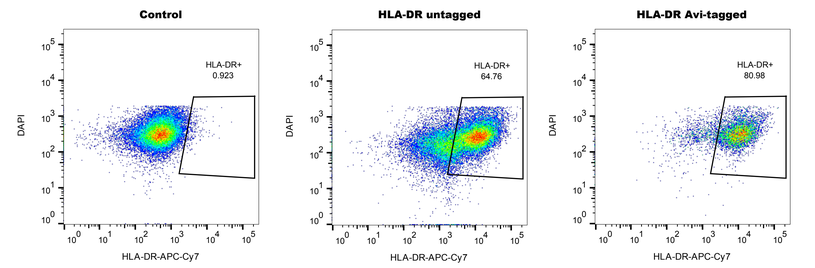

FIGURE 2 – Flow cytometric analysis of transfection.
At day 3 after transfection, cells were stained for HLA-DR using a conjugated L243 antibody. Control transfected cells (left) were used to gate live cells for expression of HLA-DR. Untagged and Avi-Tagged samples expressed high levels of surface HLA-DR. FCS files were analyzed by FlowJo.[1]

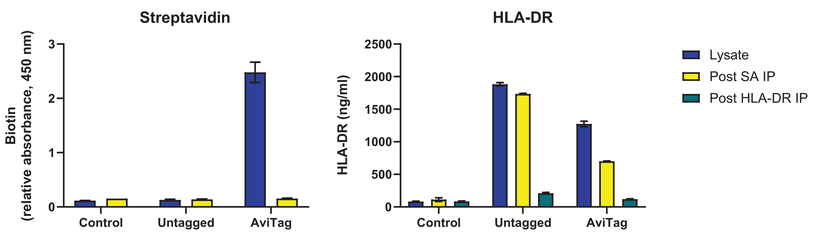

FIGURE 3 – Successful depletion of *in vivo* biotinylated MHC from lysates using streptavidin resin.
HLA-DR null cells (HL-60) were mock transfected (control) or transfected with untagged HLA-DR (α and β chains) or HLA-DR with the AviTag localization sequence (AviTag). After *in vivo* biotinylation, all samples were subjected to serial IP using streptavidin resin followed by HLA-DR antibody-conjugated resin. Biotinylated HLA-DR and total HLA-DR were assayed in lysates and flowthroughs using sandwich ELISA.

TABLE 1 – *In vivo* biotinylated HLA-DR was successfully utilized for identification of presented peptides.
LC-MS/MS was performed on the eluates from each IP, and resulting spectra were searched against the human proteome using PEAKS software.[2] Total peptide numbers identified in each sample are shown, as well as peptides not common with the background sample (Untagged, Streptavidin IP).

Parameter	Sample	Streptavidin IP	HLA-DR IP
Total peptides	Untagged HLA-DR	372	1,753
	AviTag HLA-DR	972	1,131
Non-background peptides	Untagged HLA-DR	0	1,722
	AviTag HLA-DR	748	1,103

RESULTS *(CONTINUED)*

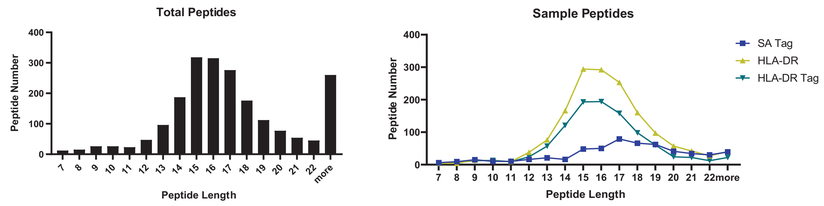

FIGURE 4 – Peptide length histograms.
Peptides identified by mass spectrometry were sorted by length and graphed as a total list (left) and separated by sample (right).

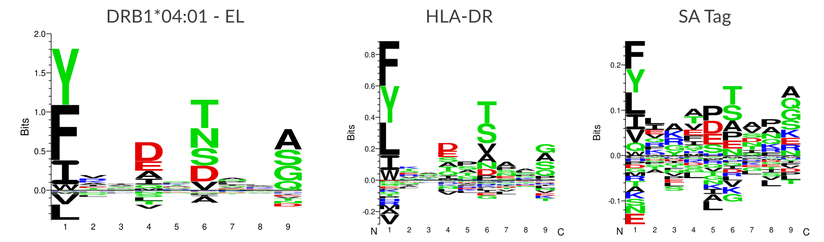

FIGURE 5 – Peptide motif analysis.
Peptides identified by mass spectrometry were submitted to GibbsCluster for unbiased clustering, using MHC class II parameters.[3] The canonical motif for HLA-DRB1*04:01 (EL – eluted ligands) is shown on the left.[4] The peptides from the untagged HLA-DR sample were clustered as shown in the middle and the peptides from the streptavidin pulldown are clustered as shown on the right.

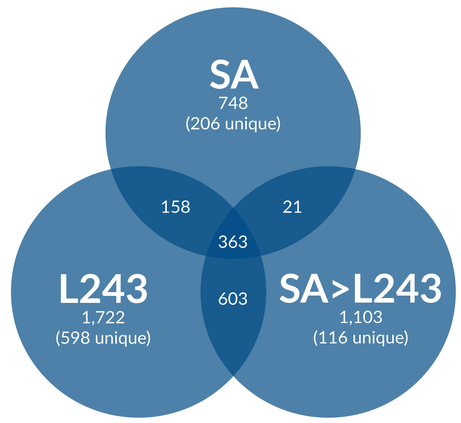

FIGURE 6 – Peptide overlap analysis.
Each peptide identified in this study was evaluated for which samples it was identified in. Total peptide numbers in each sample are indicated, and common peptides were counted.

CONCLUSIONS

1. Transiently coexpressed BirA successfully biotinylated about 50% of HLA-DR complexes containing a single BirA recognition sequence on the C-terminus of the α chain.
2. Biotinylated HLA-DR was depleted from lysates by streptavidin resin.
3. Streptavidin pulldown is compatible with mass spectrometry identification of eluted peptides.
4. A common pulldown workflow enhances the high-throughput capacity of the experimental system, enabling the analysis of multiple HLA alleles or multiple exogenous antigens in a single experiment reliably.

References

Software Tools Used:

1. FlowJo (TreeStar, Inc.)
2. PEAKS (Bioinformatics Solutions Inc.)
3. GibbsCluster (DTU Health Technology: https://services.healthtech.dtu.dk/services/GibbsCluster-2.0/)
4. NetMHCIIpan v4.3 (DTU Health Technology: https://services.healthtech.dtu.dk/services/NetMHCIIpan-4.3/)

Useful Reading:

1. Beckett, D., Kovaleva, E., and Schatz, P.J. A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Sci.* **8(4)**, 921-929 (1999).
2. Röhn, T.A., Reitz, A., Paschen, A., *et al.* A novel strategy for the discovery of MHC class II-restricted tumor antigens: Identification of a melanotransferrin helper T-cell epitope. *Cancer Res.* **65(21)**, 10068-10078 (2005).