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INSIGHTS

Target acquired: Lock-on bacterial immunopeptides

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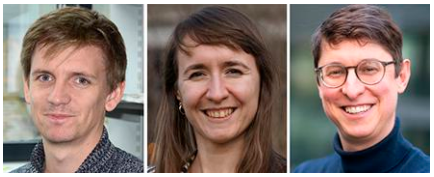
In this issue of *JEM*, Leddy et al. (https://doi.org/10.1084/jem.20250444) present PathMHC, a computationally guided mass spectrometry approach that boosts the detection of pathogen immunopeptides presented on infected cells.

Antimicrobial resistance (AMR) is an emerging global health threat, urging the need for preventive measures to reduce the use of antibiotics (Ho et al., 2025). Vaccines are effective tools to prevent infection and mitigate AMR (Micoli et al., 2021). However, the development of novel vaccines for many complex pathogens is hampered by a lack of antigen knowledge. Antigens capable of priming T cells are often especially unknown, yet T cell responses are essential for immune protection against many AMR pathogens, including *Mycobacterium tuberculosis* (*Mtb*) and other bacteria.

A powerful approach for unbiased discovery of T cell antigens is mass spectrometry (MS)–based immunopeptidomics, a technology that can identify bacterial peptides presented by the major histocompatibility complex (MHC) on the surface of infected cells. Performing such immunopeptidomics analyses on infected and noninfected control cells has facilitated antigen discovery for several bacterial pathogens, including *Mtb* (Leddy et al., 2023), *Chlamydia trachomatis* (Cormican et al., 2025), and *Listeria monocytogenes* (Mayer et al., 2022). While these modern immunopeptidomics workflows routinely identify thousands of immunopeptides in a single experiment, only a few dozen bacterial immunopeptides are typically detected against a background of thousands of host self-peptides.

Here, Leddy et al. (2025) introduce PathMHC, a computational MS approach that guides acquisition toward bacterial immunopeptides by targeted analysis of immunopeptide ions that are unique to pathogen-infected samples but absent in uninfected controls (see figure). Signal-guided acquisition has been used in MS-based proteomics for a long time, often in combination with isotopic labeling where only those peptides with heavy/light ratios exceeding a certain threshold are selected for fragmentation (Zhang and Regnier, 2002). However, such strategy has not yet been applied to immunopeptidomics. In PathMHC, isolated immunopeptides from infected and uninfected samples are first analyzed by a traditional, untargeted proteomics run that selects the most abundant peptide precursor ions for fragmentation. The acquired MS data are then used to align precursor ion signals between infected and uninfected runs based on their retention time, which enables the identification of high-quality peptide ions unique to infected samples that remained undetected in the first run. As these signals might be derived from missed bacterial immunopeptides, the peptide ions are extracted by PathMHC to construct an inclusion list of precursor ions that are actively selected and searched in a subsequent targeted MS run. This strategy thus actively focuses MS instrument time toward infection-only peptide precursors, thereby increasing the likelihood of identifying bacterial immunopeptides.

The authors demonstrate that PathMHC improves detection of bacterial peptides presented on primary human monocyte-derived dendritic cells (hMDCs) for two different mycobacterial species: *Mycobacterium smegmatis* (*Msmeg*) and *Mtb*. For



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Msmeg, a nonpathogenic mycobacterium not adapted to intracellular survival, the authors isolated MHC class II peptides from hMDCs that had phagocytosed the bacteria. Following the initial untargeted MS run, targeted acquisition of infection-only precursor ions by PathMHC almost doubled the number of identified bacterial peptides compared with two untargeted MS runs as a benchmark strategy. Next, PathMHC was applied to hMDCs infected with *Mtb*, a WHO critical AMR bacterium estimated to cause over one million deaths a year. *Mtb* is adapted to an intracellular lifestyle, where it primarily resides in phagosomes, likely restricting the accessibility of *Mtb* proteins to MHC-I antigen processing pathways. Nevertheless, PathMHC identified 19 *Mtb* peptides presented on MHC class I, including four peptides that were undetected in the initial untargeted run. Interestingly, all *Mtb* peptides were derived from type VII secretion system (T7SS) protein substrates, corroborating the authors' previous observations in primary human monocyte–derived macrophages (Leddy et al., 2023). This puts forward T7SS substrates as dominant MHC class I antigens for *Mtb*, raising

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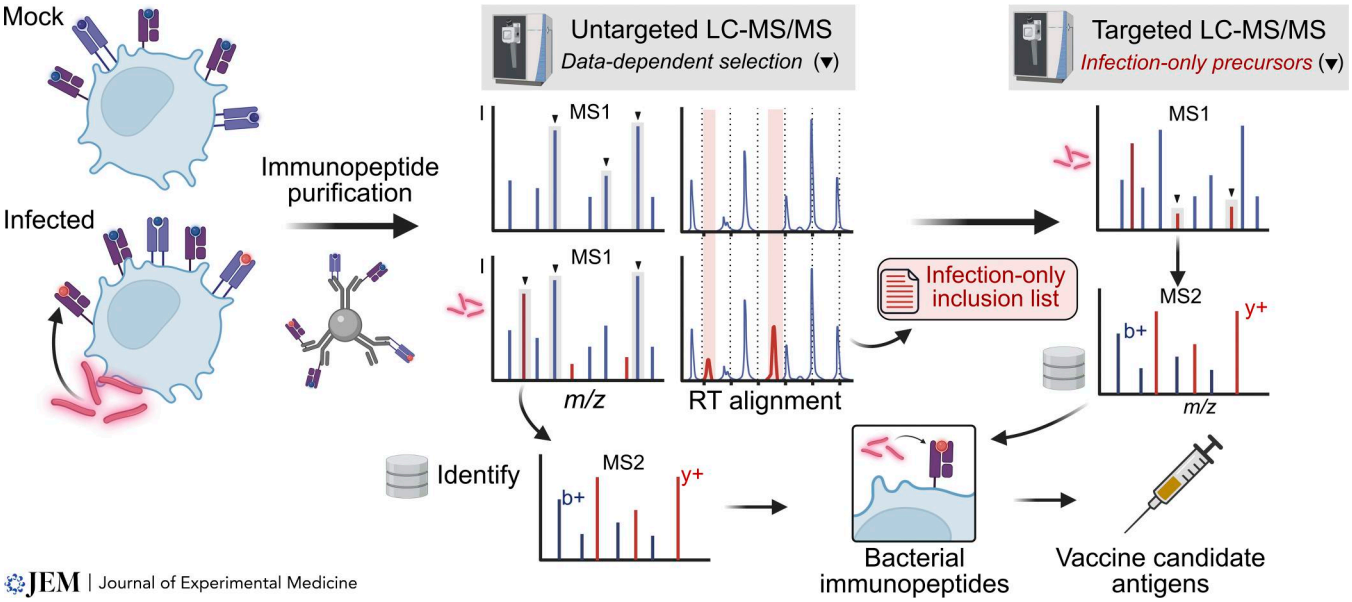

Immunopeptides isolated from infected and uninfected samples are first analyzed by traditional data-dependent acquisition LC-MS/MS. Here, host self-peptides and bacterial immunopeptides can be identified by matching b/y fragment ions of MS/MS (MS2) spectra to a host–pathogen protein database. In parallel, peptide precursor ion (MS1) features are detected, and their retention times are aligned between infected and uninfected runs by DeepRTAlign (Liu et al., 2023). High-quality precursors unique to infected samples that remained unidentified by database searching are then used to define an inclusion list for a subsequent, targeted run on the same samples. This leads to the detection of additional bacterial immunopeptides compared with the initial untargeted run, thereby empowering the discovery of potential vaccine candidates. LC-MS/MS, liquid chromatography–tandem MS. Created in BioRender. Impens, F. (2025) https://BioRender.com/599d8nw.

questions about the lack of presentation of non-T7SS proteins.

The PathMHC workflow has broad applicability. As a demonstration of this, PathMHC also enriched bacterial peptides from published untargeted MS runs of MHC-I immunopeptides isolated from SARS-CoV-2- (Weingarten-Gabbay et al., 2021) and *Listeria monocytogenes*- (Mayer et al., 2022) infected cells. Additionally, the principle of PathMHC could be implemented within diverse proteomic profiling studies where the in-depth discovery of peptides unique to a specific condition is of interest. In addition to bacterial immunopeptides in infection models, this could be a specific disease or treatment context given that appropriate controls are available. Examples include affinity purification MS experiments where specific interaction partners are only present in conditions with bait pull-down, or shotgun MS experiments where proteins of interest are only expressed in one condition. Illustrating this, along with bacterial immunopeptides, PathMHC also identified several interferon-associated host peptides unique to the *Mtb*-infected samples. PathMHC is also adaptable to different software platforms, as demonstrated by the authors using Proteome Discoverer, and to other MS instruments, such as timsTOF devices that can boost bacterial immunopeptide detection (Willems et al., 2025a).

In addition to the targeted approach leveraged by PathMHC, chemical labeling by tandem mass tags (Mayer et al., 2022), multiple search engines (Willems et al., 2025a), spectral rescoring (Willems et al., 2025a; Cormican et al., 2025), and data-independent acquisition (Willems et al., 2025b, *Preprint*) have been used to boost the detection of bacterial epitopes by immunopeptidomics. PathMHC is a valuable addition to this expanding toolbox of immunopeptidomics strategies for improved detection of pathogen antigens. Importantly, these antigens can be used in downstream vaccine development (Leddy et al., 2021; Mayer and Impens, 2021), for instance, by encoding highly presented antigens in mRNA lipid nanoparticle vaccines. Recently, such immunopeptidomics-based mRNA vaccines were shown to mount protective immune responses in mice against *Listeria monocytogenes* (Mayer et al., 2022) and the protozoan parasite *Trypanosoma cruzi* (Versteeg et al., 2024). As such, advances in bacterial immunopeptidomics can fast-forward rational next-generation vaccine development.

In this context, Leddy et al.'s immunopeptidomics results for *Mtb* can inform tuberculosis (TB) vaccine development. Despite the prevalence of this deadly infection, there is currently no effective vaccine for adults. The authors' finding that *Mtb*-derived immunopeptides were exclusively T7SS substrates suggests that these proteins may be promising vaccine targets for TB. In line with this, the only modern TB vaccine with demonstrated efficacy in clinical trials, M72/AS01E, contains the T7SS substrate PPE18 as one of the two protein antigens in the formulation. However, another protein subunit vaccine, H56:IC31, which contains the T7SS substrate EsxA, failed to prevent recurrent TB (Borges et al., 2025). As such, further investigation is needed to determine which characteristics of T7SS substrates and which vaccine formulation parameters may be important for eliciting protection against TB.

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