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Review

Immunopeptidomics for next-generation bacterial vaccine development

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Antimicrobial resistance is an increasing global threat and alternative treatments substituting failing antibiotics are urgently needed. Vaccines are recognized as highly effective tools to mitigate antimicrobial resistance; however, the selection of bacterial antigens as vaccine candidates remains challenging. In recent years, advances in mass spectrometry-based proteomics have led to the development of so-called immunopeptidomics approaches that allow the untargeted discovery of bacterial epitopes that are presented on the surface of infected cells. Especially for intracellular bacterial pathogens, immunopeptidomics holds great promise to uncover antigens that can be encoded in viral vector- or nucleic acid-based vaccines. This review provides an overview of immunopeptidomics studies on intracellular bacterial pathogens and considers future directions and challenges in advancing towards next-generation vaccines.

Highlights

Mass spectrometry-based immunopeptidomics screens allow the identification of bacterial antigens presented on infected cells.

These antigens can easily be encoded in next-generation nucleic acid-based vaccines as novel tools to tackle increasing antimicrobial resistance.

Mainly infection models with *Mycobacterium* and *Chlamydia* have been screened so far, but recent advances in both immunopeptidomics and mRNA vaccine technology prelude an increase in screens on intracellular bacterial infection models.

A need for next-generation antibacterial vaccines

With the advent of antibiotics in the first half of the 20th century, many bacterial diseases lost their devastating dominance over humankind. Commonplace outbreaks of the plague, cholera, and many other bacterial diseases claimed a death toll in the hundreds of millions. A combination of improved sanitary standards, elevated living conditions, and the availability of antibiotics massively reduced these outbreaks; however, (over)use of antibiotics has accelerated the emergence of antimicrobial resistance (AMR). Annual AMR-related deaths are predicted to skyrocket from 700 000 in 2019 to 10 million globally in 2050 [1]. Hence, the World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC) have identified pathogenic bacteria for which the AMR situation is particularly dire, including several intracellular pathogens such as *Salmonella, Shigella* spp. and *Mycobacterium tuberculosis* (Mtb) [2,3]. In addition to stringent antibiotic stewardship and fast development of novel antibacterial drugs by initiatives like the AMR Action Fund [4], vaccines are regarded as highly effective tools to mitigate resistance (recently reviewed in [5]). The prophylactic use of bacterial vaccines prevents infections, thereby reducing the need for antibiotic prescription and minimizing selective drug pressure [5]. In contrast to antibiotics, antibacterial vaccines remain effective against their target pathogen over time. Furthermore, high vaccination rates create a herd immunity that protects susceptible individuals who cannot be effectively vaccinated, like the elderly, immunosuppressed, or chronically sick people [5].

Several antibacterial vaccines have been utilized successfully in the past and are routinely used nowadays, typically providing a high degree of immunity and safety. These include vaccines against tetanus, diphtheria, and pertussis, against *Haemophilus influenzae* type B (Hib), pneumococcus, as well as meningococcus [6,7]. There are also vaccines against typhoid fever, anthrax, and cholera that are administered in, and for those traveling to, endemic areas. In contrast, for tuberculosis (TB) as a major global health burden, the only readily available and inexpensive vaccine, Bacillus Calmette–Guérin (BCG), does not offer efficient and reliable protection against all forms of the disease [8–10]. Efforts to bolster the protection afforded by BCG – by using it in combination with MVA85A (modified vaccinia Ankara 85A) – have not yielded significant improvements [11]. Most

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of the aforementioned vaccines target extracellular bacteria that replicate outside host cells, while only the tuberculosis and typhoid fever vaccines target intracellular pathogens. Being protected from the humoral, and parts of the cellular immune response, as well as several antibiotics inside host cells, intracellular bacteria often present a particular clinical challenge rendering vaccine development even more crucial [12]. Many intracellular pathogens are also capable of persisting in host cells by entering a dormant state such as Mtb, *Salmonella*, *Bartonella*, uropathogenic *Escherichia coli* or *Brucella* [13].

Many recent antibacterial vaccines, like the ones against Hib, meningococcus, and streptococcus, are conjugate vaccines with bacterial polysaccharides bound to carrier proteins. Conjugate vaccines achieve high protective efficacy, even in young children, but suffer from relatively long development times and are mainly targeted against extracellular bacteria [5,7,14]. For intracellular pathogens, the development of effective vaccines has long been held back by a lack of antigen knowledge along with the inability of most vaccine platforms to elicit strong cytotoxic responses. The latter can now be overcome by next-generation vaccines, including viral vector, DNA, and mRNA vaccines that induce both humoral and cytotoxic immune responses and allow relatively fast development times (Box 1, and reviewed in detail in [15–17]). All three approaches introduce the genetic information encoding the actual antigen into host cells for intracellular antigen synthesis, resulting in elevated cytotoxic immune responses.

The question remains, though, of which bacterial antigen(s) to encode into these vaccine platforms. In contrast to viruses, the potential antigen palette for pathogenic bacteria is significantly more diverse, with typically several thousand genes per bacterium rendering the choice of the correct antigen a challenging task. This is illustrated by the limited coverage of most intracellular bacterial pathogens in the Immune Epitope Database (IEDB, www.iedb.org) as depicted in Figure 1 [28].

Immunopeptidomics, a powerful approach for antigen discovery

Vaccine development has come a long way since Pasteur postulated his classical vaccinology approach of isolating, inactivating, and injecting disease-causing agents to protect patients. Reverse vaccinology, as spearheaded by Rino Rappuoli in 2000, utilizes full pathogen genomes

Box 1. Next-generation nucleic acid-based vaccines

Viral vectors (such as adenoviral vectors) encode the antigen(s) to be delivered, which are typically surface-exposed antigens such as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein, within a nonpathogenic viral vehicle. While viral vectors are a flexible platform, allowing the rapid adaption to new antigen targets of emerging pathogens, they might suffer from pre-existing immunity against the vector, neutralizing the vaccine before cellular uptake. This is a particular problem for booster vaccinations using the same viral vector and also the relatively costly cell-based production of viral vectors present a challenge [17]. In the case of DNA vaccines, the antigen-encoding DNA is introduced directly or lipid-encapsulated into host cells. Advantages of DNA vaccines include high stability and absence of pre-existing immunity, while reduced expression efficiency has been an issue due to the need of nuclear import before transcription into antigen-encoding mRNA and nuclear export prior to antigen translation [16]. In contrast, mRNA-based vaccines introduce the antigenic information via mRNA to omit nuclear import, allowing direct antigen translation, and like DNA vaccines, they are not subject to pre-existing immune defenses. While it has been known since the nineties that exogenous transcribed mRNA can be used to express proteins *in vivo* [18], only in recent years has mRNA emerged as a promising vaccination platform technology. *In vitro* transcribed (IVT) mRNA showed its utility as a vaccine format to promote prophylactic protection against infections with viruses such as Zika [19] and influenza [20]. A number of modifications to the vector used to produce the mRNA, as well as to the synthetic mRNA itself, have further ameliorated the biologic properties of the IVT mRNA [21]. In the past year, the SARS-CoV-2 pandemic greatly accelerated the clinical entry of next-generation vaccines, especially mRNA vaccines conferring over 90% protection against coronavirus disease 2019 (Covid-19) [22,23] This has proven that mRNA-based vaccines have evolved to safe, well-tolerated and very effective vaccines against viral infections. However, their potential in the context of intracellular bacterial infections remains largely unexplored [24–26]. mRNA vaccines therefore represent a promising alternative to conventional vaccines based on their high potency, capacity for short development time, as well as their potential for low-cost manufacture and safe administration [27].

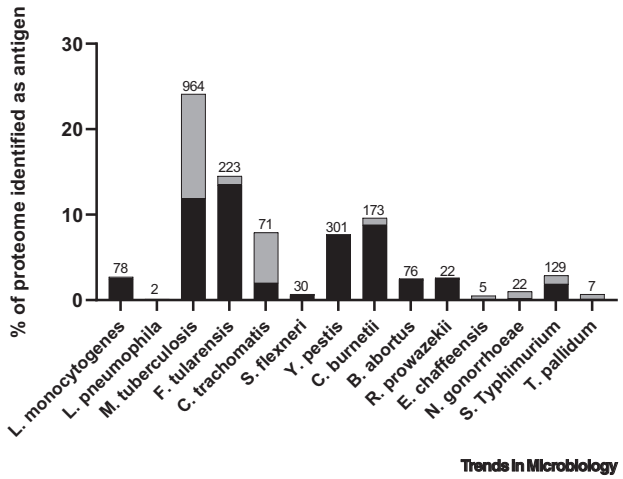

Figure 1. Antigenic coverage for intracellular bacterial pathogens. The number of protein antigens for a selection of intracellular bacterial pathogens is displayed as a fraction of each pathogen's total proteome. Black parts of the bars indicate MHC class I-presented antigens, while in gray all non-MHC class I-presented antigens are depicted. Numbers above the bars indicate the total antigen count per pathogen retrieved from the Immune Epitope Database (IEDB) on February 22, 2021 [28]. While, for Mtb, more than 20% of its protein-coding genes have been identified as antigen, for most other bacteria less than 5% of their proteins were reported as antigen. Moreover, only a subset of these antigens gives rise to MHC class I-restricted epitopes and might elicit cytotoxic immune responses. While the number of immunodominant antigens is likely much smaller than the total protein repertoire, it can be assumed that the list of bacterial antigens discovered so far is far from complete and that many antigens still await their discovery as vaccine candidate.

to infer protein sequences followed by *in silico* predictions to identify secreted and exposed surface proteins as suitable vaccine candidates [29]. Recombinantly expressed in *E. coli* or other systems, the candidate antigens' immunogenicity is evaluated in animal models prior to clinical studies. This way, a serogroup B meningococcal vaccine, as well as vaccine candidates against group B streptococcal infection, *Chlamydia*, antibiotic-resistant *Staphylococcus aureus*, and *Streptococcus pneumoniae* were successfully developed [30]. Building on the initial reverse vaccinology platform, reverse vaccinology 2.0 was gradually implemented in the past decade harnessing contemporary platforms like B cell repertoire deep sequencing, structure-based antigen design, and mass spectrometry-based proteomics [31].

Immunoproteomics, as a collection of techniques that readily allow the detection of antigenic peptides or proteins, has benefitted greatly from recent quantum leaps in mass spectrometry (MS) and data analysis [32]. Methods for investigating extracellular antigens include variants of immunocapture MS for circulating immune complexes (CICs), electroimmunoprecipitation of antibody–antigen complexes, and multiple affinity protein profiling (MAPPing) [32]. Also, serological proteome analysis (SERPA) as well as array-based approaches have been utilized for antigen analysis [32]. All of these methods, however, require antibody secretion and can therefore only detect targets of the humoral immune response. Antibody-independent detection of antigens can be realized with another type of immunoproteomic methodology known as immunopeptidomics.

Immunopeptidomics aims to detect antigens as peptides that are presented on cell surfaces via major histocompatibility complexes (MHCs), commonly termed immunopeptides, MHC-associated peptides, or MHC ligands. These immunopeptides are antigen fragments of 8–25 amino acids that are loaded intracellularly onto MHC complexes before transportation to the cell surface for T cell inspection. The two classes of MHC molecules, MHC class I and II, facilitate presentation of antigens from cytosolic and vacuolar/extracellular antigens, respectively, and they bind different T cell populations with distinct functionalities. While MHC II expression is restricted mainly to professional antigen-presenting cells (APCs), MHC I is present on all nucleated cells and binds CD8 cytotoxic T cells to initiate cell death in compromised cells. The resulting cytotoxic

immunity is particularly relevant for many intracellular pathogens and is therefore specifically spotlighted in this review (Box 2).

Following isolation and purification, MHC-presented peptides can be identified by MS. Technological quantum leaps over the past 30 years have allowed the field to mature, and identification of thousands of epitopes per study is now commonly achieved. In 1992 the laboratory of Donald Hunt successfully identified the first HLA peptide ligands from HLA allele A*0201 by liquid chromatography–tandem mass spectrometry (LC-MS/MS) [51]. Since this first study, HLA-bound peptide elution and detection by LC-MS/MS has remained the method of choice for measuring antigenic peptides. Isolation of immunopeptides can be achieved by two methods. Firstly, mild acid elution (MAE) directly from the surface of intact cells allows fast and simple immunopeptide harvest but suffers from contaminant peptide coelution and inseparability of MHC class I and II peptides [52]. Secondly, immunoprecipitation (IP) of intact MHC–peptide complexes after cell lysis requires large amounts of specific antibodies and elevated sample preparation times, while delivering improved immunopeptide enrichment and increased numbers of immunopeptide identifications [52]. Furthermore, IP allows separation of MHC class I and II peptides, and is compatible with frozen samples, while MAE is not. Unsurprisingly, IP is therefore applied for most immunopeptidomics studies (Figure 2).

The major limitation for immunopeptidomics is the large amount of starting material required, typically around several hundred million cells, or 1 g of tissue, since the abundance of immunopeptides is usually extremely low [38,53]. Hence, sample processing steps and surface exposure are to be minimized. A detailed protocol on immunopeptide isolation has been described elsewhere [53]. After isolation, immunopeptides are analyzed by LC-MS/MS, utilizing more sophisticated workflows than standard proteomics by implementation of special derivatization,

Box 2. Cellular immunity based on MHC presentation

The MHC class I pathway features proteasomal digestion of cytosolic intracellular antigens with optional further trimming by other proteases, such as endoplasmic reticulum aminopeptidase 1 and 2 (ERAP1 and 2), to a peptide length of around 8–12 amino acids, enabling loading onto MHC I complexes in the endoplasmic reticulum (ER). Introduction into the ER occurs via the transporter associated with antigen processing (TAP) before binding to MHC class I molecules and transportation of the entire complex to the cell surface via the Golgi apparatus for CD8 T cell surveillance and elimination of pathogen-infected or malignant cells. In healthy cells, class I peptides result from defective ribosomal products (DRiPs) or retiree proteins favoring abundant, short-lived proteins [33]. While standard proteasomes facilitate continuous monitoring of correct protein synthesis, immunoproteasomes occur in immune cells or upon immune stimulation. Primarily responsible for intracellular microbial protein digestion, immunoproteasomes localize to the ER and utilize altered peptide-cleavage properties, creating a distinct immunopeptidome enhancing efficient pathogen-derived antigen presentation via MHC class I [34–40]. Besides proteolysis, the proteasome might also concatenate distant protein fragments by proteasome-catalyzed peptide splicing (PCPS) forming novel, antigenic peptide sequences [41]. The abundance of spliced peptides is currently debated to be between 0.1 and 34% of all presented immunopeptides [42–44] and has been recently reviewed in [45]. Importantly, T cell responses to several intracellular bacteria-derived spliced peptides have been demonstrated [46,47]. In infection, PCPS could benefit immunity by circumventing minor antigen mutations providing stable epitopes for persistent immune responses [48].

Human MHC molecules are termed human leukocyte antigens (HLAs) and both MHC I and MHC II are each divided into classical and nonclassical HLAs. The three classical MHC I genes (HLA-A, HLA-B, HLA-C) are codominantly expressed with thousands of diverse alleles using variable peptide-binding specificities facilitating presentation of a broad antigen spectrum. Allele frequencies vary greatly, with a dozen alleles covering the majority of most local, homogeneous populations, while different ethnic groups and distant geographical regions present with diverging dominant alleles, having substantial implications for antigen prioritization and vaccine development [49].

In contrast to MHC I, MHC class II binding peptides are longer, with around 15–24 amino acids, and result from exogenous antigens endocytosed by APCs followed by subsequent endolysosomal degradation [50]. Similar to MHC I, there are three classical, highly polymorphic MHC II genes (HLA-DP, HLA-DQ, HLA-DR) creating a plethora of possible combinations of alleles.

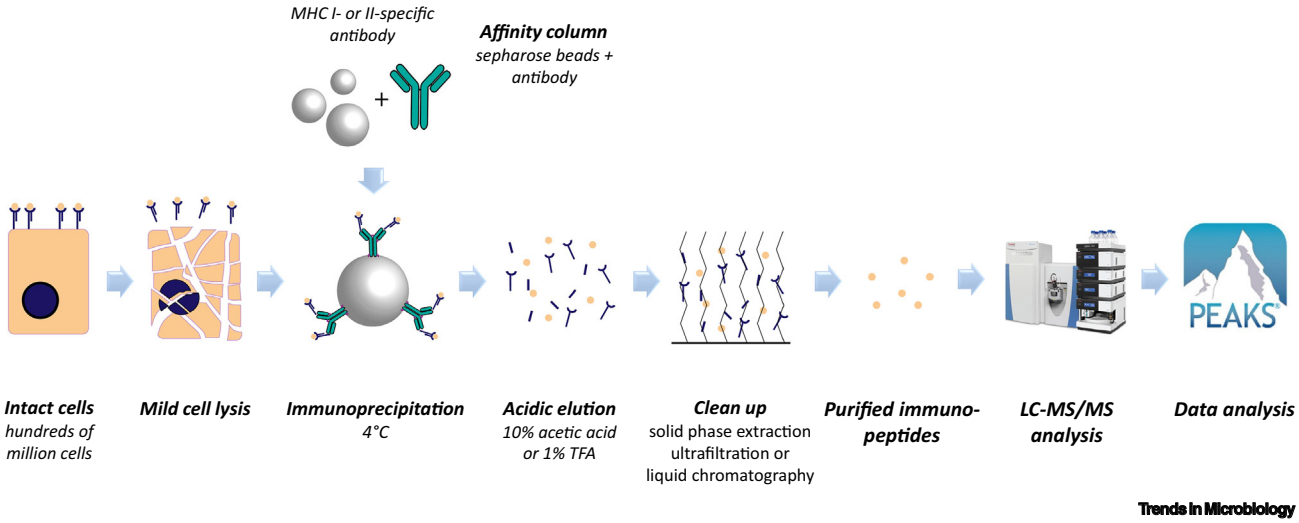

Figure 2. IP-based immunopeptidomics workflow. Cells or tissues are lysed by mild detergents in combination with sheer stress [53] and lysates are cleared and sterile-filtered to remove residual debris and/or pathogens. Cleared lysates containing intact MHC–peptide complexes are incubated with immunoaffinity columns. For human samples, the high-quality pan-class I antibody from the W6/32 hybridoma cell line is commonly used, while for all classical MHC II alleles (HLA DR, DQ, and DP) the IVA12 hybridoma-derived antibody is available. The amount of antibody required for 1E9 cells is in the mg range, posing a significant cost factor. The MHC-specific antibody is typically covalently cross-linked to protein A- or G-coated beads to avoid antibody coelution and sample contamination. Following several washes, MHC–peptide complexes are eluted from the immunoaffinity columns by acidic elution. Eluates are further purified to remove MHC molecules by liquid chromatography, ultrafiltration, or C18-based solid-phase extraction to separate short-chain hydrophilic immunopeptides from longer hydrophobic MHC molecules. For liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of purified immunopeptides, highly sensitive, high-resolution instruments and customized parameters are imperative for a successful analysis. Data analysis of the raw data can be carried out by classical proteomics software packages such as MaxQuant or more sophisticated solutions like PEAKS studio [53]. Abbreviation: TFA, trifluoroacetic acid.

fragmentation, and identification strategies, particularly for mapping immunopeptide post-translational modifications (PTMs) (Box 3).

Untargeted identification of novel bacterial antigens

Immunopeptidomics is extensively applied for tumor-associated antigen discovery, but its use in infection models is rather limited [69–74]. Next to several studies on viral and parasitic pathogens, bacterial infection models are historically understudied by immunopeptidomics. Recently however, more studies on bacterial infection models are being conducted in light of AMR emergence.

Mycobacterium tuberculosis

The first immunopeptidomics study on bacterially infected cells was reported in 2002 by the Hunt laboratory, identifying antigenic peptides from Mtb strain H37Ra [75]. From one billion infected human U937 monocyte cells expressing MHC class I HLA-A*02:01, three nested peptides from the Mtb isoniazid inducible gene protein iniB (Rv0341) were identified ($Rv0341_{33-42}$, $Rv0341_{33-44}$, and $Rv0341_{33-45}$) next to one peptide from heat-shock protein hsp65 and one peptide from galactofuranosyl transferase glfT2. The identity of the three nested peptides was confirmed by LC-MS. MHC I binding affinity, as well as immunogenicity, was confirmed by CD8 T cell-induced lysis of peptide-presenting and Mtb-infected cells.

A decade later, Cayabyab and colleagues infected C57BL/6 mice with Mtb strain H37Rv identifying MHC I peptides from splenic adherent cells [76]. From ~500 identified immunopeptides, the authors sequenced 4 peptides originating from Mtb, of which one peptide was specific for Mtb and not present in other bacteria. The authors encoded the parent protein of this peptide,

Box 3. Advanced LC-MS/MS and data analysis pipelines for immunopeptides

For immunopeptidomics, the LC-MS/MS setup needs to be well maintained and suited for identification of ultra-low abundant analytes. Nano-flow liquid chromatography (nanoLC), as one of the most sensitive separation technologies, is typically coupled to a high-resolution mass spectrometer enabling immunopeptide separation for complexity reduction before the peptide measurement. Therefore, high-performance separation columns, using sub-2 µm chromatographic beads or micropillar arrays, are ideal for peptide separation [54]. Immunopeptides are usually analyzed by LC-MS/MS in their native state, termed label-free; however, chemical derivatization and isotopic labeling were recently found to improve detectability of these short, nontryptic peptides by promoting positive charges on peptides, which are essential for successful peptide ionization during LC-MS/MS analysis [55–57]. Utilizing these techniques, efforts are ongoing to further reduce the required sample input by peptide amplification strategies [58]. MS fragmentation methods to infer immunopeptide sequence information include collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD), but also sophisticated techniques like EThcD combining electron transfer dissociation (ETD) with HCD to generate more diverse fragment ions and to improve identification rates and detection of post-translationally modified peptides [59,60]. Peptides carrying PTMs, such as phosphorylation, O-linked glycosylation, acetylation, formylation, N-myristoylation, citrullination, arginine (di)methylation, cysteinylation, and deamidation, are known to be presented via MHC class I, potentially altering peptide–MHC interactions and CD8 T cell responses [61–64]. Identification of modified immunopeptides poses a particular challenge due to their low abundance and instability. Hence, specific enrichment methodologies and enzyme inhibitors are used during lysis to prevent PTM degradation [65]. We are now only starting to understand the impact of PTMs on antigen presentation, an important subject for future immunopeptidomics studies, especially in the context of infection, since PTMs are known to play key roles in host–pathogen interactions [66]. Data analysis of MS raw data is the crucial final step of immunopeptide identification, and algorithms to do so have improved substantially in recent years. Due to the extremely variable cleavage options for proteasome-generated immunopeptides, the number of theoretical peptides for presentation is extremely high, hampering sensitive and accurate matching between mass spectra and peptide sequences using standard proteomics software. Current sophisticated approaches to circumvent this problem utilize MHC sequence motif-guided approaches, innovative false discovery rate estimation techniques, or *de novo* peptide sequencing. The latter approach avoids matching of raw spectral data against a predefined protein sequence database but matches mass differences between peptide fragments directly to amino acid masses [67,68].

5′-phosphoribosyl-glycinamide-transformylase 2 PurT (MT0401, Rv0389), in a viral vector for a prime/boost immunization experiment in mice, resulting in a strong CD8 T cell response.

In 2017, McMurtrey and colleagues reported on the immunopeptidome presented upon infection with Mtb strain H37Rv on nonclassical and low-polymorphic MHC class I HLA-E molecules [77]. Next to over 1200 host immunopeptides, 28 Mtb peptides from 13 different proteins were identified from HLA-E molecules secreted from infected human glioblastoma U373 cells [78,79]. All Mtb epitopes were confirmed by synthetic peptide LC-MS/MS, and 12 of them were recognized by T cells from healthy donors, latent or active TB patients. The most commonly recognized epitope, Rv0634A$_{19\text{-}29}$, showed restriction to HLA-E. Interestingly, eight of the Mtb antigens were either secreted or membrane-bound, which is consistent with the current belief of easier accessibility of peripheral bacterial antigens. Half of the Mtb peptides originated from the Esx protein family, crucial for the ESX-1 secretion system missing in BCG. Hence, it is suggested that loss of this secretion system and associated antigens reduces the HLA-E-displayed epitope repertoire, thereby limiting HLA-E-restricted T cell expansion in BCG vaccination.

Recently, a study by Bettencourt *et al*. on BCG-infected human THP1 macrophages reported 43 MHC I and 94 MHC II epitopes from 112 BCG proteins next to 23 894 MHC I and 17 599 MHC II host peptides [80]. In addition to standard infection conditions, the authors used interferon-gamma (IFNγ) and interleukin-10 (IL10) antibodies to increase MHC II expression, as well as heat-killed BCG, avoiding pathogen-mediated downregulation of antigen presentation. Stringent filtering along with spectral validation and T cell response measurements in peripheral blood mononuclear cells (PBMCs) from BCG-vaccinated donors, latent or active TB patients, resulted in a comprehensive dataset, including four BCG peptides with acetylated, methylated, or oxidized residues. Previous unrelated studies additionally identified palmitoylated, formylated, and biotinylated Mtb epitopes [81–83]. Again, most BCG antigens presented were associated with the cell

membrane or the bacterial periphery. BCG antigens releasing peptides detected in multiple experiments were prioritized, and further filtering for high expression levels yielded three antigens – glfT2, fas, and Ag85A – that were included in an Mtb vaccination challenge study. glfT2 and Ag85A had been identified in previous studies [75,84], and the authors also tested iniB and PPE15 as previously detected antigens [75,77,84]. BCG vaccination was followed by two boosts with viral vectors encoding the selected antigens before aerosol infection with Mtb Erdman. Encoding glfT2, fas, and iniB as single antigens resulted in a subtle, but significantly lower bacterial load in the lungs. Combined delivery of all five antigens, however, resulted in a nearly tenfold reduction in bacterial load in both lungs and spleens, indicating that especially combinations of antigens identified by immunopeptidomics hold great potential for novel vaccine formulations.

Chlamydia muridarum and *Chlamydia trachomatis*

In 2008 the laboratory of Robert Brunham reported on murine bone-marrow-derived dendritic cells (BMDCs) infected with *Chlamydia muridarum*, detecting one MHC I and 13 MHC II *Chlamydia* peptides from nine proteins besides 509 murine self-peptides [85]. *Chlamydia*-specific CD4 T cells recognized the identified MHC II epitopes, and dendritic cells (DCs), pulsed with a pool of eight MHC II peptides, provided partial protection against genital and intranasal *Chlamydia* infection. Sequence conservation assessment of the *C. muridarum* peptides in *Chlamydia trachomatis* revealed high sequence homology between the MHC II immunopeptides of all but two proteins, indicating that most epitopes might also hold antigenic relevance for *C. trachomatis* infection in human hosts. In a follow-up study, the authors used eight of the previously detected antigens and identified PmpG-1, PmpE/F-2, and RplF as immunodominant *Chlamydia* antigens providing significant protection against *C. muridarum* infection in mice [86]. Interestingly, PmpG-1, PmpE/F-2 are bacterial membrane proteins. A further study by this laboratory compared the MHC class II immunopeptidome of BMDCs pulsed with live and dead *C. muridarum* elementary bodies (EBs, a metabolically inactive spore-like form of *Chlamydia*), revealing that infection with live EBs resulted in elevated MHC class II presentation of bacterial peptides [87]. For live EBs, 45 immunopeptides from 13 proteins were identified including PmpG and PmpE/F-2, while only six peptides from three proteins were found for inactivated EBs. Immunization with live EBs also provided improved protection in a mouse model. In another study, the same group identified MHC II peptides from infected BMDCs isolated from C3H mice, instead of C57BL/6 mice [88]. From different MHC alleles, peptides from PmpG and PmpF were again identified from the C3H BMDCs. The authors also infected C57BL/6 BMDCs with *C. trachomatis*, the typical pathogen in humans, and identified only PmpG as a shared antigen with *C. muridarum* in both mouse strains. This antigen also showed immunodominance and high protection levels in mice with prolonged presentation on splenic APCs even months after clearing primary infection [89,90], representing an ideal vaccine candidate along with other Pmps [91]. Abundance, cellular location and expression kinetics were suggested to play predominant roles for pathogen protein presentation and antigenicity as the study revealed a total of four Pmps, important for adhesion to human epithelial cells, as well as MOMP, all abundant outer-membrane proteins [92,93]. As *Chlamydia* replicates almost exclusively in epithelial cells during *in vivo* infection, the Brunham laboratory recently investigated the immunopeptidome of epithelial cells infected with *C. muridarum* after IFNγ stimulation to boost MHC class I and initiate MHC class II expression [94]. The authors chose Bm1.11 murine oviduct epithelial cells due to partially matching MHC alleles with the previously studied C57BL/6 mouse BMDCs. Among 1138 MHC I and 431 MHC II peptides, 26 and four of these peptides were derived from *Chlamydia*, respectively. Surprisingly, there was no overlap with the previous immunopeptidomes from infected DCs, neither at the epitope level nor the antigen level. Furthermore, CD4 T cells from *C. muridarum*-recovered mice did not recognize any dominant MHC II-bound *Chlamydia* peptides presented on epithelial cells, having substantial implications for vaccine development.

Salmonella Typhimurium

Next to *Chlamydia*, the Brunham laboratory also applied their immunopeptidomics pipeline to *Salmonella* Typhimurium-infected mouse BMDCs, identifying 1891 MHC I and 617 MHC II peptides [95]. Some 87 MHC II peptides from 53 proteins, and 23 MHC I peptides from 23 proteins, were derived from *Salmonella*. Antigenicity validation of the top-scoring 12 MHC II peptides revealed that four peptides elicited an IFNγ response by CD4 T cells isolated from persistently infected mice.

Listeria monocytogenes

In 2018 Graham *et al*. reported an algorithm for the genome-wide prediction of MHC class II-restricted immunodominant epitopes termed BOTA (bacteria-originated T cell antigen). The authors identified MHC class II-bound peptides from *L. monocytogenes*-infected, H2-IA-expressing mouse BMDCs using an isobaric tagging LC-MS/MS approach [96]. Starting from their 3671 identified mouse immunopeptide sequences, they trained BOTA, followed by verification of the algorithm with the 48 *Listeria*-derived unique sequences. Of the 17 BOTA-predicted *Listeria* epitopes, nine were indeed identified by LC-MS/MS. In total, 48 partially nested *Listeria* peptide sequences were identified by MS, comprising 26 individual epitopes from 21 antigens. Nine of these *Listeria* peptides carried deamidated or oxidated residues, while previously only acetylated and formylated *Listeria* epitopes were described [97,98]. Against four of the top eight *Listeria* epitopes, robust CD4 T cell responses could be detected. BOTA utilizes information on protein localization, transmembrane structure, and domain distribution, and outperformed NetMHCIIpan for the prediction of the identified MHC II *Listeria* immunopeptides. BOTA also improved predictive power when combined with NetMHCIIpan for publically available data on inflammatory bowel disease (IBD) over NetMHCIIpan alone.

Francisella tularensis

Recently, Gaur *et al*. mapped MHC class I peptides from *F. tularensis* presented on infected human blood-derived immature dendritic cells (iDCs) [99]. The authors reported ten *Francisella*-derived peptide sequences from ten different antigens and validated their findings *in silico*, calculating high HLA binding affinities and antigenicity scores for the three best scoring epitopes.

From identified antigens to effective vaccines

Ongoing improvements to immunopeptidomics technology will allow deeper digging into the bacterial antigen repertoire in the future. Peptide splicing, a process generating nongenetically determined immunopeptides (Box 2), was ignored in all of the previously-mentioned studies. Since T cells can recognize bacterial spliced peptides [46,47], considering such peptides will yield more complete antigen panels. Similarly, other PTMs, such as formylation or palmitoylation (Box 3), can be found on bacterial immunopeptides, and even host cell-derived immunopeptides might display altered modifications during infection [66]. Bacterial infection can also interfere with host cell antigen presentation [80,100,101], and together with PTMs, such immunomodulatory processes should be considered in future immunopeptidomics studies. Finally, even though immunopeptidomics typically requires large amounts of starting material, recent innovations have allowed downscaling to ca. 1E7 cells, and efforts are ongoing to downscale even further [38,53,55,58]. Together with more sensitive mass spectrometers, these developments will expand the number of infection models that are accessible to immunopeptidomics.

With immunopeptidomics being applied to increasing numbers of bacterial infection models, the question arises on how to select antigens from these studies to encode in effective next-generation vaccines. Validation of identification and antigenicity of discovered epitopes is the crucial first step prior to *in vivo* testing. While anticancer vaccines encode mutated epitopes that are tumor-specific

and patient (HLA)-specific, antimicrobial vaccines typically incorporate full protein antigens rather than epitopes, ensuring HLA-independent protection of population majorities. There are currently no guidelines on how to prioritize immunopeptidomics-derived antigens as bacterial vaccine candidates; however, a few trends and principles start to surface from the previously-mentioned studies which we integrated in a proposed vaccine candidate prioritization strategy outlined in Figure 3.

First, proteins releasing most epitopes might represent immunodominant antigens. Ideally, these antigens are picked up in different studies and presented on several HLA alleles on different cell types, tissues or species, for example, the Mtb glfT2 and iniB antigens [75,77,80]. In case of little overlap, antigens detected from cell types reflecting the replicative niche during *in vivo* infection might be prioritized [94]. More sensitive future immunopeptidomics screens identifying hundreds (instead of tens) of bacterial epitopes/screen are required to further substantiate a potential link between epitope abundance and antigen immunodominance. Second, antigens eliciting good immune responses often originate from the bacterial membrane or periphery, in line with close contact of these surface proteins with the host intracellular milieu [77,80,88,96]. Third, combined delivery of multiple immunopeptidomics-discovered antigens seems to provide better protection than single antigens, allowing stable protection for different populations and omitting single antigen shifts [80,85]. Of note, also general considerations and tools for vaccine antigen selection should be taken into account, including prioritization of antigens conserved in clinical strains, exclusion of harmful antigens (e.g., bacterial toxins) and avoidance of host- and microbiome-resembling antigens to avoid autoimmunity [102].

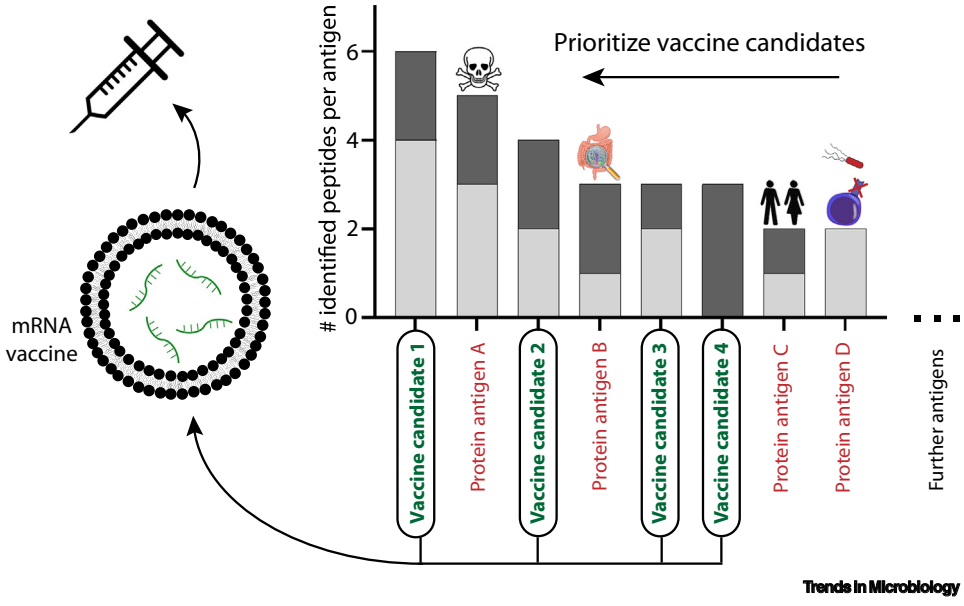

Figure 3. Proposed vaccine candidate prioritization strategy from immunopeptidomics studies. Bar chart representing the outcome of a hypothetical immunopeptidomics experiment on bacterially infected cells. The screen is carried out in two different host cell types relevant for infection represented in gray (light gray, epithelial cells; dark gray, antigen-presenting cells, APCs). The y-axis depicts the number of identified peptide epitopes for each bacterial protein antigen over the two screens. Antigens depicted in green are considered good vaccine candidates since they release the highest number of epitopes over both screens. Often these candidate antigens are derived from the bacterial periphery and are in close contact with the host intracellular milieu. Antigens in red are not considered suitable vaccine candidates due to substantial toxicity (antigen A), sequence homology to microbiome (antigen B) or host proteins (antigen C), or low T cell reactivity (antigen D). Encoding the four top candidates within a single mRNA vaccine formulation could facilitate broad immune responses, while avoiding immune escape of the pathogen by single antigen shifts.

Antigens identified by MHC class I immunopeptidomics can be encoded quite easily into vaccines against cytosolic pathogens that typically require strong cytotoxic CD8 T cell responses for infection clearance, such as *L. monocytogenes* [103]. Indeed, the encoded antigenic information will be translated into bacterial proteins in the host cell cytosol ready for MHC I presentation. For noncytosolic, vacuolar pathogens, such as *Chlamydia* or *Salmonella*, for which humoral and CD4 T cell responses are typically more important [104,105], MHC II-identified antigens can be encoded using secretion signal tags allowing uptake of the secreted bacterial antigens by professional APCs for MHC II presentation [106]. Here, care must be taken to avoid secretion-associated glycosylation potentially inferring immune tolerance [107]. Of note, for many pathogens, protective immunity relies on both cellular and humoral immunity [108,109]. While classic whole-pathogen or subunit vaccines typically elicit strong antibody responses, nucleic acid-based vaccines now allow the induction of much stronger cytotoxic responses. For pathogens such as Mtb, for which the contribution of cytotoxic responses has become clear in recent years [108], MHC class I immunopeptidomics might help to design more effective nucleic acid-based vaccines.

Concluding remarks and future perspectives

Immunopeptidomics has matured into a powerful technology for antigen discovery. Currently, the technology has been applied to only a handful of intracellular bacterial pathogens, especially *Mycobacteria* and *Chlamydia*, but with rising AMR, more studies are expected in the years to come to shed light on burning questions in the field (see Outstanding questions). Discovered antigens can be directly encoded in nucleic acid-based vaccines, constituting a versatile and powerful vaccine development pipeline. Further improvements to increase the sensitivity of immunopeptidomics studies are expected to boost the number of identified antigens and to strengthen the golden alliance with viral vector and mRNA vaccine platforms, facilitating development of effective, novel antibacterial vaccines.

Outstanding questions

Do immunopeptidomics screens on bacterial infection models hold information on which bacterial antigens to select as vaccine candidates? Can we use the abundance of identified epitopes and recurrence in different screens to prioritize their parent antigens?

Which host cell type, tissue, or organ is best suited to screen for antigens? Should we screen infected APCs or rather nonimmune (epithelial) cells for pathogens that use the latter as a replicative niche during *in vivo* infection?

Will nucleic acid vaccines against intracellular bacterial pathogens be equally effective against cytosolic (MHC I presented) or vacuolar (MHC II presented) pathogens?

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Declaration of interests

There are no interests to declare.

References

1. Mohammed, A. *et al.* (2019) *No Time to Wait: Securing the Future from Drug-resistant Infections*, UN Interagency Coordination Group on Antimicrobial Resistance (IACG)
2. Centers for Disease Control and Prevention (2019) *Antibiotic Resistance Threats in the United States, 2019*, CDC
3. Tacconelli, E. *et al.* (2018) Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* 18, 318–327
4. Clancy, C.J. and Nguyen, M.H. (2020) Buying Time: The AMR Action Fund and the State of Antibiotic Development in the United States 2020. *Open Forum Infect. Dis.* 7, ofaa464
5. Micoli, F. *et al.* (2021) The role of vaccines in combatting antimicrobial resistance. *Nat. Rev. Microbiol.* 19, 287–303
6. Mendoza, N. *et al.* (2009) Existing antibacterial vaccines. *Dermatol. Ther.* 22, 129–142
7. Poolman, J.T. (2020) Expanding the role of bacterial vaccines into life-course vaccination strategies and prevention of antimicrobial-resistant infections. *NPJ Vaccines* 5, 84
8. Kernodle, D.S. (2010) Decrease in the effectiveness of Bacille Calmette–Guerin vaccine against pulmonary tuberculosis: a consequence of increased immune suppression by microbial antioxidants, not overattenuation. *Clin. Infect. Dis.* 51, 177–184
9. Mangtani, P. *et al.* (2014) Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. *Clin. Infect. Dis.* 58, 470–480
10. Rana, A. *et al.* (2018) Recent Trends in system-scale integrative approaches for discovering protective antigens against mycobacterial pathogens. *Front. Genet.* 9, 572
11. Kashangura, R. *et al.* (2015) Effects of MVA85A vaccine on tuberculosis challenge in animals: systematic review. *Int. J. Epidemiol.* 44, 1970–1981
12. Kamaruzzaman, N.F. *et al.* (2017) Targeting the hard to reach: challenges and novel strategies in the treatment of intracellular bacterial infections. *Br. J. Pharmacol.* 174, 2225–2236
13. Byndloss, M.X. and Tsolis, R.M. (2016) Chronic bacterial pathogens: mechanisms of persistence. *Microbiol. Spectr.* 4. https://doi.org/10.1128/microbiolspec.VMBF-0020-2015
14. Avci, F. *et al.* (2019) Glycoconjugates: What it would take to master these well-known yet little-understood immunogens for vaccine development. *mSphere* 4, e00520-19
15. Zhang, C. *et al.* (2019) Advances in mRNA vaccines for infectious diseases. *Front. Immunol.* 10, 594
16. Gary, E.N. and Weiner, D.B. (2020) DNA vaccines: prime time is now. *Curr. Opin. Immunol.* 65, 21–27

17. Sasso, E. *et al.* (2020) New viral vectors for infectious diseases and cancer. *Semin. Immunol.* 50, 101430
18. Wolff, J.A. *et al.* (1990) Direct gene transfer into mouse muscle *in vivo*. *Science* 247, 1465–1468
19. Richner, J.M. *et al.* (2017) Vaccine mediated protection against Zika virus-induced congenital disease. *Cell* 170, 273–283 e212
20. Bahl, K. *et al.* (2017) Preclinical and clinical demonstration of immunogenicity by mRNA vaccines against H10N8 and H7N9 influenza viruses. *Mol. Ther.* 25, 1316–1327
21. Steinle, H. *et al.* (2017) Concise review: application of *in vitro* transcribed messenger RNA for cellular engineering and reprogramming: progress and challenges. *Stem Cells* 35, 68–79
22. Baden, L.R. *et al.* (2021) Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. *N. Engl. J. Med.* 384, 403–416
23. Polack, F.P. *et al.* (2020) Safety and efficacy of the BNT162b2 mRNA covid-19 Vaccine. *N. Engl. J. Med.* 383, 2603–2615
24. Maruggi, G. *et al.* (2017) Immunogenicity and protective efficacy induced by self-amplifying mRNA vaccines encoding bacterial antigens. *Vaccine* 35, 361–368
25. Xue, T. *et al.* (2004) RNA encoding the MPT83 antigen induces protective immune responses against *Mycobacterium tuberculosis* infection. *Infect. Immun.* 72, 6324–6329
26. Lorenzi, J.C. *et al.* (2010) Intranasal vaccination with messenger RNA as a new approach in gene therapy: use against tuberculosis. *BMC Biotechnol.* 10, 77
27. Pardi, N. *et al.* (2018) mRNA vaccines – a new era in vaccinology. *Nat. Rev. Drug Discov.* 17, 261–279
28. Vita, R. *et al.* (2019) The Immune Epitope Database (IEDB): 2018 update. *Nucleic Acids Res.* 47, D339–D343
29. Rappuoli, R. (2000) Reverse vaccinology. *Curr. Opin. Microbiol.* 3, 445–450
30. Sette, A. and Rappuoli, R. (2010) Reverse vaccinology: developing vaccines in the era of genomics. *Immunity* 33, 530–541
31. Rappuoli, R. *et al.* (2016) Reverse vaccinology 2.0: Human immunology instructs vaccine antigen design. *J. Exp. Med.* 213, 469–481
32. Fulton, K.M. *et al.* (2019) Immunoproteomics methods and techniques. *Methods Mol. Biol.* 2024, 25–58
33. Bassani-Sternberg, M. *et al.* (2015) Mass spectrometry of human leukocyte antigen class I peptidomes reveals strong effects of protein abundance and turnover on antigen presentation. *Mol. Cell. Proteomics* 14, 658–673
34. Griffin, T.A. *et al.* (1998) Immunoproteasome assembly: cooperative incorporation of interferon gamma (IFN-gamma)-inducible subunits. *J. Exp. Med.* 187, 97–104
35. Aki, M. *et al.* (1994) Iterferon-gamma induces different subunit organizations and functional diversity of proteasomes. *J. Biochem.* 115, 257–269
36. Boes, B. *et al.* (1994) Interferon gamma stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes. *J. Exp. Med.* 179, 901–909
37. Driscoll, J. *et al.* (1993) MHC-linked LMP gene products specifically alter peptidase activities of the proteasome. *Nature* 365, 262–264
38. Chong, C. *et al.* (2018) High-throughput and sensitive immunopeptidomics platform reveals profound interferongamma-mediated remodeling of the human leukocyte antigen (HLA) ligandome. *Mol. Cell. Proteomics* 17, 533–548
39. Wang, J. and Maldonado, M.A. (2006) The ubiquitin–proteasome system and its role in inflammatory and autoimmune diseases. *Cell. Mol. Immunol.* 3, 255–261
40. Rivett, A.J. *et al.* (2001) Regulation of proteasome complexes by gamma-interferon and phosphorylation. *Biochimie* 83, 363–366
41. Vigneron, N. *et al.* (2004) An antigenic peptide produced by peptide splicing in the proteasome. *Science* 304, 587–590
42. Mylonas, R. *et al.* (2018) Estimating the contribution of proteasomal spliced peptides to the HLA-I ligandome. *Mol. Cell. Proteomics* 17, 2347–2357
43. Liepe, J. *et al.* (2016) A large fraction of HLA class I ligands are proteasome-generated spliced peptides. *Science* 354, 354–358
44. Faridi, P. *et al.* (2018) A subset of HLA-I peptides are not genomically templated: Evidence for cis- and trans-spliced peptide ligands. *Sci. Immunol.* 3, eaar3947
45. Faridi, P. *et al.* (2021) Spliced HLA-bound peptides: a Black Swan event in immunology. *Clin. Exp. Immunol.* 204, 179–188
46. Platteel, A.C.M. *et al.* (2017) Multi-level strategy for identifying proteasome-catalyzed spliced epitopes targeted by CD8(+) T cells during bacterial infection. *Cell Rep.* 20, 1242–1253
47. Platteel, A.C. *et al.* (2016) CD8(+) T cells of *Listeria monocytogenes*-infected mice recognize both linear and spliced proteasome products. *Eur. J. Immunol.* 46, 1109–1118
48. Paes, W. *et al.* (2019) Contribution of proteasome-catalyzed peptide cis-splicing to viral targeting by CD8(+) T cells in HIV-1 infection. *Proc. Natl. Acad. Sci. U. S. A.* 116, 24748–24759
49. Gonzalez-Galarza, F.F. *et al.* (2020) Allele frequency net database (AFND) 2020 update: gold-standard data classification, open access genotype data and new query tools. *Nucleic Acids Res.* 48, D783–D788
50. Manoury, B. (2013) Proteases: essential actors in processing antigens and intracellular Toll-like receptors. *Front. Immunol.* 4, 299
51. Hunt, D.F. *et al.* (1992) Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* 255, 1261–1263
52. Lanoix, J. *et al.* (2018) Comparison of the MHC I immunopeptidome repertoire of B-cell lymphoblasts using two isolation methods. *Proteomics* 18, e1700251
53. Purcell, A.W. *et al.* (2019) Mass spectrometry-based identification of MHC-bound peptides for immunopeptidomics. *Nat. Protoc.* 14, 1687–1707
54. Stadlmann, J. *et al.* (2019) Improved Sensitivity in low-input proteomics using micropillar array-based chromatography. *Anal. Chem.* 91, 14203–14207
55. Pfammatter, S. *et al.* (2020) Extending the comprehensiveness of immunopeptidome analyses using isobaric peptide labeling. *Anal. Chem.* 92, 9194–9204
56. Murphy, J.P. *et al.* (2019) Multiplexed relative quantitation with isobaric tagging mass spectrometry reveals class I major histocompatibility complex ligand dynamics in response to doxorubicin. *Anal. Chem.* 91, 5106–5115
57. Chen, R. *et al.* (2018) Chemical derivatization strategy for extending the identification of MHC class I immunopeptides. *Anal. Chem.* 90, 11409–11416
58. Ramarathinam, S.H. *et al.* (2020) A peptide-signal amplification strategy for the detection and validation of neoepitope presentation on cancer biopsies. *bioRxiv* Published online June 12, 2020. https://doi.org/10.1101/2020.06.12.145276
59. Mommen, G.P. *et al.* (2014) Expanding the detectable HLA peptide repertoire using electron-transfer/higher-energy collision dissociation (EThcD). *Proc. Natl. Acad. Sci. U. S. A.* 111, 4507–4512
60. Frese, C.K. *et al.* (2012) Toward full peptide sequence coverage by dual fragmentation combining electron-transfer and higher-energy collision dissociation tandem mass spectrometry. *Anal. Chem.* 84, 9668–9673
61. Alpizar, A. *et al.* (2017) A molecular basis for the presentation of phosphorylated peptides by HLA-B antigens. *Mol. Cell. Proteomics* 16, 181–193
62. Marcilla, M. *et al.* (2014) Increased diversity of the HLA-B40 ligandome by the presentation of peptides phosphorylated at their main anchor residue. *Mol. Cell. Proteomics* 13, 462–474
63. Marino, F. *et al.* (2015) Extended O-GlcNAc on HLA class-I-bound peptides. *J. Am. Chem. Soc.* 137, 10922–10925
64. Marino, F. *et al.* (2017) Arginine (Di)methylated human leukocyte antigen class I peptides are favorably presented by HLA-B*07. *J. Proteome Res.* 16, 34–44
65. Zarling, A.L. *et al.* (2000) Phosphorylated peptides are naturally processed and presented by major histocompatibility complex class I molecules *in vivo*. *J. Exp. Med.* 192, 1755–1762
66. Ribet, D. and Cossart, P. (2010) Pathogen-mediated post-translational modifications: A re-emerging field. *Cell* 143, 694–702
67. Andreatta, M. *et al.* (2019) MS-rescue: a computational pipeline to increase the quality and yield of immunopeptidomics experiments. *Proteomics* 19, e1800357
68. Zhang, J. *et al.* (2012) PEAKS DB: *de novo* sequencing assisted database search for sensitive and accurate peptide identification. *Mol. Cell. Proteomics* 11, M111 010587

69. Bassani-Sternberg, M. and Coukos, G. (2016) Mass spectrometry-based antigen discovery for cancer immunotherapy. *Curr. Opin. Immunol.* 41, 9–17
70. Boucau, J. and Le Gall, S. (2019) Antigen processing and presentation in HIV infection. *Mol. Immunol.* 113, 67–74
71. Chen, R. *et al.* (2021) Identification of Mhc peptides using mass spectrometry for neoantigen discovery and cancer vaccine development. *Mass Spectrom. Rev.* 40, 110–125
72. Haen, S.P. *et al.* (2020) Towards new horizons: characterization, classification and implications of the tumour antigenic repertoire. *Nat. Rev. Clin. Oncol.* 17, 595–610
73. Lopez, D. *et al.* (2019) Immunoproteomic lessons for human respiratory syncytial virus vaccine design. *J. Clin. Med.* 8, 486
74. Bettencourt, P. (2020) Current challenges in the identification of pre-erythrocytic malaria vaccine candidate antigens. *Front. Immunol.* 11, 190
75. Flyer, D.C. *et al.* (2002) Identification by mass spectrometry of CD8(+)-T-cell *Mycobacterium tuberculosis* epitopes within the Rv0341 gene product. *Infect. Immun.* 70, 2926–2932
76. Cayabyab, M.J. *et al.* (2013) An unbiased peptide-wide discovery approach to select *Mycobacterium tuberculosis* antigens that target $CD8^+$ T cell response during infection. *Vaccine* 31, 4834–4840
77. McMurtrey, C. *et al.* (2017) T cell recognition of *Mycobacterium tuberculosis* peptides presented by HLA-E derived from infected human cells. *PLoS One* 12, e0188288
78. Braud, V. *et al.* (1997) The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur. J. Immunol.* 27, 1164–1169
79. Braud, V.M. *et al.* (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391, 795–799
80. Bettencourt, P. *et al.* (2020) Identification of antigens presented by MHC for vaccines against tuberculosis. *NPJ Vaccines* 5, 2
81. Busch, R. *et al.* (1990) Degenerate binding of immunogenic peptides to HLA-DR proteins on B cell surfaces. *Int. Immunol.* 2, 443–451
82. da Fonseca, D.P. *et al.* (1998) Identification of new cytotoxic T-cell epitopes on the 38-kilodalton lipoglycoprotein of *Mycobacterium tuberculosis* by using lipopeptides. *Infect. Immun.* 66, 3190–3197
83. Chun, T. *et al.* (2001) Induction of M3-restricted cytotoxic T lymphocyte responses by N-formylated peptides derived from *Mycobacterium tuberculosis*. *J. Exp. Med.* 193, 1213–1220
84. Stylianou, E. *et al.* (2018) Identification and evaluation of novel protective antigens for the development of a candidate tuberculosis subunit vaccine. *Infect. Immun.* 86, e00014–e00018
85. Karunakaran, K.P. *et al.* (2008) Immunoproteomic discovery of novel T cell antigens from the obligate intracellular pathogen *Chlamydia*. *J. Immunol.* 180, 2459–2465
86. Yu, H. *et al.* (2009) Novel *Chlamydia muridarum* T cell antigens induce protective immunity against lung and genital tract infection in murine models. *J. Immunol.* 182, 1602–1608
87. Yu, H. *et al.* (2011) Immunization with live and dead *Chlamydia muridarum* induces different levels of protective immunity in a murine genital tract model: correlation with MHC class II peptide presentation and multifunctional Th1 cells. *J. Immunol.* 186, 3615–3621
88. Karunakaran, K.P. *et al.* (2015) Outer membrane proteins preferentially load MHC class II peptides: implications for a *Chlamydia trachomatis* T cell vaccine. *Vaccine* 33, 2159–2166
89. Li, L.X. and McSorley, S.J. (2013) B cells enhance antigen-specific CD4 T cell priming and prevent bacteria dissemination following *Chlamydia muridarum* genital tract infection. *PLoS Pathog.* 9, e1003707
90. Johnson, R.M. *et al.* (2012) PmpG303-311, a protective vaccine epitope that elicits persistent cellular immune responses in *Chlamydia muridarum*-immune mice. *Infect. Immun.* 80, 2204–2211
91. Zhong, G. *et al.* (2019) National Institute of Allergy and Infectious Diseases workshop report: 'Chlamydia vaccines: The way forward'. *Vaccine* 37, 7346–7354
92. Saka, H.A. *et al.* (2011) Quantitative proteomics reveals metabolic and pathogenic properties of *Chlamydia trachomatis* developmental forms. *Mol. Microbiol.* 82, 1185–1203
93. Becker, E. and Hegemann, J.H. (2014) All subtypes of the Pmp adhesin family are implicated in chlamydial virulence and show species-specific function. *Microbiologyopen* 3, 544–556
94. Karunakaran, K.P. *et al.* (2020) Discordance in the epithelial cell-dendritic cell major histocompatibility complex class II immunoproteome: implications for *Chlamydia* vaccine development. *J. Infect. Dis.* 221, 841–850
95. Karunakaran, K.P. *et al.* (2017) Identification of MHC-bound peptides from dendritic cells infected with *Salmonella enterica* Strain SL1344: implications for a nontyphoidal *Salmonella* vaccine. *J. Proteome Res.* 16, 298–306
96. Graham, D.B. *et al.* (2018) Antigen discovery and specification of immunodominance hierarchies for MHCII-restricted epitopes. *Nat. Med.* 24, 1762–1772
97. Bubert, A. *et al.* (1994) Synthetic peptides derived from the *Listeria monocytogenes* p60 protein as antigens for the generation of polyclonal antibodies specific for secreted cell-free L. monocytogenes p60 proteins. *Appl. Environ. Microbiol.* 60, 3120–3127
98. Lenz, L.L. *et al.* (1996) Identification of an H2-M3-restricted *Listeria* epitope: implications for antigen presentation by M3. *Immunity* 5, 63–72
99. Gaur, R. *et al.* (2021) Identification of MHC class I bound peptides of *Francisella tularensis* live vaccine strain using mass spectrometry. *Eur. J. Pharm. Sci.* 158, 105651
100. Codolo, G. *et al.* (2019) *Helicobacter pylori* dampens HLA-II expression on macrophages via the up-regulation of miRNAs targeting CIITA. *Front. Immunol.* 10, 2923
101. Cajander, S. *et al.* (2018) Dynamics of monocytic HLA-DR expression differs between bacterial etiologies during the course of bloodstream infection. *PLoS One* 13, e0192883
102. Dalsass, M. *et al.* (2019) Comparison of open-source reverse vaccinology programs for bacterial vaccine antigen discovery. *Front. Immunol.* 10, 113
103. Harty, J.T. and Bevan, M.J. (1992) $CD8^+$ T cells specific for a single nnamer epitope of *Listeria monocytogenes* are protective *in vivo*. *J. Exp. Med.* 175, 1531–1538
104. Hess, J. *et al.* (1996) *Salmonella typhimurium* aroA- infection in gene-targeted immunodeficient mice: major role of $CD4^+$ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. *J. Immunol.* 156, 3321–3326
105. Gondek, D.C. *et al.* (2012) $CD4^+$ T cells are necessary and sufficient to confer protection against *Chlamydia trachomatis* infection in the murine upper genital tract. *J. Immunol.* 189, 2441–2449
106. Kou, Y. *et al.* (2017) Tissue plasminogen activator (tPA) signal sequence enhances immunogenicity of MVA-based vaccine against tuberculosis. *Immunol. Lett.* 190, 51–57
107. Ozdilek, A. *et al.* (2020) Host protein glycosylation in nucleic acid vaccines as a potential hurdle in vaccine design for nonviral pathogens. *Proc. Natl. Acad. Sci. U. S. A.* 117, 1280–1282
108. van Pinxteren, L.A. *et al.* (2000) Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. *Eur. J. Immunol.* 30, 3689–3698
109. Healey, G.D. *et al.* (2005) Humoral and cell-mediated adaptive immune responses are required for protection against *Burkholderia pseudomallei* challenge and bacterial clearance postinfection. *Infect. Immun.* 73, 5945–5951