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Breaking the deadlock in antigen discovery for *Leishmania* vaccines

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Abstract

Despite the global burden of leishmaniasis, no licensed human vaccine exists. Vaccine antigens are typically based on *in silico* predictions or their immunogenicity in animal models and cured patients. T cell epitopes have only been mapped for fewer than 2% of over 8,000 *Leishmania* proteins, with only ten proteins tested in clinical trials. Whether infected human phagocytes naturally present such antigens early on during infection, and prior to the onset of parasite-induced regulatory mechanisms, is missing. We discuss why this is critical to the induction of protective immunity, and argue that antigen availability can conclusively guide and accelerate vaccine candidate discovery. Here, emerging technologies, including immunopeptidomics, single-cell multiomics, and TCR repertoire mapping, offer transformative opportunities to redefine *Leishmania* antigen discovery.

Keywords: Vaccine, leishmaniasis, leishmania, antigen discovery

Paucity in the *Leishmania* vaccine pipeline

The leishmaniases are a group of vector-borne infectious diseases caused by dimorphic protozoan parasites of the genus *Leishmania*. The primary disease spectrum ranges from the non-lethal but disfiguring skin disease cutaneous leishmaniasis (CL) to the potentially fatal visceral leishmaniasis (VL) which affects the internal organs. Around 600,000-1 million new CL and 50,000-90,000 new VL cases are reported to the WHO annually across 98 endemic countries, leading to 18,700 deaths and 1.6 million disability adjusted life years (DALYs) lost each year, with current numbers likely grossly underestimating the true burden.[1,2] A licensed vaccine is, however, still lacking despite modelling efforts estimating demand over a 10-year period at 310-830 and 557-1400 million doses, for VL and CL prevention respectively, if a vaccine was available with 50% efficacy at 3 dollar per dose cost.[3]

Host protection against infection is dependent on Th1-type $CD4^+$ T cell activation resulting in IFN-y production that stimulates infected phagocytes to kill intracellular *Leishmania* parasites by producing nitric oxide and reactive oxygen species (ROS).[4] Support for the notion that leishmaniasis is preventable with a vaccine includes the generation of lifelong immunity after healing, with or without treatment, and the frequent prevalence of asymptomatic infection.[5,6] Vaccination proof-of-principle is evidenced by the effectiveness of **leishmanization** (see **Glossary**).[7] Despite hundreds of published vaccine studies (using heat killed organisms, live attenuated whole parasite vaccines, and subunit vaccines) that demonstrated T cell immunogenicity in pre-clinical models, less than ten *Leishmania* vaccine candidates have progressed to human trials.[8] First generation *Leishmania* vaccines composed of whole killed parasites, often adjuvanted with Bacillus Calmette–Guérin (BCG) were not efficacious in a prophylactic setting.[5,7] However, second generation vaccines, including recombinant poly-protein vaccines combined with lipid-based adjuvants, were shown to be immunogenic and safe in phase I and therapeutic phase II settings, but were not continued.[9] Likewise, a third generation adenoviral-vector vaccine was safe and immunogenic, but lacked efficacy as a stand-alone therapeutic in Sudanese patients with persistent post-kala-azar dermal

leishmaniasis.[10] Notably, these second and third generation vaccines have not been tested for prophylactic efficacy in humans due to cost and duration of phase III clinical trials, lack of defined immune correlates of protection (ICP), previously limited evidence on vaccine demand, and limited funder/commercial willingness.[3] However, recent advances in health economic evaluations, demand and use case modelling, the availability of a controlled human infection model (CHIM) and a defined (skin) test reagent, combined with the accrued safety data on several *Leishmania* antigens, significantly reduces these barriers.[3,11] Indeed, in the case of veterinary vaccines where such barriers were less evident, several prophylactic vaccines have been licensed (Leish-Tec®, CaniLeish®, LetiFend®, and recently Neoleish®).[5,12] Moreover, the immunological threshold required for prophylactic efficacy is likely to be lower than for therapeutic benefit, due to a lower parasite load and competent immune system in naïve versus diseased individuals. Even decreasing the parasite load below a critical threshold may already reduce the incidence of the most severe disease outcomes (e.g. systemic disease, non-healing lesions).

Nevertheless, despite VL elimination initiatives in Asia and Africa and the WHO's prioritization of leishmaniasis vaccines,[2,13] no vaccine trials are ongoing. The most advanced candidate (e.g. a live-attenuated centrin gene-deleted vaccine[14]) is slowly moving to Phase I trials with unguaranteed success. While its strong preclinical performance offers hope, there remains an urgent need to develop subunit vaccines. These vaccines can provide a safer, more targeted immune response (avoiding **distractopes**, polyclonal antibody activation, and vaccine-associated disease), allow immunization of vulnerable groups (such as pregnant women, immunosuppressed), and avoid interference with essential diagnostic assays.

Evidence base of MHC presentation for current *Leishmania* vaccine antigens

We argue that this paucity in leishmaniasis vaccines is, in addition to barriers discussed above, sustained by a weak evidence base on desirable antigens and the protective T cell response(s) to be targeted. For extracellular pathogens, vaccine antigens are often selected based on essential structural or functional roles (e.g. SARS-CoV-2 spike protein), as these may be less prone to **antigenic variation** and/or provide clear targets for antibodies. This rationale is less applicable to the intracellular *Leishmania* parasite, where parasite killing relies on non-specific mechanisms such as the ROS pathway and, unlike viruses, *Leishmania* does not expose its entire proteome to the host cell in order to replicate. Instead, antigen shedding and/or abundance may be more critical for persistent MHC presentation and early immune recognition by T cells. For example, phosphoenolpyruvate carboxykinase (PEPCK), a gluconeogenic enzyme without obvious structural or functional prioritization, was identified as a natural MHC ligand via **immunopeptidomics** in *L. major*-infected dendritic cells and later shown to be **immunogenic** and metabolically essential.[15] Due to the lack of specificity in host killing mechanism (e.g. ROS), selective pressure on individual antigens is also likely reduced, limiting a need for antigenic variation.

The *Leishmania* proteome consists of roughly 8200 proteins with over $>10^9$ potential MHC-I and MHC-II **epitopes**.[16] A search in the curated Immune Epitope Database (IEDB ID:5658, accessed on 25-APR-2025) on reported *Leishmania* T cell epitopes from any host organism, resulted in only 660 epitopes derived from 143 antigens that are reported in 71 publications between 1990 and 2022. Whilst IEDB may not reflect all known epitopes due to lack of reporting/publishing, this nevertheless accounts for less than 0.1% of all theoretical epitopes, with less than 2% of all *Leishmania* proteins having known T cell epitopes. Of these 432 postulated epitopes, only 228 (52.8%) were reported to be validated in humans (**Figure 1**), which is of importance as established animal and *in vitro* models are likely to differ

in antigen processing compared to human infection (due to non-human MHC, discordant disease pathology, nutrient supplies, sand-fly derived proteins, etc.).[17,18]

The majority of reported immunogenicity assays involved *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) from treated and cured patients. While these patients are expected to have developed a strong *Leishmania*-specific T cell response that contributed to successful disease outcome, by definition, these patients did not mount an initial protective response to the infection as they developed symptomatic disease. Their T cell immunogenicity profile might therefore be reflective of successful parasite escape mechanisms and (**immunodominant**) antigens that only became available through efficacious drug-induced killing of the parasite (e.g. release of intracellular parasite contents). This will obscure the protective clonotypes required in a prophylactic and untreated setting with a more limited antigen availability. In addition, *ex vivo* stimulation is conventionally performed with soluble *Leishmania* antigen (SLA) derived from **promastigotes** due to ease of preparation, yet this may omit or mask responses to key **amastigote** stage-specific antigens (e.g. A2, 2,3-trans-enoyl Co1 isomerase, nuclease P4, HASPB1, centrin, and amastins).

We should also be cautious on assuming natural MHC presentation when using T cell immunogenicity as confirmation. E*x vivo* immunogenicity screens with overlapping peptide pools may miss the exact and effective epitope length, and have been reported to induce false positive responses to non-endogenous peptides.[19] Similarly, *in vitro* MHC binding/stabilization assays that were performed for 81 (19%) of the IEDB-listed immunogenic epitopes, merely reflect stable binding but not *in vivo* endogenous antigen processing.

Mass spectrometry (MS)-based MHC immunopeptidomics is a method that allows direct assessment of naturally processed epitopes that can be eluted from MHC molecules. This approach has been performed for only 8% of the IEDB-reported epitopes. From these, Singh et al. identified 12 MHC-II

epitopes from donor-derived monocyte-derived dendritic cells that were pulsed with *L. donovani* promastigote lysate.[20] However, this identifies naturally processed epitopes from crude soluble antigens rather than those derived from infection with whole parasite. The remaining epitopes were identified from *in vitro L. major* infected bone marrow-derived dendritic cells isolated from mice.[21] Because no direct *Leishmania* epitope identifications from clinical samples were published to date, we applied immunopeptidomics on lesional punch biopsies of 27 Ethiopian CL patients across the full clinical spectrum. We identified 333 MHC-I and 247 MHC-II novel *L. aethiopica* peptides derived from 398 proteins.[22] However, as these were patients with active disease of up to 172 months duration, these data do not inform on antigen availability in an early (protective) stage of infection. Nevertheless, it is noteworthy that only one of all of these MHC-eluted candidate antigens (namely *Leishmania* elongation factor 1α (LeIF or P74)) was included in prior human vaccine trials.

To date, only 10 *Leishmania* antigens have been integrated in subunit vaccine constructs that entered clinical trials. This includes LeIF (or P74), thiol-specific oxidant (TSA), Cysteine protease A and B (CPA and CPB, respectively), Hydrophilic acylated surface protein B (HASPB), kinetoplastid membrane protein-11 (KMP-11), stress-inducible protein 1 (STI1), Amastigote stage-specific protein (A2), Nucleoside Hydrolase (NH36), and Sterol 24-c-methyltranferase (SMT). **Figure 1** depicts the limited T cell epitope evidence base for this limited set of clinically tested antigens, as derived from IEDB. This analysis raises the urgent need for a more critically focused antigen discovery and validation program to inform on future vaccine candidates. We argue that a validation of MHC presentation and consequently antigen availability in an early and untreated infection (e.g. asymptomatic infection) is pivotal to drive antigen selection in prophylactic vaccine development. A considerable amount of time and money in vaccine development could be saved by starting off with the ‘right’ antigens in subunit vaccines.

Key considerations to unlock optimal vaccine antigens

The longstanding deadlock in effective *Leishmania* T cell epitope and immunogen discovery is driven in large by the proteome size that renders classical *ex vivo* and low-throughput T cell epitope mapping approaches insufficient. Here, we discuss several key considerations to more efficiently identify optimal vaccine antigens:

Account for timing and location of antigen availability in natural infection

Leishmania replicates and resides in a parasitophorous vacuole/phagolysosome of antigen-presenting cells, where host effector mechanisms are targeted to kill the parasites. Subsequently, parasite-derived antigens are degraded to be presented via the MHC-II pathway or via the MHC-I cross-presentation pathway.[23] Similar to *Mycobacterium tuberculosis* (*Mtb*) infections, this general presentation machinery has been described to be hampered during *in vitro Leishmania* infections as a means for immune escape.[24–26] Yet, *L. mexicana* was shown to uniquely and differentially modulate the proteolytic capacity of its replication niche, in comparison to *Coxiella burnetti*, the only other human pathogen able to replicate within the mature phagolysosomal compartment of host cells.[27] However, the complete (post-)transcriptional mechanisms affecting MHC expression and avoiding hydrolytic degradation remain to be fully characterized and validated in patient samples (research gaps summarized in **Figure 2**).

Initial *Leishmania* vaccines were heavily based on immunodominant antigens in patients with active disease. Earlier studies targeted antigens such as Leishmanolysin (gp63), Kinetoplastid membrane protein-11 (KMP-11), and *Leishmania*-activated C-kinase antigen (LACK or p36) that were shown to be immunodominant in western blot analysis of infected sera from animal models.[9,28] In a similar manner, the Phosphoenolpyruvate carboxykinase $(PEPCK)_{335\text{-}351}$ epitope received much attention as it was shown to be immunodominant in murine models with peptide-specific tetramer screenings.[15]

However, immunodominance of decoy antigens might be a survival strategy of complex protozoan to avoid immune recognition of essential proteins, and as argued by many, **cryptic antigens** might be better suited for vaccines as they have less chance of selection due to immune pressure.[29] However, based on an alternative hypothesis suggested by Leddy et al. for *Mtb [30]*, it can be that *Leishmania* faces selective pressure to eliminate those epitopes that can be presented on MHCs by infected phagocytes, leaving only those epitopes that cannot be mutated without incurring other fitness costs. In addition, vaccination will confer "immunodominance" by increasing T cell frequency and may compensate for the lack of natural immunodominance. Hence, we argue that **immunoprevalent** MHC presentation in early infection is a more important criteria for antigen discovery pipelines.

The threshold for protection (or prevention of early parasite dissemination) may require recognition of a select subset of antigens presented during the early phases of infection. These are difficult to discover in treated patients, or even those untreated patients with long-term clinical presentation, as they are likely overshadowed with **immunodominant** antigens from effective parasite escape mechanisms or degraded parasites due to intracellular competition for MHC-loading. Hence, immunodominance hierarchies likely change from early to late infection, as also observed in many viral infections.[31,32] This may be further compounded by treatment-associated changes to the available antigenic repertoire compared to natural lysis of the parasite.

In addition, the antigen-specificity and immunogenicity of tissue-resident memory T cells (T_{RM}) in the skin might shed better light on natural MHC ligands in early infection compared to PBMCs used for all clinical antigen validation/discovery to date (**Figure 1**). Skin T_{RM} have been shown to be crucial for protection in mouse models by stopping further spread of the parasite in the skin/blood/organs.[33,34] Therefore, as antigen presentation and recognition is likely to differ strongly across tissues, we argue to study the cognate antigens behind the (protective) T_{RM}

populations in infected skin, spleen, bone marrow and lymph nodes biopsies/aspirates that mediates the concomitant immunity as an important step in guiding vaccine antigen selection.

The recently developed controlled human infection model (CHIM) for naturally transmitted *L. major*-infection might also present a solution to study natural antigen presentation (and vaccine-induced recognition) early during infection in an unbiased manner.

Embrace cutting-edge methodology to directly study clinical infection and overcome HLA restrictions

It is impractical to screen the large *Leishmania* proteome using donor-derived T cells and conventional assays (e.g. ELISPOT screenings). However, recent methodological advancements and analytical technologies such as immunopeptidomics[35], single-cell multiomics[36], T Cell Receptor (TCR) sequencing, DNA-barcoded pMHC multimers[37], reverse vaccinology and artificial intelligence, now enable large-scale immunogenicity screenings on limited human tissue samples (e.g. skin punch biopsies or fine needle aspirations).

Our recent immunopeptidomics analysis of lesions from Ethiopian CL patients showed that NetMHCpan prediction tools missed 20-70% of the naturally presented antigens, despite predicting millions of candidate peptides.[22] Moreover, predicated binding strength showed no correlation with actual presentation. These findings underscore the need for context-specific training data and caution in applying *in silico* tools, which currently do no capture the antigenic complexity illustrated in **Figure 2**. In addition, these tools also have restricted HLA alleles to screen for and lack dominant HLA alleles in endemic regions due to biased training data from the Global North.[38] Despite the above limitations, these current tools are highly valuable to narrow down candidates by predicting, amongst others, if prioritized antigens have highly **promiscuous binding capacity**, whether they are likely to be

immunoprevalent, or to discover T cell epitope-rich regions in antigens for multi-epitope vaccine construction.

Immunopeptidomics has seen a steep evolution in recent years, with inspiring *in vitro* examples from tuberculosis (TB)[30,39,40] and Chagas' disease[41] that identify immunoprevalent epitopes in early infection. This offers an unbiased way to map immunodominance and immunoprevalence of *Leishmania* antigens directly within infected tissue samples, as it can identify cryptic (or non-immunodominant) antigens as compared to T cell derived screens. It can additionally detect the effective length of *Leishmania* T cell epitopes and more robustly inform multi-epitope based vaccine constructs. Cognate T cells to those epitopes can then be identified with DNA-barcoded dextramers. Such dextramers allow the screening of 1000's of epitopes with as few as $1–2\times10^6$ human PBMCs and detect low avidity T cells at a frequency of 0.01%.[37] Similar high-throughput methods to link cognate antigens to their TCR sequences have been published (e.g. TetTCRseq).[42] Importantly, simultaneous analyses of i) T cell recognition to large panels of custom peptide-MHC barcoded dextramers, ii) paired TCRα and TCRβ chains, and iii) functional screening via transcriptomic and proteomic evaluations is now feasible in low-and-middle income settings, supported by device-free methodology and newer cell fixation protocols.[43]

With the rise of computational TCR prediction tools, it is now possible to investigate common features of TCRs (e.g. CDR3 regions) that are specific for a particular *Leishmania* epitope and identify determinants that may predict TCR specificity to a *Leishmania* antigen in a given TCR library.[44–46] Once generated, a sufficient large training database of *Leishmania*-specific peptide-MHC:TCR pairs would enable prediction of seen, and potentially unseen[47], *Leishmania* epitopes in more easily generated bulk TCR data sets of RNA-preserved blood or organ samples from well-described clinical cohorts in a HLA-agnostic manner. Recent advances in sequence similarity clustering and **convergence**

algorithms might even uncover potential groups of TCR clonotypes that are associated with *Leishmania* without costly multimer experiments.[45,48]

Together, this forms an affordable, high-throughput pipeline that holistically evaluates how large intracellular organisms influence epitope recognition and protective T cell immunity across all HLA types, while accounting for the disease complexity illustrated in **Figure 2**. Recently, using similar methodology and principles to timely discover high priority targets of TB vaccine development, it enabled the discovery of a small fraction of protective TCR repertoires (<1%) and their cognate antigens that were linked with control of TB infection.[49,50] One of the cognate epitopes of this TCR specificity group was identified among 27 Mtb-derived peptides generated with an *in vitro* immunopeptidomics screen, suggesting its relevance to identify the protective antigenic repertoire.[30] In parallel, Sun et al. reported that the $CD4^+$ T cells of these TB resisters showed enrichment of T_H17 and regulatory T cell-like functional programs, compared to *Mtb*-specific T cells from individuals with latent *Mtb* infection.[51]

It can not only provide insights into protective TCR clonotypes but also on distractopes, potentially by stage variation. These are well-described in a chronic CMV infection with many ineffective CMV-specific TCR clones, but also in TB where the TB10.4 antigen in specific was shown to be a dominant decoy antigen.[52] It is to be noted that *Leishmania* strains could be genetically closely related, but antigenically distinct in a similar manner as described for Dengue virus serotypes/strains.[53] Hence, discriminating the decoy from effective antigens driving organ dissemination (CL vs VL) could inform vaccine-induced attenuation of severe disease.

As there are currently no published TCR data sets for *Leishmania* antigens nor epitopes, we strongly recommend the research community to generate public and well-annotated TCR datasets to enable rapid TCR annotation and interpretation once trained tools become available. A library of *Leishmania*-

specific TCRs would also enable their tracing in a spatially-resolved manner, shedding more light on their protective or detrimental behaviour/functionality and/or restricted location.[54,55]

Antibody responses should not be overlooked

Although B-cell responses appear to play a limited role in protective immunity and may even exacerbate disease, their effector functions remain poorly understood.[56,57] *Leishmania* rapidly enters skin APCs, leaving little opportunity for antibody-mediated clearance.[58] Whether antibodies limit cell-to-cell transfer, and contribute to protection via DC maturation and antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), macrophage activation via antibody crosslinking of MHC molecules, cell lysis via complement-dependent cytotoxicity (CDC), or instead (and more likely) promote pathology through more efficient parasite internalization via antibody-dependent enhancement (ADE) as seen in Dengue virus infections[59], remains unresolved. Consequently, the relevance of antibody antigenicity in vaccine design is still uncertain.

Concluding remarks

In conclusion, a tailored *Leishmania* epitope discovery platform accounting for the complexity of antigen availability, as outlined in this viewpoint, is urgently needed to develop novel and effective vaccine candidates. This will help answer outstanding questions on the impact of timing and location of the infection on the protective antigenic repertoire, and may reveal influential distractopes underlying significant immune escape, for rational subunit vaccine design (see **Outstanding Questions**). Leveraging the integration of cutting-edge tools with the recent controlled human infection model and/or well-characterized clinical cohorts with public TCR repertoires has the potential to fundamentally advance antigen discovery for *Leishmania*. The establishment of an well-annotated pMHC:TCR library would provide a relatively quick avenue to gain crucial insights in antigen immunodominance and immunoprevalence hierarchies, as well as the role of MHC-I epitopes in

protection. Future research should also study if antibody-mediated mechanisms should be leveraged or minimized in vaccine strategies.

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Declaration of interests

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Glossary

Amastigote: The non-motile, intracellular stage of *Leishmania* found in mammalian host macrophages

Antigenic variation: The process by which pathogens alter their surface antigens to evade host immune responses, often through genetic recombination or gene expression changes.

Convergence algorithms: Computational methods that detect similar immune receptor sequences arising independently across individuals, indicating convergent immune responses

Cryptic antigens: Antigenic determinants that are normally hidden or poorly accessible to the immune system but can become exposed under certain conditions, potentially triggering immune responses.

Distractopes: decoy antigens that divert immune responses away from protective targets, reducing immune effectiveness

Immunogenic: Capable of inducing an immune response; refers to molecules (such as antigens or epitopes) that stimulate the activation of immune cells.

Immunodominance: The tendency of certain antigenic epitopes to elicit a stronger and more dominant immune response compared to others (determines epitope hierarchy)

Immunopeptidomics: mass spectrometry-based identification of MHC-eluted peptides of infected antigen-presenting cells

Immunoprevalence: The frequency with which an epitope is recognized across individuals in a population (determines epitope coverage or population-level recognition frequency)

Leishmanization: A cutaneous inoculation with live *L. major* parasites practiced during the mid-20th century that was discontinued due to safety concerns.

Promastigote: The motile, flagellated extracellular stage found in the sandfly vector

Promiscuous binding: the ability of certain peptides to bind to multiple MHC alleles due to flexible anchor residues (determines MHC-peptide interaction diversity)

T cell epitope: A short peptide fragment of an antigen presented by MHC molecules that is specifically recognized by a T cell receptor. These processed fragments are usually 8-11 amino acids and 13-25 amino acids long for MHCI and MHC II, respectively.

Figures

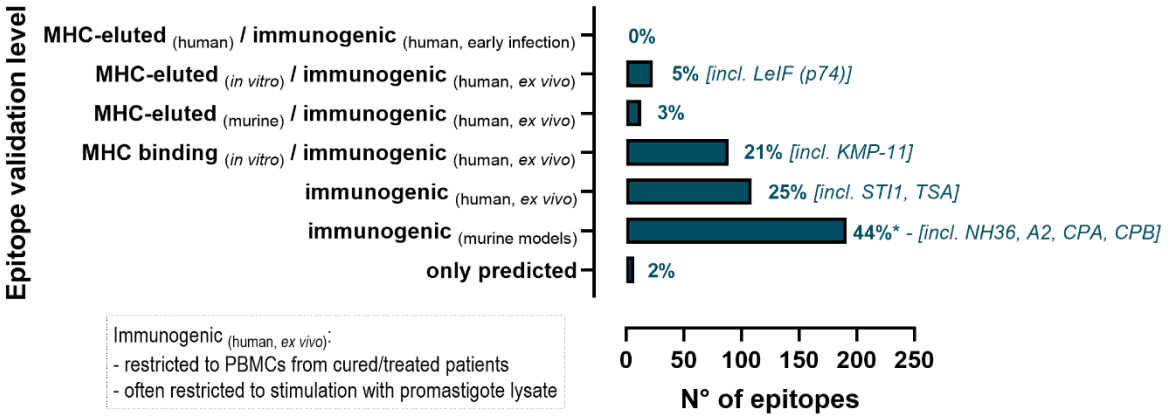

Figure 1. Evidence base for the IEDB-listed *Leishmania* T cell epitopes. Bar chart depicting the validation level of *Leishmania* T cell epitopes as registered on the immune epitope database (IEDB), ranging from most qualitative (top) to least qualitative (bottom). Bars represent the number of reported T cell epitopes, followed by their percentage from all 432 postulated T cell epitopes. The 10 antigens included so far in vaccine trials are listed next to their respective evidence level bar (with exception of HASPB and SMT that were not represented in IEDB). *15 epitopes of LdODC (Leishmania

donovani orthinine decarboxylase) and 3-ectonucleotidase antigens were subsequently retracted or flagged for image falsification.[60,61]

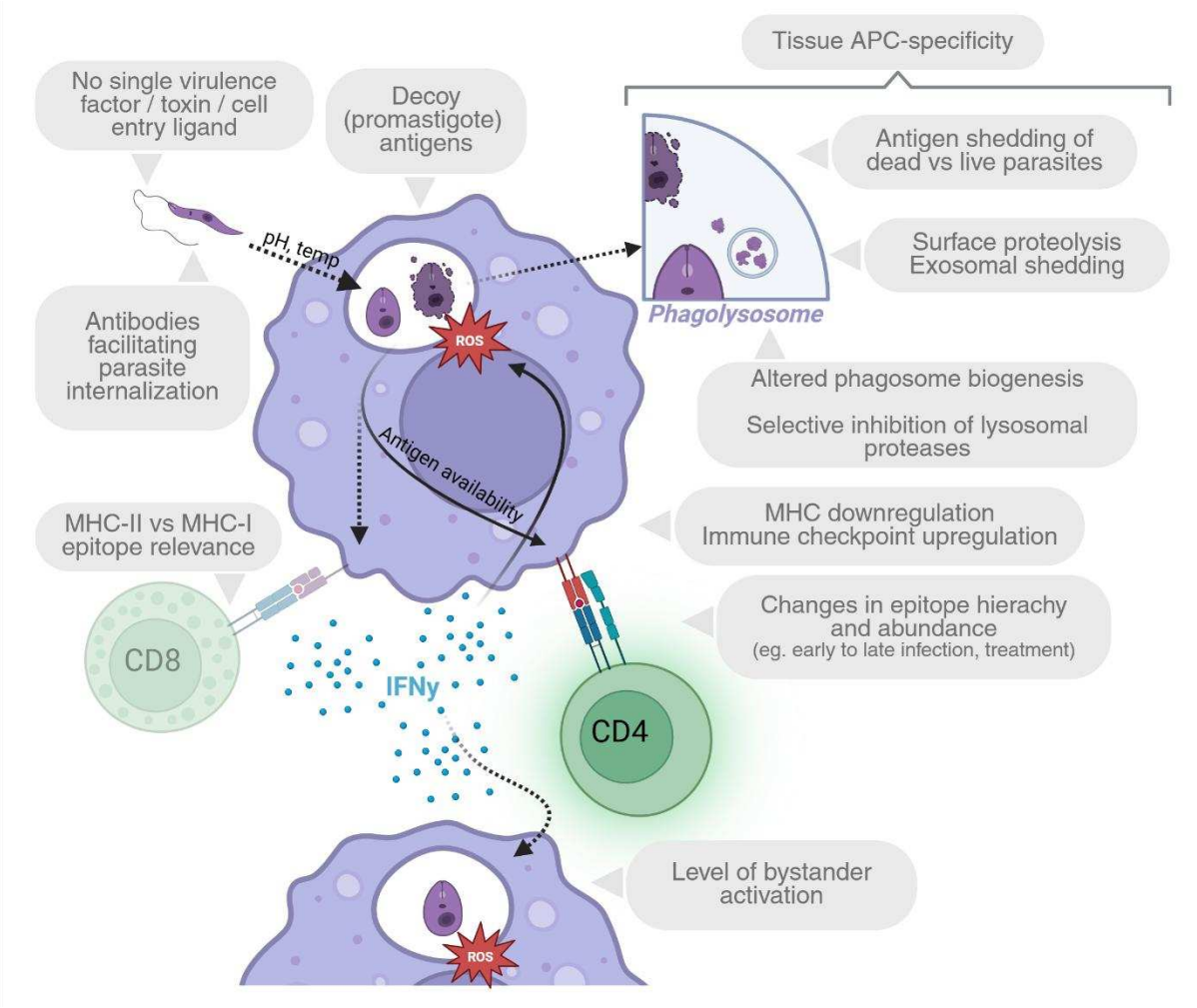

Figure 2. **Antigen accessibility and crypticity in *Leishmania* infection**. Visual representation of knowledge gaps (grey boxes) underlying the complexity of antigen accessibility and antigenic crypticity during *Leishmania* infection, highlighting the need for transformative research on T cell epitope discovery. The MHC-I/II-presented antigens will stimulate CD4+ T cells via a specific T cell receptor (TCR) resulting in IFN-γ production. The latter induces production of reactive oxygen species (ROS) in the phagolysosome to eradicate the intracellular parasite. However, little patient-derived information is available on issues of altered phagosome biogenesis/maturation, selective inhibition of lysosomal proteases, MHC downregulation or HLA-E retention (to evade natural killer cell detection), co-

stimulatory/inhibitory signaling, and the role of MHC-I epitopes in protective immunity.[62] Antigen presenting cells adapt their processing and presentation pathways to the microenvironment of each tissue, resulting in distinct antigen repertoires and MHC-peptide complexes that reflect local immune challenges. The stage variation process could also be an evasion mechanism by priming the host immune system with abundant but decoy promastigote-only antigens. Likewise, live parasites may have restricted accessibility of antigens (e.g. secreted, exosomal, surface-cleaved), but whether the distinction between antigens derived from dead versus live parasites is important to drive vaccine antigen selection is questionable.[5] Even in a non-treated infection, some dead parasites are to be found and may trigger bystander killing of live parasites in the same cell or in nearby infected cells, aiding further antigen presentation of antigens normally not released by live parasites. We do not know how early-to-late infection and treatment modulate epitope hierarchies and antigen crypticity. Created in BioRender. Adriaensen, W. (2026) https://BioRender.com/b78vw1g