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# Design and characterization of a multistage peptide-based vaccine platform to target *Mycobacterium tuberculosis* infection

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Tuberculosis (TB) is the second leading cause of death by a single infectious agent worldwide. The Bacille Calmette-Guérin (BCG), the only available vaccine against TB, provides inconsistent protection against pulmonary TB in adults, the primary source of disease transmission. Thus, a more effective vaccine is urgently needed to stop the TB epidemic. The development of a new vaccine is hindered by the complex immunopathology of *Mycobacterium tuberculosis* (*Mtb*), especially in eliciting protection against both active and latent stages of infection.

Multistage subunit vaccines, which incorporate antigens expressed in both phases, are designed to address different stages of a pathogen's life cycle or diverse aspects of the immune response in one construct. This strategy may be particularly advantageous when dealing with complex pathogens characterized by high antigen variability or propensity for mutagenesis, such as *Mtb*. However, their main limitation is that they often elicit a less robust immune response compared to traditional vaccines. In addition, they may face challenges related to bioavailability, including rapid clearance or undesired post-injection degradation. Consequently, developing subunit vaccines requires a well-designed formulation, incorporating immunostimulatory adjuvants and a delivery system to address these limitations [**1**].

**Epitope selection:**
A set of validated T cell epitopes was chosen from the available literature and synthesized using solid-phase peptide synthesis. After the screening, the 4 epitopes contained in the peptide mixture PM1 were selected for the inclusion in the vaccine platform.

***Sequence selection workflow for multi-epitope vaccine antigens***

1. **Database Research**
   Selection of MHC I and MHC II-restricted validated epitope peptides expressed in the active or latent TB stages.
2. **Epitope Peptides Synthesis**
   Solid Phase Peptide Synthesis (SPPS) - Fmoc/$^{t}$Bu strategy
3. **Cellular Uptake in Antigen-Presenting Cells (APCs) Model**
   Internalization efficiency
4. **Epitope Peptides Pools**
   The epitope peptides selected from the preliminary screening were pooled in two peptide mixtures (**PM1** and **PM2**)

**PM1**

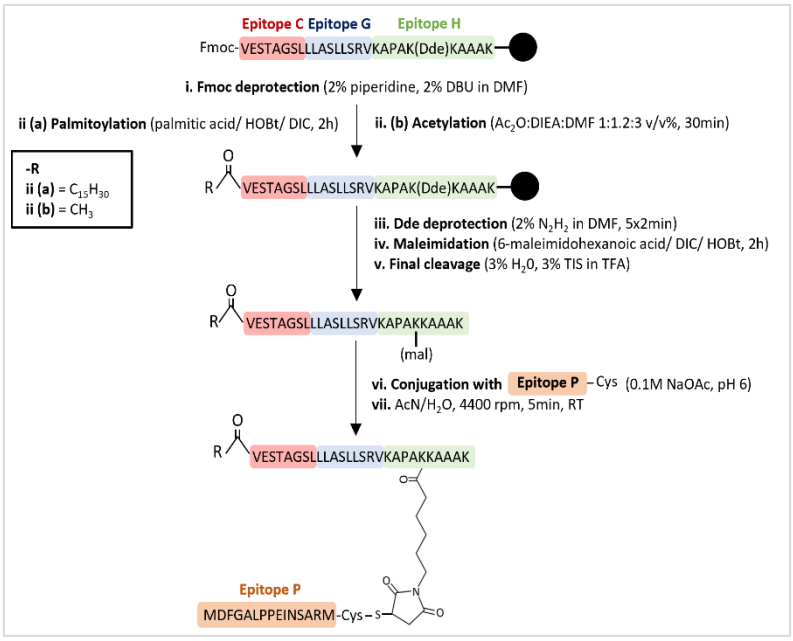
| Gene name | Epitope name | Stage | MHC |
|---|---|---|---|
| **Rv3874** | $CFP10_{32-39}$ | Active | MHC I |
| **Rv3908c** | $GlfT2_{4-12}$ | Active | MHC I |
| **Rv0475** | $HBHA_{185-194}$ | Latent | MHC I and MHC II |
| **Rv1039c** | $PPE15_{1-15}$ | Latent | MHC II |

**PM2**

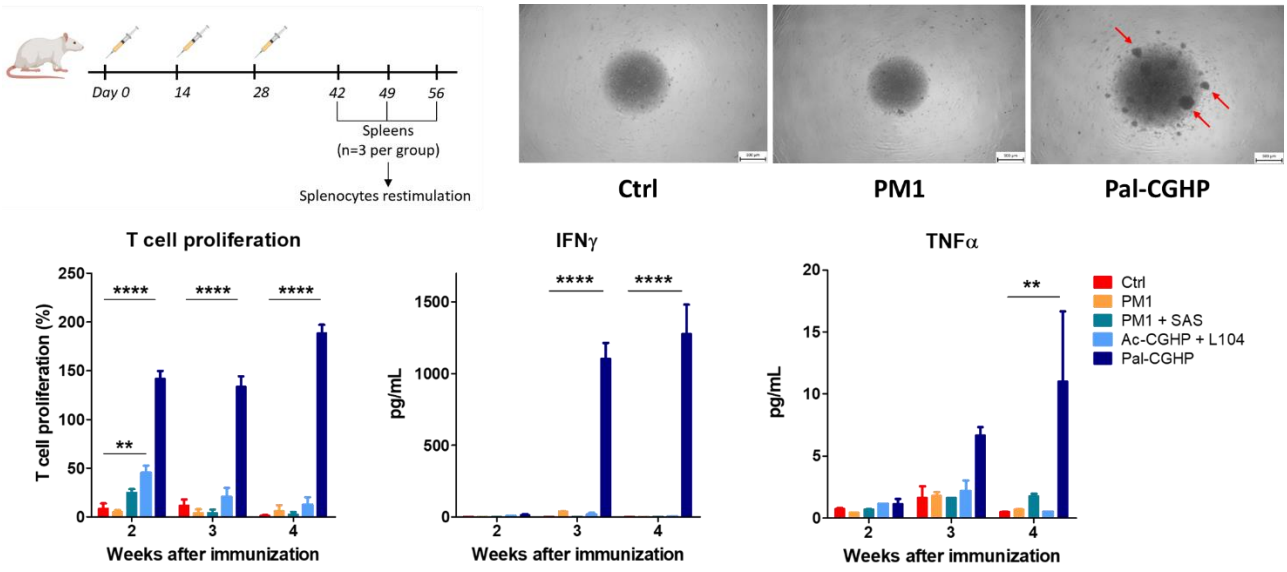
| Gene name | Epitope name | Stage | MHC |
|---|---|---|---|
| **Rv3874** | $CFP10_{32-39}$ | Active | MHC I |
| **Rv1886c** | $Ag85B_{41-48}$ | Active | MHC I |
| **Rv0125** | $Mtb32a_{309-318}$ | Latent | MHC I |
| **Rv0341** | $IniB_{33-45}$ | Latent | MHC I and MHC II |

5. **Antigenicity**
   Antigen-recall assay on human PBMCs from *Mtb*-sensitized donors

**Lipopeptide-based vaccine platform:**
The peptides included in PM1 were used to synthesize the peptide-based vaccine platforms. $CFP10_{32-39}$, $GlfT2_{4-12}$, and $HBHA_{185-194}$ were combined in one linear peptide chimera, which was derivatized with a maleimide moiety by orthogonal reaction. The $PPE15_{1-15}$ was elongated with a cysteine residue at the *C*-terminal and then conjugated to the linear peptide through the thiol-maleimide reaction. The *N*-term of the peptide chimera was either acetylated or palmitoylated to obtain the final branched conjugates (Ac-CGHP and Pal-CGHP). Palmitoylation previously demonstrated to improve the cellular uptake and the immunogenicity of peptide-based vaccines [**2**].




**Immunological evaluation.**
The Pal-CGHP conjugate, the peptide mixture PM1 in PBS, and PM1 formulated with the Sigma Adjuvant System (SAS) were administered subcutaneously to BALB/c mice three times, two weeks apart. We then measured antigen-specific splenic T cell proliferation and cytokine secretion among immunised and unimmunised animals. As expected, PM1 did not produce a noticeable immune response, likely due to intrinsic poor immunogenicity and *in vivo* stability of short synthetic peptides. Pal-CGHP immunization produced significant T cell proliferation, which slightly increased over time, with the highest levels observed four weeks post-immunization. Similarly, IFNγ and TNFα concentrations followed a time-dependent increase. In contrast to the SAS-formulated candidate, Pal-CGHP induced significant IL-22 and IL-10 production but no IL-17 response. When splenocytes from Pal-CGHP immunized mice were restimulated with the individual C/G/H/P peptides, bearing the original sequences, epitope-specific antigenicity was observed, suggesting that their chemical modification occurred in the conjugation did not compromise the epitope recognition. Thus, the results suggest that Pal-CGHP is more promising than the two tested candidates and it merits further investigation as a multi-epitope self-adjuvating vaccine platform [**3**].

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