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A matter of delivery: nanocarriers and the engineering of protective immunity in tuberculosis vaccination

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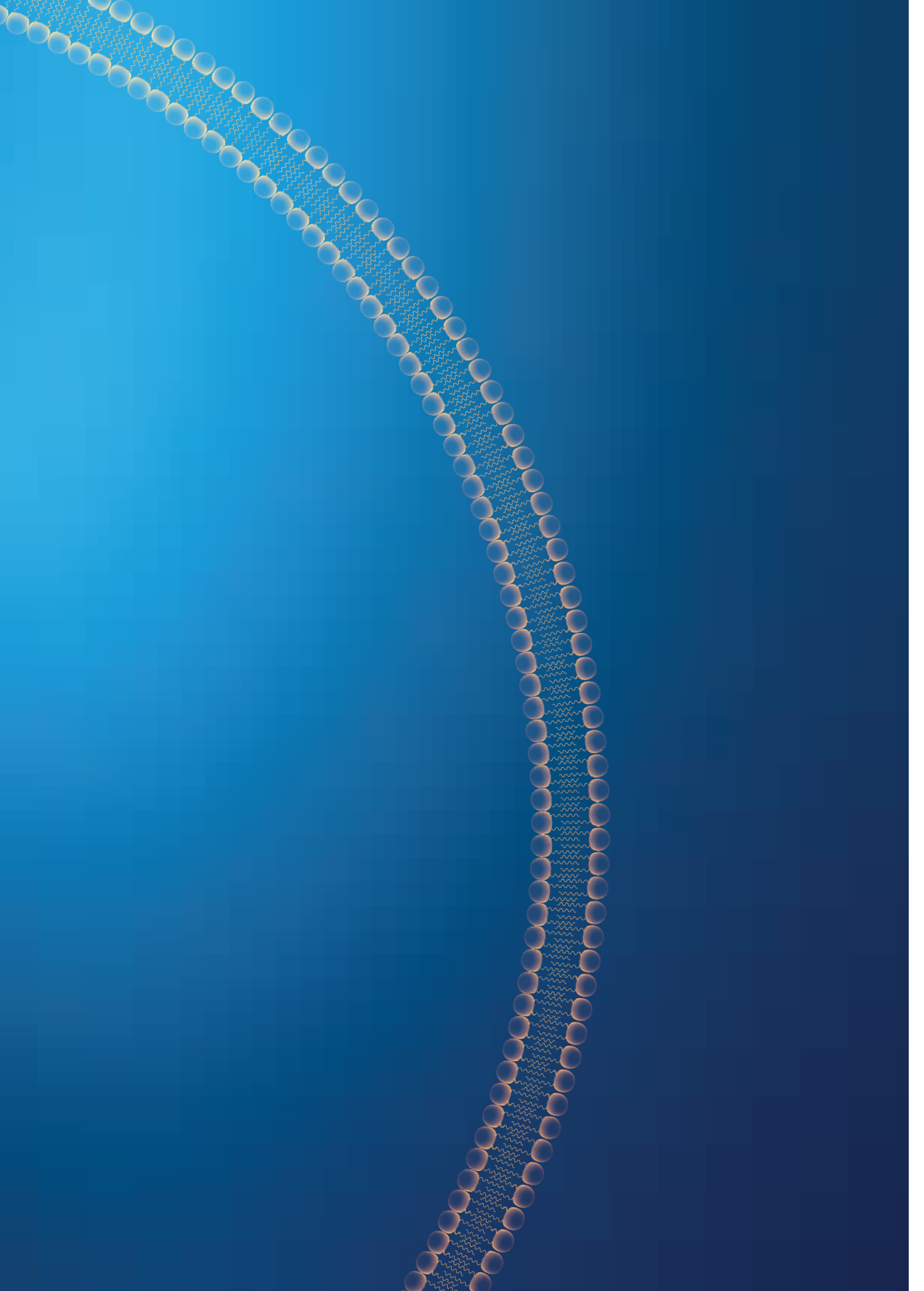
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CHAPTER 7

General discussion and future perspectives

1. INTRODUCTION: THE URGENT NEED FOR NEW TB VACCINES

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (Mtb), remains one of the most devastating infectious diseases globally. In 2023, TB regained its position as the leading cause of death from a single infectious agent, surpassing the annual toll of the COVID-19 pandemic. According to the World Health Organization (WHO), approximately 10.8 million people developed TB in 2023, and 1.25 million died from the disease, including 161,000 individuals co-infected with HIV. Furthermore, about 400,000 individuals developed multidrug-resistant or rifampicin-resistant TB (MDR/RR-TB), significantly complicating treatment options and outcomes.¹ These numbers not only highlight the persistent global burden of TB but also underscore the urgent need for more effective public health interventions, especially early diagnosis, host-directed approaches that are not affected by rising drug resistance, like vaccines.

TB disproportionately affects populations in low- and middle-income countries and is closely linked with poverty, undernutrition, and weakened healthcare systems. Moreover, individuals living with HIV are particularly vulnerable due to their compromised immunity, and TB remains the leading cause of death among people with HIV. The global situation is further complicated by the high prevalence of latent TB infection, estimated to affect about a quarter of the world population, posing a vast reservoir for future disease.^{1,2}

The Bacillus Calmette-Guérin (BCG) vaccine, derived from *Mycobacterium bovis*, remains the only approved vaccine against TB. While BCG provides reliable protection against severe pediatric TB forms, such as miliary and meningeal TB, its efficacy in preventing pulmonary TB in adolescents and adults is highly variable and generally poor.³ This limited efficacy, combined with BCG's unsuitability for use in immunocompromised populations and its inability to prevent reactivation from latent TB infection, underscores the critical need for improved vaccination strategies.

To meet the WHO's End TB Strategy goals and the United Nations' Sustainable Development Goals, there is an urgent demand for next-generation TB vaccines that are safe, effective in all age groups, affordable, and suitable for use in resource-limited settings.⁴ The recent successes of novel vaccine platforms, including protein subunits, mRNA, and viral vectors, have reinvigorated TB vaccine research. In



particular, subunit vaccines are emerging as highly promising candidates due to their safety profile and the possibility of rational antigen selection.^{5,6} When combined with advanced delivery systems such as nanoparticles (NPs), which enhance antigen stability, cellular uptake, and immune activation, these vaccines may overcome the limitations of both BCG and first-generation subunit formulations.^{7,8}

This thesis explores the design and evaluation of NP-based subunit vaccine formulations targeting TB, with a focus on enhancing immunogenicity through optimized antigen delivery. In the context of growing antimicrobial resistance and high global comorbidity with HIV, the development of safe and potent vaccine platforms is more critical than ever. This work aims to contribute to this urgent global challenge by investigating novel delivery strategies that could improve TB vaccine efficacy and offer broader insights for future vaccine development.

2. LIMITATIONS OF BCG AND THE CURRENT TB VACCINE DEVELOPMENT LANDSCAPE

Despite being used for over a century, the BCG vaccine remains inadequate for controlling TB, particularly adult pulmonary forms of the disease that drive transmission. Its protective efficacy ranges from 0 % to 80 %, with most studies in high-burden countries reporting poor effectiveness in adolescents and adults.³ This variability is attributed to multiple factors, including geographic differences, environmental mycobacterial interference, and host genetic background. Importantly, BCG does not reliably protect against reactivation of latent TB infection or multidrug-resistant TB, and it cannot be safely administered to immunocompromised individuals, such as those living with HIV.^{3,9}

In response to the limitations of BCG, significant efforts have been made to develop improved TB vaccines. These include a range of technological platforms, each with distinct advantages and challenges:

- **Live-attenuated vaccines:** These aim to improve upon BCG by retaining its ability to stimulate robust immune responses while enhancing antigen expression or attenuating virulence.¹⁰ Examples include VPM1002 and MTBVAC, both in advanced clinical trials.¹¹ Safety concerns, particularly in immunocompromised populations, remain a possible limitation.¹⁰

- **Whole-cell inactivated vaccines:** These formulations use killed mycobacteria, such as *Mycobacterium obuense* (DAR-901) or *Mycobacterium indicus pranii* (Immuvac), to stimulate broad immune responses.¹¹ While generally safe, their immunogenicity is typically weaker, requiring potent adjuvants and repeated administration.¹²
- **Subunit vaccines:** These consist of defined antigens, typically proteins or peptides, combined with adjuvants. Their safety and flexibility make them attractive, especially for vulnerable populations. However, they often suffer from low immunogenicity unless integrated into effective delivery systems.¹³ Notable candidates include M72/AS01E, which showed ~50 % efficacy in preventing progression from latent TB infection in a phase IIb trial.¹⁴ Despite growing interest, there are still relatively few adjuvants and delivery systems in late-stage clinical development for TB subunit vaccines. This gap significantly contributes to the translational bottlenecks in the field.¹⁵
- **Viral vector-based vaccines:** These use replication-deficient viral platforms such as adenoviruses or Modified Vaccinia Ankara (MVA) to deliver TB antigens. They elicit strong T-cell responses and are suitable for prime-boost regimens.¹⁶ Candidates include ChAdOx1.85A and MVA85A.¹¹ However, there are safety concerns, including rare but serious adverse effects, as seen in other viral vector-based vaccines, and some vectors face pre-existing immunity in target populations, which can limit efficacy.¹⁷
- **mRNA vaccines:** Building on the success of COVID-19 vaccines, mRNA-based TB vaccine candidates such as BNT164a1 and BNT164b1 (developed by BioNTech) are currently in early-phase clinical trials.¹⁸ These vaccines offer rapid development timelines and flexibility in antigen design. Nonetheless, this approach is in its infancy for TB, and there are still many unknowns regarding how to design mRNA vaccines that are optimized for TB-specific immune responses. More broadly, TB vaccinology remains challenging due to the complex host-pathogen interactions and the lack of well-defined correlates of protection.¹⁹

Overall, the current TB vaccine pipeline is more diverse and active than ever before. However, no new vaccine has yet replaced or supplemented BCG in routine immunization programs. Continued innovation in antigen selection, formulation,



and delivery is necessary to meet the unique immunological and logistical demands of TB vaccination, particularly in the most affected regions. The research presented in this thesis contributes to this evolving field by investigating NP-based delivery strategies that could enhance the performance of subunit vaccines and offer scalable, safe, and effective solutions for global TB control.

3. RESEARCH RATIONALE AND STUDY OBJECTIVES

While the global TB vaccine pipeline has expanded to include diverse technological platforms, the development of effective subunit vaccines remains constrained by a lack of optimized antigen delivery systems.^{8,15,20} Subunit vaccines are among the safest and most adaptable platforms, particularly suited for use in immunocompromised populations and regions with high HIV prevalence. However, their clinical translation has been hindered by inherently low immunogenicity, which necessitates sophisticated delivery systems to achieve robust, durable immune responses. Despite the promise of NPs in enhancing antigen stability, cellular uptake, and immune activation, few NP-based formulations for TB vaccines have advanced beyond early-stage research.^{8,15} Compared to the fields of oncology or COVID-19, the TB field still suffers from a significant lag in delivery system innovation and characterization.

This thesis directly addresses this critical bottleneck. It contributes a systematic investigation into how distinct NP platforms, specifically cationic liposomes, PLGA NPs, and hybrid lipid–PLGA systems, can be tailored to improve subunit TB vaccine performance. While both liposomes and PLGA NPs have been individually studied in other biomedical areas, few comparative studies exist within TB vaccinology that examine their relative efficacy, mode of action, and compatibility with rationally designed fusion protein antigens. This work is among the first to perform a side-by-side analysis of these platforms using a consistent antigen and harmonized evaluation criteria.

The antigen used throughout this research Ag85B-ESAT6-Rv2034 (AER), is a rationally selected fusion protein that combines three Mtb-derived antigens known to induce strong T-cell responses.^{21–23} By anchoring the delivery system development to this antigen, the thesis ensures biological relevance and translational potential, particularly since AER has already demonstrated protective efficacy in preclinical models.

This research explores several under-investigated aspects of NP design in TB vaccine development. First, it examines the influence of different cationic lipids on immunogenicity, an area where most studies have focused merely on charge, overlooking the potential immunomodulatory effects of specific lipid chemistries.²⁴ Second, it develops and characterizes pH-sensitive liposomes, which may facilitate improved antigen release in the acidic endosomal compartments of APCs, yet have rarely been tested in TB models.²⁵ Third, the study introduces a hybrid NP system combining lipid and polymer components to synergize the advantages of both platforms, namely, the endosomal escape capacity of the lipids and the structural rigidity and controlled release of PLGA.²³

All formulations were tested in murine models using both common subcutaneous and alternative intradermal routes of immunization, with subsequent challenge by the virulent Mtb H37Rv strain delivered intranasally. This approach not only mirrors the pulmonary route of natural infection but also enables rigorous assessment of protective efficacy and immune correlates.²⁶ The combination of intradermal and subcutaneous vaccination strategies aligns with a growing interest in heterologous prime-boost regimens, which may be essential for achieving sterilizing immunity in TB.²⁷

By integrating antigen design with state-of-the-art delivery systems and *in vivo* efficacy testing, this thesis positions itself at the interface of fundamental immunological research and translational vaccine development. It complements existing clinical efforts by addressing a neglected area of the TB vaccine field, the delivery science, and proposes NP-based solutions that could accelerate the development of potent, scalable, and safe vaccines not only for TB, but also for other intracellular pathogens requiring robust cellular immunity.

4. SUMMARY OF THE RESEARCH OF THIS THESIS

To achieve effective immune activation against Mtb, this thesis systematically explored how different NP-based platforms can improve immunogenicity, promote antigen-specific T-cell responses, and ultimately offer protection in preclinical TB models. By synthesizing the insights from both *in vitro* and *in vivo* studies, the study provides a comprehensive overview of how formulation chemistry, adjuvant co-delivery, particle characteristics, and administration routes collectively shape vaccine efficacy. The summary below highlights how these variables influenced antigen



presentation, immune polarization, memory formation, and protection outcomes, and reflects on their broader implications for the development of next-generation TB vaccines.

In the research described in **Chapter 2**, a broad panel of cationic liposomes was screened to evaluate how different cationic lipids and cholesterol content affect human APC activation and T-cell priming. This study showed that liposomes based on DOTAP and EPC, especially those with cholesterol, exhibited enhanced uptake by dendritic cells and stimulated superior antigen-specific CD4⁺ T-cell activation *in vitro*. Importantly, the findings highlighted that the immunostimulatory effects of cationic lipids go beyond their net charge and involve structural determinants that modulate membrane rigidity and immune cell engagement. This chapter underscored the significance of careful lipid selection in designing effective vaccine carriers and established DOTAP- and EPC-based formulations as promising candidates.

Building on these findings, in **Chapter 3**, the development and *in vitro* assessment of pH-sensitive liposomes designed to destabilize under acidic endosomal conditions and promote cytosolic antigen release is discussed. These formulations, particularly DOPC:DOPE:DOBAQ:EPC liposomes, showed favorable uptake by APCs and induced robust activation of human MDDCs, leading to efficient presentation to antigen-specific T-cells. The superior cytokine and chemokine induction observed for this pH-sensitive formulation marked it as a promising vector for cross-presentation and CD8⁺ T-cell priming—an essential but often elusive goal in TB subunit vaccine design.

In the study described in **Chapter 4**, the immunological potential of the optimized pH-sensitive liposomes was validated in a murine TB challenge model. When co-loaded with CpG and MPLA adjuvants, the DOPC:DOPE:DOBAQ:EPC liposomes induced potent polyfunctional CD4⁺ and CD8⁺ T-cell responses, increased activation of IL-17A-producing B- and T-cells, and significantly reduced Mtb bacterial burden in the lungs and spleens. Notably, the vaccine achieved protection at antigen and adjuvant doses much lower than those used in the soluble formulation. These findings provided *in vivo* confirmation that delivery system design can critically influence vaccine potency, antigen dose efficiency, and immune polarization.

In **Chapter 5**, the immunological performance of three nanoparticle platforms: PLGA NPs, pH-sensitive liposomes, and lipid-PLGA hybrid NPs, was compared in a head-to-head preclinical study. All three AER-loaded NP vaccines significantly

reduced Mtb burden in the lungs and spleens compared to the antigen-adjuvant mix, which conferred no protection. Although no statistically significant differences were observed between the NP groups, a consistent trend emerged: PLGA NPs showed the greatest median CFU reduction, exceeding BCG by one logarithmic unit, followed by hybrid NPs, then liposomes. This suggests a potential superiority of PLGA-based systems in mediating protective efficacy. Interestingly, immunological data did not fully correlate with protection. The liposomal formulation, which induced the weakest protection, paradoxically generated the highest abundance of T-cell responses, while the antigen-adjuvant mix elicited similar T-cell levels to PLGA NPs but did not confer protection. These findings imply that the magnitude of conventional immune readouts does not directly predict protection, highlighting the unresolved complexity of immune correlates in TB. Similar observations were made for B-cell responses and antibody titers, which did not align with protection outcomes. This chapter underscored the need for better understanding of the qualitative features of vaccine-induced immunity that are mechanistically linked to protection, rather than relying solely on the quantitative expression of specific immune markers.

The study described in **Chapter 6** assessed how the route of administration influences immune responses to a hybrid NP-based TB vaccine. Using intradermal (i.d.) delivery of the lipid–PLGA hybrid NP formulation, this study demonstrated superior induction of polyfunctional CD4⁺ and CD8⁺ T-cells, central memory-like phenotypes, and stronger B-cell activation compared to the conventional subcutaneous (s.c.) route. Moreover, i.d. immunization led to significantly higher AER-specific antibody titers and class switching toward IgG2a. These findings align with the increasing recognition of dermal APC richness and dose-sparing advantages of i.d. vaccination, suggesting that strategic delivery routes can further amplify the benefits conferred by NP-based vaccines.

Together, these five chapters demonstrate how careful modulation of NP formulation parameters, lipid composition, pH-sensitivity, polymer-lipid hybridization, adjuvant co-delivery, and administration route can shape the immune response to subunit TB vaccines in distinct and sometimes unexpected ways. While strong immunogenicity was necessary, it was not sufficient to predict protection, emphasizing the need for more refined and/or additional immunological correlates in TB vaccine research, including e.g. innate immunity markers and cell types such as NK cells.



Ultimately, this thesis contributes to a growing body of evidence that immune engineering through tailored NP design can overcome longstanding limitations in TB subunit vaccine development. It lays the groundwork for future work to identify correlates of protection, refine delivery strategies for clinical use, and extend these technologies to other intracellular pathogens requiring strong cell-mediated immunity.

5. KEY INSIGHTS

Bringing together the findings from all experimental chapters, several key take-home messages emerge that go beyond the scope of individual formulations or isolated results. While each chapter addressed specific research questions, several consistent themes and integrative insights became evident across the thesis. These cross-cutting observations highlight the broader scientific relevance of this work and help position it within the larger field of TB vaccine research and NP-based immunization strategies.

1. Delivery System Matters More Than Antigen or Adjuvants Alone

A consistent pattern observed throughout this work is that the mode of antigen delivery, whether via liposomes, PLGA NPs, or hybrid systems, has a decisive impact on both the immunological and protective outcomes. Formulations in which the same antigen (AER) and adjuvants (CpG and MPLA) were delivered in solution failed to confer protection, despite inducing T- and B-cell responses similar in magnitude to some NP-based vaccines. In contrast, all NP-based vaccines significantly reduced bacterial loads *in vivo*. This indicates that antigen delivery vehicles do more than enhance uptake; they fundamentally shape how antigens are processed and presented, which downstream immune pathways are activated, and ultimately, whether protective immunity is achieved.

2. Immune Magnitude Does Not Equate to Immune Efficacy

A second important insight is the dissociation between the magnitude of immune responses and actual protective efficacy. For instance, cationic liposomes often induced the highest T-cell abundances *in vivo* but consistently offered the weakest protection in challenge models. Meanwhile, PLGA NPs, which triggered similar or even lower T-cell frequencies, outperformed liposomes in bacterial clearance, even exceeding the licensed standard BCG vaccine in some instances. These discrepancies

suggest that qualitative aspects of the immune response, such as spatial localization, cellular phenotype, antigen persistence, or functional potential, are more important determinants of protection than bulk measurements of immune magnitude alone.

3. NP Properties Dictate Immune Quality and Protection

Physicochemical properties of NPs, including surface charge, hydrophobicity, rigidity, and particle size, emerged as powerful modulators of immune function. The depot-forming behavior of cationic lipid-containing NPs favors prolonged antigen retention at the injection site, potentially supporting extended antigen presentation.^{28,29} Meanwhile, the smaller, negatively charged PLGA NPs likely drained efficiently to lymph nodes, facilitating a different mode of immune priming.^{8,30} These formulation-dependent differences not only influenced DC activation and T-cell polarization but also correlated with distinct patterns of bacterial control. Importantly, the type of NP appeared to direct not just *how much* of an immune response was generated, but *what kind* of response predominated.

4. Protection Is Not Driven by a Single Cell Type or Marker

Another cross-cutting finding is that protection could not be linked to a singular immune cell type, cytokine, or antibody isotype. While polyfunctional CD4⁺ and CD8⁺ T-cells were consistently observed following NP vaccination, and are widely considered correlates of protection, they were also present in non-protected groups. Similarly, high levels of IgG2a antibodies, often associated with Th1-biased immunity, did not guarantee bacterial clearance. Even B-cell activation markers, which correlated with strong immunogenicity, failed to map clearly with protection outcomes. These results align with the broader consensus that TB immunity is multifactorial and likely requires a constellation of immune features rather than a single hallmark biomarker.

5. pH-Sensitive and Biodegradable Carriers Enable Dose Sparing

The incorporation of pH-sensitive liposomes or biodegradable polymeric NPs enabled substantial dose reduction without compromising efficacy. Vaccines using these delivery platforms were able to reduce the required amount of antigen and adjuvants by several folds while still inducing robust immunity and protection. This



finding has important translational implications for scalable vaccine manufacturing, cost-effective distribution, and dose-sparing strategies, especially in settings where vaccine accessibility remains a challenge.

6. I.d. Administration Enhances Immune Efficiency

Finally, i.d. delivery of hybrid NPs demonstrated superior immunogenicity over s.c. administration, especially considering very low doses of antigens, producing higher frequencies of polyfunctional T- and B-cells and increased antibody titers at reduced doses. This highlights the underutilized potential of i.d. immunization routes and supports ongoing efforts to develop minimally invasive, self-administered, or needle-free delivery systems, particularly relevant for global TB vaccine roll-out.

Conclusion

Taken together, these cross-cutting insights underscore the importance of delivery system design in determining not just immune response magnitude, but also its quality, durability, and protective potential. They also reinforce the central premise of this thesis: that rational engineering of NP-based vaccine platforms, coupled with systematic comparative evaluation, offers a powerful strategy to overcome the immunological challenges posed by TB and other intracellular pathogens. Future work should continue to explore the mechanistic basis of NP-driven immunity, optimize the route of vaccine delivery, and refine correlates of protection, paving the way for the next generation of effective TB vaccines.

6. BROADER IMPLICATIONS FOR TB VACCINE AND DELIVERY SYSTEM DEVELOPMENT

Beyond generating formulation-specific findings, this thesis provides strategic insights that may inform the broader field of vaccinology, particularly in TB but also extending to other infectious and immune-related diseases. The integrated research pipeline developed here offers a structured approach to early-stage vaccine development. This framework enhances the predictive power of preclinical studies and can be adapted across delivery technologies and disease contexts.

Importantly, several key implications emerged that go beyond the immediate study objectives:

1. For Vaccine Design and Immunology

- **Immune Magnitude $\neq$ Protection:** The consistent disconnect between the magnitude of immune responses (e.g., IFN γ -producing T-cells or high antibody titers) and actual protection challenges long-held assumptions in TB vaccine design. Our findings reinforce the need to identify immune responses with functional relevance, such as tissue-localized memory, recall capacity, or antigen persistence sensing, rather than relying on standard peripheral readouts.
- **Polyfunctionality Is not Predictive Alone:** Despite liposomes inducing the strongest polyfunctional CD4⁺ and CD8⁺ T-cell responses, they provided the weakest protection. This decouples polyfunctionality from protection and emphasizes the complexity of protective immunity in TB.
- **Delivery Platform Modulates Mechanism:** The fact that NP-based vaccines induced distinct immune profiles compared to BCG or antigen-adjuvant mixtures suggests that the delivery system actively shapes the quality and trajectory of the immune response. This finding supports a more mechanistically tailored approach to vaccine design, where innate training, mucosal targeting, or antigen persistence can be modulated intentionally.

2. For NP and Adjuvant Technology

- **Formulation as an Immunoengineering Tool:** Even minor chemical modifications, such as swapping DOTAP for EPC or altering cholesterol content, led to significant shifts in uptake, activation, and toxicity. This supports the view of NP formulation as a highly tunable lever for shaping immune responses.
- **Dose Sparing and Antigen Economy:** NP-based systems achieved protection with significantly reduced antigen and adjuvant doses. This not only enhances safety and feasibility for global immunization campaigns, but also addresses one of the main hurdles in TB vaccinology: the need for scalable, cost-effective solutions.



- **pH-Sensitive Systems Beyond Oncology:** While often used in cancer therapy, this work demonstrates that pH-sensitive liposomes can also be effective in infectious disease contexts, supporting the relevance of endosomal escape, efficient cross-presentation, and CD8⁺ T-cell activation. This may open new avenues for applying these systems to pathogens that require strong cytosolic responses.

3. For TB Research and Correlates of Protection

- **Need for New Biomarkers:** Traditional readouts such as IFN γ levels or polyfunctional T-cells did not consistently correlate with protection. This highlights the pressing need for better-defined biomarkers, potentially involving cell localization, metabolic state, or non-classical immune subsets.
- **Focus on Underexplored T-Cell Subsets:** CD4⁺ and CD8⁺ T-cells with a CD44^{lo} CD62L^{hi} phenotype emerged repeatedly in our studies as being enriched in protected animals. These may represent a functionally superior central memory subset and warrant focused investigation in future research.
- **Emerging Role of B-Cells:** We observed IL-17A- and TNF α -producing B-cell subsets, supporting a more nuanced role for B-cells in TB. These findings contribute to growing evidence that B-cells should be included in mechanistic models of protection and correlates of efficacy.

4. For Vaccine Delivery and Clinical Translation

- **Undervalued Potential of I.d. Route:** I.d. delivery yielded superior T- and B-cell responses and improved antibody subclass switching compared to the subcutaneous route, using lower doses. This suggests broader application of i.d. administration in next-generation TB vaccines, particularly in resource-constrained settings.
- **Delivery Can Rescue Antigen Performance:** The AER antigen provided no protection when delivered in solution with adjuvants, yet conferred significant protection in NP formulations. This reaffirms that antigen efficacy is not solely intrinsic but can be unlocked through optimized delivery.

- **Translatable to Other Pathogens:** The platforms and principles validated here, including cationic liposomes, pH-sensitive systems, and hybrid PLGA-lipid NPs, are readily adaptable to vaccines against other intracellular pathogens (e.g., Leishmania, HIV, Chlamydia) or even therapeutic cancer vaccines requiring potent cytotoxic responses.

5. For Experimental Design and Preclinical Strategy

- **Head-to-Head Comparisons Clarify Value:** Conducting standardized, parallel comparisons of different formulations, delivery systems, and administration routes allowed for robust identification of performance trends. This contrasts with many fragmented studies in the field and strengthens decision-making for further development.
- **Early Human-Relevant Screening Increases Predictive Value:** By incorporating human MDDC and T-cell models early in the pipeline, we enhanced the translational relevance of our candidate selection. This step could improve the efficiency and accuracy of preclinical screening in other vaccine pipelines.

A key contribution of this work is the stepwise framework developed for the design, optimization, and evaluation of NP-based vaccines:

1. Rational selection of formulation components, including antigens, lipids, polymers, and adjuvants, based on their known or hypothesized immunological properties and physicochemical compatibility.
2. Formulation development and optimization, guided by physicochemical characterization (e.g., size, charge, stability) and human in vitro immunological readouts. These assays, including APC uptake, activation, cytokine production, and T-cell stimulation, allow for early screening of immunopotency in a human-relevant system.
3. Iterative refinement, where suboptimal candidates are reformulated, e.g., by adjusting lipid composition or adjuvant content, and re-assessed to enhance desired characteristics, particularly those related to immune activation and cellular viability.



4. In vivo evaluation in a relevant small animal model, focusing not only on bacterial load reduction but also on dissecting the nature of the induced immune response, including CD4⁺, CD8⁺, B-cell, and memory subsets, to explore potential mechanisms of protection.
5. Comparative studies across multiple delivery systems, routes of administration, or dosing regimens to determine the most promising configuration for translation to more advanced models, such as guinea pigs or non-human primates, and to inform the design of early-phase clinical trials.

Together, these insights argue for a shift in focus across the vaccine development pipeline – from antigen discovery alone to a more holistic strategy that incorporates immuno-engineering, delivery and adjuvant science, and immunology. This work contributes both concrete data and methodological scaffolding to guide the development of next-generation vaccines, particularly those targeting difficult pathogens such as Mtb. The tools and lessons developed here can inform not only the future of TB vaccines but also be broadly applicable across infectious disease and immunotherapy fields.

7. APPLICABILITY BEYOND TB

The delivery platforms and immunological insights developed in this thesis hold significant translational potential far beyond the context of TB. Their versatility, safety, and capacity to fine-tune immune responses make them valuable for diverse biomedical challenges in both infectious and non-infectious settings.

Infectious Disease Vaccinology. The NP-based delivery platforms, especially pH-sensitive liposomes and lipid-PLGA hybrid systems, can be applied to a wide array of intracellular pathogens. These include *Salmonella*, *Leishmania*, *Chlamydia*, and HIV, for which cell-mediated immunity is essential. Moreover, their adaptability enables the design of rapid-response vaccines for emerging pathogens, supporting pandemic preparedness initiatives. The mucosal immune enhancement potential of these formulations also makes them suitable for respiratory and enteric vaccines delivered via intranasal or oral routes.

Cancer Immunotherapy. The platform's ability to induce polyfunctional CD8⁺ T-cell responses and support antigen-specific recall responses suggests its use in delivering tumor neoantigens. pH-sensitive and depot-forming NPs could be

tailored for endosomal escape, enhancing cytosolic delivery of peptide antigens, mRNA, or siRNA for therapeutic cancer vaccines or immunomodulation of the tumor microenvironment.

Autoimmune Disease and Immune Tolerance. Rationally designed NPs could be repurposed to induce antigen-specific tolerance by co-delivering autoantigens with tolerogenic agents.

Allergy and Asthma Immunotherapy. NP systems could deliver allergens in controlled doses to promote immune tolerance rather than hypersensitivity, offering a safer alternative to conventional desensitization. Depot-forming intradermal or microneedle-based administration routes could improve adherence and reduce adverse effects.

Drug Delivery and Gene Therapy. The principles demonstrated here, particularly pH-sensitivity and controlled release, are applicable to intracellular delivery of antimicrobials, nucleic acid-based therapies, or cytokine delivery for immune modulation. These technologies could be extended into hormone therapies or long-acting injectables requiring depot-like performance.

Trained Immunity and Immunosenescence. NP platforms could be adapted to train innate immune cells via agents like BCG derivatives, helping protect immunocompromised or elderly individuals. Intradermal delivery of immune rejuvenating agents may offer a new strategy to combat immunosenescence.

Preclinical and Translational Research Tools. The structured pipeline developed here, from rational antigen and adjuvant selection, through *in vitro* human cell testing, to *in vivo* efficacy screening, serves as a broadly applicable model for vaccine and immunotherapy development. It offers a more predictive and efficient framework for early-stage screening across disease models.

Global One Health Applications. Solid, thermostable NP-based formulations enable room temperature storage, simplifying logistics in low-resource settings. Combined with needle-free intradermal administration (e.g., microneedles or jet injectors), these vaccines could improve compliance and access, especially in mass campaigns or outbreak control settings.



In summary, the modularity and efficacy of the vaccine platforms developed in this work open a range of translational opportunities. By combining innovative materials science with rational immunological design, these systems have the potential to revolutionize how we approach prevention and treatment in a broad spectrum of medical fields.

8. LIMITATIONS OF THE STUDY

While this thesis provides important insights into NP-based subunit vaccine design for TB, several limitations should be acknowledged to contextualize the scope and impact of the findings.

A key limitation of this work is the evaluation of immune responses and protection at a single post-challenge time point. This approach restricts insights into the time dynamics of vaccine-induced immunity and may overlook critical events associated with either early clearance or long-term protection. Similarly, the use of a 7-day *in vitro* lymphocyte restimulation protocol precluded the characterization of early activation events and innate-like recall responses. Future studies incorporating longitudinal sampling at multiple time points will be necessary to capture the full trajectory of immune induction, contraction, and memory formation.

Another limitation lies in the fixed antigen and adjuvant doses used throughout the experiments. While this design allowed for direct comparison across formulations, it limits the understanding of dose–response relationships. The choice of adjuvants and their dosages, particularly CpG and MPLA, was based on literature precedent rather than systematic optimization, potentially obscuring synergistic or dose-dependent effects. Exploring a broader range of adjuvant types and concentrations could yield improved immunogenicity and better safety profiles, especially when translating these systems to clinical settings.

Additionally, although the study involved rational design of delivery platforms and incorporated multiple NP systems (PLGA, liposomes, lipid–PLGA hybrids), the chemical diversity of tested materials remained limited. Further structural modifications and mechanistic studies would be valuable to refine delivery kinetics and target cell specificity.

From an immunological standpoint, the study emphasized systemic immune responses (e.g., splenocyte analysis) and peripheral markers of T- and B-cell

activation. However, site-specific immune activity in the lungs and draining lymph nodes, crucial compartments in Mtb pathogenesis and vaccine efficacy, was not assessed. Nor were tissue-resident memory T-cells, mucosal antibody production (e.g., secretory IgA), or innate training responses characterized, all of which are increasingly recognized as important correlates of protection against respiratory pathogens. Importantly, the lack of immune response data from Mtb-challenged animals limits the ability to directly correlate protection outcomes (i.e., bacterial burden reduction) with specific immunological signatures. Without this, mechanistic conclusions regarding how each formulation mediates protection remain speculative.

The reliance on a single preclinical model – C57BL/6 mice – also limits generalizability. This strain, while convenient for mechanistic immunology studies, does not develop human-like granulomas and does not fully recapitulate latency, reactivation, or vaccine-driven pathology modulation. Validation in more predictive models, such as guinea pigs, non-human primates, or human challenge studies, will be essential to assess translational relevance.

Lastly, practical considerations relevant to future clinical deployment, such as cold-chain stability, storage conditions, needle-free delivery options (e.g., microneedle patches), and large-scale manufacturability, were not evaluated in this study. These aspects, while beyond the current thesis' scope, are vital for the ultimate implementation of new TB vaccine candidates, particularly in low-resource settings.

In summary, while the current research advances our understanding of NP-based TB vaccine strategies, its limitations underscore the need for continued optimization of formulation, dose, delivery route, immunological readouts, and model systems. Addressing these gaps will enhance the translational potential of next-generation vaccines and strengthen their pathway toward clinical development.

9. FUTURE DIRECTIONS

The findings of this work open multiple avenues for future research that span mechanistic exploration, formulation refinement, and translational advancement. While not all directions are required to move this technology forward into clinical development, each represents a unique opportunity to answer distinct scientific questions or optimize the vaccine platform for different use cases. The appropriate



next steps will depend on the specific research goals, whether they relate to elucidating mechanisms of protection, optimizing immune responses, or advancing toward clinical application.

1. Deeper Immunological Characterization

As stated above, one key limitation of this study was the single post-vaccination time point used for immune assessment. Future studies should incorporate longitudinal analyses to track the kinetics of immune priming, contraction, and memory formation over time. Additionally, sampling from mucosal tissues, particularly the lung, could provide insights into tissue-resident responses that may be more relevant for TB control than those observed in peripheral lymphoid organs. Analysis of mucosal IgA, bronchoalveolar lavage samples, and lung-resident T-cell subsets could provide valuable information on localized immunity. Expanding B-cell and antibody profiling to include affinity maturation, class switching, and functional assays may also help clarify their contributions to protection.

2. Mechanistic Studies and Correlates of Protection

Further mechanistic dissection is warranted to define the immune pathways responsible for protection. Immune subset depletion experiments (e.g., CD4⁺, CD8⁺, B-cells, or IL-17-producing cells) and adoptive transfer studies could help clarify the relative importance of different cell types. Additionally, transcriptomic and proteomic profiling of lymphocytes or APCs after vaccination could reveal novel pathways linked to efficacy. Evaluating markers of trained immunity or metabolic reprogramming in myeloid cells may uncover non-classical correlates of protection beyond canonical Th1 immunity.

3. Formulation and Antigen Optimization

While this study explored select formulations and adjuvant combinations, a broader screen could identify improved variants. Future work could involve systematic dose titration studies to determine the minimum effective doses of antigen and adjuvants. Incorporation of novel immunostimulants (e.g., cGAMP, QS-21, or TLR7/8 agonists) into the existing platforms could enhance immunogenicity or skew responses toward specific profiles. Modular antigen design, including epitope mapping or combining more antigens targeting both latent and active Mtb stages, may further improve the breadth and depth of immune coverage.

4. Delivery Route and System Innovation

The demonstrated advantages of intradermal delivery suggest that future studies should explore its broader applicability, including via microneedle patches or solid-form vaccines for thermostable, field-ready use. Rational engineering of NP surfaces, e.g., using receptor-targeting ligands or optimizing lipid composition, could improve cellular targeting, endosomal escape, and depot effects. Additionally, exploring mucoadhesive delivery systems could help target mucosal immune compartments more effectively.

5. Advanced Preclinical Studies

To bridge the translational gap between murine models and human tuberculosis, evaluation in more predictive animal models is essential. Non-human primates provide superior physiological and immunological relevance, especially for studying granuloma architecture, latency, and reactivation. Other intermediate models, such as rabbits and humanized mice, may also offer valuable insights, particularly in capturing aspects of human-like immune responses or pathophysiological features absent in conventional mouse models. Performing comparative challenge studies against leading clinical-stage vaccines (e.g., M72/AS01_E) could help benchmark performance and support go/no-go decisions for clinical development.

6. Toward Clinical Translation

If lead formulations continue to show promise, future work should focus on manufacturing under GMP conditions and evaluating formulation stability under different temperature and humidity conditions. Scalability, batch-to-batch reproducibility, and shelf-life under non-refrigerated conditions will be critical for deployment in low-resource settings. Early dialogue with regulatory agencies and global health organizations (e.g., WHO) can help define development milestones and trial readiness. Dose-sparing, intradermal, or even self-administrable formats may offer logistical advantages that accelerate real-world implementation.

These future directions represent a flexible roadmap for further development of the NP-based TB subunit vaccines described in this thesis. Depending on the chosen



research priorities, mechanistic insight, formulation innovation, or translational readiness, different branches of this pipeline may be pursued independently or in combination.

10. FINAL CONCLUSIONS

This thesis presents a comprehensive body of work on the rational design, development, and evaluation of NP-based subunit vaccines for TB. By combining antigen engineering, smart delivery systems, *in vitro* human immune models, and mouse challenge studies, this research demonstrates how formulation science can significantly enhance vaccine efficacy and provide a path forward for next-generation TB vaccines.

The findings illustrate that NP delivery systems, specifically PLGA NPs, pH-sensitive liposomes, and lipid–PLGA hybrid particles, offer not only enhanced antigen delivery but also allow precise modulation of immune responses. All three NP platforms significantly reduced Mtb bacterial burden in mice, with PLGA NPs showing the greatest reduction, even outperforming the benchmark BCG vaccine in median lung protection. This underscores the potential of subunit vaccines when paired with well-engineered delivery vehicles.

Importantly, the data challenge traditional assumptions in vaccinology. High frequencies of polyfunctional T-cells or elevated antibody titers did not reliably predict protection, revealing a disconnect between immune magnitude and functional efficacy.³¹ These findings advocate for a shift toward identifying more mechanistically relevant correlates of protection, including central memory-like T-cell subsets, B-cell-derived cytokines, and tissue-resident immunity.

This work also proposes and validates a translational research pipeline that begins with rational formulation design, progresses through *in vitro* human immunological assessment, and culminates in comparative *in vivo* studies that inform clinical strategy. This iterative framework can be adapted for other infectious diseases and even non-infectious indications where immune precision and antigen sparing are critical.

Beyond TB, the platforms and design principles established here are broadly applicable to vaccine development against other intracellular pathogens, as well as

for cancer immunotherapy and mucosal vaccines. The study highlights the utility of i.d. delivery and pH-sensitive systems as underutilized yet powerful strategies for improving vaccine accessibility, efficacy, and global deployment.

In conclusion, this thesis provides both concrete advances in TB vaccine research and generalizable insights for immunoengineering. It reinforces that subunit vaccines, when paired with intelligent delivery platforms, are a viable and scalable path forward for combating TB and potentially many other diseases where conventional vaccine approaches have fallen short.

11. REFERENCES

1. World Health Organization. *Global Tuberculosis Report 2024*. (2024).
2. Schrager, L. K., *et al.* The status of tuberculosis vaccine development. *Lancet Infectious Diseases* 20, e28–e37 (2020).
3. Brewer, T. F. Preventing Tuberculosis with Bacillus Calmette-Guérin Vaccine: A Meta-Analysis of the Literature. *Clinical Infectious Diseases* 31, S64–S67 (2000).
4. World Health Organization. *The End TB Strategy*. (2015).
5. Christensen, D. *et al.* Cationic liposomes as vaccine adjuvants. *Expert Review of Vaccines* 10, 513–521 (2011).
6. Moyle, P. M. & Toth, I. Modern Subunit Vaccines: Development, Components, and Research Opportunities. *ChemMedChem* 8, 360–376 (2013).
7. Lai, R. *et al.* Key advances in vaccine development for tuberculosis—success and challenges. *NPJ Vaccines* 2023 8:1 8, 1–10 (2023).
8. Duong, V. T. *et al.* Towards the development of subunit vaccines against tuberculosis: The key role of adjuvant. *Tuberculosis* 139, 102307 (2023).
9. Ottenhoff, T. H. M. & Kaufmann, S. H. E. Vaccines against Tuberculosis: Where Are We and Where Do We Need to Go? *PLoS Pathogens* 8, e1002607 (2012).
10. Minor, P. D. Live attenuated vaccines: Historical successes and current challenges. *Virology* 479–480, 379–392 (2015).
11. Tuberculosis Vaccine Initiative. Pipeline of vaccines - TBVI. <https://www.tbvi.eu/what-we-do/pipeline-of-vaccines/>.
12. William Tong, C. Y. Different Types of Vaccines. *Tutorial Topics in Infection for the Combined Infection Training Programme* (2019).
13. Ghattas, M. *et al.* Vaccine Technologies and Platforms for Infectious Diseases: Current Progress, Challenges, and Opportunities. *Vaccines* 9:12, 1490 (2021).



14. Tait, D. R. *et al.* Final Analysis of a Trial of M72/AS01 E Vaccine to Prevent Tuberculosis. *New England Journal of Medicine* 381, 2429–2439 (2019).
15. Franco, A. R. & Peri, F. Developing New Anti-Tuberculosis Vaccines: Focus on Adjuvants. *Cells* 10:1, 78 (2021).
16. Ewer, K. J. *et al.* Viral vectors as vaccine platforms: from immunogenicity to impact. *Current Opinion in Immunology* 41, 47–54 (2016).
17. Lundstrom, K. Viral Vectors in Gene Therapy: Where Do We Stand in 2023? *Viruses* 15:3, 698 (2023).
18. BioNTech. Safety and Immune Responses After Vaccination With Two Investigational RNA-based Vaccines Against Tuberculosis in BCG Vaccinated Volunteers, Trial ID BNT164-02. <https://clinicaltrials.biontech.com/trials/BNT164-02>.
19. Larsen, S. E. *et al.* Tuberculosis vaccines update: Is an RNA-based vaccine feasible for tuberculosis? *International Journal of Infectious Diseases* 130, S47–S51 (2023).
20. O'Hagan, D. T. *et al.* Towards an evidence based approach for the development of adjuvanted vaccines. *Current Opinion in Immunology* 47, 93–102 (2017).
21. Commandeur, S. *et al.* The in vivo expressed Mycobacterium tuberculosis (IVE-TB) antigen Rv2034 induces CD4+ T-cells that protect against pulmonary infection in HLA-DR transgenic mice and guinea pigs. *Vaccine* 32, 3580–3588 (2014).
22. Szachniewicz, M. M. *et al.* Cationic pH-sensitive liposome-based subunit tuberculosis vaccine induces protection in mice challenged with Mycobacterium tuberculosis. *European Journal of Pharmaceutics and Biopharmaceutics* 203, 114437 (2024).
23. Szachniewicz, M. M. *et al.* Evaluation of PLGA, lipid-PLGA hybrid nanoparticles, and cationic pH-sensitive liposomes as tuberculosis vaccine delivery systems in a Mycobacterium tuberculosis challenge mouse model – A comparison. *International Journal of Pharmaceutics* 666, 124842 (2024).
24. Szachniewicz, M. M. *et al.* Intrinsic immunogenicity of liposomes for tuberculosis vaccines: Effect of cationic lipid and cholesterol. *European Journal of Pharmaceutical Sciences* 195, 106730 (2024).
25. Szachniewicz, M. M. *et al.* Cationic pH-sensitive liposomes as subunit vaccine delivery systems against tuberculosis: effect of liposome composition on cellular innate immune responses. *International Immunopharmacology*. *Manuscript submitted for publication* (2024).

26. Geluk, A. *et al.* A multistage-polyepitope vaccine protects against *Mycobacterium tuberculosis* infection in HLA-DR3 transgenic mice. *Vaccine* 30, 7513–7521 (2012).
27. Szachniewicz, M. M. *et al.* Intradermal versus subcutaneous immunization: Effects of administration route using a lipid-PLGA hybrid nanoparticle tuberculosis vaccine. *European Journal of Pharmaceutical Sciences* 205, 106995 (2025).
28. Henriksen-Lacey, M. *et al.* Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. *Journal of Controlled Release* 142, 180–186 (2010).
29. Henriksen-Lacey, M. *et al.* Comparison of the depot effect and immunogenicity of liposomes based on dimethyldioctadecylammonium (DDA), 3 β -[N-(N',N'-dimethylaminoethane)carbonyl] cholesterol (DC-Chol), and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP): Prolonged liposome retention mediates stronger Th1 responses. *Molecular Pharmaceutics* 8, 153–161 (2011).
30. Rao, D. A., Forrest, M. L., Alani, A. W. G., Kwon, G. S. & Robinson, J. R. Biodegradable PLGA based nanoparticles for sustained regional lymphatic drug delivery. *Journal of Pharmaceutical Sciences* 99, 2018–2031 (2010).
31. Caccamo, N. *et al.* Multifunctional CD4+ T cells correlate with active *Mycobacterium tuberculosis* infection. *European Journal of Immunology* 40, 2211–2220 (2010).

