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

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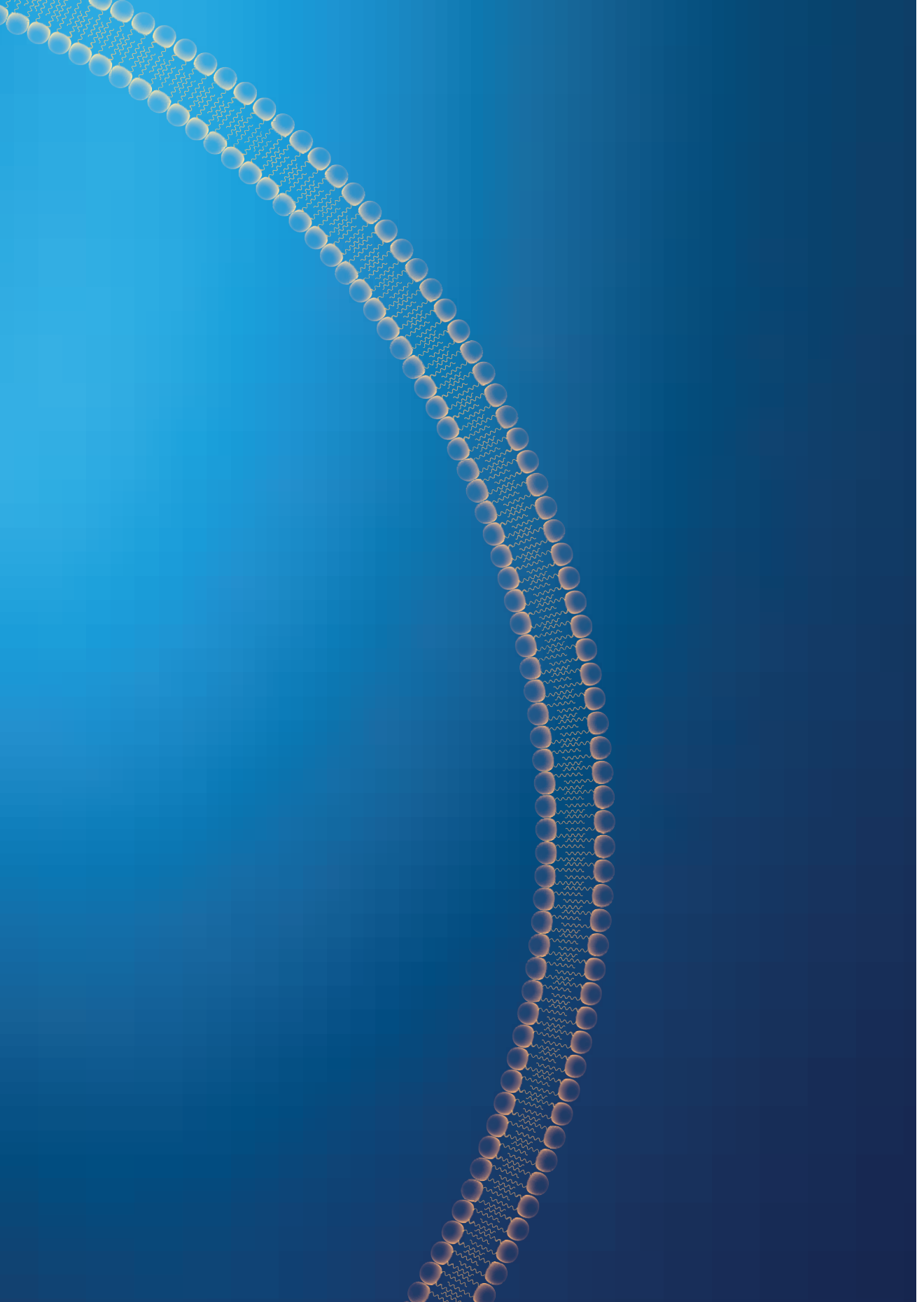

CHAPTER 1

General introduction and thesis outline

1. TUBERCULOSIS – AN OVERVIEW

Tuberculosis (TB) is a contagious airborne disease that is both preventable and curable in modern times. Evidence of human TB infection dates back to the Neolithic era, around 9,000 years ago.[1] The causative agent, *Mycobacterium tuberculosis* (Mtb), has co-evolved with humans for many millennia. It is theorized that Mtb accompanied humans for 6 to 70 thousand years,[2] allowing it to emerge as a sophisticated pathogen capable of surviving in its host for years without symptoms. Currently, the World Health Organization (WHO) estimates that about a quarter of the global population is latently infected with Mtb, with 5-10 % of these individuals likely to develop active TB.[3]

In 2023, TB likely regained its position as the world's leading cause of death from a single infectious agent, after being surpassed by COVID-19 for three years, and caused nearly twice as many deaths as HIV/AIDS.[4] It also significantly contributed to deaths related to antimicrobial resistance. An estimated 10.8 million people, including 1.3 million children, contracted TB that year, with eight countries: India, Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh, and the Democratic Republic of the Congo, accounting for over two-thirds of the global total.[4] COVID-19 disruptions between 2020 and 2023 led to a 4.6 % increase in the TB incidence rate, reversing nearly two decades of annual declines of about 2 %, and resulted in nearly 700,000 additional TB deaths.[4]

Global efforts have made significant strides in combating TB, saving an estimated 79 million lives since 2000. In 2023, 8.2 million people were newly diagnosed with TB, the highest number since WHO monitoring began in 1995. Despite these efforts, there remains a substantial gap between the estimated number of TB cases and those diagnosed, with approximately 2.7 million people undiagnosed or unreported in 2023.[4]

Several new modalities are now in the pipeline to alleviate the TB burden. As of January 2024, the Tuberculosis Vaccine Initiative (TBVI) reports 16 prophylactic TB vaccines and one therapeutic vaccine in clinical trials.[5] Additionally, WHO notes that 28 drugs for TB treatment are in clinical trials, including 18 new compounds, two with

accelerated approval, one recently approved by the US FDA, and seven repurposed drugs. There are also at least 29 trials and studies evaluating drug regimens and delivery models for TB preventive treatment.[6]

2. VACCINES

A vaccine is a biological preparation that enhances immunity against a specific pathogen. The primary goal of vaccination is to protect those at risk, including children, the elderly, immunocompromised individuals, people with chronic diseases, and those in disease-endemic areas. Vaccines against infectious diseases contain an agent resembling a disease-causing microorganism, made from weakened or killed forms of the pathogen, its toxins, or components such as nucleic acids or proteins. This agent stimulates the immune system of the host to recognize, destroy, and "remember" the pathogen for future defense. They are widely recognized as the most effective and cost-efficient means of combating infectious diseases. Their success in eradicating smallpox and rinderpest underscores their efficacy.[7] Various types of vaccines are in use and research.

Since the discovery of the smallpox vaccine in 1798, numerous vaccines have become available. According to the WHO, vaccines can currently prevent 25 life-threatening infectious diseases, helping people of all ages live longer, healthier lives.[8] Immunization prevents 3.5 to 5 million deaths annually, [9] and more vaccines against 16 infectious diseases are in development.[8]

3. SIMPLIFIED MECHANISM OF ACTION OF VACCINES

Regardless of the type, all vaccines operate through a common general mechanism: they introduce antigens into the body to train the immune system to recognize and respond to a specific pathogen (e.g., *Mtb*) or disease state (e.g., cancer). These antigens can take various forms, that instructs host cells to produce antigenic proteins.[10]

The primary goal of vaccination is to deliver these antigens to specialized immune cells known as antigen-presenting cells (APCs), such as dendritic cells (DCs), which play a central role in initiating adaptive immune responses. Depending on the form of the antigen, APCs process it via different intracellular pathways. Protein

and peptide antigens are typically degraded into smaller fragments called epitopes. These epitopes are then loaded onto specialized surface receptors known as major histocompatibility complexes (MHCs),[11] which come in two forms:

- **MHC Class I**: presents antigens to $CD8^+$ cytotoxic T cells.
- **MHC Class II**: presents antigens to $CD4^+$ helper T cells.

In humans, MHC molecules are referred to as human leukocyte antigens (HLAs).[11]

To effectively initiate an immune response, vaccines must also include components that stimulate APC activation. These are known as adjuvants and may consist of compounds derived from microbes or synthetic molecules designed to mimic such compounds. The activation of APCs is crucial, as it ensures effective antigen processing and presentation to T and B lymphocytes.[11]

Once activated, APCs migrate from the site of antigen encounter (typically the site of vaccine injection) to the nearest draining lymph nodes via the lymphatic system. In the lymph nodes, they present antigens to naïve T cells and B cells, facilitating the initiation of the adaptive immune response. This interaction not only triggers the production of disease-specific effector immune cells but also establishes immunological memory, which enables a faster and more robust immune response upon subsequent exposure to the pathogen or disease antigen.[11]

The process of antigen presentation involves three essential signals:

1. **Signal 1** – Antigen Recognition: APCs present processed epitopes on MHC molecules to the T-cell receptor (TCR) on naïve T cells (MHC I to $CD8^+$ T cells; MHC II to $CD4^+$ T cells).
2. **Signal 2** – Co-stimulatory Activation: APCs express co-stimulatory molecules (e.g., CD80, CD86) that interact with receptors on T cells (e.g., CD28), providing the necessary secondary activation signal.
3. **Signal 3** – Cytokine Signaling: APCs secrete cytokines that further direct T-cell differentiation and functional polarization (e.g., toward Th1, Th2, or cytotoxic responses).[12]

Following successful antigen presentation, T and B cells undergo activation, clonal expansion, and differentiation into effector and memory cells. This leads to the

generation of memory T cells and memory B cells, which are central to long-term immunity. Upon future encounters with the pathogen or cancer antigen, these memory cells enable the immune system to mount a rapid and effective response.[11]

This coordinated process of antigen delivery, immune activation, and memory formation is the fundamental goal of all prophylactic and therapeutic vaccines.[10]

4. SOLE LICENSED TB VACCINE – BCG

Despite the success of traditional vaccines, effective vaccines for several infectious diseases, including TB, remain elusive. The only available TB vaccine, *Mycobacterium bovis* bacille Calmette–Guérin (BCG), has been in use since 1921 and is recommended at birth in over 180 countries.[13] Unfortunately, BCG provides inconsistent and often insufficient protection, ranging from 0 to 80 %,[14] particularly against the transmissive pulmonary form of TB in adolescents and adults.[15–17] It is more effective in preventing disseminated and meningeal TB in children.[18,19] This protection is thought to be partly mediated by BCG-induced trained immunity, which enhances the nonspecific responsiveness of innate immune cells and may contribute to broader early-life protection.[20,21]

The variable efficacy of the BCG vaccine against adult pulmonary TB is likely influenced by several factors.[22] BCG's effectiveness may wane with age, though evidence varies widely across studies.[23–25] BCG may be less effective in endemic areas due to exposure to environmental non-tuberculous mycobacteria (NTM), especially in tropical regions where BCG efficacy is inversely related to NTM prevalence.[26,27] Exposure to NTM may theoretically elicit immune responses that nullify BCG benefits in certain populations.[22] Differences in BCG vaccine preparations and genetic variability of different strains have also been proposed as factors,[28] though there is insufficient evidence to support this hypothesis.[29–32] Finally, host factors, including sociological factors, genetics, and environmental exposures, likely contribute to variable BCG efficacy.[33–36]

5. TB VACCINE CANDIDATES

This inadequacy of the BCG vaccine has prompted the development of new vaccine candidates, with 17 currently in various stages of clinical trials. These candidates employ various innovative vaccine technologies, including viral vector platforms, subunit (protein-based) vaccines, inactivated, and live-attenuated strains, to

hopefully offer more robust and consistent protection against TB across different demographic groups. A selection of vaccine candidates in a preclinical phase of development was summarized in Table 1, while vaccines that are in clinical trials were described in Table 2.

Table 1. TB vaccine candidates in a preclinical phase of development that are listed by the Working Group on New TB Vaccines (WGNV),[37] part of the Stop TB Partnership and Tuberculosis Vaccine Initiative (TBVI)[5] grouped by the type of vaccine technology as of June 2025.

Vaccine	Description	Additional information
	Mycobacterial – live attenuated	
MtbDsigH Tulane University and Texas Biomedical Research Institute	Mtb clinical isolate CDC1551 lacking SigH gene (RNA polymerase σ-H factor) regulating stress-response resulting in poor scavenging of ROS.[38,39] In macaques, it induced strong central memory $CD4^+$ and $CD8^+$ responses and induced protection in lethal Mtb challenge.[38]	- Prevention of Mtb infection or sustained infection - Prevention of TB disease - Adolescents, adults, children, and infants - Mucosal administration - Non-human primate models
AY035 Anyong Dingye Biotechnology Ltd.	Recombinant BCG-Japan over-expressing *M. bovis* allele of phoP-phoR displaying enhanced immunogenicity and protection in C57BL/6 mice and guinea pigs.[40]	- Prevention of Mtb infection or sustained infection - Prevention of TB disease - Adolescents, adults -Intramuscular administration - Guinea pigs and mice models

BCG::ESAT6-PE25SS James Cook University	Recombinant BCG-Danish constructed by fusing gene esxA to the general secretion signal for the mycobacterial type VII secretion pathway protein PE25. It secretes full-length ESAT-6 via the ESX-5 secretion system. In mice, it induced immune responses and protection against a clinical Mtb isolate Beijing 17919.[41]	- Prevention of TB disease - Immunotherapy/shortening TB treatment and prevention of TB recurrence - Adolescents, adults, children, and infants - Mucosal administration - Mice and rabbit models
Mycobacterium microti ATCC 35782 Institut Pasteur Paris	ATCC 35872 vole isolate lacking a specific RD1^{mic} region encoding the ESX-1 type VII secretion system. Shown to be completely inoffensive in highly susceptible SCID mice surpassing BCG and as effective in protection against Mtb as BCG in guinea pigs.[42]	- Prevention of TB disease - Elderly and people living with HIV - Intradermal administration - Guinea pigs and mice models
BCG::ESX-1Mmar Institut Pasteur Paris	Recombinant BCG Pasteur 1173P2 transformed with pRD1-2F9 and pESX-1 (*M. marinum*) cosmids expressing ESX-1 type VII secretion system. It displayed robust immunogenicity and superior protection relative to the parental BCG against Mtb H37Rv and hypervirulent HN878 and M2.[43]	- Prevention of TB disease - Prevention of Mtb infection or sustained infection, and prevention of TB recurrence - Adolescents, adults, children, and infants - Intradermal administration - Guinea pigs and mice models

BCGΔBCG1419c Centro de Investigación y Asistencia en Tecnología y diseño del Estado de Jalisco, A.C. (CIATEJ)	Recombinant BCG Pasteur ATCC 35734, lacking antibiotic-resistance markers and the BCG1419c gene (encoding cyclic di-GMP phosphor-diesterase), has been demonstrated to induce better protection against TB-induced lung pathology during the chronic stages of infection compared to BCG and improved immune responses profile.[44,45]	- Prevention of TB disease - Prevention of Mtb infection or sustained infection - Adolescents, adults, children, infants, people living with HIV, with/ without Mtb infection - Subcutaneous administration - Guinea pigs and mice models
BCG w/MK-2206 Institut Pasteur de Tunis (IPT)	Immunization with BCG Pasteur strain 1173P2 followed 24 hours later by administration of anti-cancer Akt inhibitor, MK-2206 shown to induce enhanced protection of BCG in Mtb aerosol-challenged mice and guinea pigs.[46]	- Prevention of Mtb infection or sustained infection - Prevention of TB disease - Adolescents, adults, children, and infants - Subcutaneous/intradermal administration - Guinea pigs and mice models
BCG-ZMP1 University of Zürich	Recombinant BCG-Danish lacking zmp1 gene encoding a zinc metalloprotease linked to inhibition of phagolysosome maturation by preventing inflammasome activation.	- Prevention of Mtb infection or sustained infection - Prevention of TB disease - Adolescents, adults, children, and infants

	It showed enhanced immunogenicity *in vitro* and *in vivo*.[47]	- Subcutaneous administration - Guinea pigs, mice, and cattle models
Subunit – protein-based		
CysVac2/Advax University of Sydney	Fusion protein antigen comprising Ag85B and CysD formulated with Advax adjuvant, a particulate polysaccharide delta-inulin adjuvant containing CpG. It induced IL-17-dependent protection against aerosol Mtb infection in C57BL/6 mice.[48]	- Prevention of Mtb infection or sustained infection - Prevention of TB disease - Adolescents and adults - Mucosal administration - Mice models
Spore-FP1 St George's University of London	Inactivated *Bacillus subtilis* spores coated with the FP1 fusion protein comprising Ag85B, Acr/HspX, and the N-terminal domain of HBHA antigen, and formulated with Poly(I:C). Intended as a mucosal boost vaccine in BCG-immunised hosts. Shown to be highly protective in guinea pigs but failed to improve protection in non-human primates.[49]	- Prevention of Mtb infection or sustained infection - Prevention of TB disease - Adolescents, adults, children, and people with Mtb infection - Mucosal administration - Guinea pigs, mice, and non-human primates models

Spray Dried ID93+GLA-SE Access to Advanced Health Institute (AAHI)	Fusion protein antigen comprising Rv3619, Rv1813, Rv3620, and Rv2608 (ID93), formulated with GLA-SE adjuvants, a squalene emulsion containing gluco-pyranosyl lipid A (GLA) (Toll-like receptor 4 agonist).[50]	- Prevention of TB disease - Prevention of Mtb infection or sustained infection - Adolescents, adults, elderly - Mucosal administration - Mice models
	Vector vaccine	
BCG, ChadOx/ MVA 5Ag University of Oxford	A replication-deficient recombinant chimpanzee adenovirus expressing four antigens from the mycobacterium Esx-5a system (PPE15, PE8, EsxI, EsxJ) and Ag85A. It enhanced BCG efficacy in mice and showed promise in non-human primates.[51]	- Prevention of Mtb infection or sustained infection - Prevention of TB disease - Adolescents and adults - Mucosal/intramuscular administration. - Mice and non-human primates models
Lm::Mtb9Ag University of California (UCLA)	A live attenuated *Listeria monocytogenes* vector (Lm ΔactA ΔinlB prfA) with two major virulence gene deletions and a mutation that enhances T-cell immunity, expressing nine Mtb protein antigens: Mpt64, TB10.4, ESAT-6, CFP10, Ag85B, EsxN, PPE68, EspA, and TB8.4. It demonstrated efficacy	- Prevention of TB disease - Prevention of Mtb infection or sustained infection, prevention of TB recurrence - Adolescents, adults, elderly, and people without Mtb infection

	against Mtb aerosol challenge in BALB/c, C57BL/6 mice, and guinea pigs.[52]	- Subcutaneous administration - Guinea pigs, mice, and non-human primate models

Table 2. TB vaccine candidates in a clinical phase of development grouped by the phase of clinical trial as of June 2025.[53]

Vaccine	Description	Additional information
	Phase 1	
AdHu5Ag85A Vector vaccine McMaster University, CanSino Biologics	A recombinant type 5 human adenovirus vector encoding Ag85A antigen. Mucosal but not intramuscular administration induced polyfunctional airway tissue-resident $CD4^+$ and $CD8^+$ T-cells in humans after a single dose. It was demonstrated to be safe and well tolerated.[54]	- Prevention of Mtb infection or sustained infection - Prevention of TB disease - Adolescents and adults - Mucosal/intramuscular administration - NCT02337270 - Completed in September 2021
BNT164(a1, b1) mRNA vaccine BioNTech SE	Two multi-antigen RNA vaccine candidates formulated with lipid nanoparticles.[55]	- Prevention of TB disease - Adolescents and adults - Intramuscular administration - NCT05537038 - NCT05547464 - Ongoing

H107e/CAF10b Subunit protein-based vaccine Statens Serum Institut	Fusion protein construct consisting of 8 non-BCG-cross-reactive antigens (PPE68, ESAT-6, EspI, EspC, EspA, MPT64, MPT70, and MPT83) in a cationic liposomal formulation containing DDA, monomycoloyl glycerol (MMG) and CpG. It was shown to induced protection when co-administrated with BCG-Danish in mice, and induced robust Th1/Th17 responses in Cyno-molgus macaques.[56–58]	- Prevention of TB disease - Adolescents, adults, infants, People living with HIV, with/without Mtb infection - Intramuscular administration - NCT06050356 - Ongoing
TB/FLU-05E Vector vaccine Smorodintsev Research Institute of Influenza	A recombinant attenuated influenza vector (Flu/THSP) expressing a truncated NS1 protein and Mtb antigens TB10.4 and HspX proteins. In BCG-prime with mucosal TB/FLU-05E boost regimen protected mice and guinea pigs from Mtb better than BCG alone.[59,60]	- Prevention of Mtb infection or sustained infection - Prevention of TB recurrence - Adolescents, adults, and children - Intranasal administration - NCT05945498 - Completed in September 2023

Phase 2a		
AEC/BC02 Subunit protein-based vaccine Anhui Zhifei Longcom Biopharmaceutical Co., Ltd.	Mixture of Ag85B with ESAT-6-CFP10 fusion protein and adjuvant BC02 comprising CpG and aluminum hydroxide. In mice showed protective immune responses but failed to control TB in guinea pigs.[61] In a therapeutic approach combined with isoniazid and rifapentine lowered CFUs better than antibiotics alone.[62]	- Prevention of TB disease - Adults, elderly, and people with Mtb infection - Intramuscular administration - NCT05284812 - Ongoing
ChAdOx1.85A + MVA85A Vector vaccine University of Oxford	Prime with simian adenovirus ChAdOx1.85A expressing Ag85A followed by boost with vaccinia virus Ankara MVA85A expressing Ag85A. In mice, BCG boosted with ChAdOx1.85A and then boosted with MVA85A improved BCG efficacy.[63] In humans, in phase 1 induced strong cellular and humoral responses.[64]	- Prevention of TB disease - Prevention of Mtb infection or sustained infection and prevention of TB recurrence - Adolescents, adults, children, and infants - Intramuscular administration - NCT03681860 - Completed in May 2021
ID93+GLA-SE (QTP101) Subunit protein-based vaccine Quratis, NIAID/NIH	Fusion protein antigen comprising Rv3619, Rv1813, Rv3620, and Rv2608 (ID93), formulated with GLA-SE adjuvants, a squalene emulsion containing gluco-pyranosyl lipid A (GLA) (Toll-like receptor	- Prevention of TB disease - Prevention of Mtb infection or sustained infection and Prevention of TB recurrence - Adolescents and adults

	4 agonist).[50] In BCG-vaccinated healthy adults, vaccine induced antigen-specific cellular and humoral immune res-ponses.[65]	- Intramuscular administration - NCT03806686 - Completed in June 2020
	Phase 2b	
BCG (Revaccination) Live attenuated vaccine Bill & Melinda Gates Medical Research Institute	Revaccination with AJVaccines' BCG SSI (Danish 1331) of QuantiFERON-TB Gold Plus (QFT)-negative individuals. In the phase 2b trial, BCG revaccination reduced the rate of sustained QFT conversion with an efficacy of 45.4 % in a high transmission setting.[66]	- Prevention of Mtb infection or sustained infection - Adolescents and children - Intradermal administration - NCT04152161 - Ongoing
DAR-901 Inactivated vaccine Dartmouth, St. Louis University	Whole-cell booster of BCG, comprising of non-tuberculous *Mycobacterium obuense* grown by broth fermentation of SRL 172 strain and heat-inactivated.[67] In phase 1 trial, it induced vaccine-specific Th1 polyfunctional effector memory immune responses.[68] In phase 2b, it did not prevent initial or persistent IGRA conversion. However, vaccin-ated IGRA converters showed	- Prevention of TB disease - Adolescents, adults, and people living with HIV - Intradermal administration - NCT02712424 - Completed in February 2020

	enhanced responses to ESAT-6.[69] It may be later tested for prevention of TB instead of Mtb infection.	
RUTI® Inactivated vaccine Archivel Farma, S.L	RUTI is a preparation comprising detoxified cellular H37Rv Mtb nanofragments formulated in liposomes (size 100 nm).[70] In mice, it inhibited the growth of BCG Pasteur *ex vivo*.[71] In phase 2 trial, in LTBI ($HIV^{-/+}$) patients pre-treated for 1 month with isoniazid polyantigenic responses were observed.[72]	- Immunotherapy and shortening TB treatment - Improving TB cure rates, prevention of TB disease and recurrence - Adolescents, adults, people with active TB, MDR-TB, and Mtb infection - Subcutaneous administration - NCT04919239 - Ongoing
Phase 3		
BCG (Travel vaccine) Live attenuated vaccine Henry M. Jackson Foundation for the Advancement of Military Medicine	Glutamate BCG Japan (Tokyo 172) administrated as a single dose to adults with no history of prior BCG vaccination or Mtb infection traveling to countries with high TB burden.	- Prevention of Mtb infection or sustained infection - Adults - Intradermal administration - NCT04453293 - Ongoing

GamTBvac Subunit protein-based vaccine Ministry of Health of the Russian Federation	Fusion protein construct comprising Ag85A-ESAT-6-CFP10 fused with dextran-binding domain from *Leuconostoc mesenteroides*. The construct was formulated with adjuvants consisting of dextran covered in CpG. In mice and guinea pigs, it showed robust immunogenicity and protection against H37Rv aerosol and intravenous challenges as an effective BCG booster.[73] In phase 1 trial, it induced robust cellular and humoral vaccine-specific immune responses.[74]	- Prevention of TB disease - Prevention of TB recurrence - Adults - Subcutaneous administration - NCT04975737 - Ongoing
Immuvac (MIP) Inactivated vaccine Cadila Pharmaceuticals, Indian Council of Medical Research	Immuvac is a heat-killed suspension of a non-pathogenic, cultivable atypical *Mycobacterium Indicus Pranii* (MIP) in saline, originally formulated against leprosy. It has been clinically evaluated as a treatment against category II TB, Gram-negative sepsis, non-small cell lung cancer, HIV, muscle-invasive bladder cancer, and COVID-19 with promising results.[75]	- Prevention of TB disease - Adolescents and adults - Intradermal administration - CTRI/2019/01/017026 - Ongoing

M72/AS01E Subunit protein-based vaccine GSK Vaccines, Bill & Melinda Gates Medical Research Institute	Fusion protein M72 comprising of Mtb antigens Mtb32A and Mtb39A formulated with $AS01_E$ adjuvant, which is a liposomal formulation containing MPLA, *Quillaja saponaria* Molina, fraction 21 (QS-21; a triterpene glycoside), and cholesterol. In a phase 2b trial, the vaccine induced 49.7 % protection against TB after 3 years in a TB-endemic population.[76]	- Prevention of TB disease - Adolescents, adults, people living with HIV, people with/without Mtb infection - Intramuscular administration - NCT06062238 - Ongoing
MTBVAC Live attenuated vaccine Biofabri, Bharat Biotech, University of Zaragoza, IAVI, TBVI, HIV Vaccine Trials Network	MTBVAC is an attenuated clinical Mtb strain Mt103 in which phoP and fadD26 virulence genes were deleted. It contains RD1 region absent in BCG, which contains 23 % of the known human T-cell epitopes present in Mtb. In the phase 1b trial, it induced robust polyfunctional Th1 responses in neonates.[77]	- Prevention of TB disease - Adolescents, adults, and infants - Intradermal administration - NCT04975178 (infants up to 7 days of age) - Ongoing
VPM1002 Live attenuated vaccine Serum Institute of India, Vakzine Projekt Management GmbH	BCG vaccine strain which is urease C-deficient, and expresses listeriolysin from *Listeria monocytogenes* (rBCGΔureC:Hly) to improve the immunogenicity and safety. In phase 2 trial, it was safe and immunogenic; however, IFNγ	- Prevention of Mtb infection or sustained infection - Prevention of TB disease and recurrence - Adolescents and adults

production, polyfunctional $CD4^{+}$, and $CD8^{+}$ T-cell responses were inferior compared to BCG in neonates.[78]	- Intradermal administration - CTRI/2019/01/017026 - Ongoing

6. TYPES OF TB VACCINES

Vaccines can be broadly classified into two categories: live (capable of replication) and non-live (incapable of replication). This classification distinguishes vaccines that use attenuated, replicating strains of the relevant pathogen, like the BCG vaccine, from those that consist of only pathogen components, such as antigens in the form of proteins, peptides, or genetic material (DNA or mRNA encoding antigens). These non-live vaccines may deliver these components via micro- or nanoparticles (NPs), or through vector organisms like viruses or bacteria, or they may use inactivated whole organisms.[79]

Another key distinction is between prophylactic and therapeutic vaccines. Prophylactic vaccines are designed to prevent infection, disease onset, or transmission of the pathogen. In contrast, therapeutic vaccines aim to treat existing infections or support standard treatments, such as antibiotics, by enhancing the immune response.[79]

6.1 Live-attenuated Whole-cell Vaccines

Live-attenuated vaccines are engineered to replicate just enough in a healthy individual to generate a strong immune response without causing significant disease symptoms. Crucially, these vaccine strains should not establish a persistent infection in the host and should be naturally cleared by the immune system without the need for medical intervention. Achieving this balance is vital, as it ensures that the pathogen replicates sufficiently to stimulate robust immunity while remaining attenuated enough to avoid causing illness.[79]

However, this technology faces several challenges. The manufacturing process is labor-intensive and requires stringent quality control to ensure consistent efficacy and safety, resulting in higher production costs.[80] Additionally, the use of these vaccines in immunocompromised individuals, particularly in TB-endemic regions

where HIV co-infection is common, poses significant safety risks. [81] Another major limitation is the reliance on cold chain logistics. In regions where TB vaccines are most needed, maintaining refrigeration throughout the distribution process is extremely difficult, compounded by frequent electricity shortages.[82] It is worth noting that cold chain dependency is a common challenge across nearly all vaccines.

Despite these challenges, live-attenuated vaccines offer significant advantages. They can provoke a complex and diverse immune response by presenting a wide range of antigens and pathogen-associated molecular patterns (PAMPs), closely mimicking a natural infection and providing long-lasting protection.[79]

The smallpox vaccine, which used the live cowpox vaccinia virus, is the most notable example of a live-attenuated vaccine. Developed by Edward Jenner in 1796, it was the first vaccine ever created. Its effectiveness and relative safety led to its refinement, mass production in the 20th century, and the implementation of a global vaccination program.[83] This effort culminated in the complete eradication of smallpox, officially declared by the WHO in 1980, making smallpox the only human disease to be eradicated.[84] Other examples of live-attenuated vaccines include those for influenza, measles, mumps, rubella, rotavirus, oral polio, and BCG.[80]

Currently, new live-attenuated whole-cell TB vaccine candidates are being developed as prophylactic vaccines to potentially replace BCG vaccination in neonates. These candidates are also under evaluation as post-exposure vaccines in adults to prevent TB recurrence. Examples of such vaccines currently in the clinical trials are: BCG revaccination and travel programmes, MTBVAC, and VPM1002.

6.2 Inactivated Vaccines

Inactivated vaccines are formulated using pathogens that have been killed or rendered non-infectious through chemical or physical methods, such as heat or radiation. These vaccines cannot replicate or cause disease but retain many structural components of the original pathogen, including key antigens and PAMPs. This allows them, in theory, to elicit a broad immune response targeting multiple antigens.[79]

Pathogens used for vaccine production are cultured and inactivated under strictly controlled and validated conditions to ensure both safety and consistent

immunogenicity. Failure to achieve complete inactivation could leave viable pathogenic remnants, posing a safety risk, though modern manufacturing standards make this highly unlikely.[79]

Since inactivated vaccines cannot replicate in the host, they are considered safer than live-attenuated vaccines and are suitable for use in immunocompromised individuals. However, their inability to replicate also results in weaker and less durable immune responses, typically dominated by humoral (antibody-mediated) immunity. To overcome this limitation, inactivated vaccines often require the addition of adjuvants to enhance immunogenicity and multiple booster doses to establish and maintain protective immunity. Advanced adjuvant systems may also improve the induction of cellular immune responses, but this remains a challenge compared to live vaccines.[79]

The first inactivated vaccines were developed in the late 19th century for diseases such as cholera, plague, and typhoid fever. Today, inactivated vaccines are licensed for the prevention of several diseases, including influenza, polio (Salk vaccine), rabies, hepatitis A, pertussis (as part of acellular pertussis vaccines),[85] and some COVID-19 vaccines (e.g., CoronaVac, BBIBP-CorV).

While inactivated vaccines are not the primary focus of TB vaccine development, several candidates in the pipeline utilize this approach:

- **DAR-901**: Derived from *Mycobacterium obuense*, developed for TB prevention.[69]
- **RUTI®**: A therapeutic vaccine based on detoxified fragments of *Mtb* (H37Rv strain) encapsulated in liposomes, intended to shorten treatment duration and prevent relapse.[71]
- **Immuvac (MIP)**: Based on *Mycobacterium indicus pranii*, explored for both TB prevention and as an immunotherapeutic agent.[75]

6.3 Genetic Vaccines

Genetic vaccines work by delivering genetic material encoding specific antigens into host cells, prompting those cells to produce the target antigens internally. This, in turn, stimulates the immune system to recognize and respond to the pathogen.

The genetic material used in these vaccines is typically either DNA or messenger RNA (mRNA). Delivery can be achieved through various platforms, including gene delivery systems such as NPs or viral vectors.[79]

Genetic vaccines can be classified into three main types:

- **DNA Vaccines**: Utilize plasmid DNA to encode antigens that have to be transcribed to mRNA by the host to produce antigens.
- **mRNA Vaccines**: Use mRNA to directly instruct cells to produce antigens.
- **Viral Vector Vaccines**: Employ modified viruses (usually non-replicating) to deliver genetic material encoding antigens.[86]

While DNA vaccines have been explored in various infectious disease contexts, they are not a significant focus in current TB vaccine development efforts and will not be further discussed here.

In contrast, mRNA vaccines and viral vector vaccines are actively being investigated as innovative platforms for TB vaccines due to their potential to induce robust cellular immunity, which is critical for protection against *Mtb*.

6.3.1 Vector Vaccines

Viral vector vaccines utilize genetically engineered viruses to deliver genetic material encoding specific antigens into host cells. These vectors are modified to be replication-deficient, ensuring they cannot cause disease. Once inside the host cells, the delivered genetic material directs the production of antigens, which stimulate the immune system to mount a response against the target pathogen, not the vector itself. This approach effectively mimics natural infection, promoting robust immune responses.[87]

The selection of viral vectors depends on several critical factors. Pre-existing immunity in the target population is particularly important, as prior exposure to certain viruses, such as common human adenoviruses, can reduce vaccine efficacy by neutralizing the vector before it delivers its genetic payload. Additionally, the type of immune response induced by the vector is a key consideration. For diseases like cancer, HIV, and TB, strong cellular immunity is essential, especially the activation

of cytotoxic $CD8^+$ T cells. Other important factors include the vector's safety profile, particularly for immunocompromised individuals, and the feasibility of large-scale manufacturing and long-term stability of the vaccine.[87]

Adenoviruses are among the most widely used viral vectors. These double-stranded DNA viruses include human adenovirus serotypes 5 (Ad5) and 26 (Ad26), as well as the chimpanzee-derived ChAdOx1 vector. They have been employed in several COVID-19 vaccines, including Oxford–AstraZeneca's ChAdOx1 nCoV-19, Janssen's Ad26.COV2.S, Sputnik V (which uses both Ad26 and Ad5), and Convidecia, developed by CanSino Biologics. Adenovirus vectors are also being explored in TB vaccine candidates, such as AdHu5Ag85A and ChAdOx1.85A combined with MVA85A. These vectors are known for inducing strong cellular and humoral immune responses; however, pre-existing immunity to human adenoviruses remains a significant limitation, potentially reducing vaccine effectiveness.[88]

Modified Vaccinia Ankara (MVA), a highly attenuated poxvirus, is another widely used viral vector. It has an excellent safety profile and is particularly effective at inducing strong $CD4^+$ T-helper cell responses, although its ability to stimulate cytotoxic $CD8^+$ T-cell responses is generally weaker than that of adenoviruses. MVA is used in licensed smallpox and mpox vaccines and has also been incorporated into TB vaccine candidates, such as the heterologous prime-boost strategy using ChAdOx1.85A followed by MVA85A.[89]

Vesicular stomatitis virus (VSV), a negative-strand RNA virus, has been successfully used as the vector in the Ervebo Ebola vaccine (rVSV-ZEBOV), the first approved vaccine for Ebola. VSV-based vectors are highly immunogenic, inducing both strong cellular and humoral immune responses. However, there are some concerns regarding safety, particularly related to potential neurovirulence, which has limited their broader application.[90]

The measles virus, another negative-strand RNA virus, has also been investigated as a vaccine vector for diseases such as HIV, TB, and chikungunya. It shows promise in inducing long-lasting immunity based on preclinical research. However, the widespread global immunity to measles resulting from routine vaccination significantly limits its effectiveness as a vector, as most individuals would rapidly neutralize the vector before it can deliver its genetic material.[91]

Cytomegalovirus (CMV), a large DNA virus, is being explored experimentally as a viral vector, particularly for chronic infections like HIV and TB. CMV has a unique ability to establish long-term antigen expression and induce robust and persistent $CD8^+$ T-cell memory responses. Despite these promising characteristics, no vaccines using CMV as a vector have been approved for human use to date, and research remains at the preclinical stage.[92]

As of June 2025, six viral vector vaccines against infectious diseases have been approved for human use. Four of them target COVID-19: the Oxford–AstraZeneca vaccine (ChAdOx1 nCoV-19 / AZD1222), Janssen's Ad26.COV2.S, Sputnik V (Gam-COVID-Vac), and Convidecia from CanSino Biologics. In addition, two vaccines have been approved for the prevention of Ebola virus infection. Zabdeno/Mvabea, a two-dose regimen using Ad26.ZEBOV and MVA-BN-Filo vectors, has been approved by the European Commission.[93]

Viral vector vaccines have played a critical role in combating global infectious disease threats such as COVID-19 and Ebola. Their ability to induce strong immune responses, particularly cellular immunity, makes them highly valuable platforms for both current and future vaccine development efforts, including TB.

6.3.2 mRNA Vaccines

mRNA vaccines are a class of genetic vaccines that utilize synthetic messenger RNA (mRNA) to direct the production of protein-based antigens within host cells. Once delivered into the cytoplasm, the mRNA is translated by the host cell's ribosomes into the encoded antigen. These antigens are then processed by the cell, initiating a cascade of immune processes leading to the activation of adaptive immunity, including the generation of effector cells and long-lasting immunological memory.[79]

Because mRNA is highly unstable and susceptible to enzymatic degradation, it must be delivered using specialized delivery systems. The most widely used and clinically validated delivery platform is lipid NPs (LNPs), which protect the mRNA and facilitate its uptake by host cells.[94]

Although mRNA vaccine technology has been under investigation for several decades, the first mRNA vaccines were only recently approved during the COVID-19 pandemic, marking a significant milestone in vaccine development. As of 2025, approved mRNA vaccines are available for the prevention of COVID-19 and

respiratory syncytial virus (RSV). However, numerous mRNA vaccine candidates are currently in clinical trials targeting a range of other diseases, including TB, influenza, cytomegalovirus, and various cancers.[95]

Among these candidates are BNT164a1 and BNT164b1, investigational mRNA-based TB vaccines developed by BioNTech SE. Both vaccines employ multi-antigen mRNA sequences formulated with LNPs.[96,97] These candidates are currently being evaluated in a two-part, randomized, placebo-controlled, observer-blind, Phase Ib/IIa clinical trial conducted in South Africa and Mozambique. The trial is designed to assess safety, tolerability, and immunogenicity across up to four different dose levels, with the goal of determining a safe and effective dose for a three-dose vaccination schedule in healthy, BCG-vaccinated adult argue that mRNA vaccines re in a way also subunit vaccine.t volunteers.[55]

6.4 Subunit Vaccines

Subunit vaccines are a class of vaccines that contain only selected, purified components of a pathogen, such as proteins, peptides, polysaccharides, or polysaccharide–protein conjugates, that are specific to the pathogen and capable of eliciting an immune response. These components are chosen for their antigenic properties, meaning they can be recognized by the immune system and trigger an adaptive immune response. While subunit antigens can be directly extracted from cultured pathogens, they are more commonly produced synthetically or through recombinant DNA technology, allowing for precise manufacturing and quality control.[98]

In the context of therapeutic cancer vaccines, subunit vaccines often utilize tumor-specific antigens or neoantigens, which are short peptides or proteins derived from mutations unique to cancer cells. These neoantigens help train cytotoxic $CD8^+$ T cells to recognize and destroy malignant cells while sparing healthy tissue, leveraging the immune system's ability to distinguish "non-self" from "self."[99]

The first subunit vaccine approved for human use was the hepatitis B vaccine, which consists of recombinant hepatitis B surface antigen (HBsAg). Since then, subunit vaccines have become a cornerstone of modern vaccinology, with examples including vaccines against human papillomavirus (HPV), pertussis (acellular pertussis), and pneumococcal disease (using polysaccharide-protein conjugates).[100]

Because subunit vaccines do not contain whole organisms or innate immune stimulatory molecules, they require the addition of adjuvants – immunostimulatory compounds that activate APCs and enhance the magnitude and quality of the immune response. Without appropriate immune activation, subunit vaccines risk eliciting suboptimal responses, such as anergy (non-responsiveness), immune tolerance, or ineffective immune polarization. Additionally, they often require multiple doses or booster shots to achieve durable and protective immunity.[101]

One of the major advantages of subunit vaccines over live-attenuated or inactivated whole-pathogen vaccines is their safety profile. Because they contain no replicating organisms or infectious materials, they pose minimal risk of reversion or infection and are well-suited for immunocompromised populations, such as individuals living with HIV. This is particularly important in regions where both HIV and other infectious diseases like TB are endemic, and safe, effective vaccines are critically needed.[102]

Subunit vaccines can also be rationally designed and tailored to direct the immune response toward specific effector mechanisms (e.g., Th1, Th2, or cytotoxic T-cell responses). They are typically more stable, easier to store and transport, and less expensive to manufacture at scale than many traditional vaccine platforms.[102]

A crucial element in the success of subunit vaccines is formulation, particularly the integration of advanced delivery systems. These platforms are designed to improve antigen stability, target delivery to specific cells (such as DCs), and optimize the kinetics of antigen release. Delivery systems can be biological (e.g., viral vectors or engineered bacteria) or synthetic, with the latter category including polymers, antibodies, liposomes, micelles, and microparticles or NPs. Synthetic, non-replicating systems are generally preferred for subunit vaccine delivery due to their safety and versatility.[102]

Subunit vaccines are a major focus in TB vaccine development, with several candidates in clinical trials. H107e/CAF10b (Statens Serum Institut) contains eight non-BCG-cross-reactive antigens in cationic liposomes with DDA, MMG, and CpG, showing strong Th1/Th17 responses in macaques and protection in mice.[56–58] AEC/BC02 (Anhui Zhifei) combines Ag85B, ESAT-6-CFP10, CpG, and a traditional adjuvant aluminum hydroxide.[61,62] ID93+GLA-SE (Quratis) includes a four-antigen fusion with a TLR4 agonist emulsion and induced immune responses in BCG-vaccinated adults.[65] GamTBvac (Russian Ministry of Health), a fusion of Ag85A, ESAT-6, and CFP10 with

dextran-CpG adjuvant, showed protection in animal models and strong responses in a phase 1 trial.[73,74] M72/AS01E (GSK) combines Mtb32A and Mtb39A with a liposomal MPLA/QS-21 adjuvant, offering 49.7 % protection over three years in a phase 2b trial.[76]

The main topic of this thesis is protein subunit vaccines formulated with NP-based delivery systems, representing a promising strategy to enhance immunogenicity, reduce antigen dose requirements, and improve vaccine outcomes.

7. NP-BASED VACCINES

NPs have become indispensable components in the design and delivery of modern vaccines. Among the most prominent examples are lipid NPs, which played a central role in the success of mRNA-based COVID-19 vaccines such as Comirnaty (Pfizer–BioNTech) and Spikevax (Moderna).[103,104] NPs are typically defined as fine particulate structures with at least one dimension in the nanometer range, most commonly between 1 and 100 nanometers. However, in applied biomedical fields, especially drug and vaccine delivery, functional definitions are more relevant. These definitions consider particles up to approximately 200–250 nanometers as NPs when they exhibit unique behaviors at the biological level, such as enhanced uptake by APCs, that are not observed at larger scales.[105]

In the context of immunization, particles below this size threshold are more efficiently internalized by APCs, including dendritic cells and macrophages. This enhanced uptake is central to their utility in vaccine formulations. Beyond passive transport, NPs offer a range of benefits. Their small size and surface properties enable them to traverse biological barriers and target specific immune cell populations. Some NPs possess intrinsic immunostimulatory properties, allowing them to function as adjuvants in addition to delivery vehicles. Moreover, NPs can co-deliver multiple functional components, such as antigens, molecular adjuvants, targeting ligands, and immunomodulatory agents, within a single structure. This multifunctionality allows for the precise tuning of the immune response, including directing it toward desired T-cell profiles or enhancing mucosal immunity.[102,105]

A major advantage of NP-based vaccine systems is their capacity to reduce the antigen dose required to elicit protective immunity. By enhancing antigen presentation and delivery to key immune cells, NPs often enable significant dose-sparing, which

has cost and scalability benefits. Additionally, they can prolong antigen retention at the injection site, acting as a depot that maintains antigen exposure over time and thereby strengthens the immune response. NPs can also be engineered to control the kinetics of antigen release, enabling burst release, sustained release, or environmentally triggered release (e.g., pH- or enzyme-dependent), further improving immunogenicity and therapeutic precision. Importantly, they protect labile vaccine components such as proteins, peptides, or nucleic acids from degradation during manufacturing, storage, and transport in the body.[102,105]

A wide range of NP types is currently under investigation for vaccine applications. Inorganic NPs, including gold, silica, carbon-based structures, aluminum salts (alum), calcium phosphate, and magnetic NPs, have been studied both for their delivery capacity and as immunostimulatory adjuvants. However, organic NPs are more commonly used due to their superior biocompatibility, flexibility in formulation, and easier regulatory pathways. These include polymeric NPs (e.g., PLGA), liposomes, solid lipid NPs, niosomes, micelles, dendrimers, immunostimulatory complexes (ISCOMs), virus-like particles (VLPs), and nanoemulsions. Each type presents unique physicochemical characteristics and biological behavior, offering distinct advantages for specific vaccine strategies, though also posing formulation challenges.[106]

Several licensed vaccines incorporate NPs as delivery systems or adjuvants. mRNA vaccines such as Comirnaty and Spikevax use lipid NPs to encapsulate and protect their mRNA cargo. mRESVIA, Moderna's RSV vaccine, also uses this technology. Protein subunit vaccines have also benefited from NP technologies. Nuvaxovid (Novavax COVID-19 vaccine) uses Matrix-M, a saponin-based NP adjuvant, in combination with recombinant spike proteins assembled into NPs. Skycovione (SK Bioscience) employs self-assembling protein NPs displaying the receptor-binding domain of SARS-CoV-2, combined with AS03, an oil-in-water emulsion-based adjuvant that enhances immunogenicity.[104]

VLP vaccines represent another well-established NP-based platform. Gardasil and Gardasil 9, both HPV vaccines, use recombinant L1 proteins assembled into VLPs that mimic the structure of the native virus but lack genetic material, offering a high safety profile and strong immunogenicity. Cervarix, another HPV vaccine, incorporates VLPs of HPV types 16 and 18 and uses the AS04 adjuvant, which combines monophosphoryl lipid A (MPLA) with aluminum hydroxide.[107] The herpes

zoster vaccine Shingrix employs the AS01B adjuvant system, which includes MPLA and the saponin QS-21 encapsulated in liposomes. Similarly, the malaria vaccine RTS,S/AS01 (Mosquirix) uses the AS01 liposomal adjuvant system to deliver *Plasmodium falciparum* antigens effectively.[108]

In the field of TB vaccine research, NPs offer particular promise for enhancing subunit vaccine performance. TB antigens are typically weakly immunogenic on their own and require delivery platforms that enhance antigen uptake. NP systems under active investigation in TB vaccine development include LNPs, liposomes, polymeric NPs (PNPs), such as those based on poly(lactic-*co*-glycolic) acid (PLGA), and nanoemulsions (NEs). These platforms are often combined with potent adjuvants to overcome the limitations of traditional BCG vaccination and to develop novel prophylactic or therapeutic TB vaccines.[102]

7.1 LNPs

LNPs are a versatile and biocompatible class of nanocarriers widely used in pharmaceutical and biomedical research. These nanoscale delivery systems are primarily composed of lipids that self-assemble into structures capable of encapsulating a wide variety of therapeutic agents, including nucleic acids, small molecules, and proteins. Their lipophilic nature, combined with the ability to incorporate hydrophilic and amphiphilic compounds, makes LNPs uniquely suited for overcoming biological barriers such as enzymatic degradation and cellular membranes.[106]

The term "lipid nanoparticles" often encompasses a range of different lipid-based NPs such as liposomes, solid LNPs (SLNs), nanostructured lipid carriers (NLCs), lipid NEs, and lipid–polymer hybrid NPs. However, in the context of nucleic acid delivery, especially mRNA vaccines, the term refers specifically to a formulation of ionizable lipid-based NPs with a core–shell structure optimized for delivering nucleic acids into cells.[104]

LNPs have gained increasing attention due to their ability to address critical challenges in drug delivery. Their nanoscale size facilitates transport across biological barriers, including the blood–brain barrier, and enhances uptake by target tissues. LNPs

exhibit high biocompatibility and are generally well-tolerated. In dermatological applications, their composition allows for good skin compatibility and improved penetration through the stratum corneum, the outermost layer of the skin.[104,106,109]

A major milestone in LNP technology was reached in 2018 with the approval of Onpattro (patisiran), the first siRNA-based drug using LNPs as a delivery vehicle. This formulation uses PEGylated ionizable lipids to deliver siRNA targeting transthyretin for the treatment of hereditary transthyretin-mediated amyloidosis. The approval of Onpattro marked a turning point, demonstrating that LNPs could be safely and effectively used in human gene-silencing therapies.[109]

The most prominent breakthrough in LNP technology came with the emergency use authorization and subsequent full approval of mRNA-based COVID-19 vaccines in 2020. Both the Moderna (Spikevax) and Pfizer–BioNTech (Comirnaty) vaccines employ LNPs to encapsulate and protect fragile mRNA strands encoding the SARS-CoV-2 spike protein. These LNPs are specifically designed for intracellular mRNA delivery: they consist of ionizable lipids that are neutrally charged at physiological pH but become positively charged in the acidic environment of the endosome, facilitating endosomal escape and cytoplasmic release of the mRNA payload. These formulations also include helper lipids (such as cholesterol and phospholipids) and PEGylated lipids to stabilize the NPs and extend circulation time. [104] The same LNP technology by BioNTech is utilized in their two multi-antigen mRNA TB vaccine candidates, BNT164a1 and b1, currently in phase 1a/2b clinical trial.

Unlike traditional solid-core NPs, LNPs used in mRNA vaccine formulations are ionizable amorphous liquid-disordered nanostructures, more accurately described as vesicle-like carriers. They do not form solid crystalline cores; rather, they possess a disordered internal phase that encapsulates the nucleic acid.[110]

7.2 Liposomes

Liposomes are spherical, self-assembling nanocarriers composed of one or more lipid bilayers enclosing an aqueous core. These bilayers are typically made from phospholipids, such as phosphatidylcholine, and often incorporate cholesterol to enhance membrane stability and fluidity. The amphiphilic nature of lipids allows the spontaneous formation of bilayers in aqueous environments, with hydrophilic head groups oriented outward and hydrophobic tails facing inward. This unique structure

enables liposomes to encapsulate both hydrophilic compounds (within the aqueous core) and hydrophobic agents (within the lipid bilayer), making them highly versatile drug and vaccine delivery systems.[111]

The physicochemical and biological behavior of liposomes can be finely tuned by altering their size, surface charge, lipid composition, and surface chemistry. For example, surface modifications such as PEGylation (the addition of polyethylene glycol chains) can improve circulation time and reduce immune recognition, while cationic or ionizable lipids can enhance interactions with negatively charged cell membranes and facilitate intracellular delivery. This adaptability has led to extensive use of liposomes across medicine, cosmetics, nutrition, and nanotechnology.[111]

Liposomes are among the earliest developed nanocarriers in pharmaceutical science. They were first described in 1961 by British hematologist Alec Douglas Bangham at the Babraham Institute in Cambridge, who recognized their structural similarity to biological membranes. This discovery sparked widespread research into their potential as both model systems for cell membranes and carriers for therapeutic agents.[112] In 1981, the first human trial of a liposome-encapsulated drug, liposomal cytarabine, was initiated, highlighting the promise of liposomes for improving drug delivery and pharmacokinetics.[113]

The clinical value of liposomes lies in their ability to enhance drug bioavailability, modify pharmacokinetics, reduce systemic toxicity, and enable targeted delivery. This is especially beneficial for potent but toxic chemotherapeutics. The first FDA-approved liposomal drug, Doxil® (pegylated liposomal doxorubicin), was approved in 1995 for the treatment of Kaposi's sarcoma, and later indicated for ovarian and breast cancers. Doxil dramatically improved the safety profile of doxorubicin by reducing cardiotoxicity. In 1997, AmBisome® (liposomal amphotericin B) was approved for systemic fungal infections, offering potent antifungal activity with significantly reduced nephrotoxicity compared to conventional formulations.[109]

Beyond oncology and antifungal therapy, liposomes have gained particular relevance in the field of vaccine delivery. Cationic and ionizable liposomes are of special interest due to their ability to enhance uptake by APCs, such as DCs, and their capacity to act as immunostimulatory adjuvants. Their surface charge, size, lipid composition, and structural rigidity can all be modified to optimize uptake,

biodistribution, and immune activation. Positively charged liposomes, in particular, interact favorably with negatively charged cell membranes and enhance endosomal escape, facilitating intracellular delivery of antigens and nucleic acids.[111]

Liposomes can efficiently encapsulate a wide range of vaccine-related cargo, including peptides, proteins, protein conjugates, and nucleic acids. The ability to co-deliver antigens along with targeting ligands, stimuli-responsive lipids, and adjuvants allows for the design of advanced vaccine formulations tailored to elicit desired immune responses. Their modularity also permits the targeting of specific tissues and the fine-tuning of release kinetics.[111]

A milestone in liposomal vaccine adjuvant technology was achieved with the development of AS01, a liposome-based adjuvant system used in Shingrix®, the recombinant herpes zoster vaccine approved in 2015. AS01B, the specific formulation in Shingrix, combines liposomal MPLA with the saponin QS-21, resulting in robust humoral and cellular immunity and superior efficacy compared to earlier zoster vaccines. In the same year, Mosquirix® (RTS,S/AS01), the first malaria vaccine to receive regulatory approval (via a positive opinion from the European Medicines Agency), also employed the AS01 adjuvant system, marking another significant application of liposomal delivery in infectious disease prevention.[108]

In the TB vaccine pipeline, two candidates utilize liposomal delivery systems. H107e/CAF10b (Statens Serum Institut) employs cationic liposomes formulated with dimethyldioctadecylammonium (DDA), monomycoloyl glycerol (MMG), and CpG.[56–58] M72/AS01E (GSK Vaccines) incorporates the $AS01_E$ adjuvant, a liposomal formulation containing monophosphoryl lipid A (MPLA), *Quillaja saponaria* Molina fraction 21 (QS-21), and cholesterol.[76]

7.3 Polymeric NPs (PNPs)

PNPs are nanoscale carriers composed of natural or synthetic polymers and have emerged as one of the most adaptable and promising platforms for drug and vaccine delivery. Their polymeric composition allows for a high degree of control over key structural features, including size, shape, surface chemistry, and surface charge, making them ideal candidates for targeted and responsive delivery systems. The remarkable chemical versatility of polymers enables the incorporation of a wide range of functional groups, ligands, and responsive moieties, allowing PNPs

to interact selectively with biological environments, trigger cargo release under specific conditions, and localize effectively within tissues or even subcellular compartments.[114,115]

PNPs can encapsulate, incorporate, or adsorb therapeutic agents, including antigens, adjuvants, and small-molecule drugs, within or onto their matrix. This provides protection from enzymatic degradation in physiological fluids and improves the bioavailability of labile compounds. The release of cargo from PNPs can be precisely engineered through modifications to polymer composition and surface properties. Controlled or stimuli-responsive release can be triggered by external factors such as pH changes, temperature, light, magnetic fields, or enzymatic activity, which is especially useful in pathological microenvironments like tumors or infected tissues. Additionally, the surface of PNPs can be functionalized with targeting ligands, such as peptides, antibodies, or carbohydrates, to achieve receptor-mediated uptake by specific cell types.[115]

A wide range of polymers has been investigated for NP fabrication. Natural polymers such as chitosan, alginate, dextran, and gelatin offer biocompatibility and biodegradability, often with intrinsic biological activity. Synthetic polymers, including poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), polycaprolactone (PCL), polyethylene glycol (PEG), and polymethacrylates, provide highly tunable physicochemical properties and are well characterized in terms of safety and degradation. Many of these polymers can be combined into copolymers to further tailor their release kinetics, mechanical strength, and biological interactions.[115]

In the field of vaccinology, PNPs offer multiple advantages. Their structure allows for the co-delivery of antigens and adjuvants, enabling synchronized immune activation. The rigid nature of many PNPs can promote enhanced uptake by antigen-presenting cells (APCs) compared to softer lipid-based systems. Moreover, their ability to sustain antigen release over extended periods enables prolonged immune stimulation, which is particularly beneficial for building strong immunological memory. PNPs can also be engineered to be mucoadhesive, a critical feature for the development of mucosal vaccines, administered via oral, nasal, or pulmonary routes, where residence time at mucosal surfaces significantly impacts vaccine efficacy.[115]

Despite their strong potential, PNP vaccines have not yet achieved regulatory approval for human use. In the TB vaccine pipeline, however, the GamTBvac candidate

incorporates a PNP-based adjuvant composed of a core of diethylaminoethyl (DEAE)-dextran, a polycationic dextran derivative, coated with CpG oligonucleotides.[73] While no PNP vaccines are currently licensed, polymer-based delivery systems are already in clinical use in other therapeutic fields, underscoring their translational promise. Several injectable controlled-release formulations based on PLGA microspheres have been approved and are commercially available. These include Lupron Depot® (approved in 1989), which delivers leuprolide acetate for the treatment of prostate cancer through sustained hormone suppression, and Sandostatin LAR® Depot, used for acromegaly and neuroendocrine tumors. Other examples include Risperdal Consta® for schizophrenia and Bydureon® for type 2 diabetes mellitus. Although these formulations are based on microscale rather than nanoscale carriers, they exemplify the clinical viability of polymer-based systems and underscore the future potential of PNPs in vaccine development.[115]

7.4 Nanoemulsions (NEs)

NEs are colloidal dispersions composed of two immiscible liquids, typically oil and water, stabilized by surfactants and co-surfactants. Characterized by droplet sizes ranging from approximately 20 to 200 nanometers, NEs form translucent or opaque systems with unique physicochemical properties that enhance the delivery of both hydrophobic and hydrophilic therapeutic agents. Depending on the internal and external phases, NEs can be classified into oil-in-water (O/W), water-in-oil (W/O), or multiple emulsions such as W/O/W and O/W/O systems.[106]

The advantages of NEs in drug and vaccine delivery are numerous. Their small size increases surface area and improves absorption, leading to enhanced bioavailability of poorly soluble drugs. NEs also provide protection from enzymatic degradation, enable controlled and stimuli-responsive release, and can be administered via multiple routes, including oral, intravenous, intranasal, pulmonary, ocular, and transdermal. In particular, their ability to protect labile compounds in harsh environments (such as gastric pH or enzymatic conditions) makes them useful for oral formulations. For parenteral applications, NEs act as protective carriers, improving drug stability and targeting efficiency while reducing systemic toxicity.[116]

Polymer- or lipid-stabilized NEs can be further functionalized with ligands, including antibodies, peptides, or small molecules, enabling targeted delivery through

receptor-mediated uptake. Moreover, NEs can be engineered to respond to biological stimuli such as pH, enzymes, or temperature, triggering cargo release in specific tissues or intracellular compartments.[116]

In addition to drug delivery, NEs have demonstrated utility in gene and vaccine delivery. Compared to traditional liposomes, certain NE systems have shown superior gene transfer efficiency, likely due to improved stability and membrane penetration. Photosensitizer-loaded NEs have also been explored for photodynamic therapy, addressing issues such as poor solubility, nonspecific toxicity, and self-aggregation of conventional formulations.[106]

In vaccinology, NEs serve three primary functions: as antigen delivery vehicles, as immune potentiators (adjuvants), and as mucosal delivery platforms. Their nanoscale properties facilitate antigen uptake APCs, promote both humoral and cellular immune responses, and support co-delivery of antigens and immunostimulants. NEs are particularly promising for mucosal vaccines delivered intranasally or sublingually, where they enable efficient absorption and immune activation without the need for needles. Early applications include mucosal vaccines for influenza and HIV, which progressed to clinical trials. Ongoing research continues to explore their use for diseases such as hepatitis B and anthrax. [106]

Two NE-based adjuvants have been approved for human use [106]. MF59®, developed by Chiron (now part of CSL Seqirus), is a squalene-based O/W NE with an average droplet size of approximately 160 nm. It is stabilized with Tween 80 and Span 85 and was the first NE adjuvant to receive regulatory approval, granted in Europe in 1997.[117] MF59 is currently used in seasonal and pandemic influenza vaccines, such as Fluad®.[106] It enhances immune responses by promoting the recruitment of immune cells to the injection site and activating antigen-presenting cells (APCs).

AS03, developed by GlaxoSmithKline (GSK) and approved in Europe in 2009, is another O/W adjuvant with a droplet size around 150 nm. Its formulation includes squalene, DL-α-tocopherol (vitamin E), and polysorbate 80. AS03 is used in vaccines such as Pandemrix®, Arepanrix®, and Skycovione (in South Korea). The combination of squalene and vitamin E induces local inflammation and potent APC activation, with a notable dose-sparing effect.[118]

In the clinical trials, a TB vaccine candidate ID93+GLA-SE (QTP101) (Quratis) utilizes an NE-based adjuvant. The GLA-SE adjuvant is a stable O/W NE composed of squalene and glucopyranosyl lipid A (TLR4 agonist).[50]

8. MOTIVATION AND RATIONALE FOR THE STUDY

Despite global efforts to control TB, the disease remains a leading cause of death from a single infectious agent, particularly in low- and middle-income countries. Although the BCG vaccine offers protection against severe forms of TB in children, its efficacy in preventing pulmonary TB in adults is inconsistent and limited. This has led to an urgent demand for novel, more effective TB vaccines. Among the strategies currently under exploration, subunit vaccines offer several advantages in terms of safety and versatility. However, subunit vaccines are often poorly immunogenic and require delivery and adjuvant systems to enhance their efficacy. NP-based delivery platforms, particularly those using liposomes and biodegradable polymers, are being investigated to address these challenges.

A critical knowledge gap in the field concerns the optimization of NP vaccine delivery systems, particularly regarding the physicochemical properties of the carriers and how these properties influence the immune response. Although liposomes and PLGA-based particles have been studied in various biomedical contexts, their specific immunological impact in TB vaccine applications remains poorly understood. In particular, limited information exists on how different cationic lipids, cholesterol content, or pH-sensitivity affect the functionality of liposomal vaccine formulations. Similarly, the comparative performance of liposomes, PLGA NPs, and hybrid particles combining both systems has not been systematically evaluated in TB models.

The overarching goal of this research was to address these knowledge gaps by systematically developing, optimizing, and evaluating multiple NP-based vaccine platforms carrying a fusion protein antigen (Ag85B-ESAT6-Rv2034, AER) previously shown to confer protection in mouse and guinea pig models. We aimed to:

- Investigate the role of different cationic lipids in liposome formulations to determine whether various cationic lipids affect immunogenicity beyond simply providing a positive charge.
- Study the underexplored area of pH-sensitive liposomes in TB vaccine development: formulation, stability, and immunogenicity.

- Translate these new findings *in vivo* in a biologically relevant mouse model of intranasal TB infection using the established strain H37Rv of Mtb.
- Explore other understudied NP systems in TB vaccine research, such as PLGA NPs, and pH-sensitive lipid-PLGA hybrid NPs, *in vivo* in a side-by-side comparison.
- Explore the effects of alternative routes of vaccine administration, such as intradermal delivery.

Together, these studies aimed to form a comprehensive body of work that elucidates how rational design and engineering of NP-based delivery and adjuvant systems influence immune responses to subunit vaccines. By identifying and validating critical parameters such as lipid composition, pH-sensitivity, hybrid NP architecture, and administration route, this research addresses key challenges in TB vaccine development. These findings offer practical insights for future preclinical and clinical efforts aimed at creating safer, more effective TB vaccines and may also inform vaccine design strategies for other challenging infectious diseases.

9. SCOPE AND OUTLINE OF THE THESIS

This thesis explores the rational design, development, and evaluation of NP-based delivery systems to enhance the efficacy of subunit vaccines against TB. The primary focus is on optimizing physicochemical parameters of liposomal and polymeric NPs to improve antigen delivery, immune activation, and protection in preclinical models.

The scope of the work encompasses both *in vitro* and *in vivo* studies. It begins with investigating how specific lipid components within cationic liposomes affect DC activation and immunogenicity. Subsequent chapters explore pH-sensitive liposomes designed to enhance cytosolic delivery of antigens and their immunological effects both *in vitro* and in a mouse model of TB. This is followed by a direct comparison of three NP platforms – liposomes, PLGA particles, and lipid-PLGA hybrid NPs – to evaluate their performance as vaccine carriers. The final study addresses the effect of vaccine administration route (subcutaneous vs. intradermal) and dose reduction using hybrid NPs, with the goal of improving vaccine practicality and accessibility.

The thesis is structured as follows:

- **Chapter 1**: General introduction and thesis outline.
  Provides background on TB as a global health threat, limitations of current vaccines, and the rationale for subunit and NP-based vaccine strategies.

- **Chapter 2**: Intrinsic immunogenicity of liposomes for tuberculosis vaccines: Effect of cationic lipid and cholesterol.[119]
  Investigates the immunogenic effects of various cationic lipids and cholesterol content in liposome formulations using DCs.

- **Chapter 3**: Cationic pH-sensitive liposomes as tuberculosis subunit vaccine delivery systems: Effect of liposome composition on cellular innate immune responses.[120]
  Focuses on the design and *in vitro* evaluation of pH-responsive liposomes for delivering TB fusion protein antigen.

- **Chapter 4:** Cationic pH-sensitive liposome-based subunit tuberculosis vaccine induces protection in mice challenged with Mycobacterium tuberculosis.[121]
  Assesses immunogenicity and protective efficacy of the liposomal vaccine compared to BCG and antigen-adjuvant mixtures.

- **Chapter 5**: 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.[122]
  Compares three NP delivery platforms for their ability to induce immune responses and reduce bacterial burden in mice.

- **Chapter 6**: Intradermal versus subcutaneous immunization: Effects of administration route using a lipid-PLGA hybrid nanoparticle tuberculosis vaccine.[123]
  Explores alternative administration routes and reduced antigen dosing to improve immunogenicity and practical applicability.

- **Chapter 7**: General Discussion and Future Perspectives.
  Summarizes key findings, discusses implications for TB vaccine development, and outlines directions for future research.

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