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REVIEW

RNA vaccines: The dawn of a new age for tuberculosis?

Junli Li[a,b,c,d*], Dong Liu[e,f*], Xiaochi Li[a,b,c,d], Jiazheng Wei[g], Weixin Du[a,b,c,d], Aihua Zhao[a,b,c,d], and Miao Xu[a,b,c,d]

[a]Division of Tuberculosis Vaccine and Allergen Products, Institute of Biological Product Control, National Institutes for Food and Drug Control, Beijing, China; [b]State Key Laboratory of Drug Regulatory Science, National Institutes for Food and Drug Control, Beijing, China; [c]Key Laboratory for Quality Research and Evaluation of Biological Products, National Medical Products Administration (NMPA), Beijing, China; [d]Key Laboratory of Research on Quality and Standardization of Biotech Products, National Health Commission (NHC), Beijing, China; [e]Graduate School of Guangzhou Medical University, Guangzhou Medical University, Guangzhou, China; [f]Guangzhou Laboratory, Guangzhou, China; [g]College of Life Sciences and Biopharmaceuticals, Shenyang Pharmaceutical University, Shenyang, China

ABSTRACT

Since 2019, there has been a growing focus on mRNA vaccines for infectious disease prevention, particularly following the emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV -2). mRNA vaccines offer advantages such as rapid production and the ability to induce robust cellular and antibody responses, which are essential for combating infections that require cell-mediated immunity, including Tuberculosis (TB). This review explores recent progress in TB mRNA vaccines and addresses several key areas: (1) the urgent need for new TB vaccines; (2) current advancements in TB vaccine development, and the advantages and challenges of mRNA technology; (3) the design and characteristics of TB mRNA vaccines; (4) the immunological mechanisms of TB mRNA vaccines; (5) manufacturing processes for TB mRNA vaccines; and (6) safety and regulatory considerations. This interdisciplinary review aims to provide insights for researchers working to address critical questions in TB mRNA vaccine development.

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The imperative need for advancing novel TB vaccines

Tuberculosis (TB) remains a significant challenge in global public health. According to the 2024 edition of the 'Global Tuberculosis Report' published by the World Health Organization (WHO),[1] there are projected to be 10.8 million new TB cases globally in 2023, reflecting an incidence rate of 134 per 100,000 individuals. This marks a slight increase from the 10.7 million reported in 2022 and surpasses the figures of 10.4 million in 2021 and 10.1 million in 2020. The 30 countries with a high burden of tuberculosis account for approximately 87% of the estimated global incidence, with five nations – India (26%), Indonesia (10%), China (6.8%), the Philippines (6.8%), and Pakistan (6.3%) – collectively representing over half (56%) of this global burden. With a total of 1.25 million deaths worldwide, TB has reemerged as the leading cause of death attributable to a single infectious disease globally, resulting in nearly double the fatalities compared to HIV/AIDS. In response to this growing crisis, the WHO launched its 'End TB Strategy' in 2014, which includes integrated, patient-centered care and prevention, robust policy frameworks, support systems, and a strong emphasis on research and innovation. The strategy aims to achieve a 95% reduction in TB mortality and a 90% reduction in TB incidence by 2035, compared to levels recorded in 2015, thereby alleviating the catastrophic financial burden on affected families. The development of innovative prophylactic vaccination regimens that target either infection prevention or disease mitigation has the potential not only to save numerous lives globally but also to significantly reduce morbidity, lower associated treatment costs, and potentially mitigate the rise of drug-resistant strains. Currently, the most widely used preventive measure against TB is the intradermal administration of the *Bacillus Calmette-Guérin* (BCG) vaccine. However, the availability of the BCG vaccine has recently been limited due to various factors. While BCG vaccination in newborns provides effective protection against severe forms of TB, such as miliary and meningeal TB, this protection lasts only about 10 years post-birth and diminishes over time, leaving adults inadequately protected. In addition to new infections, challenges such as disease reactivation from latent TB, the emergence of drug resistance, and co-infections with HIV pose significant threats to affected populations.

Although new antibiotic strategies are currently under development,[2–6] many existing approaches have proven inadequate when used alone to address this widespread disease.[7,8] Over the past five years, nearly 500,000 individuals infected with *Mycobacterium tuberculosis* (Mtb) annually have developed resistance to the first-line medication rifampicin, with approximately 80% of these cases exhibiting multi-drug-resistant (MDR) traits.[9–11] In 2023, it is estimated that there will be 400,000 new patients globally diagnosed with MDR/rifampicin-resistant tuberculosis (accounting for 3.7% of all

CONTACT Aihua Zhao zhaoaihua@nifdc.org.cn; Miao Xu xumiao@nifdc.org.cn Division of Tuberculosis Vaccine and Allergen, Institute of Biological Product Control, National Institutes for Food and Drug Control, No. 31, Huatuo Rd., China Bio-medicine Park (CBP), Daxing District, Beijing 102269, China.
*These authors contributed equally to this work.

cases), with a resistance rate of 3.2% among initially treated patients and as high as 16% among those who have undergone retreatment. Innovative strategies to combat antimicrobial resistance are particularly beneficial for immunocompromised patients and those undergoing immunosuppressive therapies. 'Strengthening research & fostering innovations' is a crucial component of the overarching End TB Strategy, as achieving the ambitious goals set for 2035 depends on embracing pioneering technologies. The complex nature of Mtb, along with its diverse pathogenic phases, highlights the urgent need for the development of cost-effective yet potent vaccines designed either to prevention of infection (POI) or to preemptively avert the prevention of disease (POD).[12]

Current status of novel TB vaccine development strategies, advantages and challenges associated with mRNA-based TB vaccines

Various research strategies have been devised for developing TB vaccines tailored to different populations. These strategies primarily focus on three main categories: replacing BCG with a new primary preventive TB vaccination; enhancing prevention following BCG immunization; and developing a vaccine for the prevention of latent TB infection from progressing to active disease.[2] Currently, there are 15 TB vaccines undergoing clinical trials worldwide, including six in Phase III trials, five in Phase II, and four in Phase I, down from 16 in 2023. The pipeline includes candidates to prevent TB infection and TB disease, and candidates to help improve the outcomes of treatment for TB disease. The research and development technology platform primarily encompasses viral vector vaccines, recombinant protein/subunit vaccines, recombinant BCG/live mycobacterium vaccines, inactivated mycobacterium vaccines, and nucleic acid-based vaccines (Figure 1). However, further extensive clinical studies are necessary before any of these candidates can be approved for public use. The rapid advancements in RNA-based vaccines have the potential to significantly impact this evolving field, a prospect that has generated considerable excitement.

Over recent decades, in vitro transcription (IVT) has become a widely adopted method for designing and producing messenger RNA (mRNA). Since 2019, the focus in the mRNA field has shifted from targeting cancers or genetic disorders characterized by deficient key proteins to combating infectious diseases, particularly following the emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This shift is largely due to the inherent advantages of mRNA vaccines. Firstly, mRNA-based therapeutics mitigate concerns

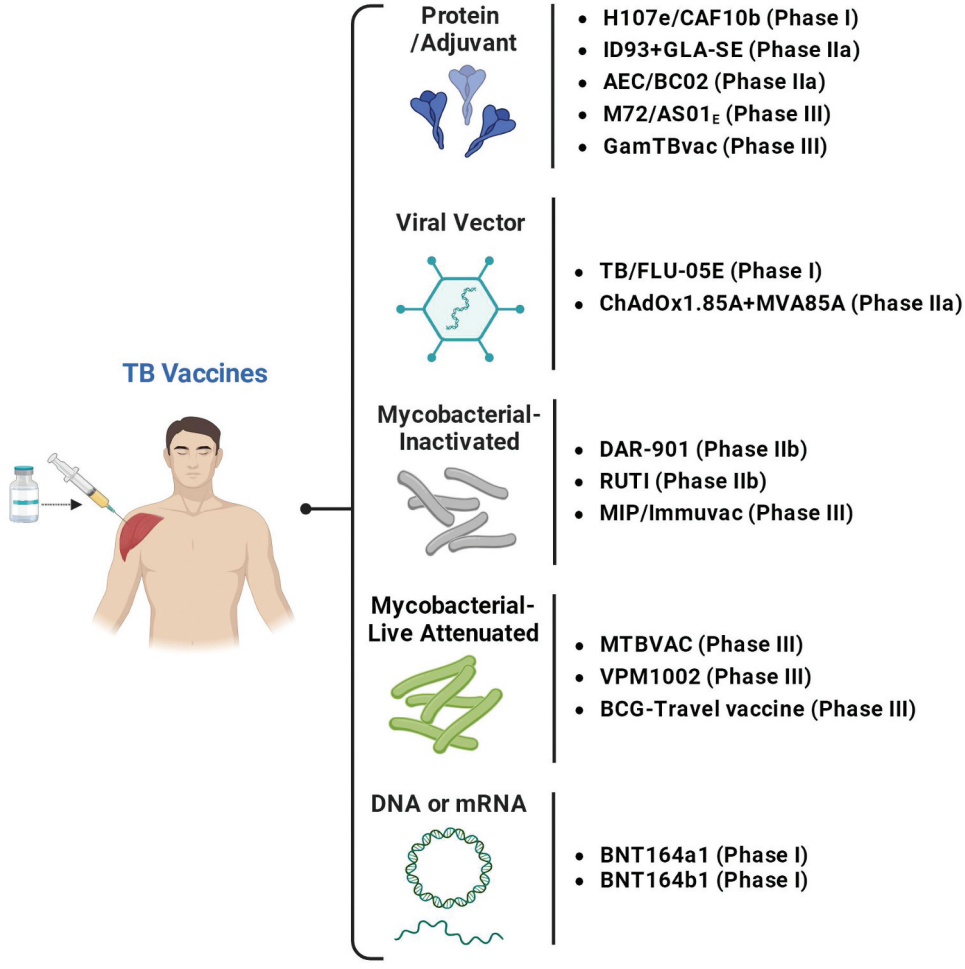

Figure 1. Overview of current TB vaccine candidates in clinical trials. The pipeline provides data on TB vaccine candidates in clinical development, but does not include information on candidates that have been withdrawn, terminated, or are in preclinical development.

associated with infection or endotoxin risks stemming from mutation reversion or incomplete attenuation, which are prevalent challenges with live attenuated and inactivated vaccines. Moreover, unlike DNA vaccines that necessitate nuclear entry and pose risks of genomic integration, or viral vector vaccines that present insertional mutagenesis risks, mRNA vaccines circumvent these potential complications entirely. Secondly, mRNA vaccines induce host cells to express target proteins, leading to properly folded proteins with the necessary post-translational modifications, an advantage over in vitro-manufactured protein-based vaccines. Thirdly, these target proteins are directly presented on MHC class I and II molecules, eliciting strong cellular and humoral immune responses, which are particularly effective in cell-mediated pathogen clearance, as seen in TB. Additionally, the production of clinically viable mRNAs involves a standardized IVT process using DNA templates and various enzymes in a cell-free system, enabling short development timelines, ease of mass production, and consistent quality control. Following the publication of the SARS-CoV-2 sequence, Moderna successfully developed its first vaccine candidate in just 28 days, completing all phases of clinical trials (phases 1–3) within a few months, and subsequently distributing millions of vaccine doses.[13] The entire process for the BioNTech and Pfizer COVID-19 vaccine (BNT162b2), from initiation to receipt of Emergency Use Authorization (EUA), also lasted only about 10 months. This production process also facilitates the efficient generation of a diverse range of antigen-specific mRNA formulations. In 2021, the WHO and the Medicines Patent Pool (MPP) launched the mRNA Vaccine Technology Transfer Programme in South Africa, with Afrigen Biologics & Vaccines designated as the central hub. In April 2023, technology transfer partners, alongside international stakeholders from vaccine manufacturers in various countries, convened in South Africa to assess advancements in mRNA vaccine development and identify potential vaccine targets.[14] Simultaneously, the WHO assembled a global panel of experts to evaluate the feasibility and potential benefits of mRNA-based TB vaccines. Experts agree that the mRNA platform offers a significant opportunity to accelerate the development and approval of new candidates for TB vaccination.

The mRNA-lipid nanoparticle (LNP) vaccine has received considerable attention due to its promising results in clinical trials. However, the notably increased incidence of common adverse events, such as fatigue, chills, myalgia, headache, and myocarditis, particularly following booster vaccinations, raises urgent concerns about the safety profile of this vaccine platform. Comprehensive analysis and reanalysis of clinical trial data from Pfizer and Moderna indicate an elevation in the risk of serious adverse events (SAEs) associated with these COVID-19 vaccines. Collectively, individuals receiving mRNA vaccinations exhibited a 16% increased risk of SAEs.[15,16] The population receiving TB vaccinations includes individuals co-infected with HIV or those with latent tuberculosis infection (LTBI), necessitating increased scrutiny of these heightened side effects in the development of TB mRNA vaccines. More alarmingly, this adverse effect may intensify the skepticism among individuals who are already ambivalent about the advantages of vaccination. Certain novel TB vaccines, such as those that have replaced BCG as the primary immunization strategy, predominantly target neonates due to parental concerns regarding their children's health, cautious vaccine adoption, and a pervasive distrust toward vaccines and vaccination policies. Moreover, the expedited development of innovative mRNA vaccines – alongside rampant misinformation surrounding these advancements – reluctance among healthcare professionals to advocate for them, and an absence of effective communication channels for disseminating information on vaccination further complicate efforts to promote these groundbreaking vaccines.

Moreover, a significant bottleneck in mRNA production lies in the effective scaling of operations. Most equipment currently employed for mRNA synthesis repurposes existing biotechnological apparatus designed for much larger scales than those required for mRNA production. Although mRNA production is well-suited to standardization and platformization, many processes are presently executed across multiple stages using specialized machinery. Therefore, there is an urgent need for the development of integrated and systematic solutions for mRNA processing. Furthermore, although mRNA vaccines utilize cell-free production systems and theoretically have the potential to be more cost-effective than alternative vaccine methodologies, they often incur higher production costs in practice. This discrepancy may reflect a balance between input costs, benefits, and risks associated with developing TB vaccines, which are intrinsically more complex. With regard to packaging and delivery technologies, mRNA faces significant challenges in cellular entry due to its negative charge and susceptibility to rapid degradation by nucleases, such as RNase. Currently, LNP-based strategies are employed in mRNA vaccines to mitigate some of these issues, alongside other methods that involve base modifications or innovative mRNA designs. However, a major challenge associated with nanostructured technology is its inherent complexity, which encompasses numerous potential components and presents various clinical outcomes that remain poorly understood. Furthermore, these advanced technologies and their corresponding patent disclosures often lack transparency. While companies continue to secure funding from private investors, they have largely withheld detailed scientific information, thereby establishing substantial technical barriers for other developers attempting to establish TB mRNA vaccines.

Returning to the Mtb bacterium and the host itself presents another challenge: the intricate nature of this organism makes it unlikely that the strategies used in viral mRNA vaccine development can be directly replicated for TB mRNA vaccines, given Mtb's approximately 4,000 genes.[17] This genetic complexity starkly contrasts with the relatively simple structure of viral genomes and is further complicated by the uncharacterized genetic diversity observed among different Mtb strains.[18] There is currently no evidence to suggest that a single target antigen or a limited number of target antigens play a decisive role in providing protection. Consequently, should the development of a multi-sequence mRNA vaccine be considered for tuberculosis? This represents one of the significant challenges in both production and quality control. Similarly, considering the complex interactions between Mtb and its host, distinct populations exhibit varied responses to Mtb infection,

including heterogeneous subgroups within both alveolar macrophage and monocyte-derived macrophages lineages. To date, advancements in developing effective tuberculosis vaccines have been impeded by an absence of reliable biomarkers for predicting vaccine efficacy and outcomes.[19–22] Consequently, reliance solely on assays such as interferon-gamma release assay (IGRA) or Mycobacterium growth inhibition assay (MGIA), along with analogous biological data from vaccine development initiatives, proves inadequate in substantiating their predictive capacity for immune protection.[19,20] In the context of tuberculosis, if disease outcomes are influenced by the biological characteristics of host macrophages[23]—where distinct populations play pivotal roles in either controlling or facilitating bacterial proliferation – it raises a critical question regarding whether variability among these macrophage subpopulations should be regarded as a significant factor in TB mRNA vaccine development.

In 2023, BioNTech capitalized on its successes with COVID-19 mRNA vaccinations to rapidly advance the development of its polyvalent TB mRNA candidate BNT164. This candidate is formulated from a combination of acute phase, recovery phase, and latent stages of infection, with the coding region specificity comprising Ag85A (with amino acids 1–41 omitted), ESAT-6, M72, VapB47, Hrp1, RpfA, RpfD, and HbhA antigen sequences. However, the specific modifications involved in the BNT164 vaccine antigen have not yet been disclosed. Currently, BNT164 is the only TB mRNA vaccine in phase I clinical trials (NCT05537038; NCT05547464), designed to evaluate safety and immunogenicity following three injections (BNT164a1 and BNT164b1), thereby establishing a foundation for subsequent phases of clinical investigation. The trial involves 120 healthy adults aged between 18 to 55 years and is expected to complete phase I by September 2025. However, whether this mRNA vaccine approach represents a new era in the fight against TB remains a significant international question worthy of consideration.

Potential antigen of TB mRNA vaccine

The complexity of Mtb lies in its lack of clear target antigens, which poses a significant challenge for the development of mRNA vaccines.[24] The traditional vaccine development strategy relies on identifying and targeting specific antigens that can trigger protective immune responses. However, the antigen diversity and immune escape mechanism of Mtb make this process exceptionally difficult.[25] Although mRNA vaccine technology has shown great potential in other fields, its success largely relies on precise antigen selection. The uncertainty of target antigens for Mtb increases research and development costs, prolongs the development cycle, and may affect the efficacy and safety of the final vaccine.[26] Therefore, identifying and verifying effective target antigens of tuberculosis is a key step to promote the development of mRNA vaccine. Among the clinical candidate vaccines for tuberculosis (TB), whether using viral vector vaccine technology, recombinant protein vaccine technology or nucleic acid vaccine technology, screening effective tuberculosis antigen is a crucial step. The names, length, and functions of clinical candidate vaccine antigens are shown in Table 1.

GamTBvac is a recombinant protein vaccine that clones synthetic genes corresponding to the Mtb antigen sequences Ag85a, ESAT6, and CFP10 into the prokaryotic expression vector pET28a. The prokaryotic expression vector includes a D-D-D-binding domain (DBD) and a Gly Ser spacer from Leuconostoc. Two recombinant plasmids (DBD1-AG85a (pL107cc) and DBD1-ESAT6-CFP10 (pL177cc)) were constructed, each encoding a chimeric gene consisting of the nucleotide sequence of the DBD gene, Gly Ser spacer region, and the nucleotide sequence of AG85a or ESAT6-CFP10 Mtb antigen.[27] The phase II clinical trial results of healthy adults vaccinated with BCG showed that GamTBvac vaccine induces antigen-specific interferon gamma release, CD4+T cells expressing Th1 cytokines, and IgG response.[28]

The target antigen of the recombinant protein vaccine M72/AS01E is Mtb72F, which is a recombinant fusion protein based on Mtb22A (Rv0125 encoding PepA) and Mtb39A (Rv1196 encoding PPE18).[29] In order to improve the long-term stability of purified Mtb72F, a point mutation was introduced in Mtb22A, replacing serine 706 with alanine 706. This inactivated the assumed active site of serine protease, resulting in the fusion protein M72 [(30)]. In addition, adding methionine to the N-terminus of M72 polypeptide chain and adding two histidine residues increased the expression level of the protein.[30]

AEC/BC02 is a subunit vaccine composed of recombinant Mtb Ag85b protein, ESAT6-CFP10 fusion protein, and BCG CpG complex adjuvant BC02. It is mainly used for the preventive treatment of latent infected populations.[31] The ID93+GLA-SE recombinant protein vaccine concatenates the Rv3619c, Rv1813c, Rv3620c, and Rv2608 genes to prepare a fusion protein.[32] ChAdOx1.85A+MVA85A is a viral vector vaccine that optimizes the target antigen Ag85A codon for mammalian expression, and uses the Ag85A gene and tissue type plasminogen activator (t-PA) signal peptide as a single coding sequence.[33]

The TB/Flu-05E viral vector vaccine directly links the TB10.4 antigen to the 124 amino acids of NS1 (of influenza virus); After insertion of HspX antigen into the “self protein hydrolysis” site of 2A and IgK signal peptide. The length of the chimeric NS124-TB10.4-HspX gene fragment is 1720 bp.[34]

The H107e/CAF10b recombinant protein vaccine consists of 8 Mtb antigens, with ESAT-6 repeated four times: PPE68-[ESAT-6] - EspI - [ESAT-6] - EspC - [ESAT-6] - EspA - [ESAT-6] - MPT64-MPT70-MPT83. Among them, EspI (Rv3876) protein is missing 75–294 amino acids to achieve large-scale expression.[35]

The goal of preventive vaccines is to generate immune memory so that they can respond quickly and effectively when encountering pathogens in the future. The selection of target antigens usually prioritizes molecules that can trigger specific and persistent immune responses, especially antigens that can activate T cells and B cells.[36] M. tuberculosis is an intracellular parasite that infects antigen-presenting cells in the lungs, including macrophages and dendritic cells, before developing into a predetermined infection during the granulomatous stage.[37] Therefore, the development of tuberculosis vaccine mainly focuses on stimulating cell-mediated immune response, especially screening antigenic epitopes that can activate T cells.[38] These T cell epitopes typically bind to major histocompatibility complex (MHC) molecules on antigen-

Table 1. Clinical candidate vaccine antigen.

Antigen name	Function[a]	Length	Family	Vaccine
Ag85a	(1) The high affinity of mycobacteria for fibronectin; (2) Help to maintain the integrity of the cell wall; (3) Catalyze the transfer of a mycoloyl residue from one molecule of alpha, alpha-trehalose monomycolate (TMM) to another TMM, leading to the formation of TDM; (4) Mediates triacylglycerol (TAG) formation	338aa	A85 antigen family	ChAdOx1.85A +MVA85A GamTBvac BNT164
Ag85b	Same as Ag85a	325aa	A85 antigen family	AEC/BC02
TB10.4	Promoting intracellular bacterial growth	96aa	WXG100 family. ESAT-6 subfamily	TB/FLU-05E
HspX	May slow down the growth rate of M.tuberculosis in culture and by extension during macrophage infection.	144aa	small heat shock protein (HSP20) family	
ESTA-6	Modulates the host's immune response to infection and facilitates bacterial escape into the host cytoplasm	95aa	WXG100 family. ESAT-6 subfamily	GamTBvac AEC/BC02 H107e/CAF10b BNT164
CFP-10	Involved in translocation of bacteria from the host (human) phagolysosome to the host cytoplasm	100aa	WXG100 family. CFP-10 subfamily	GamTBvac AEC/BC02
Mtb32a	Serine protease, conserved protein	355aa	/	M72/AS01E BNT164
Mtb39a	Maybe a key virulence factor for the survival of *Mycobacterium tuberculosis* cells	391aa	PPE family	M72/AS01E BNT164
PPE42	Maybe involved in directing the host toward development of a more humoral type of immune response	580aa	PPE family	ID93+GLA-SE
EsxV	Possible virulence factors.	94aa	WXG100 family. ESAT-6 subfamily	
EsxW	Possible virulence factors.	98aa	WXG100 family. CFP-10 subfamily	
Rv1813	/	143aa	/	
PPE68	Plays a major role in RD1-associated pathogenesis, and may contribute to the establishment and maintenance of M.tuberculosis infection	368aa	PPE family	H107e/CAF10b
EspI	Required to repress ESX-1-mediated secretion under low ATP conditions	666aa	Type VII secretion system ESX-1 associated protein	
EspC	(1) Required for ESX-1 function; (2) Required for either stability or expression of EspA	103aa	EspC family	
EspA	Required for secretion of EsxA (ESAT-6) and EsxB (CFP-10) and for virulence. Involved in translocation of bacteria from the host (human) phagolysosome to the host cytoplasm	392aa	Type VII secretion system ESX-1 associated protein	
MPT64	Cellular response to starvation	228aa	RsiV family	
MPT70	Cell adhesion	193aa	FAS1 structural domain	
MPT83	Cell surface glycolipid proteins	220aa	FAS1 structural domain	
VapB47	Antitoxin component of a type II toxin-antitoxin (TA) system	99aa	phD/YefM antitoxin family	BNT164
Hrp1	Unlike some other CBS-domain containing proteins does not seem to bind AMP	143aa	CBS domain	
RpfA	(1) Factor that stimulates resuscitation of dormant cells; (2) Has peptidoglycan (PG) hydrolytic activity; (3) Stimulates growth of stationary phase M.bovis (a slow-growing Mycobacterium), reduces the lag phase of diluted fast-growers *M. smegmatis* and Micrococcus luteus	407aa	Transglycosylase family. Rpf subfamily	
RpfD	(1) Factor that stimulates resuscitation of dormant cells; (2) Has peptidoglycan (PG) hydrolytic activity; (3) Stimulates growth of stationary phase M.bovis (a slow-growing Mycobacterium), reduces the lag phase of diluted fast-growers *M. smegmatis* and Micrococcus luteus	154aa	Transglycosylase family. Rpf subfamily	
HbhA	(1) Required for extrapulmonary dissemination; (2) Promotes hemagglutination of erythrocytes of certain host species; (3) Induces mycobacterial aggregation	199aa	/	

Note: a: Information regarding the functionality of various antigens in clinical vaccine candidates is obtained from the UniProt database (https://www.uniprot.org/).

presenting cells (APCs), enabling T cells to recognize and respond to these antigens, thereby triggering targeted immune responses.[39]

There are currently multiple TB antigens in the research and development stage. Liu et al. found that PstS1 and its T cell epitope (PstS1p) can significantly increase the level of antigen-specific IgG antibodies and induce strong T cell proliferation and Th1 type immune response. Compared with the traditional antigen Ag85B, PstS1p shows more outstanding performance in promoting cell-mediated immune responses, especially in stimulating the differentiation of CD4+ T cells and the expression of IFN-γ.[40] Rv0674 had good performance in serological test with sensitivity and specificity of 77.1% and 81.1%, respectively. While it shows poor sensitivity and specificity of 26.23% and 79.69% for IFN-γ tests. Moreover, the cytokine profile and IgG isotype characterized Rv0674 as a Th1/Th2-mixed-type protective immunity with the predominance of Th1 cytokines.[41] Wan et, al. constructed a T-cell epitope-rich tripeptide-splicing fragment (nucleotide positions 131–194, 334–377, and 579–643) of Rv2201 (also known as the 72 kDa AsnB) from the Mtb genome, ultimately yielding the recombinant protein Rv2201–519 in Escherichia coli BL21 (DE3).[42] The result show that Rv2201–519 triggered a strong Th1 response, with increased antigen-specific IgG and higher IFN-γ/IL-4 ratios. It also induced more IL-6 and activated CD4+ and CD8+ T cells. In addition, multiple studies have screened T cell epitopes of Mtb and identified immunogenic epitopes that can activate T cells, especially CD4+ and CD8+ T cells, thereby triggering an immune response. Cecilia et, al. studied 21 active tuberculosis patients using a library containing 20,610 Mtb derived peptides, and identified 137 unique T cell epitopes, mainly from cell wall and cell process antigens.[43] The study found that 16% of these epitopes can be recognized by two or more participants and are associated with the rapidly differentiating Mtb gene. The study also developed a peptide pool (ATB116) specifically recognized by active tuberculosis (ATB) patients, which can distinguish ATB patients from IGRA+/IGRA – individuals with over 60% sensitivity and over 80% specificity, demonstrating its potential as a diagnostic tool. In addition, the study also observed changes in T cell response to ATB116 during ATB treatment, indicating that these epitopes may be associated with disease activity rather than protective immune response. A total of 79 whole-genome samples of Mtb strains isolated from Indian patients were analyzed, and 905 human CD4+ T cell epitopes from the IEDB database were screened .[44] Ultimately, 64 mutated T-cell epitopes (mTCEs) were identified, 89% of which were novel discoveries. Among these, the mutated epitopes significantly impacted the functional secretion o IFN-γ and/or IL-2 by CD4 + T cells. Notably, gain-of-function mutations increased HLA-DR binding affinity, while loss-of-function mutations reduced it. With the advancement of artificial intelligence, an increasing number of researchers are using bioinformatics software to predict tuberculosis T-cell epitopes. Zhang et al. used four bioinformatics software tools (DNAStar, SYFPEITHI, RANKPEP, and NetMHC IIpan) to predict CD4+ T cell epitopes of protective antigens from Mtb (including CFP10, 38KDa, ESAT6, Ag85A, and TB27.4).[45] Epitopes were comprehensively screened based on their affinity scores with HLA-DRB1 × 0701, protein structure, hydrophobicity/hydrophilicity, and surface accessibility. Five novel candidate CD4+ T cell epitope peptides – P39, P50, P40, P185, and P62—were predicted and validated. Among them, P39 and P62 demonstrated strong immunogenicity, effectively inducing CD4+ T cell proliferation, promoting the secretion of IFN-γ, TNF-α, and IL-2, and suppressing the secretion of IL-10. The combination of P39 and P62 (P39+P62) showed higher sensitivity (95.83%) and specificity (97.91%), indicating its potential application in tuberculosis diagnosis and vaccine development. In addition to T-cell epitopes of Mtb, other proteins of the bacterium have also been screened and widely applied in the development of tuberculosis vaccines. Alireza et al. used the GlycoPP and IEDB databases to identify and predict the O- and N-glycosylation sites of Mtb protein extracts, and the BepiPerd-2.0 server to predict B cell epitopes.[46] The research identified 293 glycoproteins and found that some of them, such as Rv0954, LpqN (Rv0583), PPE68 (Rv3873), Phosphorylated binding proteins (Rv0932c), PPE61 (Rv3532), and LprA (Rv1270c) have high glycosylation percentages, and these glycoproteins are present in Mtb plays an important role in survival, antigenicity and immunogenicity, and may be a new candidate for tuberculosis vaccine and a therapeutic target.

At present, the potential vaccine antigens for tuberculosis still need further research. A deep understanding of the functions of proteins related to Mtb and their mechanisms of action during infection is crucial for us to screen and identify more effective antigens. In the future, we should be committed to in-depth research and screening of these antigens to optimize the antigen design of mRNA vaccines, so as to improve the protective efficacy and adaptability of vaccines and provide more effective strategies for the prevention and treatment of tuberculosis.

TB mRNA vaccine platforms

mRNA acts as an intermediary between the transcription of protein-coding DNA and the synthesis of proteins by ribosomes within the cytoplasm. There are three distinct therapeutic modalities that utilize mRNA technology: (1) Cellular therapy, which involves transfecting cells with mRNA ex vivo to alter their phenotype or function before reintroducing them into the patient. (2) Replacement therapy, where mRNA is directly administered to patients to compensate for defective genes or proteins or to provide therapeutic proteins. (3) Vaccination, where healthy individuals are vaccinated with specific antigen-encoding mRNAs to elicit protective immunity.

Although mRNA was discovered in 1961, it took several decades of scientific research and experimental studies to uncover its potential as a promising platform for developing both preventive and therapeutic vaccines. The successful development of the mRNA vaccine for SARS-CoV-2 in 2020 has significantly advanced this platform. Two such vaccines, mRNA-1273 and BNT162b2, have received approval from the U.S. Food and Drug Administration (FDA), have been administered to hundreds of millions of people, and have demonstrated high protective efficacy of over 90%.[47] This success opens new avenues for TB vaccine development,

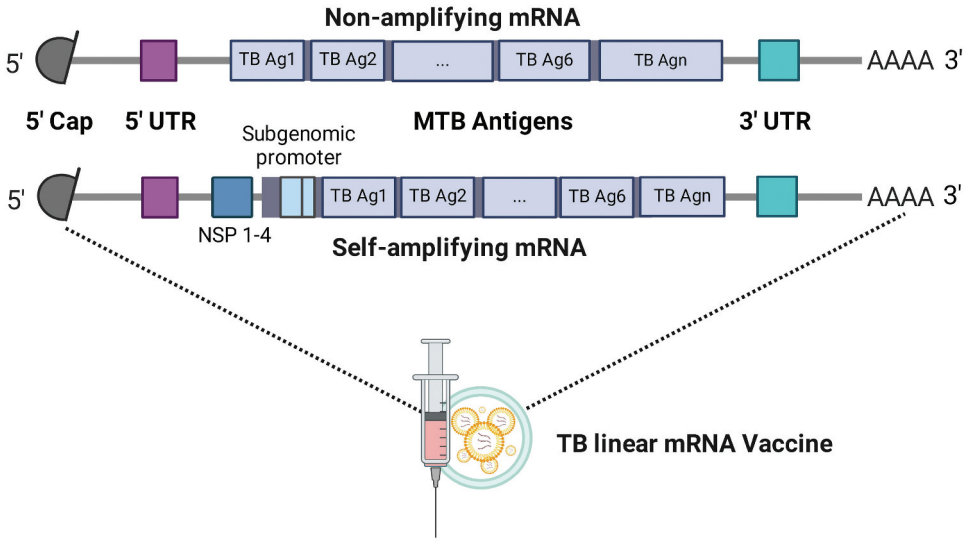

Figure 2. A comparison of TB linear mRNA vectors. Both conventional non-amplified and self-amplifying mRNAs share fundamental components, including a cap, 5′ UTR, 3′ UTR, and poly(a) tail of varying lengths. In addition to these components, self-amplifying RNA (saRNA) also encodes four non-structural proteins (nsP1–4) and a subgenomic promoter derived from the genome of the alphavirus genome.

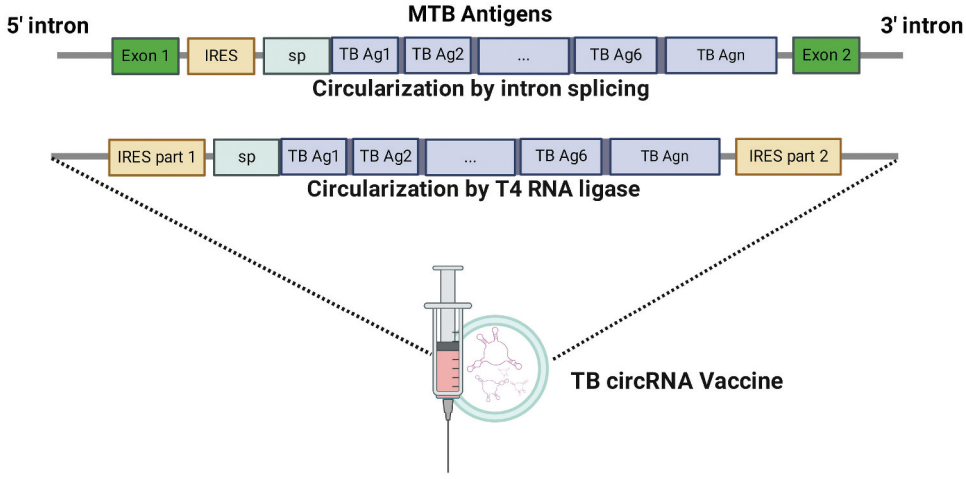

Figure 3. A comparison of TB circular RNA. Pre-circRNA was synthesized by IVT, and then TB circRNA was generated by two cyclization strategies: enzyme synthesis and intron self-splicing.

where traditional approaches have faced significant challenges. Currently, two main types of mRNA are being studied for use in TB vaccines: linear mRNA vaccines and circular RNA vaccines (Figures 2 and 3). These vaccines utilize the host cell's translational machinery to produce the target antigen and initiate an adaptive immune response.

TB linear mRNA vaccine

Non-amplifying TB mRNA vaccine

Conventional non-amplified mRNA (NAM) platforms have been extensively studied for their applications in in vitro RNA synthesis for vaccines. A typical TB NAM construct comprises a cap, 5′-untranslated regions (UTRs), open reading frames (ORFs) that encode TB vaccine antigens, 3′-UTRs, and a poly (A) tail (Figure 2). These components are crucial for mRNA maturation, transport, and the initiation of translation, as well as for preventing premature degradation of the mRNA.[48–52] Additionally, chemical modifications of nucleotides within the ORF, such as thiouracil, methylcytosine and pseudouracil, can mitigate the inherent immune responses against the mRNA.[53–56] Base editing and optimizing the sequence to increase the C/G ratio can also enhance the stability of TB mRNA. However, such modifications to bases and nucleotides may alter the mRNA's secondary structure, potentially affecting protein folding and T cell epitope presentation, which could reduce immunogenicity. While these features are advantageous for preclinical studies, including quality control during NAM-based TB vaccine development, there remains a need to extend the duration and increase the levels of in vivo mRNA expression to induce a robust immune response against intracellular bacteria.

Self-amplifying TB mRNA vaccine

Self-amplifying TB mRNA (SAM) represents an evolution of traditional messenger RNA technology, designed to overcome

its limitations through intracellular amplification. By employing RNA-dependent RNA polymerases (RdRp) derived primarily from alphaviruses and related agents,[57] these innovative vaccines enhance the production of TB antigens within host cells via amplified RNA delivery[58] (Figure 2). In contrast to conventional mRNA vaccines, SAM vaccines are characterized by their larger size and more complex structure. In addition to the standard components of mRNA, SAM vaccines incorporate additional sequences encoding viral nonstructural proteins that enable intracellular replication of the mRNA, leading to prolonged and higher levels of antigen expression. SAM constructs include an extended ORF encoding key elements for RdRp activity, such as nonstructural proteins 1 to 4 (Nsp1–4), as well as specific TB genes under the control of subgenomic promoters. The various functions of these components, including mRNA capping, NTPase/helicase/protease activities, macrodomain interactions, and RdRp activity, contribute to the assembly of the replication complex during translation.[59–61] Several strategies are employed to achieve optimal antigen expression, including DNA plasmid-based systems, virus-like particles, and in vitro transcription techniques. Although antigen production in SAM vaccines begins gradually, the sustained presence of antigens significantly prolongs stimulation periods for antigen-presenting cells (APCs).[62] However, a major limitation of these vaccines is their long and complex sequences, which often exceed 9 kb, mainly due to the extensive Nsp1–4 sequences that span approximately 7 kb. To address this, a trans-amplification strategy (taRNA) using bipartite RNA vectors has been developed to reduce the overall nucleotide length while enhancing stability.[63,64] The efficient expression driven by non-replicating RNA encoded replicase in taRNA systems has shown comparable efficacy to conventional monopartite SAM models in mouse influenza challenge models.[65] Additionally, the conserved pathogen-associated molecular patterns (PAMPs) of viral vectors pose challenges for controllability, raising concerns about immunogenicity-related antibody responses in previously exposed individuals.[66]

TB circular RNA vaccine

In addition to linear mRNAs, circular RNAs (circRNAs) represent a significant alternative for the design of TB mRNA vaccines. Currently, there are no TB circRNA vaccines in clinical development.[67] Linear RNA precursors (pre-circRNAs) serve as the foundation for circRNA vaccine formulation, necessitating the incorporation of multiple elements to establish comprehensive vaccine characteristics and functionalities. Internal ribosome entry sites (IRESs) and ORFs constitute the essential components of circRNA vaccines.[68] For mRNA, its 5‘and 3’ UTRs facilitate the recruitment of RNA-binding proteins (RBPs), thereby enabling translation initiation and enhancing mRNA stability through a 5‘cap structure and a 3’ poly(A) tail.[69,70] In contrast, circRNA does not require UTR structures or a 5‘cap and 3’ poly(A) tail;[71,72] however, regions flanking IRES-ORF can be engineered as UTR analogs to mimic RBP binding sites and enhance translation efficiency. For instance, the introduction of N6-methyladenosine (m6A)-modified motifs – specifically RRACH where R denotes G or A while H signifies A, C or U – facilitates the recruitment of YTHDF3 along with translation initiation factors eIF4G2 and eIF3A.[73,74] As a nucleoside modification that emulates endogenous RNA properties, m6A also serves to diminish immunogenicity while improving circRNA stability.[75]

Pre-circRNA was synthesized using IVT, followed by the generation of circRNA through various cyclization methodologies. Currently, the preparation of circRNA predominantly relies on two principal cyclization strategies: enzymatic synthesis and intron self-splicing (Figure 3). The enzymatic synthesis approach primarily utilizes RNA ligases, such as T4 RNA ligase, to catalyze the formation of 3’–5’ phosphodiester bonds.[68] Intron self-splicing mechanisms can be further classified into type I and type II categories, both capable of establishing a properly arranged intron-exon (PIE) system. By integrating effective techniques for enhancing cyclization rates, such as incorporating homologous arm sequences, it is feasible to achieve cyclization of sequences up to 5 kb in length.[76,77] The resultant cyclic RNA consists of precursor RNA, circular RNA, and nicked fragments; therefore, the identification and purification processes for circRNA are critical steps in ensuring the quality and safety of circRNA vaccines.[78]

Currently, circRNA can be administered in either its unencapsulated form or encapsulated within delivery vehicles such as LNPs.[79] The unmodified circular RNA vaccine does not require any delivery system and is directly injected as a circular RNA solution. This method capitalizes on APCs at the injection site, particularly dendritic cells, which employ DC-mediated macroendocytosis for the intradermal administration of naked mRNA. Subsequently, circRNA expresses antigens with the assistance of dendritic cells. Alternative mRNA delivery strategies, including poly nanoparticles, cationic nanoemulsions, and exosomes among others, may also facilitate circRNA transport. While LNPs are currently predominant in use, they present several limitations such as low biocompatibility associated with organic solvents, specific storage requirements, and restricted routes of administration.[80] As circRNA vaccines advance, the development of next-generation delivery systems becomes essential.

In comparison to the corresponding modified linear RNA, circRNA demonstrates enhanced stability within a specific temperature range due to its circular structure, which is less susceptible to degradation by exonucleases.[81] Current linear RNA vaccines necessitate stringent storage and transportation conditions,[82] whereas circRNA vaccines encapsulated in LNPs can be stored at 4°C for a minimum of four weeks and at room temperature for approximately two weeks.[83] This characteristic is particularly advantageous for the development of TB vaccines, especially when introducing these novel vaccines into less developed countries or regions severely affected by TB epidemics. Furthermore, circRNA exhibits an extended half-life in animal models, requiring only low immunogenic doses to achieve sustained protein expression, thereby facilitating continuous immune system activation and providing enduring immune protection.[84] This positions circular RNA as a promising candidate in TB vaccine development.

Additionally, circRNA vaccines exhibit lower immunogenicity compared to linear mRNA vaccines, suggesting they may elicit fewer adverse reactions and enhance vaccine safety and tolerance.[85] CircRNA vaccines have demonstrated encouraging therapeutic and prophylactic effects against COVID-19,[86] malignant melanoma,[87] and monkeypox virus.[88] However, the advancement and production of circRNA vaccines remain in their nascent stages and encounter numerous technical challenges. For circRNA vaccines, the cyclization rate significantly influences vaccine immunogenicity. Thus, it is imperative to enhance the cyclization efficiency of linear RNA precursors while establishing suitable purification methods to eliminate impurities. Moreover, given the unique characteristics of circRNA vaccines, existing quality control methodologies may not be entirely applicable. Therefore, new approaches must be developed to ensure vaccine quality and safety.

Mechanisms of mRNA-based TB vaccines

The precise mechanisms underlying mRNA vaccines, particularly the mRNA-based TB vaccine, have not yet been fully elucidated. However, it is widely accepted that, when administered through parental routes (such as intramuscular, subcutaneous, or intradermal injections), mRNA vaccines can transfect either nonimmune cells (via the myocyte pathway) or immune cells (via the APC pathway) in the vicinity of the injection site (Figure 4).

Transfection of nonimmune cells results in the production of Mtb target antigens which are subsequently degraded by proteasomes. After degradation, antigen-derived epitopes form complexes with major histocompatibility complex (MHC) class I molecules, facilitating antigen presentation to $CD8^+$ cytotoxic T cells and thereby establishing cellular immunity. In 1990, Wolff JA et al.[89] first demonstrated that mRNA could directly transfect muscle cells in vivo, leading to the expression of the encoded protein. Subsequent studies have further shown that intramuscular injection of mRNA-encoding reporter genes results in protein expression in myocytes.[90–92] It has also been reported that when naked or encapsulated mRNA is delivered via a gene gun or other delivery methods, expression predominantly occurs in somatic cells.[93,94]

mRNA vaccines can also transfect tissue-resident immune cells, primarily APCs such as DCs and macrophages.[95] The parenteral administration of TB mRNA vaccines can induce local immune responses at the injection sites,[96,97] recruiting immune cells and facilitating the transfection of tissue-resident

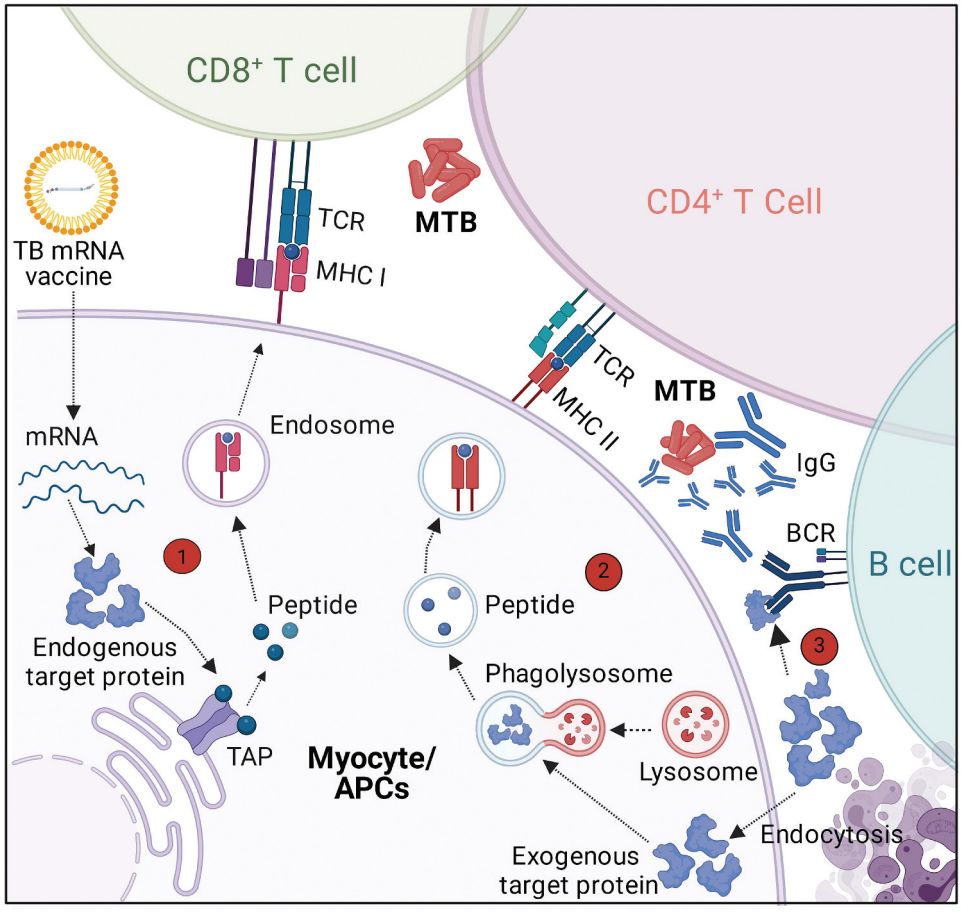

Figure 4. Mechanisms underlying the adaptive immune responses elicited by TB mRNA vaccines. The TB mRNA vaccines are enclosed in nanoparticles and internalized by myocytes or antigen-presenting cells. The mRNA is released into the cytoplasm upon escaping from endosomes, where it is then translated into antigenic proteins by ribosomes. Subsequently, endogenous antigens are degraded into polypeptides by the proteasome and presented to $CD8^+$ T cells via MHC I (①). Additionally, secreted antigens can be internalized by cells, degraded within lysosomes, and presented on the cell surface to helper T cells via MHC class II for $CD4^+$ T cell recognition (②). Simultaneously, the secreted exogenous target protein can also directly stimulate B cells to generate anti-tuberculosis antibodies targeting Mtb (③).

immune cells such as DCs and macrophages.[54] Similar to the myocyte pathway described earlier, the transfection of these immune cells by TB mRNA leads to antigen presentation via MHC class I molecules, resulting in the maturation of CD8+ T cells. There is preliminary evidence suggesting that SAM vectors may also be capable of in vivo transfection of APCs.[98] Furthermore, antigen expression predominantly occurs in muscle fibers following administration with lipid-based delivery systems, raising questions about the capability of somatic muscle cells to initiate $CD8^+$ T cell responses.[99]

In addition, APCs process antigens through MHC class II pathways, leading to the activation of $CD4^+$ T helper cells following their transfection. Due to their robust antigen-presenting capabilities, DCs are particularly receptive to RNA transfection. They initiate adaptive immune responses by internalizing and proteolytically processing Mtb antigens, which are then presented on MHC class II molecules.

Chemistry, manufacturing, and controls (CMC) of TB mRNA vaccines

Manufacture of TB mRNA vaccines

The development of new vaccines is often a lengthy and expensive process, primarily due to the absence of standardized procedures. To ensure the production of safe, effective, and high-quality vaccines, it is essential to utilize equipment, facilities, and procedures that adhere to Good Manufacturing Practice (GMP) guidelines. Currently, viral vectors and nucleic acid technologies are two advanced platforms in vaccine development, providing the versatility and features needed to accelerate both vaccine development and production. However, viral vector vaccines are associated with high costs and complex manufacturing processes. In contrast, DNA vaccines, a subset of nucleic acid vaccines, often exhibit limited immunogenicity, reducing their suitability for certain clinical applications. mRNA technology offers distinct advantages, as modifications to the coding antigen do not alter the physicochemical properties of the mRNA backbone. This allows for greater flexibility and simplification in the production of mRNA vaccines, rendering them a more attractive option compared to traditional vaccines and even DNA vaccines.

To produce TB mRNA vaccines with specific quality attributes, a series of precise manufacturing steps must be followed. The entire manufacturing platform typically includes multiple stages, such as the upstream processing stage for the enzymatic synthesis of mRNA and the downstream processing stage for the purification of the mRNA product. These steps are integrated with LNP formulation and fill-finish processes to complete the production of the final TB mRNA vaccine product (Figure 5).

Plasmid DNA (pDNA) is a critical precursor in the production of mRNA vaccines, serving as a template for mRNA synthesis. In the manufacturing process, the sequence encoding the target antigen is designed and optimized based on the Mtb genome, followed by the construction of a DNA plasmid containing this sequence. Subsequently, the necessary DNA stock solution is prepared through several steps, including amplification, purification, and quality assessment. The pDNA is then linearized and used as a template for IVT to produce the desired mRNA. In addition to linearized DNA, mRNA synthesis requires the inclusion of several components, such as RNA polymerase, nucleotide triphosphate (NTP) substrates, a polymerase cofactor ($MgCl_2$), and a pH buffer

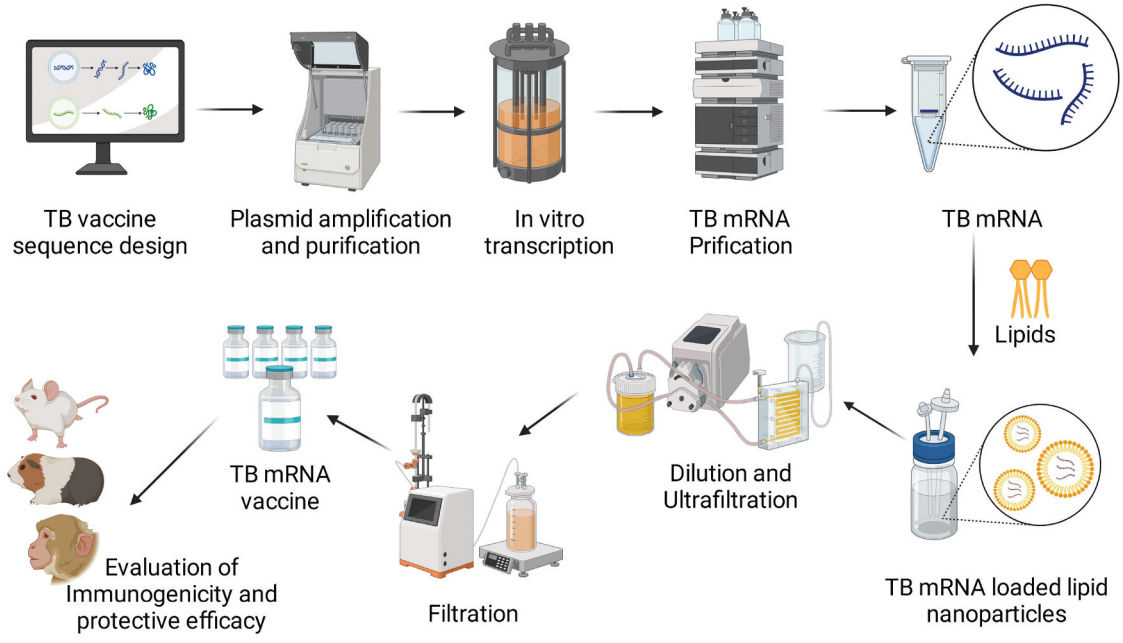

Figure 5. A schematic diagram of the TB mRNA vaccine preparation process. The production of TB mRNA involves a two-step process that includes both synthesis and purification. The mRNA sequence can be generated via either a single-step or a two-step enzymatic approach. In the latter case, the two-step enzymatic reaction consists of in vitro transcription of the DNA template to produce an RNA polymerase transcript, which can subsequently be capped using vaccinia capping enzyme along with a methyl donor. This procedure concludes with purification to eliminate the DNA template, followed by TFF for buffer exchange and concentration, and finally sterile filtration through a 0.2 µm filter.

containing polyamines and antioxidants.[100,101] These modifications significantly reduce reaction times compared to traditional vaccine production methods, minimize contamination risks, and enable standardized production processes that are independent of the specific antigens encoded in the template. To enhance mRNA stability, it is necessary to incorporate a cap structure at the 5' end of the mRNA; however, using cap analogs can affect production costs, particularly in large-scale manufacturing.[102] Therefore, it is crucial to conduct a comprehensive cost analysis of the one-step capping method (capping during the IVT process) and the two-step capping method (capping after IVT) production methods. Producing mRNA through IVT under current Good Manufacturing Practice (cGMP) conditions presents substantial challenges compared to the production of mRNA vaccines for preclinical studies using commercial kits at the laboratory scale. This difficulty arises from the limited availability and high procurement costs of specialized IVT components that must be sourced from certified suppliers to ensure they are animal-free and meet GMP-grade standards.[103,104]

The purification of the mRNA stock solution is a critical step in the downstream process, as the purity of the final mRNA vaccine directly affects its safety and efficacy. Achieving high purity and yield requires effective purification methods at the preparation scale. The mRNA stock solution contains not only the desired target product but also various impurities, including enzymes, NTPs, DNA templates, and aberrant mRNA products generated during the IVT process. Conventional laboratory-scale purification methods, such as DNase digestion to remove residual DNA and lithium chloride (LiCl) precipitation to eliminate process-related impurities, are insufficient for removing product-associated impurities (e.g., double-stranded RNA (dsRNA) and truncated RNA fragments). To effectively eliminate these impurities, which can negatively affect mRNA performance, advanced techniques such as affinity chromatography, tangential flow filtration (TFF), size exclusion chromatography (SEC), ion pair reverse-phase chromatography (IPC), and ion exchange chromatography (IEC) are employed to purify the mRNA stock solution.[105–111] To protect the active mRNA component from degradation before cellular uptake, LNPs are used for encapsulation. This process involves mixing various lipid components in specific ratios and dissolving them in ethanol or other organic solvents. The mRNA is dissolved in water and then diluted with an acidic buffer to achieve the desired concentration. These two phases are then uniformly mixed using a microfluidic device to rapidly produce nucleic acid lipid nanoparticles encapsulating the mRNA. Subsequent ultrafiltration is performed to remove the organic phase and any unencapsulated free components from the mRNA-LNP solution. Additionally, necessary excipients may be added to enhance long-term stability during storage.

TB mRNA vaccine safety and quality

To enhance the production and preparation of mRNA, there is currently a focus on establishing robust manufacturing processes that ensure consistent, high-quality products. However, it is crucial to define key process steps and standards for product quality control in order to enable rigorous quantification and characterization of the product. Therefore, developing and refining regulatory guidelines for assessing the quality, safety, and efficacy of RNA vaccines is essential.

In August 2020, the Center for Drug Evaluation (CDE) of the National Medical Products Administration (NMPA) issued the "Technical Guidelines for Pharmaceutical Research of Novel Coronavirus Preventive Vaccine (Draft Edition)."[112] These guidelines provide comprehensive guidance for the design, development, production, final activity evaluation, and clinical research considerations of novel coronavirus mRNA vaccines in special emergencies. In December of the same year, the WHO released guidance on "Evaluation of the quality, safety and efficacy of messenger RNA vaccines for the prevention of infectious diseases: regulatory considerations."[113] More recently, the United States Pharmacopeia (USP) released the "Analytical Procedures for Quality of mRNA Vaccines and Therapeutics-Draft Guidelines: 3rd Edition" (with earlier versions issued in February 2022 and April 2023). This enhanced focus on mRNA quality control indicators provides more specific analysis methods and reference standards. These documents serve as guidelines for developing testing procedures related to mRNA vaccines, which aim to enhance public confidence in innovative products such as mRNA vaccines and therapies by ensuring their safety, efficacy, and controlled quality. Additionally, they establish a set of common quality analysis methods based on verifying the authenticity of mRNA active ingredients while controlling impurities. This can support developers, manufacturers, regulatory agencies, and national control laboratories worldwide in conducting relevant research.

The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q8 guideline introduces the concept of Quality by Design (QbD), emphasizing that the objective of drug development is to design a high-quality product along with a manufacturing process that consistently delivers the intended performance. The scientific knowledge and insights obtained during drug development and production help establish the design space and the product quality control system.[114] For mRNA vaccines, the nucleic acid structure of the mRNA and the properties of the liposome-encapsulated formulations define key quality control attributes, such as mRNA sequence and integrity, content and purity, capping efficiency, poly(A) tail structure or length, among others. In May 2022, the CDE of NMPA issued the "Technical Guidelines for Pharmaceutical Research and Evaluation of in Vitro Gene Modification Systems (Draft Edition),"[115] which further elaborated on these aspects. Non-clinical studies on lipid delivery systems must consider lipid toxicity and the potential induction of autoreactive antibodies in humans; the quality studies for these systems should focus on meeting Critical Quality Attributes (CQAs) similar to those for conventional nanomedicines. Specific attention should be given to parameters such as zeta potential, particle size distribution, pH, encapsulation efficiency, mRNA integrity post-encapsulation, functional performance and content following encapsulation, as well as mRNA release. These parameters are critical quality indicators that directly affect the vaccine's

Quality	Attribute	Reference Analytical Procedures	Plasmid DNA	RNA Drug Substance	RNA Drug Product
Identity	DNA sequence identity confirmation	Restriction map, Sanger sequencing	✓		
	Homologous regions including LTRs, and ITRs	Restriction map, Sanger sequencing, PCR	✓		
	mRNA sequence identity confirmation	Sanger sequencing, HTS, RT-PCR		✓	✓
	Identity of lipids	RP-HPLC-CAD			✓
Content	DNA Plasmid concentration	SEC-HPLC, UV	✓		
	RNA concentration	RT-PCR, RT-dPCR, UV		✓	✓
	RNA encapsulation efficiency	Fluorescence-based assay		✓	✓
	Lipid content	RP-HPLC-CAD			✓
Integrity	mRNA intactness	CE, CGE, AGE		✓	
	LNP size and polydispersity	DLS			✓
	RNA size and integrity	CGE			✓
Purity	DNA Plasmid purity	UV	✓		
	Plasmid DNA topology (% Supercoiled)	AGE, CGE, AEX-HPLC	✓		
	Residual host protein	ELISA, BCA, Lowry	✓		
	Residual host DNA	qPCR	✓		
	Residual host RNA	AGE, RP-HPLC, RT-PCR	✓		
	Residual antibiotic (kanamycin etc.)	ELISA, MS, RP-HPLC	✓		
	mRNA purity	IP-RP-HPLC		✓	
	5′capping efficiency	LC-MS/MS, IP-RP-HPLC, RP-LC-MS/MS		✓	✓
	3′poly(A) tail length	IP-RP-HPLC, LC-MS/MS		✓	✓
	dsRNA	Immunoblot, ELISA		✓	✓
	Aggregate quantitation	SEC-HPLC		✓	✓
	Percentage of fragment mRNA	IP-RP-HPLC		✓	✓
	Residual DNA template	qPCR		✓	✓
	Quantitation of free/non-incorporated nucleosides	RP-LC-MS/MS		✓	✓
	residual NTP and capping agent	AEX-HPLC		✓	
	residual T7 RNA polymerase content	ELISA		✓	✓
Potency	Expression of target protein	Cell-based assay		✓	✓
Safety	Endotoxin	ChP, USP <85>	✓	✓	✓
	Bioburden	ChP, USP <61>, <62>, <1115>	✓	✓	✓
	Sterility	ChP, USP <71>	✓		✓
	Mycoplasma	ChP, USP <63>	✓		
Other	Appearance	ChP, USP <790>	✓	✓	✓
	Residual solvents	ChP, USP <467>		✓	✓
	pH	ChP, USP <791>	✓	✓	✓
	Osmolality	ChP, USP <785>			✓
	Subvisible particles	ChP, USP <787>			✓
	Extractable volume	ChP, USP <1>, <698>			✓
	Container closure integrity	ChP, USP <1207>			✓

Figure 6. Characterization and release testing criteria for plasmid DNA, mRNA drug substances and mRNA drug products. PCR: Polymerase chain reaction, HTS: High throughput sequencing, RT-PCR: Reverse Transcriptase-PCR, RP-HPLC-CAD: Reversed phase-high performance liquid chromatography with charged aerosol detector; SEC-HPLC: Size exclusion high performance liquid chromatography, UV: Ultraviolet Spectroscopy; Reverse-transcription digital PCR: RT-dPCR, CE:Capillary electrophoresis, CGE: Capillary gel electrophoresis, AGE:Agarose gel electrophoresis, DLS:Dynamic light scattering, AEX-HPLC: Anion exchange high-performance liquid chromatography, ELISA: Enzyme-linked immunosorbent assay, BCA: Bicinchoninic acid, MS: Mass spectroscopy, IP-RP-HPLC: Ion pair reversed-phase high-performance liquid chromatography, LC-MS/MS: Liquid chromatography mass spectroscopy, RP-LC-MS/MS: Reverse-phase liquid chromatography mass spectroscopy, USP: United States Pharmacopeia

efficacy and safety. Currently, the quality control measures for mRNA vaccines, as reported in relevant guidelines and literature, primarily address three key areas: plasmid DNA, mRNA drug substance, and mRNA drug product (Figure 6).

Future outlook

Scientific and technological innovation constitutes a fundamental component of the 'End-TB Strategy' and is essential for achieving the TB control targets outlined in the United Nations Sustainable Development Goals. The Global Strategy for TB Research and Innovation, adopted during the 72nd World Health Assembly in 2020, emphasizes the importance of creating an enabling environment for scientific research by mobilizing both national and international funding for TB research, maximizing the potential of data sharing, and enhancing global efforts to ensure equitable access to the benefits of research and innovation. In 2021, WHO introduced an assessment checklist for TB research and innovation[116] aimed at guiding countries in reforming their policies and interventions by aligning their implementation of this global strategy with local contexts. Tuberculosis mRNA vaccine represents the new direction of tuberculosis prevention, and has the advantages of high efficiency, rapid development and safety. By encoding specific antigens of Mtb, mRNA vaccines can accurately stimulate immune responses and quickly respond to strain variations and drug resistance issues by adjusting sequences. In addition, mRNA vaccines are not integrated into the host genome, have high safety, and have standardized production processes, making them easy to scale up. They are expected to be widely used worldwide. However, vaccine research and development is a systematic process that encompasses several stages, including laboratory design, preliminary exploration and verification of protection, non-clinical safety evaluation, pilot process research, quality assessment, and standardization. This is followed by phase I, II, and

III clinical studies to further evaluate the safety and efficacy of vaccines in humans. The entire process necessitates multidisciplinary and multi-systematic approaches, including basic TB research, vaccine development, quality control and regulation, and clinical evaluation.

The financial burden of advancing a mRNA vaccine from pilot studies to clinical trials is substantial, and the duration of phases I through III in human clinical trials is protracted, involving numerous subjects and rife with uncertainties. The assessment of preclinical efficacy for mRNA vaccines plays a crucial role in estimating their development trajectory during research and development processes. Currently, there exists no standardized evaluation platform for assessing the preclinical effectiveness of TB mRNA vaccines, resulting in a lack of comparability and credibility among findings from various laboratories. Furthermore, an accepted immunological surrogate endpoint for evaluating TB mRNA vaccine efficacy is still lacking. As a result, effectiveness assessments continue to rely on ultimate protection rate indices, which further complicates the challenges associated with TB mRNA vaccine development. Additionally, some teams may lack experience in TB mRNA vaccine product development and have a limited understanding of the regulatory frameworks governing this process, as well as insufficient capabilities to implement comprehensive quality control strategies throughout the entire product lifecycle. Therefore, it is imperative to enhance regulatory scientific research within the domain of TB mRNA vaccines by establishing novel tools, methodologies, and standards for safety assessment as well as efficacy and quality control evaluations pertaining to new TB mRNA vaccines. This will facilitate breakthroughs in critical evaluation technologies essential for translating innovative research into practice, while also supporting review processes that guide research and development (R&D) efforts aimed at developing new TB mRNA vaccines and promoting the application of research outcomes. Concurrently, through regulatory scientific research, we aim to establish a credible platform for evaluating mRNA vaccine effectiveness. This platform will enable the objective assessment of mRNA vaccines developed by various teams, using preliminary efficacy data based on standardized criteria. It will help identify mRNA vaccines with genuine potential for further research, thereby mitigating risks associated with mRNA vaccine development and enhancing confidence among enterprises to pursue transformation efforts. Additionally, it aims to prevent the waste of human resources, materials, finances, and time.

Conclusion

According to the Treatment Action Team's 2022 TB Vaccine Pipeline report, "TB vaccine development is not a 100-day sprint but an endurance marathon." This lengthy, perilous, and costly development process can diminish funders' interest; as a result, many TB vaccine candidates spend more time in preclinical trials than in clinical trials. However, this trend is predictable. Although TB mRNA vaccine faces challenges such as complex immune escape mechanism and delivery efficiency, with the progress of technology and clinical trials, TB mRNA vaccine is expected to become an important tool to end tuberculosis, providing new hope for achieving the goal of "a world without tuberculosis." In the near future, it is anticipated that innovative TB mRNA vaccine research teams, incubation centers, manufacturing enterprises, regulatory agencies, and clinical trial units will be effectively integrated to establish a formidable synergy of "production, education, research, monitoring, and application." This integration will facilitate the accelerated advancement of novel TB mRNA vaccines.

Disclosure statement

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Notes on contributor

Dr. Miao Xu, Chief Expert of Quality Control of Biologicals at the National Institutes for Food and Drug Control (NIFDC), a member of the 12th National Pharmacopoeia Committee, and a member of the WHO Biological Products Standardization Expert Advisory Committee, has extensive experience in developing new methods and standards for the quality evaluation of vaccines and other biological products. In the field of tuberculosis (TB) diagnosis, she pioneered a novel neoantigen skin test diagnostic method, which is recognized as one of the three most advanced developments globally in this domain and has received international acclaim. Additionally, her team made significant contributions to the precise immunoprophylaxis of TB by developing mouse and guinea pig models with varying drug resistance profiles, infection routes, and stages of disease progression. These models have been instrumental in the pharmacodynamic evaluation of TB vaccines, facilitating the advancement of China's first novel adjuvant subunit TB vaccine into clinical trials. Leveraging the platform of the WHO Collaborative Center for Biological Product Standardization, Dr. Xu actively participates in the preparation and revision of international standards for biological products.

Author contributions

Conceptualization, J.L., A.Z., and M.X.; Data collection, J.L., X.L., J.W., D. L., and W.D.; writing – original draft preparation, J.L.and D.L.; writing – review and editing, A.Z. and M.X. All authors have read and agreed to the published version of the manuscript.

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