

Challenges and opportunities in mRNA vaccine development against bacteria

Ilke Aernout^{1§}, Rein Verbeke^{1§}, Fabien Thery^{2,3}, Patrick Willems^{2,3,4,5}, Uri Elia^{6,7,8,9,10}, Stefaan C. De Smedt¹, Rino Rappuoli¹¹, Dan Peer^{6,7,8,9}, Francis Impens^{2,3,12*}, Ine Lentacker^{1*}

1. Ghent Research Group on Nanomedicines, Ghent University, Ghent, Belgium
2. VIB-UGent Center for Medical Biotechnology, VIB, Ghent, Belgium
3. Department of Biomolecular Medicine, Ghent University, Ghent, Belgium
4. VIB-UGent Center for Plant Systems Biology, VIB, Ghent, Belgium
5. Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
6. Laboratory of Precision Nanomedicine, Shmunis School of Biomedicine and Cancer Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel
7. Department of Materials Sciences and Engineering, Iby and Aladar Fleischman Faculty of Engineering, Tel Aviv University, Tel Aviv, Israel
8. Center for Nanoscience and Nanotechnology, Tel Aviv University, Tel Aviv, Israel
9. Cancer Biology Research Center, Tel Aviv University, Tel Aviv, Israel
10. Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Ness-Ziona 76100, Israel
11. Biotechnopolo di Siena, Italy
12. VIB Proteomics Core, VIB, Ghent, Belgium

§/* These authors contributed equally

*Corresponding Authors: Francis.Impens@vib-ugent.be and Ine.Lentacker@UGent.be

Abstract

The global surge in antimicrobial resistance presents a critical threat to public health emphasizing the urgent need for the development of new and more effective bacterial vaccines. Since the success of mRNA vaccines during the COVID-19 pandemic, this vaccine strategy has rapidly advanced, with most efforts focused on cancer immunotherapy and targeting viral pathogens. Recently, mRNA vaccines have entered the early phases of clinical development for bacterial diseases. However, bacteria present greater biological complexity compared to viruses, posing additional challenges for vaccine design, such as antigen selection, immune response, and mRNA construct design. In this Review, we discuss the critical aspects in the development of bacterial mRNA vaccines from antigen selection to construct design. We also highlight the current preclinical landscape and discuss remaining translational challenges and future potential for mRNA vaccines against bacterial infections.

1. Introduction

Antibiotics have been successfully used to treat bacterial infections for decades; however, recently antimicrobial resistance (AMR) is rising to dangerously high levels¹⁻⁴. Bacterial vaccines represent an attractive tool to combat AMR as they can confer protection against infection and disease, while preventing the emergence and transmission of bacterial infections. This directly impacts on antibiotic prescription and overuse, and might thus limit the selection and dissemination of antibiotic resistant strains^{5,6}.

Bacterial vaccines are typically based on whole-cell vaccines (inactivated or live attenuated pathogens), polysaccharides alone or conjugated to proteins, and protein subunit vaccines. Whole-cell bacterial vaccines have the advantage of inducing immunity against a wide repertoire of bacterial antigens. Within this category, live attenuated vaccines are often more effective compared to inactivated vaccines because they retain the ability to replicate, thereby inducing a broader immune response. This feature, however, limits their use in immunocompromised individuals. Several successful bacterial subunit vaccines have been licensed in recent years. Among them, glycoconjugate vaccines, created by covalently linking polysaccharides to an antigenic protein, have proven particularly effective and cost-efficient in preventing bacterial infections, such as *Haemophilus influenzae* type B, *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Salmonella typhi*⁷. These vaccines also contribute to herd immunity by reducing transmission within the population⁸. Other successful bacterial vaccines have been based on protein subunits, including inactivated toxins, as for diphtheria and tetanus vaccines, or virulence factors as for the serogroup B meningococcus or can be combined, as in the acellular pertussis vaccine. In addition, innovative approaches are currently in development including engineered outer membrane vesicles (OMVs), derived from Gram-negative bacteria, and whole cell vaccines developed using synthetic biology, as reviewed elsewhere⁹. Novel immune stimulants have also been investigated to enhance T-cell-mediated immunity, boost immunogenicity, and counteract immunosenescence in bacterial subunit vaccines. One example is the clinically approved AS01 adjuvant, a liposomal formulation of monophosphoryl lipid A with the purified saponin QS-21¹⁰. As a key final step in the development of the first new anti-tuberculosis vaccine in over a century, a phase III clinical trial is currently evaluating the efficacy of a vaccine candidate, composed of the M72 recombinant fusion protein—comprising two *Mycobacterium tuberculosis* (Mtb) antigens (Mtb32A and Mtb39A)—formulated with the AS01 adjuvant (a liposome-based adjuvant)¹¹. This vaccine is aimed at preventing pulmonary tuberculosis in adults and adolescents. Earlier clinical data have already demonstrated that the M72, formulated with AS01E, which enhances immune responses, provided over 50% protection against active pulmonary tuberculosis disease in Mtb-infected adults¹².

In addition to these approaches, the recent COVID-19 pandemic has demonstrated that mRNA vaccines can become next generation approaches against infectious diseases due to their proven efficacy and safety. Their rapid manufacturing process also enables fast responses to emerging pandemics¹³. mRNA vaccines work by delivering genetic instructions to the body's cells for the temporary production of protein antigens. This is achieved by using lipid nanoparticles (LNPs) as a non-viral delivery system to transport the synthetic mRNA into cells. While mRNA vaccines continue to demonstrate success against viral diseases, the development of mRNA vaccines against bacterial disease has been more challenging. Unlike viruses, bacteria express thousands of proteins

complicating the selection of suitable antigen(s)¹⁴. While viral proteins tend to be more compatible and easier for host cells to translate, –having evolved to exploit host cellular machinery, – bacterial proteins encoded by mRNA may be poorly translated in mammalian cells. Additionally, the intracellular trafficking, processing, and post-translational modifications of these foreign proteins may substantially affect their stability and immunogenicity. While both humoral and cellular immunity play a role in protection against bacteria, their relative importance can vary depending on the bacterial species. Vaccine development against extracellular bacteria primarily focuses on inducing effective humoral responses; therefore, bacterial proteins or polysaccharides that are surface-exposed or secreted are commonly regarded as potential vaccine candidates. However, this requires the secretion of stable bacterial proteins to enable recognition and binding to B-cell receptors, leading to antibody production. While antibodies may provide some protection against intracellular bacteria, especially before cellular entry, these bacteria become less accessible to antibodies once they reside within host cells. Therefore, cellular immunity becomes important by directly killing infected cells. Cellular CD8⁺ and CD4⁺ T cell responses are triggered by short antigenic peptides which are presented by MHC class I or II molecules, respectively, on antigen-presenting cells. For loading onto MHC-I receptors, cytosolic peptides are degraded by the proteasome then translocated into the endoplasmic reticulum (ER) where they are loaded onto MHC-I receptors. MHC class II molecules bind peptides that are derived from proteins degraded in the endocytic pathway. Peptide loaded MHC class I and II molecules are then transported to the cell membrane to present their cargo to CD8⁺ and CD4⁺ T cells.

Below we discuss major challenges in bacterial mRNA vaccine design, including antigen identification and selection, as well as the impact of translational and post-translational processing of bacterial antigens in the eukaryotic cell. We also provide examples and strategies for tailoring mRNA construct design to promote either humoral or cellular immunity. Finally, we provide an overview of the current landscape of pre-clinical and clinical development of bacterial mRNA vaccines and discuss potential solutions to improve the translatability and immunogenicity of bacterial mRNA vaccines.

2. Challenges in bacterial antigen selection and post-translational processing.

While cellular and subunit vaccines involve the direct engulfment of entire bacterial pathogens or purified bacterial proteins by antigen presenting cells (APCs), mRNA vaccines work by inducing the host's own cells to produce bacterial protein antigens encoded by the mRNA. Since most protein-based bacterial vaccines are composed of several purified proteins, mRNA vaccines may have the advantage of easily encoding different protein antigens in multivalent formulations. This flexibility is particularly valuable given the antigenic complexity of bacterial pathogens which will most likely require multivalent vaccine strategies to combat variant strains and to adeptly navigate variable antigen expression in bacteria during different stages of infection¹⁵. Reversible switches of gene expression (phase variation)^{16–18} and expression of distinct (surface) antigens (antigenic variation)^{19–21} are often associated with virulence and therefore important factors to consider. However, designing mRNA-based bacterial vaccines also comes with several challenges. Many bacterial

antigens are complex multi-subunit proteins which fold in bacterial cells often requiring specific chaperones. These proteins might not correctly fold inside mammalian host cells. Moreover, host cells may glycosylate proteins, a modification which is usually absent in bacteria. In addition to these challenges, mRNA vaccines cannot be used to encode non-protein antigens such as bacterial polysaccharides, excluding the possibility to make bacterial conjugate vaccines that can elicit protective antibody responses against the polysaccharide capsule of certain bacteria²².

As with different vaccine platforms, the development of mRNA based bacterial vaccines hinges on the careful selection of antigens that are efficiently expressed and correctly folded within host cells. Ideal antigens are typically low molecular weight protein subunits capable of self-assembly without requiring chaperones and designed to avoid unwanted glycosylation. An overview of current methodologies that can be applied for bacterial antigen discovery is provided in **Box 1** and **Fig.1a,b**. Computational immunology has emerged as a powerful tool for predicting vaccine candidates in a cost-effective and rapid manner^{23,24}. While these approaches might drastically shorten the list of potential vaccine candidates, they might still yield dozens of antigen candidates requiring experimental validation. Immunopeptidomic screens offer a complementary strategy which integrates data from diverse cellular and infection models¹⁴. Recently, we used immunopeptidomics to identify antigens presented on two human cell lines infected with *Listeria monocytogenes* and identified 13 bacterial antigens represented by two or more immunopeptides²⁵. After excluding potential toxic antigens, seven candidates were retained and encoded in mRNA vaccine formulations to test their protective efficacy in a mouse model of *Listeria* infection. Interestingly, the antigen with the highest number of epitopes, an uncharacterized bacterial surface protein, LMON_0149, provided the highest level of protection. Moreover, we observed a positive correlation between the number of identified immunopeptides per antigen and the degree to which they reduced bacterial numbers following immunization²⁵. This finding suggests that the number of immunopeptides identified through immunopeptidomics experiments could serve as a valuable criterion for prioritizing bacterial vaccine candidates, though further studies are required to confirm this. The flexibility of mRNA vaccine platforms could further accelerate antigen discovery approaches as their rapid and scalable production enables the evaluation of the protective efficacy of many different protein antigens, a concept recently termed reverse vaccinology 3.0²⁶. For example, inactive sequence variants of bacterial toxins such as the non-pore forming E262K variant of listeriolysin O, used in the aforementioned *Listeria* study, can be quickly evaluated.

BOX 1: Methods for untargeted bacterial antigen discovery

*Recent technological advances enable direct experimental detection of bacterial antigens. Contemporary mass spectrometry (MS)-based proteomics workflows allow the sensitive identification of even low abundant bacterial peptides presented by MHC class I or II molecules on infected host cells^{14,27}. This method, also referred to as immunopeptidomics, has recently proven effective in identifying bacterial immunopeptides derived from the intracellular bacterial pathogens *Mycobacterium tuberculosis*²⁸⁻³⁰ (Mtb) and *Listeria monocytogenes*^{25,31} presented on the surface of infected cells. Using a diverse range of host cell types with differing HLA alleles along with sampling at various stages of infection can help increase the diversity of bacterial epitopes detected. In addition to MHC class I-presented peptides, surface-exposed bacterial proteins^{32,33} or those bound to circulating antibody-antigen complexes³⁴ can also be detected by MS, further informing vaccine antigen candidate selection. An alternative approach is proteome-wide T-cell immunoreactivity*

screening, for instance by recording *ex vivo* cytokine production when exposing patient peripheral blood mononuclear cells (PBMCs) to synthetic peptide libraries spanning 4,000 *Mtb* ORFs³⁵. Various screening strategies are also used to identify antigen targets of specific T-cell receptors (TCRs) (reviewed elsewhere³⁶), such as TCRs associated with *Mtb* infection^{37,38}. In addition to experimental methods, computational immunology can steer rational selection or prioritization of bacterial antigens starting from gene or protein sequences, an approach generally referred to as reverse vaccinology^{23,24,39}. Protein features such as surface exposure, adhesin probability and others can be used to identify antigen candidates^{40,41}. Additional considerations include possible variable expression¹⁶ and conservation of antigens across strains of the pathogen species³⁹. Alternatively, machine learning (ML) algorithms trained on curated sets of protective bacterial antigens aim to score and rank the antigenicity of bacterial proteins^{42,43}. In addition to antigen prediction, B- and T-cell epitopes can be predicted by a multitude of bioinformatic tools (reviewed elsewhere^{44,45}) and can assist in antigen selection^{40,46}, but also immunopeptide annotation^{25,28}, the rational design of peptide pools^{47,48} and synthetic multi-epitope mRNA vaccines^{49,50}. While immunoinformatics is being increasingly adopted, caution is warranted, as the available models or imposed selection criteria may not generalize well for certain pathogens or populations^{51–53}. Following their prediction or identification, prioritization of bacterial antigens as vaccine candidates is a critical step¹⁴. Toxic antigens should be avoided or rendered inactive, for example, as demonstrated for the pore-forming agent listeriolysin-O²⁵. Additionally, as HLA alleles occur at different frequencies in different human populations, antigens targeted by HLA alleles carried at high frequency in the targeted population should also be prioritised⁵⁴. Conversely, sharing of cross-reactive epitopes between pathogens and gut bacteria may influence the composition of intestinal microbiota as well as vaccine efficacy, as reported for the BCG vaccine⁵⁵.

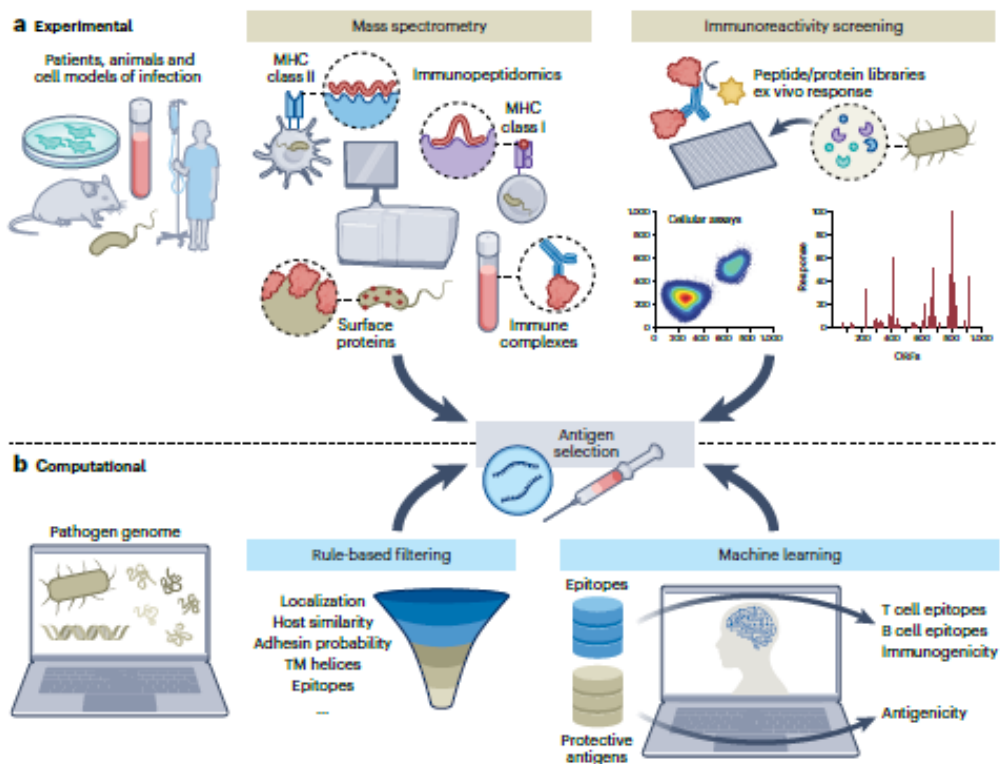


Fig 1: Antigen discovery methods in bacterial vaccine development. **a.** Antigens can be identified experimentally using samples from infected patients, animals or cell models of infection. Experimental methods include mass spectrometry (MS)-based detection of antigens via purification and identification T-cell epitopes presented on MHC molecules (immunopeptidomics), circulating immune complexes or bacterial surface proteins. Alternatively, various immunoreactivity-based screening methods can reveal immunodominant bacterial antigens. **b.** Computational immunology methods can facilitate antigen discovery by applying rule-based filtering to bacterial proteins based on known features of existing antigens to prioritize potential antigen vaccine candidates. Additionally, machine learning (ML) models trained on experimental immunology data can be used to score and rank possible protective antigens, and presented epitopes, among other relevant features such as antigenicity. Figure created with BioRender.com.

In contrast to viruses which rely on host translational machinery upon infection, bacteria have their own cellular machinery for protein production. The first issue that arises from this distinction is that bacterial antigen sequences delivered through mRNA vaccines may be poorly translated in the eukaryotic host, although this can be partially resolved by codon optimization⁵⁶. Vaccine development against extracellular bacteria mainly focuses on inducing effective humoral responses, by targeting surface-exposed or secreted bacterial proteins as potential antigen candidates. This strategy requires stable bacterial protein secretion for recognition and binding to B-cell receptors. To support this in mRNA vaccine constructs, the native bacterial secretion signal is often replaced with a mammalian secretion signal to ensure proper protein processing in the mammalian host cells. This approach was used in a study on the development of a self-amplifying mRNA vaccine against *Streptococcus pyogenes* and *S. agalactiae*. When the truncated BP-2a (pilus 2a backbone protein) antigen was compared with the same antigen preceded by the signal sequence of the murine Ig κ chain, higher antibody titers and enhanced protection were observed when the signal sequence was encoded⁵⁷. A study on *Pseudomonas aeruginosa* also found higher antibody responses by fusing the signal peptide (SP) sequence of human Tissue Plasminogen Activator (htPA) to PcrV, a *Pseudomonas aeruginosa* antigen⁵⁸. Similarly, in a study by Messou et al. on *Salmonella*, enhanced protein expression was obtained for most mRNA-encoded antigens when adding the SP sequence of human Ig K. However, this strategy did not prove to be effective for all bacterial proteins⁵⁹. Replacing the native SP sequence was also tested in a recent study by the Peer lab, where an mRNA vaccine was developed for the extracellular bacterium *Yersinia pestis*, encoding its capsule antigen, the F1 protein⁶⁰. In contrast to the recombinant F1 protein vaccine, which was immunogenic, the corresponding mRNA-encoded antigen fused to a mammalian SP sequence originating from the IgG κ light chain, yielded cellular but no humoral responses. In this case it was hypothesized that post-translational modifications may have suppressed or masked critical immunogenic epitopes, thereby reducing B-cell recognition of the bacterial epitopes. This, in turn, may result in weaker humoral responses and only partial protection against the bacteria compared to the recombinant protein vaccine.

In addition, it is well-known that post-translational processes also vary substantially between prokaryotic and eukaryotic cells. Bacterial proteins expressed by host cells may thus differ structurally from their native bacterial counterparts due to post-translational and co-translational modifications, with glycosylation being the most notable example. Glycans added by the host may cause structural differences or sterically hinder the recognition of B-cell epitopes in the bacterial antigen. Glycans can also generate new target sites for receptors that may be involved in stimulatory and inhibitory immune pathways (reviewed by Ozdilek et al.⁶¹). By utilizing bioinformatic tools such

as NetNglyc and NetOglyc, one can predict potential glycosylation sites within bacterial vaccine targets and design mutated antigens accordingly⁶². The significant impact host glycosylation can have on the immune response generated by nucleic acid vaccines, was demonstrated for the *Mycobacterium tuberculosis* (Mtb) surface protein, Ag85A⁶³. Upon immunization in mice, glycosylation impaired humoral and even cellular immunity compared to the native bacterial protein. However, immunogenicity could be restored through mutagenesis of the N-glycosylation site. Most importantly, the authors hypothesized that this might, at least partially, explain the clinical failure of two viral vector-based vaccine candidates encoding the Mtb antigen, MVA85A^{64,65} and AERAS-402⁶⁶. A study by Muir et al. further highlights the complexity associated with expressing bacterial proteins in mammalian cells⁶⁷. In that study, the authors aimed to enhance the production and secretion of a bacterial enzyme, chondroitinase ABC, which is not naturally secreted by mammalian cells in its native gene form. They demonstrated that the removal of some glycosylation sites resulted in increased protein secretion. However, two other glycosylation sites were identified as essential for the production and secretion of the active enzyme. This indicates that some degree of glycosylation might even be necessary for the secretion of the bacterial protein. Interestingly, in the study by Kon et al., vaccination with an mRNA vaccine encoding *Y. pestis* F1 protein devoid of a secretion signal, resulted in robust IgG titers providing full protection in a lethal *Y. pestis* Kimberley 53 challenge mice model⁶⁰. This suggests that the F1 protein was secreted into the extracellular environment via alternative routes, collectively known as unconventional protein secretion. These pathways, which may include stress-induced membrane pore formation and secretion of membrane-bound organelles, do not involve transition of the protein through the Golgi apparatus and endoplasmic reticulum (ER), and therefore avoid post-translational modifications (PTMs) and other protein alterations that occur in the traditional secretory pathway⁶⁸. Collectively, these studies suggest that the use of mammalian SP sequences and manipulation of host glycosylation pathways are key factors that warrant further investigation as they influence the fate of translated bacterial proteins, the resulting immune effects and ultimately the degree of protective efficacy achieved.

3. Optimizing mRNA construct design for improved cellular immunity

The generation of pathogen-specific antibodies is generally considered essential for disease protection, however, cellular immunity also plays a crucial role, especially in the case of intracellular bacteria like Mtb^{69,70}. Both CD4⁺ and CD8⁺ cytotoxic T lymphocytes (CTLs) can contribute to host defense through direct killing of infected cells upon antigen recognition^{71,72}. Activated CD4⁺ and CD8⁺ T-cells also produce cytokines, including interferon- γ (IFN- γ), which can stimulate (infected) macrophages, inducing autophagy and intracellular production of nitric oxide, leading to the apoptosis of bacteria-infected macrophages⁷³. When mammalian cells are transfected by mRNA vaccines, without SP, the encoded protein is expressed in the cytosol where proteins are typically degraded by the ubiquitin-proteasome system⁷⁴. In the case of bacterial virulence factors that are normally secreted or injected in the host cell during pathogen infection, adaptation to the eukaryotic host cell environment might result in relatively stable expression of such encoded antigens⁷⁵. Other bacterial proteins, especially cell wall or membrane proteins-, may quickly degrade in mammalian cells due to improper folding or for other reasons. Such unstable expression and fast proteasomal degradation might be beneficial for MHC Class I presentation and the activation of CD8⁺ T-cell

responses since defective ribosomal products, short-lived and non-canonical proteins, constitute a significant portion of the immunopeptidome⁷⁶⁻⁷⁸. In a study conducted by us, although the mechanism was not fully elucidated, vaccination with the bacterial surface protein LMON_0149, encoded without a SP in an mRNA construct, conferred protection against *Listeria monocytogenes* in a mice challenge study. This protection could well be the result of such unstable protein expression²⁵, especially since protective immunity against this intracellular pathogen strongly depends on CD8⁺ T-cell responses⁷⁹. This has further been exploited in the context of a mycobacterial DNA vaccine where ubiquitin fusion constructs with the mycobacterial antigen MPT64 were encoded to target ubiquitin-proteasomal degradation and favour cytotoxic T-cell stimulation, while no humoral responses were detected⁸⁰.

Since CD4⁺ and CD8⁺ T-cells synergize to generate immunity against intracellular bacteria, antigens and vaccine design should be selected to optimally target and elicit both T-cell subsets. As mRNA-encoded antigens are expressed in the cellular cytoplasm, it is expected that they will mainly be presented in the context of MHC I complexes⁸¹. However, it has been shown that MHC II molecules can also acquire access to endogenously synthesized antigens within specialized endo-lysosomal compartments⁸². Therefore, fusion constructs of antigens with HLA class II sorting signals have been evaluated for their ability to guide cytoplasmic antigens to these compartments, mainly in the context of viral vaccine design. More specifically, those HLA class II sorting signals contain endocytic-sorting motifs present at the cytoplasmic domain of endosomal or lysosomal transmembrane proteins that are recognized by trafficking components and guide these proteins into endo-lysosomal compartments⁸². Some examples are the sorting signals derived from the transmembrane protein invariant chain (Ii) stabilizing and controlling the intracellular transport of MHC class II molecules⁸³, or the lysosomal associated membrane protein (LAMP-1), present in the endo-lysosomal compartment^{84,85}. Importantly, the LAMP-1-mediated transport of cytoplasmic proteins to the endo-lysosomal compartment is dependent on their translocation to the endoplasmic reticulum (ER) which is guaranteed by including the LAMP-1 SP in the construct (**Fig 2.**)⁸⁵. Kreiter et al. explored an alternative strategy that entailed inserting the genetically encoded antigen in between the SP sequence and the cytoplasmic domain of MHC I (MITD), which was pivotal for endo-lysosomal targeting and cross-presentation of exogenously derived antigens in dendritic cells^{86,87}. This resulted in enhanced MHC I and II presentation of the antigen, and CD8⁺ and CD4⁺ T-cell activation, respectively⁸⁶. However, it remains unclear whether the SP sequence also routes the protein to the extracellular space after which reinternalization and MHC II loading can take place⁸⁸. Notably, these constructs were used to design BioNTech's clinical cancer vaccines^{89,90}. Finally, it has been shown that coupling the Fc fragment of IgG to the mRNA encoded antigen can indirectly target antigens to the MHC II compartment as secreted proteins will be reinternalized by Fcγ receptor mediated endocytosis (**Fig 2.**)⁹¹. In addition, it has been reported that this can extend the plasma half-life of proteins⁹², but it is not clear to what extent this contributed to the enhanced specific immune responses observed after Fc-conjugation in design of a SARS-CoV-2 recent and *Y. pestis* vaccine^{60,93}. Taken together, these studies illustrate that depending on the targeted bacteria and desired immune response, several approaches involving different mRNA construct designs can be explored to promote specific intracellular pathways and thereby steer adaptive immune responses, with varying degrees of success (**Fig 3.**).

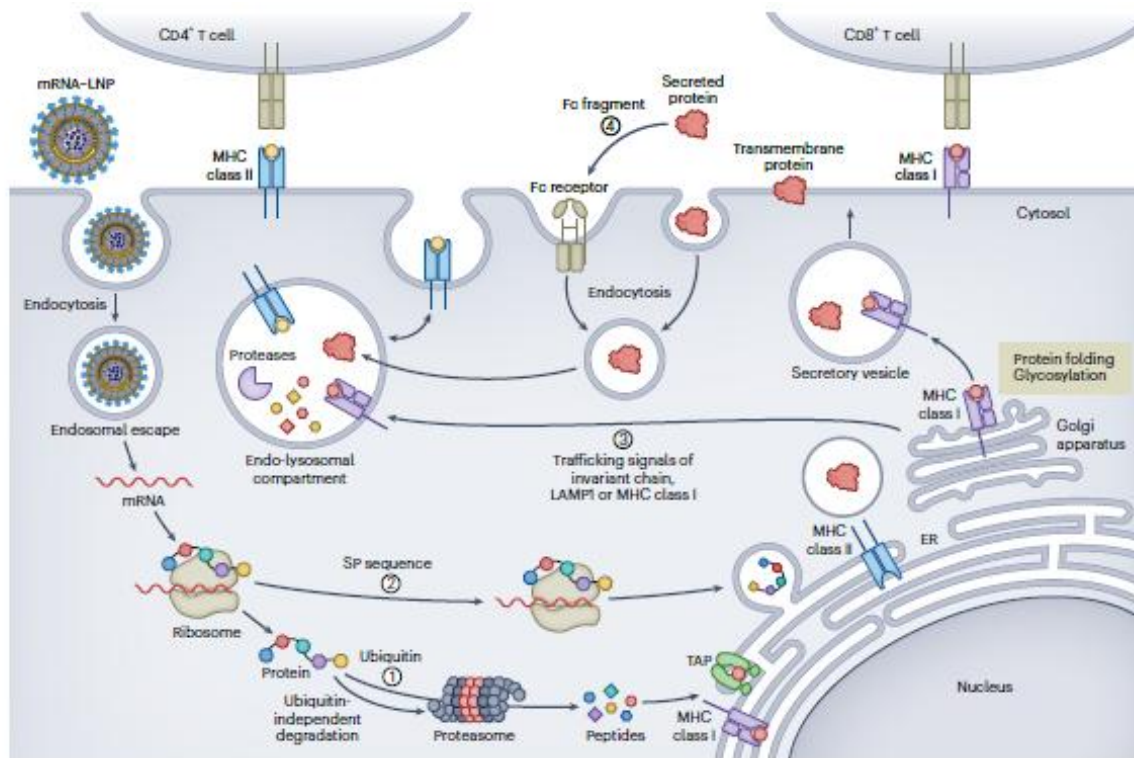


Fig 2: Inclusion of different signaling peptides and trafficking motifs directs intracellular processing and localization of the mRNA-encoded antigen after ribosomal translation.

- (1) Cytosolic proteins are targeted to the ubiquitin-proteasome pathway or alternative ubiquitin-dependent pathways for degradation. Subsequently, protein fragments can be loaded on MHC-I complexes within the endoplasmic reticulum (ER), then trafficked to the cell surface where they can activate CD8⁺ T-cells.
- (2) Proteins with a signaling peptide are imported in the ER and guided into the secretory pathway. In this process, proteins undergo conformational changes and can be subject to post-translational modifications, including glycosylation.
- (3) Proteins linked to trafficking motifs derived from invariant chain, LAMP-1, MHC-I, or others are guided into the endo-lysosomal compartments. After degradation, protein fragments can be loaded in MHC I and MHC II complexes and delivered to the cell surface, stimulating CD8⁺ and CD4⁺ T-cells respectively.
- (4) Proteins linked to a Fc fragment can become reinternalized through Fc mediated endocytosis entering endo-lysosome compartments where they are degraded and loaded onto MHC-II molecules, then trafficked to the cell surface where they can mainly activate CD4⁺ T-cells.

Figure created with BioRender.com.

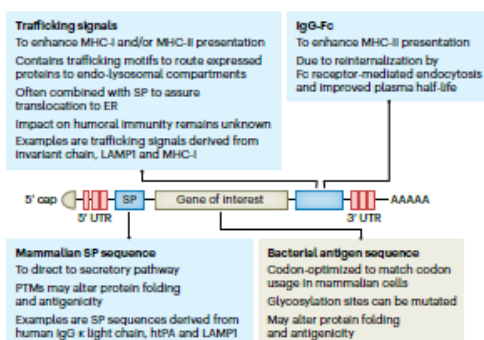


Fig 3: Optimization of mRNA construct design to enhance protein expression or to direct expressed antigens towards specific antigen presentation pathways. These approaches can aid in tailoring adaptive immune responses to mRNA vaccines depending on the desired correlates of protection of the bacterial disease, but come with certain disadvantages (exclamation-mark). *Lysosomal associated membrane protein 1 (LAMP-1)*, *Major histocompatibility complex (MHC)*, *Signal*

4. Current status of bacterial mRNA vaccines

While numerous studies have explored the use of mRNA vaccines against viral targets, only a limited number of publications have shown protective efficiency of mRNA vaccines against bacterial infections. **Table 1** summarises bacterial mRNA vaccines that have been pre-clinically evaluated (**Table 1A**) or that have entered clinical evaluation (**Table 1B**). As discussed above, mRNA vaccines have shown promise in preclinical models of various bacterial diseases caused by *Y. pestis*^{60,94}, *L. monocytogenes*^{25,95}, *S. pyogenes* and *S. agalactiae*⁵⁷, *P. aeruginosa*^{58,96}, and *S. Typhimurium*⁵⁹. Other bacterial diseases being evaluated at the pre-clinical stage are also listed in **Table 1A**.

In contrast, only a few vaccines have entered the phase of clinical evaluation. This encompasses Moderna's Phase I/II clinical trial (NCT05975099) which aims to evaluate two mRNA vaccines against Lyme disease caused by *Borrelia burgdorferi*. This bacterial pathogen is transmitted to humans through the bite of infected ticks. Lyme disease is now recognized as the most common vector-borne disease in both Europe and North America and there is no vaccine available⁹⁷. Moderna's first candidate, mRNA-1982, encodes the outer surface protein A serotype 1 (OspA SR1) of *B. burgdorferi*, while mRNA-1975 is a heptavalent vaccine encoding OspA Serotypes 1-7, designed to induce protection against four major *Borrelia* species causing Lyme disease in the US and Europe⁹⁸. Interestingly, vaccine-induced antibodies directed against the OspA antigen can neutralize bacteria within the midgut of the tick while feeding and, as such, inhibit transmission of the bacteria from the tick to the host. In a pre-clinical mouse study, an OspA-encoding mRNA-LNP vaccine was found to be superior to an alum-adjuvanted OspA protein vaccine in inducing humoral and cellular immunity, thereby showing successful protection against *B. burgdorferi* infection after a single immunization⁹⁹. Notably, an effective alum-adjuvanted recombinant OspA protein vaccine (LYMERix[®]) developed by GlaxoSmithKline was already put on the market in 1998 but was discontinued after only 3 years due to declining sales. Indeed, LYMERix[®] suffered from lack of acceptance as it was suggested that OspA was cross-reactive with human lymphocyte function-associated antigen-1 (hLFA-1) potentially causing arthritic symptoms in vaccinated individuals¹⁰⁰. This was later disproven by several independent studies¹⁰¹.

In collaboration with West Virginia University, Moderna also recently reported on a multivalent mRNA vaccine targeting both *Bordetella pertussis* antigens, and diphtheria and tetanus toxoids. mRNA constructs for the *pertussis* and tetanus antigen were optimized with SPs of either IgK or bovine prolactin, whereas the native signal sequence was maintained in the diphtheria antigen construct. Some of the antigens were additionally modified with mutations to avoid N-linked glycosylation sites, and / or to eliminate toxin-related activities. Prime-boost vaccination with a 6- to 10-valent mRNA vaccine resulted in similar levels of protection in a murine *B. pertussis* challenge model compared to 1/20th of the human dose of either acellular multivalent pertussis vaccine (DTaP) or whole cell pertussis vaccine. Additionally, they found that the mRNA vaccine elicited more balanced Th1 and Th2 immunity in contrast to DTaP that induced a Th2 polarized response, which was evident from higher IgG2a and IgG2b antibody levels, and IFN- γ producing CD4⁺ T helper response¹⁰². This is important, as Th1- and Th17-mediated immune responses are known to be essential for bacterial clearance and long-lasting protection against *B. pertussis*²².

In 2023, TB ranked as the world's leading cause of death from a single infectious agent disease¹⁰³. The live attenuated vaccine, bacillus Calmette-Guérin (BCG), has been used since 1921, and remains the only licensed vaccine against Mtb. Although effective in young children, BCG is less effective in adults, especially in low-and middle-income countries (LMICs)¹⁰⁴. Larsen et al. evaluated the efficacy of the fusion protein ID91 encoded in self-amplifying RNA complexed through a nano structured lipid carrier (NLC) against Mtb in a preclinical setting. Of interest in these studies is that by heterologous vaccination of RNA and protein subunit vaccines, promising protective results were obtained, which could be explained by the stronger induction of multifunctional T cells¹⁰⁵. BioNTech also announced that they are working on a mRNA vaccine against Mt¹⁰⁶. In the patent filed by BioNTech (WO 2024/028445 A1), the following antigens are listed: Ag85A, ESAT6, VapB47, Hrp1, RpfA, RpfD, Mtb32a, Mtb39a and HbhA, covering antigens present at various stages of infection. Different combinations of these antigens were evaluated, encoded as fusion proteins or co-formulated as single mRNA strands. In addition, the use of alternative signal peptides and/or transmembrane and cytoplasmic domains of MHC I's were screened, resulting in variable cellular and humoral responses against the different antigens. This indicates the complexity of predicting the impact of vaccine construct design on intracellular processing and immunogenicity. Based on the patent information, it is likely that BioNTech combined 4 different mRNA strands in their final vaccine, each encoding a fusion protein of 2 antigens preceded by the signal peptide of MHC I¹⁰⁷.

Using two different clinical studies, BioNTech aims to compare vaccine candidates against Mtb containing either unmodified mRNA (BNT164a1) or N1-methylpseudouridine (m1Ψ) -modified mRNA (BNT614b1). These vaccines will be administered to either BCG naïve, non-tuberculosis exposed subjects (NCT05537038, Phase Ia trial in Germany) or BCG vaccinated healthy volunteers, who will be stratified by interferon gamma release assay (IGRA) (NCT05547464, Phase Ib trial in countries in Africa and Asia, including Republic of South Africa, Mozambique, and Republic of the Philippines). The fact that BioNTech is retesting the role of mRNA modifications in their Mtb candidates is also intriguing, given that m1Ψ-modified mRNA was chosen over unmodified mRNA and self-amplifying mRNA in the development of their COVID-19 mRNA vaccine¹⁰⁸. The main distinction in their formulations is in their delivery systems. It should be noted that the unmodified mRNA is complexed in spleen-targeting lipoplexes for intravenous administration, similar to BioNTech's ongoing cancer clinical trials (NCT04526899, NCT04534205, NCT05557591, NCT03815058, NCT05968326). In contrast the m1Ψ-modified mRNA is formulated in LNPs containing ionizable lipids and is delivered intramuscularly. Separately, the replacement of uridines is critical in immune recognition of the mRNA. The replacement of normal uridines in *in vitro* transcribed (IVT) mRNA with naturally occurring modified uridines, such as m1Ψ, enables mRNA to evade intracellular RNA sensors. This prevents type I interferon (IFN)-mediated antiviral immune responses, resulting in enhanced translation capabilities and improved tolerability compared to its unmodified counterparts, as extensively summarized elsewhere^{109,110}. Notably, Mulroney et al. recently reported that the incorporation of m1Ψ in IVT mRNA affects ribosome stalling and thereby can lead to 1+ frameshifting¹¹¹. Consequently, a certain amount of the translation proteins can be altered, which in turn could potentially result in off-target T-cell and antibody responses. This highlights an additional complexity in the design of mRNA sequences when m1Ψ is incorporated, not indicating yet what the clinical outcome will be. For this reason, BioNTech's choice of putting forward both RNA platforms for clinical testing likely originates from their observation that they achieved similar protective

effects in a Mtb challenge model in mice, however, neither vaccine showed superiority to BCG vaccination.

Taken together, comparative clinical studies like those currently conducted by BioNTech, may provide new directions for the design of mRNA vaccines against intracellular bacteria, which will likely require a multifaceted immune response to provide adequate protection.

Table 1 | Overview of bacterial mRNA vaccines that underwent a preclinical evaluation or that are currently under clinical evaluation

Targeted bacterial pathogen	Intra- or extracellular	Type of RNA	Target antigen and RNA construct	Formulation	Administration route	Publication date	Ref.
Under development							
<i>M. tuberculosis</i>	Facultative Intracellular	Unmodified	MPT83	Naked	IM	2004	108
<i>M. tuberculosis</i>	Facultative Intracellular	Unmodified	Hsp65	Naked	IN	2010	109
<i>S. pyogenes</i> and <i>S. agalactiae</i>	Extracellular	saRNA	LOdm or murine Ig k ss + BP-2a	Cationic nano-emulsion	IM	2017	28
<i>S. typhimurium</i>	Facultative Intracellular	Nucleoside-modified	Human Ig k ss + antigen (Mig14 , OmpC / F/L , StyB , SseB , CpoB or T1855)	LNP	IM	2018	30
<i>Chlamydia trachomatis</i>	Obligate Intracellular	saRNAs	MOMP	CAFs with RB48, 3M-052 or Poly I:C adjuvants	IM	2019	110
<i>Staphylococcus aureus</i>	Extracellular	Nucleoside-modified	h-TPA ss + AdsA + MITD	mRNA-InstantFECT nanocomplex	IM versus SC	2020	111
<i>B. burgdorferi</i>	Extracellular	Nucleoside-modified	19ISP (derived from <i>Ixodes scapularis</i>)	LNP	ID	2021	112
<i>L. monocytogenes</i>	Facultative Intracellular	Nucleoside-modified	LMON_0149 , _276 , _0442 , _1501 , _2272 , _1065 or LLO_E262K	α -Galactosylceramide adjuvanted cationic lipoplexes	IV	2022	25
<i>M. tuberculosis</i> / <i>Mycobacterium avium</i>	Facultative Intracellular	saRNA	Fusion protein of exV , RpfD , PPE60 and Ag85B	LION	IM	2023	76,113
<i>Y. pestis</i>	Facultative Intracellular	Nucleoside-modified	ss-devoid or human Ig k chain ss Cp- caf1 and/or human IgG-Fc domain	LNP	IM	2023	31
<i>P. aeruginosa</i>	Extracellular	Nucleoside-modified	OprF-1 or h-TPA ss PcrV	LNP	IM	2023	67
<i>P. aeruginosa</i>	Extracellular	Nucleoside-modified	h-TPA ss PcrV	LNP	IM	2023	29
<i>B. burgdorferi</i>	Extracellular	Nucleoside-modified	OspA	LNP	IM	2023	70
<i>Y. pestis</i>	Facultative Intracellular	saRNA	F1 and V antigen	LNP	IM	2023	65
<i>Rhodococcus equi</i>	Facultative Intracellular	Nucleoside-modified	Equine-specific ss VapA	LNP	IM versus IN (nebulized)	2023	114
<i>L. monocytogenes</i>	Facultative Intracellular	Nucleoside-modified	LMON_0149	LNP	IM	2024	66
<i>B. pertussis</i>	Extracellular	Nucleoside-modified	Mammalian Ig k ss or bovine prolactin ss or native ss + antigen (PTX-S1 , FHA3 , FIMD2/3 , PRN , DT , TT , RTX , TCFA , SPHB1 and/or BRKA)	LNP	IM	2024	73
<i>Clostridioides difficile</i>	Obligate anaerobe and spore-forming	Nucleoside-modified	IL-2 ss TodA , TodB , PPEP1 and CdeM	LNP	IM	2024	115
Under clinical evaluation							
<i>B. burgdorferi</i>	Extracellular	Nucleoside-modified	OspA SR1 or OspA SR1-7	LNP	IM	2023	NCT05975099
<i>M. tuberculosis</i>	Facultative Intracellular	Unmodified versus nucleoside-modified	Multivalent undisclosed	LNP	IM	2023	NCT0547464 NCT05537038

Target antigens are highlighted in bold. 19ISP, 19 *Ixodes scapularis* salivary proteins; AdsA, adenosine synthase A; Ag85B, antigen85B; BP-2a, pilus 2a backbone protein; BRKA, BrkA autotransporter; CAF, cationic adjuvant formulation; CdeM, exosporium morphogenetic protein CdeM; Cp-**caf1**, circular permuted F1 capsule antigen; CpoB, cell division coordinator protein; DT, diphtheria toxin; exV, exonuclease V protein; FHA3, forkhead-associated domain protein 3; FIMD2/3, outer membrane usher protein FimD2/3; Hsp65, heat shock protein 65; h-TPA, human TPA; ID, intradermal; IgG-Fc, crystallizable fragment of immunoglobulin G; IL-2, Interleukin 2; IM, intramuscular; IN, intranasal; IV, intravenous; LION, lipid inorganic nanoparticle; LLO, listeriolysin O; LMON, *L. monocytogenes*; LOdm, double-mutated streptolysin-O; MITD, MHC class I trafficking domain; Mig14, migration inhibitor gene 14; MOMP, major outer membrane protein; MPT83, *Mycobacterium tuberculosis* protein 83; Omp, outer membrane protein; OprF-1, outer membrane porin F; PcrV, *P. aeruginosa* V antigen; PPE60, proline-proline-glutamic acid protein 60; PPEP1, Pro-Pro endopeptidase 1; PRN, pertactin autotransporter; PTS-S1, pertussis toxin S1 subunit; RpfD, resuscitation-promoting factor; RTX, repeats-in-toxin exoprotein; saRNA, self-amplifying RNA; SC, subcutaneous; SPHB1, autotransporter subtilisin-like protease; SlyB, outer membrane lipoprotein SlyB; SR, serotype; ss, secretion signal; SseB, *Salmonella* secreted effector B; TodA, N-acetylglucosaminyltransferase TodA; TodB, glucosyltransferase TodB; TCFA, autotransporter TcfA; VapA, virulence associated protein A; TT, tetanus toxin D.

Table 1: Overview of bacterial mRNA vaccines that underwent a preclinical evaluation (section A) or that are currently under clinical evaluation (section B).

Intramuscular (IM), Heat shock protein 65 (Hsp65), Self-amplifying RNA (SaRNA), double-mutated Streptolysin-O (Lodm), pilus 2a backbone protein (BP-2a), Cationic adjuvant formulation (CAF), Secretion signal (SS), Outer membrane protein (Omp), lipid nanoparticle (LNP), major outer membrane protein (MOMP), adenosine synthase A (AdsA), Human TPA (hTPA), MHC class I trafficking domain (MITD), Subcutaneous (SC), 19 Ixodus scapularis salivary proteins (19ISP), Intradermal (ID Listeria monocytogenes (LMON), Listeriolysin O (LLO), Alpha Galactosylceramide (α -GC), Intravenous (IV), resuscitation-promoting factor (RpfD), antigen85B (Ag85b), Lipid InOrganic Nanoparticle (LION), circular permutated F1 capsule antigen (cp-caf1), crystallizable Fragment of immunoglobulin G (IgG-Fc), Outer membrane porin F (OprF-I) P. aeruginosa V-antigen (PcrV), Interleukin 2 (IL-2), Pro-Pro endopeptidase 1 (PPEP-1), Outer surface protein A (OspA), virulence associates protein A (VapA). Intranasal (IN), serotype (SR).

5. Concluding remarks and outlook

Recent efforts by industry and academic groups indicate that bacterial diseases have become a desired target for mRNA vaccine development potentially offering an alternative to traditional bacterial vaccines and antibiotics. In addition to their proven efficacy for COVID-19, mRNA vaccines are attractive in terms of the flexibility of their design, speed of production and scalability but we also anticipate some specific challenges that will need to be overcome in the development of mRNA vaccines against bacteria to compete with other vaccine platforms.

First, selecting the right antigen(s) is a challenging task for a bacterial vaccine. Although we envision that recent technological advances in both experimental and computational methods will boost bacterial antigen discovery in the years to come, antigen prioritization for further preclinical validation will remain a critical step. An interesting question is whether the speed and flexibility of mRNA vaccine production could allow more functional screenings of different antigen combinations or fusion constructs in comparison with subunit platforms. It is clear that the antigenicity and immunogenicity of bacterial mRNA vaccines is substantially influenced by the expression, processing and transportation of the encoded antigen in the transfected host. Consequently, several approaches to optimize antigen expression and/or presentation of bacterial proteins in mammalian cells, such as the use of trafficking- and secretion signals and bio-informatic tools to predict and overcome host glycosylation are under evaluation. However, results obtained so far suggest that their impact on protein stability, intracellular trafficking and antigen presentation is rather unpredictable and may require proper validation for each specific antigen, thereby further hindering antigen screening. Moreover, predicting an optimal mRNA construct design is further complicated by the fact that for many bacterial diseases, the immune correlates of protection are largely unknown. In their recent review, Rappuoli and colleagues emphasize that advances in systems biology, which integrate data from multiple 'omics technologies, offers unique prospects for the in-depth characterization of protective immune responses. Combined with the ability to rapidly generate and produce various mRNA constructs, this approach can be utilized to establish a protective "target immune profile" for specific bacteria. In turn, this could help shape future mRNA vaccine designs against bacterial pathogens¹²⁰.

A key aspect to consider might be the obligate or facultative intracellular lifestyle of certain bacteria, including important human pathogens such as *Mycobacteria*, *Shigella* or *Salmonella* species with rising AMR⁹. Once intracellular, these bacteria can adopt a vacuolar or cytosolic lifestyle¹²¹, however, how this affects the downstream induction of effective immune responses and the implications of this for mRNA vaccine design are not yet known. In the case of virulence effector proteins that are often secreted or injected by these bacteria into the host cell cytosol, further research should elucidate how such virulence factors can be encoded in mRNA vaccines to elicit the most protective response. For *Mtb*, recent immunopeptidomics and proteome-wide peptide screens revealed substrates of the type VII secretion systems as major antigens (for example, ESAT-6)^{28,35}, however, it remains unclear whether encoding such effector antigens in mRNA vaccines should mimic cytosolic delivery (without SP) or rather result in endocytic targeting (with SP and/or sorting motif) as common practice in the current vaccines under clinical evaluation.

A key advantage of mRNA vaccines compared to subunit vaccine platforms is that mRNA vaccine design enables different bacterial antigens to be encoded concurrently. Both mRNA vaccines that are currently in clinical trials against *Mtb* and *B. burgdorferi* comprise multivalent formulations to include multiple antigens or serotypes (**Table 1**). Current data do not always clarify whether fusion constructs are preferred and how immunodominance, the phenomenon where certain epitopes elicit a stronger immune response than others, might impact these multivalent vaccination strategies. One additional challenge is that this might require the use of higher mRNA vaccine doses to accomplish sufficient immunogenicity, compared to the current COVID-19 mRNA vaccines encoding a single antigen¹⁰². The authors acknowledged that dosing also complicates proper comparisons between mRNA vaccines and other vaccine platforms as it is often difficult to exactly define the amount of protein that is produced after mRNA vaccination. To this end it is uncertain whether mRNA vaccines will be beneficial compared to vaccines currently considered gold standards. Based on BioNTech's patent information no superiority to BCG was seen with their two mRNA formulations in preclinical studies. Additionally, any protection that may have been conferred by application of the mRNA booster following BCG prime vaccination was not reported¹⁰⁷.

Since mRNA vaccines against bacteria are in their infancy, it remains debatable whether the current generation of mRNA-LNP vaccines will be sufficiently capable of inducing effective and durable immune protection against bacteria. The COVID-19 mRNA vaccines were characterized by high titers of neutralizing antibodies and superior vaccine efficacy in the early period of evaluation, but several studies indicated that humoral immunity and protection rapidly waned over 6 months. However, it was suggested that mRNA vaccine-induced T-cell responses are more long-lived and may potentially last for years^{122,123}. High-risk populations, such as elderly and immunocompromised individuals, typically exhibited weaker responses to primary COVID-19 mRNA vaccination and needed booster shots to achieve adequate protection^{124,125}. More recently, a single dose of Moderna's mRNA vaccine candidate (mRNA-1345) against respiratory syncytial virus (RSV) was found to be safe and effective against RSV-associated lower respiratory tract disease and RSV-associated acute respiratory disease among a study population of those who were 60 years and older, including frail adults, albeit persons with certain immunocompromising conditions were not included in this trial¹²⁶. Since these vaccines are developed for seasonal viral outbreaks, durability may be a more critical consideration when developing vaccines against bacterial pathogens.

More research is also needed to fully understand the effects of mRNA vaccines on innate immune cells. Attenuated vaccines like BCG or *Salmonella* Typhi strain TY21a are well known for inducing an innate form of immunological memory, termed trained innate immunity^{127,128}. This has been attributed to the reprogramming of epigenetic and transcriptional processes of myeloid progenitors in the bone marrow, resulting in an elevated innate immune response towards homologous or even heterologous pathogens¹²⁹. Studies so far have shown that although mRNA vaccination resulted in a transcriptional upregulation of innate and antiviral gene signatures in circulating monocytes¹³⁰, there is no evidence of long-lasting trained immunity, or beneficial non-specific effects induced by the COVID-19 mRNA vaccines¹³¹⁻¹³⁴. Notably, Hellgren et al. recently suggested that pre-existing adaptive immunity formed by primary vaccination or infection may better explain the augmented innate immune response observed after mRNA vaccination, rather than there being a trained innate immune effect. Although mRNA vaccines possess an inherent adjuvant activity generating robust adaptive immune activation¹⁰⁹, we believe that the incorporation of immune adjuvants in mRNA

vaccines could be considered to strengthen or broaden the innate immune activation^{25,135}. One example is the use of lipid nanoparticles to formulate mRNA vaccines, which provides opportunities to incorporate bacterial glycolipid antigens, as we previously demonstrated^{95,136,137}.

Another major challenge for the future of antibacterial mRNA vaccines is vaccine inequity. AMR has the highest burden in countries in a low-resource setting. These countries have suffered disproportionately from vaccine unavailability due to socio-economic disparities⁴. For example, modelling estimated that during the COVID-19 pandemic, vaccine inequity was estimated to account for more than 50% of deaths in LMICs¹³⁸. While mRNA vaccines can be produced quickly at large scale, they were not distributed equally globally during the COVID-19 pandemic. Amongst many reasons, mRNA vaccines had the limitations of (ultra-)cold chain requirements and were relatively expensive compared to other vaccine platforms. However, many efforts have been made to produce thermostable formulations to avoid cold chain needs, such as by optimization of mRNA structure¹³⁹ and the development of lyophilized products^{140,141}. In addition, several initiatives have been launched for local mRNA vaccine production hubs and training of local personnel in LMICs, which may improve access to mRNA vaccine technology in the future¹⁴². With rising levels of AMR, the importance of bacterial vaccines will also increase in high-income countries. Depending on remaining treatment options, mRNA vaccines against multi-resistant hospital-acquired infections might become in scope, protecting immunocompromised or surgery patients for the duration of their hospitalization, for instance against ESKAPE pathogens (a group of 6 bacterial species known for high virulence and antibiotic resistance). mRNA vaccine technology offers the option to customize (for example, to specific strains circulating in the hospital) or even personalize such vaccines, similar to neo-antigen encoding cancer vaccines.

Taken together, the flexibility and rapid development of mRNA vaccines, along with ongoing efforts towards more equitable and affordable production and distribution, provide bright prospects for mRNA vaccines, especially in the context of preparedness to tackle emerging infectious diseases, including those caused by bacterial pathogens. While the first mRNA vaccines against bacterial pathogens are under clinical evaluation, many more are in early or late-stage preclinical development. These studies will help to fill remaining gaps in our knowledge on mRNA vaccine design, educating on the optimal ways to enhance expression and presentation of the encoded bacterial antigen(s). Moreover, we expect that continuing research on the adaptive and innate immune response induced by mRNA vaccines, preferably in targeted populations, will teach us more about the differences between mRNA platforms, and how they could potentially compete with other advancing vaccine technologies.

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Competing interests

D.P. receives licensing fees (to patents on which he was an inventor) from, invested in, consults (or on scientific advisory boards or boards of directors) or Founder and hold shares or conducts sponsored research at TAU for the following entities: ART Biosciences, BioNTech SE, Earli Inc., Geneditor Biologics Inc. Kernal Biologics, Merck, Newphase Ltd., NeoVac Ltd., RiboX Therapeutics, Roche, SirTLabs Corporation, Teva Pharmaceuticals Inc. All other authors declare no competing financial interests.

Author Information

Ghent Research Group on Nanomedicines, Ghent University, Ghent, Belgium

Ilke Aernout, Rein Verbeke, Stefaan C. De Smedt & Ine Lentacker

VIB-UGent Center for Medical Biotechnology, VIB, Ghent, Belgium

Fabien Thery, Patrick Willems & Francis Impens

Department of Biomolecular Medicine, Ghent University, Ghent, Belgium

Fabien Thery, Patrick Willems & Francis Impens

VIB-UGent Center for Plant Systems Biology, VIB, Ghent, Belgium

Patrick Willems

Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium

Patrick Willems

Laboratory of Precision Nanomedicine, Shmunis School of Biomedicine and Cancer Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

Uri Elia & Dan Peer

Department of Materials Sciences and Engineering, Iby and Aladar Fleischman Faculty of Engineering, Tel Aviv University, Tel Aviv, Israel

Uri Elia & Dan Peer

Center for Nanoscience and Nanotechnology, Tel Aviv University, Tel Aviv, Israel

Uri Elia & Dan Peer

Cancer Biology Research Center, Tel Aviv University, Tel Aviv, Israel

Uri Elia & Dan Peer

Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Ness-Ziona 76100, Israel

Uri Elia

Biotecnopolo di Siena, Italy

Rino Rappuoli

VIB Proteomics Core, VIB, Ghent, Belgium

Francis Impens

Contributions

I.A., R.V., P.W., F.T., F.I. and I.L.: conceptualization, visualization, drafting and review of the manuscript. U.E., S.C.D.S., R.R. and D.P.: review and editing.

Corresponding authors

Correspondence to Francis Impens or Ine Lentacker.

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