



Universiteit
Leiden
The Netherlands

Towards the development of synthetic vaccines against tuberculosis

Marino, L.

Citation

Marino, L. (2022, June 7). *Towards the development of synthetic vaccines against tuberculosis*. Retrieved from <https://hdl.handle.net/1887/3307434>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3307434>

Note: To cite this publication please use the final published version (if applicable).

1

General introduction

1

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB), is responsible for the death of around 1.4 million people every year.¹ Although there is a commercially available vaccine, the *bacillus of Calmette Guérin* (BCG), an attenuated version of *Mycobacterium bovis*, studies have shown that this is not always effective and it can cause disseminated disease in immunocompromised individuals.^{2,3} A safe and effective vaccine is required to contain and, possibly, eradicate *Mtb*. Technological advances accomplished in the fields of chemistry and immunology offer the opportunity to discover efficient, safe and economical vaccines. The overarching goal of this Thesis is to devise synthetic strategies for the generation of novel, rationally designed synthetic vaccines against TB.

Immune response upon pathogen encounter and vaccination

The immune system is a defense mechanism against pathogens which can be exploited in vaccination to reduce the chance of developing a life-threatening disease. The immune system is described as consisting of an innate and an adaptive part.⁴

The innate immune system is a fast-acting defense mechanism that all animals have. It comprises the complement system of proteins, natural killer cells and several professional phagocytes (such as monocytes, macrophages and dendritic cells). In the course of this introduction, the focus will be placed on the role and function of macrophages and dendritic cells as key cellular targets for the design of a vaccine. Macrophages and dendritic cells express pattern recognition receptors (PRRs) on their cellular surface. PRRs are able to recognize components of pathogens that are common among different microorganisms and that are known as pathogen-associated molecular patterns (PAMPs). Upon PAMP recognition, a macrophage or dendritic cell can engulf the pathogen by phagocytosis. Phagocytosis can lead to direct killing of the pathogen and/or release of messenger cytokines and chemokines. Besides their role as professional phagocytes in immune responses, macrophages and dendritic cells are key players in bridging innate and adaptive immune responses *via* a process known as antigen presentation (see Figure 1 for a graphical depiction of the two systems and the key role of macrophages and dendritic cells in bridging the innate and adaptive immune response). Molecular components of a pathogen, called antigens, get processed and presented to other immune cells, such as T cells which are part of the adaptive immune system. For this function, macrophages and dendritic cells are grouped in the family of antigen presenting cells (APCs) together with other immune cells (monocytes, B cells).

Antigen presentation is a fundamental aspect of the adaptive immune system, which is typical of vertebrates. The adaptive immune response is very specific to a certain antigen, and its activation is functional to the development of immunological memory, which is the goal of prophylactic vaccination. The adaptive immune system comprises plasma cells, T cells, B-cells and antibodies.

Antibodies circulate in the blood of immunized individuals and are produced by B-cells upon B-cell activation. They bind to extracellular bacteria and viruses tagging them for destruction. Several subclasses of antibodies have been identified in humans and mice, which are the two organisms used in the research in this Thesis for the evaluation of novel vaccine modalities. Immunoglobulin G (IgG) is the major antibody type found in the blood of humans, representing about 75% of total immunoglobulins. Table 1 provides an overview of the existing antibody IgG subclasses and their properties to initiate complement activation and triggering of FcγR-expressing cells, mechanisms that can eventually result in destruction of the invading pathogen.^{5,6}

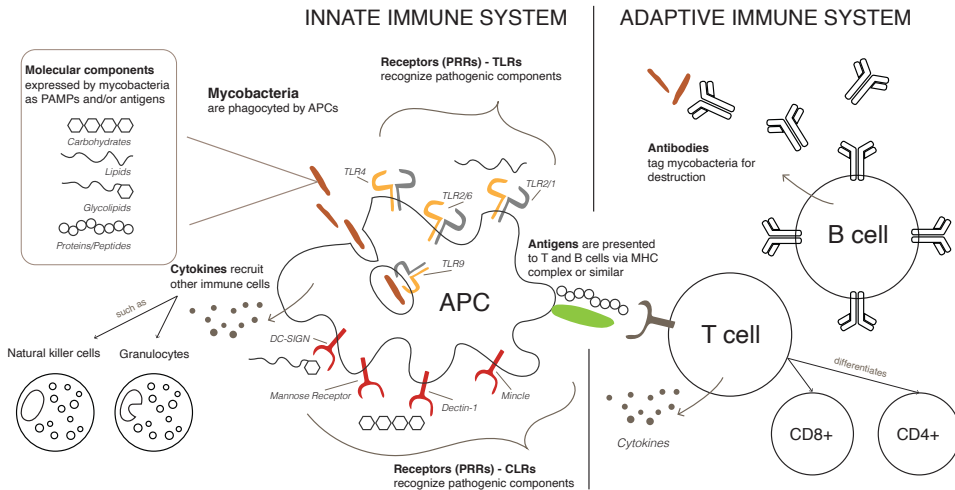


Figure 1 – Graphical depiction of key players of innate and adaptive immune response with focus on central role of antigen presenting cells (APCs) to bridge innate and adaptive system. The left side shows three cellular components of the innate immune system: APCs, natural killer cells (NKs) and granulocytes. These are activated during the innate immune response to mycobacteria. The recognition of mycobacteria by APCs is mediated by pattern recognition receptors (PRRs), capable of binding pathogen associated molecular patterns (PAMPs), such as carbohydrates, lipids, glycolipids and proteins or peptides. PRRs proven to interact with mycobacterial components are here depicted and classified in two categories: Toll-like receptors (TLRs), such as TLR2/1, TLR2/6, TLR4, TLR9, and C-type lectin receptors (CLRs), such as Mincle, Dectin-1, Mannose Receptor and DC-SIGN. Recognition and binding of mycobacterial molecular components to TLRs and CLRs results in activation of specific signaling pathways which result in production of cytokines to recruit other immune cells, such as NK and granulocytes. Additionally, APCs are able to detect and present other mycobacterial components that are phagocytosed and loaded onto MHC proteins – or MHC-like molecules such as CD1. This process is key to activation of the adaptive immune system. The right side of the figure depicts three fundamental players of the adaptive immune response: T cells, B cells and antibodies.

Table 1 - Properties of the murine and human IgG subclasses. Ability to fixate the complement and affinity to the Fcγ receptor are selected functions which provide an indication of the ability of an antibody to tag a pathogen for destruction through activation of either the complement system or phagocytosis. Properties of murine IgG2c are not shown as this subclass is not well characterized.^{5,6}

Murine IgG antibodies			Human IgG antibodies		
IgG subtype	Complement fixation	Affinity to Fcγ receptor	IgG subtype	Complement fixation	Affinity to Fcγ receptor
<i>IgG1</i>	-	+	<i>IgG1</i>	++	+++
<i>IgG2a</i>	++	+++	<i>IgG2</i>	+	+
<i>IgG2b</i>	++	+++	<i>IgG3</i>	+++	++++
<i>IgG3</i>	++	-	<i>IgG4</i>	-	++

1

Although antibodies can tag extracellular pathogens for phagocytic uptake, they cannot easily access pathogens once these are inside a cell. T cells, on the contrary, have the ability to recognize infected cells and destroy them. Different classes of T cells exist and their roles span from T-helper lymphocytes (CD4⁺) to cytotoxic T lymphocytes (CD8⁺). When APCs present antigens to T cells, the latter can identify the antigen-protein complex via T cell receptors (TCRs) if the right co-stimulatory signals and cytokines are present.⁷ Although both CD4⁺ and CD8⁺ T cells are activated via antigen presentation, the mechanism of antigen presentation differs for the two. CD8⁺ T cell activation requires processing of intracellular antigenic proteins or peptides and loading on the major histocompatibility complex class I (MHC-I).⁸ CD4⁺ T cell activation requires engulfment of extracellular proteins or peptides, processing, and loading of the antigen on the major histocompatibility complex class II (MHC-II).⁹ Other T cell classes have been discovered, some of which contain TCRs able to interact with lipid antigens presented on MHC class I like cluster of differentiation-1 (CD1) molecules.¹⁰ T cell differentiation into further subtypes producing specific cytokines is functional to the diversification of the immune-response and the recruitment of different immune cells. Depending on the type of cytokines released by T-helper cells, the cellular adaptive immune response can be classified further.

In TB vaccination, relevant T cell responses include Th1- and Th17-cellular responses.¹¹ These cells produce cytokines, including IL-2, IFN- γ , TNF- α and IL-17 that stimulate macrophages and dendritic cells to upregulate phagocytosis and antigen presentation. Notably, Th1 and Th17 cellular responses have been regarded as markers of protection. The suppression of IFN- γ and IL-17 production has been shown to increase TB susceptibility.¹²⁻¹⁵ On the other end of the spectrum are Th2 immune responses, which are characterized by production of IL-4 and IL-10, and are associated with latent TB infection, reactivation and advanced TB.¹⁶

The selection of an antigen is of fundamental importance when designing a vaccine against a certain pathogen because it determines the specificity of the immunological memory. Molecules derived from *Mtb*, or synthetically made on the basis of the natural components, can be employed to render a vaccine specific against TB. However, an antigen alone is usually not sufficient to efficiently activate the immune system.¹⁷ Therefore, the antigen needs to be delivered together with one or more immune stimulatory agents, called adjuvants when they are included in a vaccine formulation. The main role of an adjuvant is to amplify and direct the responses to the antigen towards specific cell subsets, such as dendritic cells and macrophages, or certain compartments within the cell.

Immunological memory is generated when memory B or T cells are generated after prior exposure to the specific antigen and antigen-specific T cells and antibodies rapidly increase in the circulation after exposure to the specific antigen (humoral component of the immunological memory).

Table 2 - Properties of selected cytokines that are produced upon mycobacterial infection and that can be monitored to evaluate vaccine immunogenicity. The first two columns summarize the origin and functions of each cytokine. The last two columns provide an indication of the susceptibility to mycobacterial infection in the absence or alteration of the expression of each cytokine in either mice (knock-out) or humans (genetic deficiencies).^{18,19}

	Cytokine in relation to the immune system		Susceptibility to <i>Mtb</i> infection	
	produced by:	recognized functions:	Cytokine-knockout mice	Humans with disregulated cytokine expression
IL-6	monocytes, DCs, B cells, fibroblasts and endothelial cells	↓ inhibitory activity towards Th1 and Treg function; ↑ promotes Th2 and Th17 differentiation	enhanced susceptibility during early <i>Mtb</i> infection	genetic variation in IL-6 gene associated with TB disease
IL-10	DCs, macrophages, Th0, Th1, Th2 and T regs phenotypes	↓ deactivation of macrophages; ↓ downregulation of Th1 and NK immune-responses	similar susceptibility to <i>Mtb</i> infection	N/A
IL-12	DCs, macrophages, B cells	↑ induction of IFN- γ production and polarization to Th1 responses; promotion of macrophage and NK cell activity	enhanced susceptibility to <i>Mtb</i> infection	patients with genetic defects in IL-12/IFN- γ pathway are more susceptible
IFN-γ	macrophages, Th1, CTL, NK cells	↑ promotion of antigen presentation and recruitment of CD4 ⁺ and CD8 ⁺ T cells; promotion of B cell, macrophage, NK activity	enhanced susceptibility to <i>Mtb</i> infection	patients with genetic defects in IL-12/IFN- γ pathway are more susceptible
TNF-α	DCs, macrophages, Th1, some Th2 and some CTL phenotypes	↑ induction of NO production; ↑ contribution to granuloma formation; promotion DC activity	enhanced susceptibility to <i>Mtb</i> infection	TNF- α neutralization correlates with an increased risk of reactivation of latent tuberculosis
IL-17	Th17 cells; dependent on IL-23, IL-1beta, tgfbeta and IL-6.	↑ recruitment of neutrophils; ↑ contribution to granuloma formation	N/A	N/A

Through targeting of certain cells or compartments within APCs it's possible to promote the induction of specific cytokines and chemokines to attract other immune cells or promote the diversification of T cell response, eventually leading to immunological memory. Table 2 provides an overview of the functions of selected cytokines that are involved in immune-responses against TB. These cytokines were chosen on the basis of their origin (which immune cells produces them), their relevance in the recruitment of phagocytes or polarization of T cell responses. Additionally, their significance for the susceptibility to *Mtb* infection in cytokine knock-out mice and cytokine-deficient individuals is defined.

Current vaccines against *Mtb*

The first vaccine employed against *Mtb* was developed in Lille at the Pasteur Institute through attenuation of the virulent *Mycobacterium bovis* and used for the first time in humans back in 1921.²⁰ This was the first step towards the widespread use of the BCG vaccine, the sole *Mtb* vaccine licensed and in use today. The administration of the BCG vaccine, however is connected to the risk of disseminated BCG-osis, a disease occurring particularly in immunocompromised vaccinated infants (due to HIV infection), and occasionally in immune-compromised adults. BCG's main drawback is its limited and varying (0-80%) efficacy in providing protection against TB in adults. These challenges could be overcome by developing new vaccination strategies that are more efficacious, safer and preferably both.^{21,22} In the past 100 years, six different strains of BCG have been employed and there is evidence suggesting that the outcome of immunization may be related to which strain is used and/or batch to batch variation.²³ Controversy regarding the efficacy of the BCG vaccine in adults and immune-compromised individuals has prompted the investigation of other vaccination approaches.^{2,3,23} Moreover, the immunological mechanisms of protection after BCG vaccination have not been completely elucidated.

For a long time, TB vaccines were designed and evaluated with a focus on the induction of cellular Th1 responses on the assumption that this mechanism is primarily responsible for BCG-induced protection.²⁴ The key role of IFN- γ and Th1 cellular responses was deduced from the observation that a Th1 immune response was detected in BCG-immunized infants, and the additional observation that IFN- γ -secreting BCG-specific T cells were associated with protection against active TB in the following three years of life.²⁵⁻²⁷ However, there is little evidence that IFN- γ and Th1 cellular responses are the only correlates of BCG-induced protection. The CD4⁺ Th1 paradigm of protection has been subsequently challenged, especially after recognizing the low performance of Th1-inducing vaccine candidates once they reached human clinical trials.^{20,28-30} For example, the MVA85A vaccine, which induced robust Th1 antigen-specific T cell responses in infants and adults, failed to provide protection against incident *Mtb* infection or active disease.^{31,32}

Currently, there are 15 vaccine candidates in various stages of clinical trials,² which can be grouped in three main categories: attenuated/inactivated/recombinant pathogens, virally vectored- and recombinant protein subunit vaccines. The first category includes among others a recombinant BCG vaccine (VPM1002), a non-tuberculous mycobacterium (DAR-901), and a live genetically double attenuated vaccine based on a human isolate of *Mtb* (MTBVAC), as shown in Table 3. Advantages of using this vaccine modality include the simultaneous delivery of multiple epitopes and PAMPs, which can induce a strong pro-inflammatory response in the host and act via several different immune stimulating and modulating mechanisms, leading to

both innate and adaptive immune responses.³³ On the other hand, attenuated and inactivated micro-organisms might present safety concerns due to the intrinsic risk of mutation, reversion and contamination.³⁴ Even when using genetic modification approaches to inactivate genes, the problem of balancing immunogenicity and reactogenicity persists.³³

The second category includes virally vectored vaccines, with the most studied vectors being adenoviral vectors.³⁵ Currently there are two such vaccines in clinical trials against TB (see Table 3) and they include an adenovirus serotype 5 (Ad5Ag85A) and a recombinant chimpanzee adenoviral vector, both expressing an *Mtb* protein antigen. This vaccine modality presents similar advantages to whole-cell vaccines, delivering multiple PAMPs simultaneously. However, preexisting immunity in humans due to natural adenoviral infections can significantly reduce uptake of the vaccine by APCs as a consequence of neutralization of the viral vector by virus-neutralizing antibodies.³⁶

The third category, comprising subunit vaccines, is characterized by the use of (macro)molecules (proteins and peptides) derived from *Mtb* as molecular antigens in combination with other components, such as PAMPs (TLR4, TLR9), aluminum hydroxide or additional lipids assembling into liposomal bilayer/particles/emulsions. An advantage of both the second and third strategies is the reduction of safety concerns, together with the potential to change the antigen(s) included.^{37,38} An important characteristic of subunit vaccines, and in particular synthetic subunit vaccines, is the chance for step-wise improvements to optimize vaccine formulation in terms of, for example, solubility and delivery kinetics, together with the opportunity to target specific immune cells or cell compartments. An example of the potential of subunit vaccines can be seen in the recent study using M72/AS01E, showing a vaccine efficacy of 54% for the prevention of TB development in latently-infected individuals.^{39,40}

Table 3 - Vaccines against *Mtb* in different phases of clinical trials. The vaccines are grouped in three categories: viral, subunit and whole-cell. For each vaccine an indication of the stage of clinical evaluation is provided. Viral vector and type of whole-pathogen are specified for the viral and whole-pathogen vaccines. For subunit vaccines, details of the antigen(s) including the targeted PRR and delivery system are provided.

		Vaccine	Viral vector	Protein/peptide antigen	PAMP adjuvant	Delivery system	Whole-pathogen	Ref.
Phase II	Whole-cell	MTBVAC					attenuated <i>M. tuberculosis</i>	41
		RUTI					inactivated <i>M. tuberculosis</i>	42
		DAR-901					inactivated <i>M. bovis</i>	43
Phase III	Whole-cell	VPM1002					recombinant BCG	44
		MIP					inactivated <i>M. indicus pranii</i>	45
		Vaccae					inactivated <i>M. vaccae</i>	46
Phase I	Viral	Ad5Ag85A	adenovirus serotype 5					47
		ChadOx1 + Ag85A	chimpanzee adenovirus					
		AEC + BC02		Ag85B and ESAT6-CFP10	TLR9			49
Phase II	Subunit	GamTBVac		modified-Ag85B and ESAT6-CFP10	TLR9	dextran nanoparticles		50
		ID93 + GLA-SE		Rv1813, Rv2608, Rv3619 and Rv3620	TLR4	oil-in-water emulsion		51
		H1:IC31		Ag85B and ESAT-6	TLR9	cationic particles		52
		H4:IC31		Ag85B and TB10.4	TLR9	cationic particles		53
		H56:IC31		Ag85B, ESAT-6 and Rv2660c	TLR9	cationic particles		54
		M72 + AS01E		Mtb39A and Mtb32A	TLR4	liposome		40

Synthetic organic chemistry to generate effective & economical subunit vaccines

Synthetic organic chemistry offers the tools for the generation of highly pure, well defined and modifiable vaccine components that can be used to create new generations of vaccines: synthetic subunit vaccines. Currently, one challenge associated with the design of synthetic subunit vaccines against TB is the requirement for a good understanding of the processes and players involved in mycobacterial infection-induced immune responses. A thorough understanding of these processes is key to the definition of relevant antigenic targets, adjuvants and delivery systems for targeting relevant cell populations and their responses.^{55,56} The rapid advances in the field of molecular biology will possibly pave the way to the definition of many new molecular targets that can be employed in the rational design of vaccines.

Molecules from the cell wall of mycobacteria can be used for the development of new vaccine components, such as antigens and adjuvants (see Figure 2). To date, the most studied antigens from *Mtb* are proteins or peptides, as evident from their widespread use in TB vaccines that are currently in clinical trials (see Table 3). However, a plethora of antigenic lipids or glycolipids are present on the cell surface of *Mycobacterium tuberculosis* and some of them have antigenic properties, being recognized by the human immune system as foreign.^{57,58} An example is provided by β -mannosylphosphomycoketide (MPM), a glycolipid shown to act as epitope for the CD1c receptor (Chapter 2), a protein expressed on B cells and subsets of dendritic cells and required for subsequent presentation to T cells.^{59–62}

Unfortunately, technical advances in glycobiology have been slower as compared to those that allowed the study of RNA and proteins. RNA and proteins sequences can be elucidated from the complementary DNA and their functions can be more easily understood due to the ready access to synthetic structures.^{63,64,65,66} The processing and presentation of protein-derived peptides by the MHC I and II systems has been extensively researched.^{7,67} In addition to their proven antigenicity, proteins and peptides are commonly incorporated in vaccines due to the availability of scalable and efficient synthetic methods for their production, such as the use of automated solid phase synthesis for peptides⁶⁴ and recombinant methodologies or chemical ligation for proteins^{68,69}. The development of automated synthetic strategies for the generation of key glycans is currently hampered by the requirements for numerous different building blocks.⁷⁰ Nevertheless, glycolipids remain interesting targets and synthetic routes may be devised and optimized that render their production feasible and scalable, especially when the natural structures are simplified and the fundamental epitopes discovered.

Independently of the chosen antigen, to induce a long-lasting strong immune response it is necessary to deliver the antigen together with relevant immunostimulatory molecules. The co-delivery of proteins or peptides with immune adjuvants, such as PAMPs, has proven to be a successful method to overcome their inherent poor immunogenicity as single entities, as well as to increase and direct the type of immune response.^{17,37,71}

The subunit vaccines against *Mtb* that are currently in clinical trials all make use of TLR4 or TLR9 ligands, which have been shown to induce strong cellular immunity with Th1-polarized immune responses. One such TLR4 ligand is employed in the TB M72/AS01E vaccine formulation by Glaxo Smith Klein, notably the monophosphoryl lipid A (MPL), a synthetic glycolipid whose structure is derived from the *Salmonella minnesota* lipopolysaccharide.³⁹ The use of TLR9 ligands in the IC31 cationic particles developed by Intercell AG in the context of TB relies on the use of ODN1a, a synthetic oligodeoxynucleotide.⁵²⁻⁵⁴ Expanding the research on novel vaccine adjuvants outside the current focus on TLR4 and TLR9 activators is a strategy that can improve the development of rationally designed vaccines.

By understanding the molecular mechanism of action for different PAMPs and by defining the required immune responses, combinations of multiple PAMPs can be employed in search for a synergistic stimulating effect. Another important parameter in the design of subunit vaccines is the type of formulation and delivery system. Examples of vaccine formulations that are approved for use in humans include oil in water emulsions and liposomal formulations.⁷² These formulations ensure that antigen and adjuvants are co-delivered to the target immune cells, resulting in efficacious activation of APCs. However, they are relatively unstable and suffer from the requirement of cold chain storage, increasing costs and waste and decreasing accessibility of the vaccine to low-income countries.

These problems can be solved by the development of systems that rely on chemical bonds and not physical interactions, with chemical bonds being intrinsically more stable. Fully synthetic single molecule vaccines can be rationally designed to include antigen(s) and adjuvant(s) that are chemically linked to each other. To reach the point where this vaccine strategy can be employed, more fundamental and applied research is required. In this Thesis, efforts towards the development of fully synthetic vaccines are provided with the aim of generating knowledge and insights into this class of simple and stable vaccines against TB.

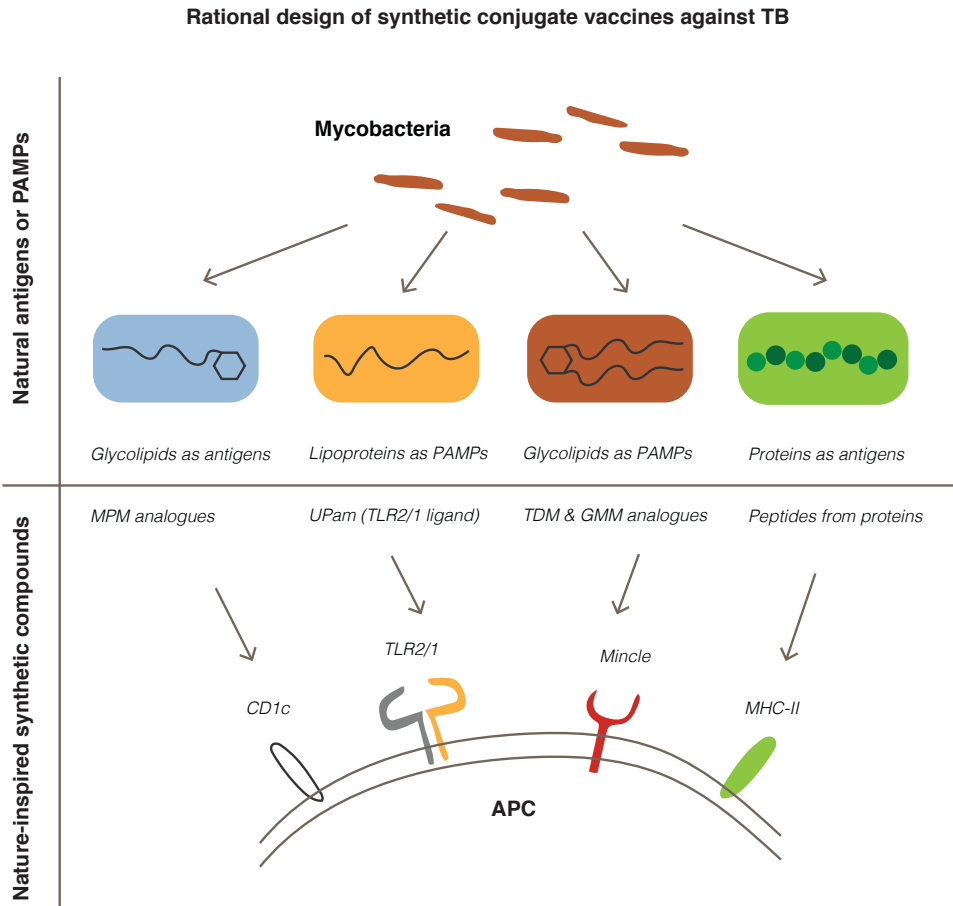


Figure 2 – Graphical depiction of rational design strategy to vaccine design in this Thesis. The rational design of vaccines begins with antigen discovery and the selection of relevant PAMPs, immunostimulatory molecules expressed by the pathogen that are recognized by PRRs, such as TLR2/1 and Mincle. TLR2/1 and Mincle get activated upon engagement with mycobacterial lipoproteins and glycolipids, respectively. Natural antigens expressed by mycobacteria and recognized by the human immune system include glycolipids and proteins/peptides. Synthetic chemistry can be employed to generate simplified/stabilized and biologically active analogues of the natural immunogenic structures. These nature-inspired synthetic compounds can be assayed for their ability to induce desired immune responses.

Thesis outline

The aim of this Thesis is to exploit synthetic chemistry to better understand processing of natural glycolipid antigens (Chapter 2), and to generate single molecule vaccines (Chapters 3 & 4), in which well-defined antigens and molecular adjuvants are combined. Co-stimulation by two synthetic PAMPs is investigated with the aim to probe synergistic immune activation (Chapter 5).

Chapter 2 applies rational antigen design to create a stabilized mannose phosphomyketide (MPM) that stimulates human T cell responses against the natural *Mtb*-derived glycolipid. Through the development of versatile synthetic strategies three stabilized MPM analogues were generated and tested for their antigenicity and cross-reactivity with nature-identical MPM. Overall, this work has provided detailed insights into presentation of MPM by CD1c.

Chapter 3 presents the first biologically active conjugate vaccine containing a peptide covalently linked to a synthetic analogue of the *Mtb*-derived glycolipid trehalose dimycolate (TDM) showing the *in vivo* efficacy of these constructs. The design and synthetic strategy for the generation of four TDM-inspired glycolipids is described, followed by the *in vitro* characterization of the glycolipid-derived conjugates. Finally murine experiments demonstrate the ability of one of the constructs to reduce the bacterial load in the spleen, correlating to strong humoral immune responses.

In **Chapter 4** the activation of TLR2 is explored in UPam-peptide conjugates for the induction of antimycobacterial responses in the context of different peptide epitopes. Three conjugates were generated, that have been shown to induce strong activation of human dendritic cells and macrophages *in vitro*. Further *in vitro* testing suggests that antigen presentation to T cells was not affected by the conjugation to the TLR-ligand. Finally, one conjugate was used to immunize mice with preliminary data indicating generation of humoral and cellular responses.

In **Chapter 5** two synthetic ligands, that are able to interact with Mincle and TLR2 were generated and assayed *in vitro* in search of functional synergies. It was shown that at certain concentrations a synergistic effect of the two ligands could be achieved, leading to increased cytokine production by human monocyte-derived dendritic cells. T cell antigen presentation experiments were also performed, suggesting that co-stimulation could not further increase presentation.

Finally, **Chapter 6** contains a summary of the results reported in this Thesis together with a discussion on the next steps required for the further development, refinement and future implementation of synthetic methods in *Mtb* vaccine development.

References

1. Geneva: World Health Organization. Global tuberculosis report 2020. 2020.
2. Cho T, Khatchadourian C, Nguyen H, Dara Y, Jung S, Venketaraman V. A review of the BCG vaccine and other approaches toward tuberculosis eradication. *Hum Vaccines Immunother.* 2021 Mar 26;1–17.
3. Foster M, Hill PC, Setiabudiawan TP, Koeken VACM, Alisjahbana B, Crevel R. BCG-induced protection against *Mycobacterium tuberculosis* infection: Evidence, mechanisms, and implications for next-generation vaccines. *Immunol Rev.* 2021 May;301(1):122–44.
4. Kenneth M. Murphy. *Janeway's Immunobiology*, 8th Edition. 2012.
5. Collins AM. IgG subclass co-expression brings harmony to the quartet model of murine IgG function. *Immunol Cell Biol.* 2016 Nov;94(10):949–54.
6. Vidarsson G, Dekkers G, Rispens T. IgG Subclasses and Allotypes: From Structure to Effector Functions. *Front Immunol.* 2014 Oct 20;5.
7. Neefjes J, Jongsma MLM, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol.* 2011 Dec;11(12):823–36.
8. Williams A, Peh CA, Elliott T. The cell biology of MHC class I antigen presentation: Williams et al : MHC class I antigen presentation. *Tissue Antigens.* 2002 Jan;59(1):3–17.
9. Roche PA, Furuta K. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat Rev Immunol.* 2015 Apr;15(4):203–16.
10. Brigl M, Brenner MB. CD1: Antigen Presentation and T Cell Function. *Annu Rev Immunol.* 2004 Apr;22(1):817–90.
11. Lyadova IV, Panteleev AV. Th1 and Th17 Cells in Tuberculosis: Protection, Pathology, and Biomarkers. *Mediators Inflamm.* 2015;2015:1–13.
12. Dalton D, Pitts-Meek S, Keshav S, Figari I, Bradley A, Stewart T. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science.* 1993 Mar 19;259(5102):1739–42.
13. Kaufmann SHE. Protection against tuberculosis: cytokines, T cells, and macrophages. *Ann Rheum Dis.* 2002 Nov;61 Suppl 2:ii54-58.
14. Qiu L, Huang D, Chen CY, Wang R, Shen L, Shen Y, et al. Severe Tuberculosis Induces Unbalanced Up-Regulation of Gene Networks and Overexpression of *IL-22*, *MIP-1 α* , *CCL27*, *IP-10*, *CCR4*, *CCR5*, *CXCR3*, *PD1*, *PDL2*, *IL-3*, *IFN- β* , *TIM1*, and *TLR2* but Low Antigen-Specific Cellular Responses. *J Infect Dis.* 2008 Nov 15;198(10):1514–9.

15. Segueni N, Tritto E, Bourigault M-L, Rose S, Erard F, Le Bert M, et al. Controlled *Mycobacterium tuberculosis* infection in mice under treatment with anti-IL-17A or IL-17F antibodies, in contrast to TNF α neutralization. *Sci Rep*. 2016 Dec;6(1):36923.
16. Ashenafi S, Aderaye G, Bekele A, Zewdie M, Aseffa G, Hoang ATN, et al. Progression of clinical tuberculosis is associated with a Th2 immune response signature in combination with elevated levels of SOCS3. *Clin Immunol*. 2014 Apr;151(2):84–99.
17. Guy B. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol*. 2007 Jul;5(7):396–7.
18. Romero-Adrian TB. Role of cytokines and other factors involved in the *Mycobacterium tuberculosis* infection. *World J Immunol*. 2015;5(1):16.
19. Hossain MdM, Norazmi M-N. Pattern Recognition Receptors and Cytokines in *Mycobacterium tuberculosis* Infection—The Double-Edged Sword? *BioMed Res Int*. 2013;2013:1–18.
20. Tran V, Liu J, Behr MA. BCG Vaccines. In: *Molecular Genetics of Mycobacteria*. Washington, DC, USA: ASM Press; 2015. p. 49–59.
21. Fine PEM. Variation in protection by BCG: implications of and for heterologous immunity. *The Lancet*. 1995 Nov;346(8986):1339–45.
22. Whitlow E, Mustafa AS, Hanif SNM. An Overview of the Development of New Vaccines for Tuberculosis. *Vaccines*. 2020 Oct 5;8(4):586.
23. Dockrell HM, Smith SG. What Have We Learnt about BCG Vaccination in the Last 20 Years? *Front Immunol*. 2017 Sep 13;8:1134.
24. Ahmed A, Rakshit S, Adiga V, Dias M, Dwarkanath P, D’Souza G, et al. A century of BCG: Impact on tuberculosis control and beyond. *Immunol Rev*. 2021 May;301(1):98–121.
25. Vekemans J, Amedei A, Ota MO, D’Elios MM, Goetghebuer T, Ismaili J, et al. Neonatal bacillus Calmette-Guérin vaccination induces adult-like IFN-gamma production by CD4+ T lymphocytes. *Eur J Immunol*. 2001 May;31(5):1531–5.
26. Marchant A, Goetghebuer T, Ota MO, Wolfe I, Ceesay SJ, De Groote D, et al. Newborns develop a Th1-type immune response to *Mycobacterium bovis* bacillus Calmette-Guérin vaccination. *J Immunol Baltim Md 1950*. 1999 Aug 15;163(4):2249–55.
27. Fletcher HA, Snowden MA, Landry B, Rida W, Satti I, Harris SA, et al. T-cell activation is an immune correlate of risk in BCG vaccinated infants. *Nat Commun*. 2016 Apr 12;7:11290.
28. Andersen P, Woodworth JS. Tuberculosis vaccines – rethinking the current paradigm. *Trends Immunol*. 2014 Aug;35(8):387–95.

29. Achkar JM, Casadevall A. Antibody-Mediated Immunity against Tuberculosis: Implications for Vaccine Development. *Cell Host Microbe*. 2013 Mar;13(3):250–62.
30. Levitz SM, Golenbock DT. Beyond Empiricism: Informing Vaccine Development through Innate Immunity Research. *Cell*. 2012 Mar;148(6):1284–92.
31. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *The Lancet*. 2013 Mar;381(9871):1021–8.
32. Ndiaye BP, Thienemann F, Ota M, Landry BS, Camara M, Dièye S, et al. Safety, immunogenicity, and efficacy of the candidate tuberculosis vaccine MVA85A in healthy adults infected with HIV-1: a randomised, placebo-controlled, phase 2 trial. *Lancet Respir Med*. 2015 Mar;3(3):190–200.
33. Dougan G, Hormaeche C. How bacteria and their products provide clues to vaccine and adjuvant development. *Vaccine*. 2006 Apr;24:S13–9.
34. Vetter V, Denizer G, Friedland LR, Krishnan J, Shapiro M. Understanding modern-day vaccines: what you need to know. *Ann Med*. 2018 Feb 17;50(2):110–20.
35. Liniger M, Zuniga A, Naim HY. Use of viral vectors for the development of vaccines. *Expert Rev Vaccines*. 2007 Apr;6(2):255–66.
36. Tatsis N, Ertl HCJ. Adenoviruses as vaccine vectors. *Mol Ther*. 2004 Oct;10(4):616–29.
37. Moyle PM, Toth I. Modern Subunit Vaccines: Development, Components, and Research Opportunities. *ChemMedChem*. 2013 Mar;8(3):360–76.
38. Bobbala S, Hook S. Is There an Optimal Formulation and Delivery Strategy for Subunit Vaccines? *Pharm Res*. 2016 Sep;33(9):2078–97.
39. Van Der Meeren O, Hatherill M, Nduba V, Wilkinson RJ, Muyoyeta M, Van Brakel E, et al. Phase 2b Controlled Trial of M72/AS01E Vaccine to Prevent Tuberculosis. *N Engl J Med*. 2018 Oct 25;379(17):1621–34.
40. Tait DR, Hatherill M, Van Der Meeren O, Ginsberg AM, Van Brakel E, Salaun B, et al. Final Analysis of a Trial of M72/AS01E Vaccine to Prevent Tuberculosis. *N Engl J Med*. 2019 Dec 19;381(25):2429–39.
41. Clark S, Lanni F, Marinova D, Rayner E, Martin C, Williams A. Revaccination of Guinea Pigs With the Live Attenuated *Mycobacterium tuberculosis* Vaccine MTBVAC Improves BCG's Protection Against Tuberculosis. *J Infect Dis*. 2017 Sep 1;216(5):525–33.
42. Vilaplana C, Montané E, Pinto S, Barriocanal AM, Domenech G, Torres F, et al. Double-blind, randomized, placebo-controlled Phase I Clinical Trial of the therapeutical antituberculous vaccine RUTI®. *Vaccine*. 2010 Jan;28(4):1106–16.

43. von Reyn CF, Lahey T, Arbeit RD, Landry B, Kailani L, Adams LV, et al. Safety and immunogenicity of an inactivated whole cell tuberculosis vaccine booster in adults primed with BCG: A randomized, controlled trial of DAR-901. PLOS ONE. 2017 May 12;12(5):e0175215.
44. Nieuwenhuizen NE, Kulkarni PS, Shaligram U, Cotton MF, Rentsch CA, Eisele B, et al. The Recombinant Bacille Calmette–Guérin Vaccine VPM1002: Ready for Clinical Efficacy Testing. Front Immunol. 2017 Sep 19;8:1147.
45. Sharma SK, Katoch K, Sarin R, Balambal R, Kumar Jain N, Patel N, et al. Efficacy and Safety of *Mycobacterium indicus pranii* as an adjunct therapy in Category II pulmonary tuberculosis in a randomized trial. Sci Rep. 2017 Dec;7(1):3354.
46. Yang X-Y, Chen Q-F, Li Y-P, Wu S-M. *Mycobacterium vaccae* as Adjuvant Therapy to Anti-Tuberculosis Chemotherapy in Never-Treated Tuberculosis Patients: A Meta-Analysis. PLoS ONE. 2011 Sep 6;6(9):e23826.
47. Zhu F-C, Hou L-H, Li J-X, Wu S-P, Liu P, Zhang G-R, et al. Safety and immunogenicity of a novel recombinant adenovirus type-5 vector-based Ebola vaccine in healthy adults in China: preliminary report of a randomised, double-blind, placebo-controlled, phase 1 trial. The Lancet. 2015 Jun;385(9984):2272–9.
48. Wilkie M, Satti I, Minhinnick A, Harris S, Riste M, Ramon RL, et al. A phase I trial evaluating the safety and immunogenicity of a candidate tuberculosis vaccination regimen, ChAdOx1 85A prime – MVA85A boost in healthy UK adults. Vaccine. 2020 Jan;38(4):779–89.
49. Lu J, Chen B, Wang G, Fu L, Shen X, Su C, et al. Recombinant tuberculosis vaccine AEC/BC02 induces antigen-specific cellular responses in mice and protects guinea pigs in a model of latent infection. J Microbiol Immunol Infect. 2015 Dec;48(6):597–603.
50. Vasina DV, Kleymenov DA, Manuylov VA, Mazunina EP, Koptev EY, Tukhovskaya EA, et al. First-In-Human Trials of GamTBvac, a Recombinant Subunit Tuberculosis Vaccine Candidate: Safety and Immunogenicity Assessment. Vaccines. 2019 Nov 1;7(4):166.
51. Penn-Nicholson A, Tameris M, Smit E, Day TA, Musvosvi M, Jayashankar L, et al. Safety and immunogenicity of the novel tuberculosis vaccine ID93 + GLA-SE in BCG-vaccinated healthy adults in South Africa: a randomised, double-blind, placebo-controlled phase 1 trial. Lancet Respir Med. 2018 Apr;6(4):287–98.
52. Mearns H, Geldenhuys HD, Kagina BM, Musvosvi M, Little F, Ratangee F, et al. H1:IC31 vaccination is safe and induces long-lived TNF- α +IL-2+CD4 T cell responses in M. tuberculosis infected and uninfected adolescents: A randomized trial. Vaccine. 2017 Jan;35(1):132–41.
53. Nemes E, Geldenhuys H, Rozot V, Rutkowski KT, Ratangee F, Bilek N, et al. Prevention of *M. tuberculosis* Infection with H4:IC31 Vaccine or BCG Revaccination. N Engl J Med. 2018 Jul 12;379(2):138–49.

54. Luabeya AKK, Kagina BMN, Tameris MD, Geldenhuys H, Hoff ST, Shi Z, et al. First-in-human trial of the post-exposure tuberculosis vaccine H56:IC31 in *Mycobacterium tuberculosis* infected and non-infected healthy adults. *Vaccine*. 2015 Aug;33(33):4130–40.
55. Stewart E, Triccas JA, Petrovsky N. Adjuvant Strategies for More Effective Tuberculosis Vaccine Immunity. *Microorganisms*. 2019 Aug 12;7(8):255.
56. Duthie MS, Windish HP, Fox CB, Reed SG. Use of defined TLR ligands as adjuvants within human vaccines. *Immunol Rev*. 2011 Jan;239(1):178–96.
57. Jackson M. The Mycobacterial Cell Envelope--Lipids. *Cold Spring Harb Perspect Med*. 2014 Oct 1;4(10):a021105–a021105.
58. Mendelson M, Walters S, Smith I, Kaplan G. Strain-specific mycobacterial lipids and the stimulation of protective immunity to tuberculosis. *Tuberculosis*. 2005 Sep;85(5–6):407–13.
59. Moody DB, Ulrichs T, Mühlecker W, Young DC, Gurcha SS, Grant E, et al. CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. *Nature*. 2000 Apr;404(6780):884–8.
60. de Jong A, Arce EC, Cheng T-Y, van Summeren RP, Feringa BL, Dudkin V, et al. CD1c Presentation of Synthetic Glycolipid Antigens with Foreign Alkyl Branching Motifs. *Chem Biol*. 2007 Nov;14(11):1232–42.
61. Ly D, Kasmar AG, Cheng T-Y, de Jong A, Huang S, Roy S, et al. CD1c tetramers detect ex vivo T cell responses to processed phosphomycoketide antigens. *J Exp Med*. 2013 Apr 8;210(4):729–41.
62. Roy S, Ly D, Li N-S, Altman JD, Piccirilli JA, Moody DB, et al. Molecular basis of mycobacterial lipid antigen presentation by CD1c and its recognition by T cells. *Proc Natl Acad Sci*. 2014 Oct 28;111(43):E4648–57.
63. Damha MJ, Zabarylo S. Automated solid-phase synthesis of branched oligonucleotides. *Tetrahedron Lett*. 1989 Jan;30(46):6295–8.
64. Albericio F. Developments in peptide and amide synthesis. *Curr Opin Chem Biol*. 2004 Jun;8(3):211–21.
65. Varki A. Biological roles of glycans. *Glycobiology*. 2017 Jan;27(1):3–49.
66. Ferreira SS, Passos CP, Madureira P, Vilanova M, Coimbra MA. Structure–function relationships of immunostimulatory polysaccharides: A review. *Carbohydr Polym*. 2015 Nov;132:378–96.
67. Mantegazza AR, Magalhaes JG, Amigorena S, Marks MS. Presentation of Phagocytosed Antigens by MHC Class I and II: Presentation of Phagocytosed Antigens. *Traffic*. 2013 Feb;14(2):135–52.

68. Cox MMJ. Recombinant protein vaccines produced in insect cells. *Vaccine*. 2012 Feb;30(10):1759–66.
69. Kent SBH. Total chemical synthesis of proteins. *Chem Soc Rev*. 2009;38(2):338–51.
70. Bojar D, Powers RK, Camacho DM, Collins JJ. SweetOrigins: Extracting Evolutionary Information from Glycans. *Bioinformatics*; 2020 Apr.
71. Black M, Trent A, Tirrell M, Olive C. Advances in the design and delivery of peptide subunit vaccines with a focus on Toll-like receptor agonists. *Expert Rev Vaccines*. 2010 Feb;9(2):157–73.
72. Baldwin SL, Bertholet S, Reese VA, Ching LK, Reed SG, Coler RN. The Importance of Adjuvant Formulation in the Development of a Tuberculosis Vaccine. *J Immunol*. 2012 Mar 1;188(5):2189–97.