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EXPERT REVIEW

Mycobacterium Tuberculosis and Interactions with the Host Immune System: Opportunities for Nanoparticle Based Immunotherapeutics and Vaccines

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ABSTRACT Tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains a deadly infectious disease. The thin pipeline of new drugs for TB, the ineffectiveness in adults of the only vaccine available, i.e. the Bacillus Calmette-Guerin vaccine, and increasing global antimicrobial resistance, has reinvigorated interest in immunotherapies. Nanoparticles (NPs) potentiate the effect of immune modulating compounds (IMC), enabling cell targeting, improved transfection of antigens, enhanced compound stability and provide opportunities for synergistic action, via delivery of multiple IMCs. In this review we describe work performed in the application of NPs towards achieving immune modulation for TB treatment and vaccination. Firstly, we present a comprehensive review of *M. tuberculosis* and how the bacterium modulates the host immune system. We find that current work suggest great promise of NP based immunotherapeutics as novel treatments and vaccination systems. There is need to intensify research efforts in this field, and rationally design novel NP immunotherapeutics based on current knowledge of the mycobacteriology and immune escape mechanisms employed by *M. tuberculosis.*

KEY WORDS immunotherapeutic nanoparticles · immunotherapy for tuberculosis · *Mycobacterium tuberculosis* · nanoparticle based host directed therapy · nanoparticles and vaccination

ABBREVIATIONS

AA	Arachidonic acid
AG	Arabinogalactan
APCs	Antigen presenting cells
BCG	Bacillus Calmette-Guerin
CaM	Calmodulin
CLRs	C-type lectin receptors
CORVET	Core vacuole/endosome tether
DCs	Dendritic cells
EEA1	Early endosomal antigen 1
ER	Endoplasmic reticulum
HBHA	Heparin binding hemagglutinin adhesion protein
HIV	Human immunodeficiency virus

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IMCs	Immune modulating compounds
INH	Isoniazid
LAMP	Lysosome-associated membrane proteins
LBPA	Lysobisphosphatidic acid
LM	Lipomannan
LTBI	Latent tuberculosis infection
Man-LAM	Mannosylated-lipoarabinomannan
MAs	Mycolic acids
MDR-TB	Multi-drug resistant tuberculosis
MHC	Major histocompatibility complex
MPs	Macrophages
NLRs	Nod-like receptors
NPs	Nanoparticles
NRP	Non-replicating persistent
PA	Phosphatidic acid
PAMPs	Pathogen associated molecular patterns
PG	Peptidoglycan
PI5P	Phosphatidylinositol 5-phosphate
PIM	Phosphatidylinositol mannoside
PIP3	Phosphatidylinositol 3-phosphate
PLGA	Poly(lactide)co-glycolide
PRRs	Pattern recognition receptors
PS	Phosphatidylserine
PtpA	Protein tyrosine phosphatase
RIF	Rifampicin
ROS/RNS	Reactive oxygen and nitrogen species
S1P	Sphingosine-1-phosphate
SPK	Sphingosine kinase
TB	Tuberculosis
TDR-TB	Totally-drug resistant tuberculosis
TLRs	Toll-like receptors
VPS33B	Vacuolar Protein Sorting 33B
WHO	World Health Organization
XDR	Extensively-drug resistant
XDR-TB	Extensively-drug resistant tuberculosis

INTRODUCTION

Tuberculosis (TB) remains a deadly infectious disease. In 2017, about 10 million people became ill with TB and there were 1.6 million deaths from this disease. Over 60% of cases arose from seven countries with India leading the count, followed by Indonesia, China, The Philippines, Pakistan, Nigeria and South Africa (1). Worldwide, about 1.7 billion people are estimated to be living with asymptomatic TB infection. To underscore the seriousness with which governments consider this pandemic, in September 2018, a high level United Nations General Assembly meeting was held with the goal of discussing unified approaches to ending the TB pandemic by the year 2035 (2,3).

TB is primarily acquired following inhalation of aerosolized *Mycobacterium tuberculosis* (*M. tuberculosis*) bacilli and the majority of TB cases are pulmonary in nature (1). Drug treatment of TB is intensive, requiring daily intake of a cocktail of 'first-line' antibiotics for at least 6 months to achieve a cure. In cases where *M. tuberculosis* has become resistant to the drugs isoniazid (INH) and rifampicin (RIF) (known as multi-drug resistant tuberculosis (MDR-TB)), treatment using 'second-line', more toxic drugs which include injectables, for up to 18 months is required (4). The increasing global incidence of MDR-TB (defined as resistance to a fluoroquinolone and one injectable drug such as amikacin) has resulted in greater attention placed on the judicious use of antibiotics, and towards the development of new drugs with novel mechanisms of action to avoid generation of drug resistant *M. tuberculosis* strains. However, despite these efforts, drug resistance remains uncurtailed and more severe forms of resistance known as extensively drug resistant tuberculosis (XDR-TB) have been detected (1,5).

M. tuberculosis is primarily an intracellular pathogen and the macrophage is the major host cell (6). The bacterium possess an innate ability to suppress the antimicrobial response of the macrophage. The survival strategies of *M. tuberculosis* within macrophages, which are detailed in this review, primarily involve prevention of phagosome maturation and an attenuation of pro-inflammatory responses (7,8).The current body of knowledge of the survival strategies employed by *M. tuberculosis* within the immune system, coupled with decreasing effectiveness of conventional antibiotics and a rise in drug resistant strains has led to revived interest in developing immunotherapies for TB. Within this context, immunotherapies encompass approaches in which compounds with immune modulating activity are administered in order to 'activate' immune cells to become a hostile environment for intracellular *M. tuberculosis*. A number of immune modulating compounds (IMCs) are at various stages of development and range from lipids and polysaccharides, cytokines and drugs such as metformin and albendazole (5,9). Engineered nanoparticles (NPs) have been employed to effectively deliver IMCs to immune cells and this application is discussed further in this review.

Vaccination is one of the most effective strategies for disease prevention. Unfortunately, the only vaccine available against *M. tuberculosis*, i.e. the Bacillus Calmette-Guerin (BCG) vaccine, has very limited effect against adult pulmonary TB (10). Therefore, developing novel, effective vaccination strategies alongside new treatment modalities is a promising strategy to eradicate TB globally. Numerous vaccine candidates for TB are currently in the clinical trial pipeline (11). However, most vaccines do not show strong immunogenicity and lack innate ability to be delivered to appropriate sites for optimal immune stimulation. In this regard, NPs are

being applied to achieve optimum induction of robust innate and adaptive immune responses and to target antigens to immune cells and facilitate transfection and this is discussed later.

The goal of this review is to stimulate intensified research to develop immunotherapeutic NPs for TB treatment and vaccination. To facilitate the reader's entry into this field, we firstly provide a comprehensive review of *M. tuberculosis* and how it modulates the host innate and adaptive immune systems. We then describe current work on the application of immunotherapeutic NPs towards *M. tuberculosis* eradication and vaccination.

THE MYCOBACTERIOLOGY OF *M. TUBERCULOSIS*

In 1882 Robert Koch successfully isolated and identified *M. tuberculosis* as the causative agent of TB (12). *M. tuberculosis* is one of more than a hundred closely related species within the genus *Mycobacterium*. This genus is divided into two groups, i.e. non-tuberculous mycobacteria that are made up of non-pathogenic or opportunistic, fast-growing pathogens including *M. smegmatis* and the *M. tuberculosis* complex comprising mainly of slow-growing, disease-causing species such as *M. leprae* and *M. tuberculosis* (13,14). *M. tuberculosis* is a gram-variable, contagious rod shaped pathogen varying in diameter and length between 0.3–0.5 μm and 1.5–4.0 μm, respectively (15). These aerobic-to-facultative anaerobes are metabolically very adaptable and can readily switch from a carbohydrate to a fat diet in an attempt to adjust to the evolving host cell conditions (16).

M. tuberculosis is surrounded by a characteristically thick and waxy cell envelope containing interconnected polymers of mycolic acids (MAs), arabinogalactan (AG) and peptidoglycan (PG) (17,18). This unique envelope renders *M. tuberculosis* hydrophobic; a trait primarily attributed to the presence of the MAs which are long chain fatty acids of up to 90 carbon atoms in length (19). The hydrophobic membrane acts as a permeability barrier towards various hydrophilic and lipophilic compounds making *M. tuberculosis* inherently resistant to antibiotics (17,18,20,21). However, the inherent resistance cannot solely be attributed to the impermeable membrane since experiments have shown that drugs are able reach cytotoxic levels within cells (20). This stresses the important contribution of other virulence factors such as efflux pumps and drug degrading enzymes towards the intrinsic resistance of *M. tuberculosis* (17,20,22).

An additional pathway in which *M. tuberculosis* proves to be problematic towards the cure of TB, is its ability to enter a non-replicating persistent (NRP) state enabling it to survive within the host until conditions are more favourable (20,23). Persister cells is a simple descriptive term used for tubercle bacilli within the NRP state and these cells are phenotypically and reversibly tolerant towards antibiotics (24). Most conventional antibiotics are designed to target cellular functions important for microbial growth and proliferation in actively replicating cells; however, NRP cells are thought to be metabolically quiescent characterised by a thickening of cell walls, a decrease in protein synthesis and transcription rates, and a low metabolic state with ATP levels up to 5-fold lower compared to actively replicating cells (25). This automatically eliminates common antibiotic targets which renders these cells tolerant towards various antibiotics if they remain within the NRP state. Only a small number of bacterial cells enter the NRP state and Keren *et al.* (26). reported that the persister fraction of an inoculum exposed to antibiotics was only around 1%. This low generation frequency together with their transient nature is the reason knowledge of persister cells is limited and the exact mechanisms by which they enter and exit this state is still unclear (27). Various factors are alleged to induce persister cell formation including presence of an acidic environment, growth-limiting by-products such as acetate and nutrient and oxygen depletion (28). Persisters are believed to be the cause of latent TB infections (LTBI) that is defined by a non-contagious, clinically asymptomatic state (24). Approximately 5–10% of persons infected with *M. tuberculosis* will eventually develop primary active TB and 90–95% will remain latently infected, not because the bacilli gets eradicated but is effectively controlled within granulomatous structures (29). LTBI is a major obstacle in the control of TB due to the chance of disease activation once the cells exit the NRP state and proliferate. Consequently, latently infected persons are the pool of future infections. The possibility of persister cells being present ensures that anti-TB treatment regimens extend over long periods of time aggravating an already rigorous antibiotic course.

CURRENT TB THERAPY AND DRUG RESISTANCE

Treatment is administered as a 'cocktail' of several antibiotics, each targeting various mycobacterial functions at relatively high doses as a preventative measure against acquisition of resistance. Treatment of drug-susceptible TB is a 6-month regimen based on a minimum of 4 first-line antibiotics (INH, RIF, ethambutol and pyrazinamide) during the initial 2-month intensive phase (4). The course for drug-resistant TB extends to around 18 months of 4 s-line core drugs (later-generation fluoroquinolone such as moxifloxacin, an injectable aminoglycoside such as amikacin plus ethionamide or prothionamide, terizidone or cycloserine, linezolid and clofazimine) with an intensive phase of at least 8 months. Elevated doses of the antibiotics are used and cause

severe side effects including ototoxicity, hepatotoxicity, hyperuricemia and neuropsychiatric problems (30). The current lengthy treatment regimens and side effects are a major cause of patient non-compliance and consequently failure of TB treatment and the manifestation of drug resistance (5).

M. tuberculosis rapidly acquires resistance to antibiotics (Fig. 1), and it is estimated that this rate is similar for both bacterial cells in both active and NRP state. Therefore it is proposed that mycobacterial mutations occur in a time-dependent manner instead of a replication-dependent manner (25). Contrary to most bacterial species, resistance is not attributed to horizontal gene transfer and is completely reliant on independently acquired chromosomal mutations and non-chromosomal events such as the production of drug modifying and inactivating enzymes, along with the presence of a MA-rich membrane and efflux pumps (22,31). Fitness costs frequently accompany these resistance mutations through secondary mutations on different loci, however, *M. tuberculosis* remains fully virulent and successfully fixes resistance mutations in consecutive populations (31).

The contribution of antibiotic resistance towards the TB epidemic has led to the restricted use of the most recently discovered anti-TB drugs (i.e. bedaquiline and delamanid) to retain efficacy and to maintain low levels of resistance. Clinical studies are also on-going to optimize use of existing TB drugs by investigating various dose and treatment duration options (32,33).

THE INTERACTION OF *M. TUBERCULOSIS* WITH THE MACROPHAGE AND MECHANISMS OF SURVIVAL

M. tuberculosis is primarily transmitted via inhalation of aerosolized bacilli and establishes infection within the lung. Bacilli are detected by resident immune cells including alveolar macrophages, dendritic cells (DCs) and neutrophils. An innate immune response is initiated through selective binding to pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and Nod-like receptors (NLRs) (6,34).

PRRs recognize polysaccharide-like structures present on *M. tuberculosis* known as pathogen associated molecular patterns (PAMPs), specifically mannosylated-lipoarabinomannan (Man-LAM), lipomannan (LM) and phosphatidylinositol mannoside (PIM) (6). PAMP motifs are highly conserved within species and are used as unique identifiers of an invading pathogen. The fate of ingested *M. tuberculosis* in immune cells can alternate between complete eradication, latent containment within a granuloma or the successful suppression of immune functioning and consequent transmission by *M. tuberculosis*.

PHAGOSOME MATURATION AND PHAGOLYSOSOME FORMATION

PAMP recognition of PRRs leads to phagocytic uptake of *M. tuberculosis* by macrophages (MPs) (35). Engagement of PRRs initiates formation of pseudopod-like structures around the bacterium that seal at the tips and form an intracellular vesicle known as a phagosome (36). The subsequent maturation stages are characterised by fusion of the phagosome with various endosomal and lysosomal compartments that alters its protein and enzymatic composition and initiates the desired antimicrobial activity. Key stages include the early phagosome, late phagosome and eventual formation of the phagolysosome (36).

M. tuberculosis can successfully prevent phagosome maturation and persist within vesicles characterised by continuous association with Rab5, the absence of PI3P and sphingosine kinase (SPK), low V-ATPase levels, a near neutral pH, and the active retention of coronin-1. Early phagosomes fuse with early endosomes and acquire Rab5, which recruits the hVPS34 kinase that together with other molecules, leads to the cyclic accumulation of PI3P on the phagosomal membrane. PI3P is a membrane trafficking regulatory lipid believed to be an important docking site for various proteins specifically the early endosomal antigen 1 (EEA1) and the class C core vacuole/endosome tether (CORVET) complex; these are central role players in membrane fusion and the ensuing phagosome maturation and phagolysosome formation stages (37–39).

However, reports have shown that PI3P is absent on the phagosomal membranes containing live *M. tuberculosis* but is continuously present on those that harbour dead cells (40). *M. tuberculosis* prevents accumulation of PI3P through direct interference with the hVPS34 kinase responsible for PI3P production or through the secretion of SapM, a PI3P hydrolysing enzyme (39,40). The transition from an early phagosome to a late phagosome is characterised by replacement of Rab5 with Rab7, the acquisition of lysosomal enzymes delivered in transport vesicles and the accumulation of lysosome-associated membrane proteins (LAMP1 and 2) necessary for phagolysosome formation (41). The late phagosome becomes a more hydrolytic and oxidative compartment suited for cargo degradation. Rab7 is important for centripetal movements and mediates the switch from a CORVET complex to the homotypic fusion and vacuole-sorting (HOPS) complex important for late endosomal fusion (36,37). *M. tuberculosis* produces PtpA, a protein tyrosine phosphatase, which dephosphorylates and inactivates the host protein Vacuolar Protein Sorting 33B (VPS33B), a regulator of membrane fusion. Inactive VPS33B cannot generate GTP-activated Rab7 hence blocking phagosome maturation and PL fusion (36,37,39,41).

Following *M. tuberculosis* ingestion, V-ATPase is rapidly recruited to the phagosomal membrane gradually acidifying the intraphagosomal compartment through inward

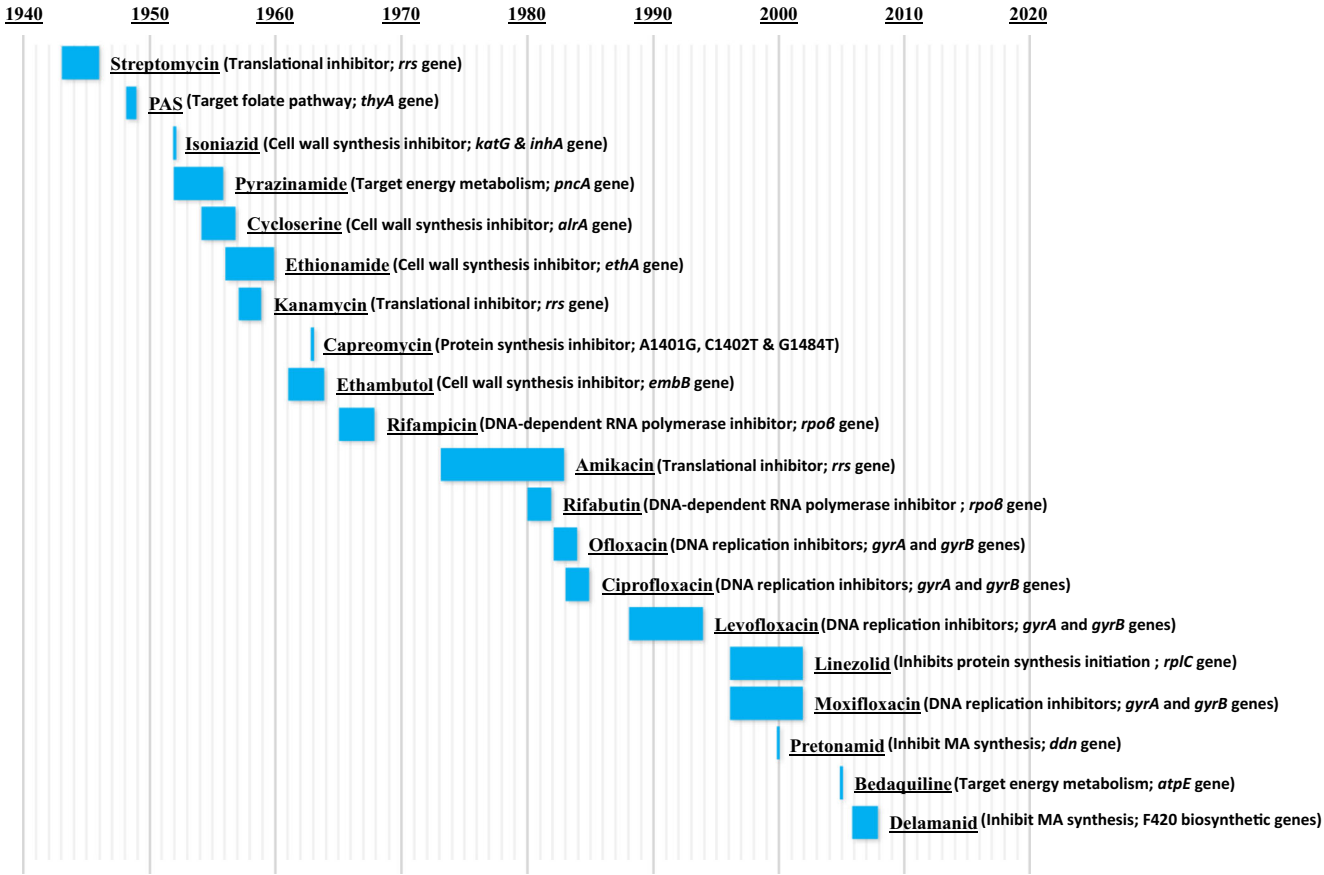

Fig. 1 Timeline illustrating period (in years) between antibiotic discovery and resistance acquisition. The brackets list the mode of action of the antibiotic and the primary genes involved in acquired resistance to the specific antibiotic. Image adapted from Calitz *et al*. (unpublished).

pumping of protons (H^+) (37). *M. tuberculosis* selectively excludes V-ATPase from the phagosomal membrane and arrests the internal acidification process at a pH of approximately 6.4; much higher than the intended pH (≤ 5) of the late phagosome necessary for downstream functioning of various phagosomal proteases and lysosomal enzymes (23,41,42). Exactly how *M. tuberculosis* excludes V-ATPase is an ongoing debate but Queval *et al.* (42) demonstrated possible strategies *M. tuberculosis* exploits to target the V-ATPase complex. Their studies have shown that the CISH protein is actively recruited to *M. tuberculosis* -containing phagosomes and actively leads to the ubiquitination and subsequent degradation of V-ATPase (42). Additionally, Wong *et al.* (43) also showed a direct link between PtpA and its ability to bind directly to the H subunit of the V-ATPase complex. Binding to the unit actively prevents the trafficking of this enzyme to *M. tuberculosis* -containing phagosomes. A low pH in mature phagosomes is important to ensure optimal enzymatic functioning of degrading lysosomal enzymes such as cathepsin D that are delivered once the phagosome fuses with the lysosome.

Calcium (Ca^{2+}) mobilization is associated with microbial ingestion. Increases in cytosolic Ca^{2+} levels are marked by the binding of Ca^{2+} with calmodulin (CaM) that activate CaMKII (8,44).This signalling cascade leads to the activation of hVPS34 that catalyses PI3P production and the consequent binding of EEA1 to PI3P, promoting membrane fusion and phagosome maturation (8). Firstly, *M. tuberculosis* can suppress sphingosine kinase (SPK) therefore blocking the increase in macrophage cytosolic Ca^{2+} levels (44). Macrophage ingestion of inactivated *M. tuberculosis* cells activates SPK resulting in the translocation of the enzyme to the phagosome membrane. SPK phosphorylates sphingosine, yielding sphingosine-1-phosphate (S1P) that induces an increase in Ca^{2+} from endoplasmic reticulum (ER) stores (44). Secondly, in contrast to the prior mentioned inhibiting effects, *M. tuberculosis* can also effectively exploit the increase in Ca^{2+} levels to prolong survival. *M. tuberculosis* actively retains coronin-1 on the phagosomal membrane leading to the Ca^{2+}-dependent activation of calcineurin and consequently, the direct prevention of phagosome-lysosome fusion (40). Notably, Jayachandran *et al.* (40) showed that coronin-1 dependent Ca^{2+} mobilization is independent of SPK. This might be due to the differences observed in

internalization between opsonized and non-opsonized *M. tuberculosis* which activates different downstream signalling pathways.

The final stage of bacterial destruction is the fusion of the late phagosome with lysosomal compartments mediated by various soluble NSF attachment protein receptors (SNAREs) (37). The resulting phagolysosome becomes acidic (pH 4.5) and the degradative capacity is enhanced through acquisition of various hydrolytic enzymes such as cathepsin (37,41). At this stage antimicrobial effects are further elevated through an increased production of reactive oxygen and nitrogen species (ROS/RNS) augmented by the NADPH oxidase (NOX) complex, recruited to the phagosome membrane throughout the maturation process, and the inducible nitric oxide synthase (iNOS) (23,36,37). NOX, in particular NOX2, transfers electrons from NADPH to intra-phagosomal oxygen forming superoxide anions. These anions dismutate to form hydrogen peroxide and other toxic ROS. In addition, iNOS generates nitrate and nitrite that reacts with nitrous acid at a low pH producing nitric oxide and nitrogen dioxide. Nitric oxide and superoxide radicals can finally come together and form the highly toxic peroxynitrite (23). These reactive radicals are important for pathogen eradication; however, *M. tuberculosis* can avert toxicity through the production of proteins involved in detoxification and damage repair. The primary strategy employed by *M. tuberculosis* is the secretion of KatG, a catalase-peroxidase that catabolizes peroxides within the phagosome (23,39). Oxidative stress can be further subdued by LAM which can scavenge free oxygen radicals (23). In addition, to inhibit phagolysosome fusion, *M. tuberculosis* secretes PknG, a eukaryotic homolog and kinase acting protein, into the cytosol of the macrophage. PknG phosphorylates a currently unknown host molecule that acts as a mediator in membrane fusion hence suppressing lysosomal delivery of the phagosome by the host factor (39). Fig. 2 summarizes the major immune regulatory strategies exploited by *M. tuberculosis*.

CYTOKINE MOBILIZATION AND GRANULOMA FORMATION

Uptake of *M. tuberculosis* by macrophages leads to secretion of various cytokines and chemokines. These cytokines and chemokines act in concert and lead to increased vascular permeability, mediate systemic effects such as fever and the recruitment of various inflammatory cells (45). The macrophage cytokine profile is made up of pro-inflammatory cytokines including IFN-γ, TNF-α, IL-2, IL-6, IL-12, IL-18, IL-23 and anti-inflammatory cytokines IL-27 as well as IL-4, IL-10, IL-13 and TGF-β (45,46). The different cytokine groups activate different macrophage phenotypes differentiating between a degrading pro-inflammatory macrophage with a Th1 cell cytokine environment or a macrophage phenotype characterised by a "resting" state with low microbicidal effects and a Th2 cytokine profile (anti-inflammatory). Macrophage activation through pro-inflammatory cytokines is necessary to obtain effective bactericidal properties; however, it is important that these pro-inflammatory cytokines are produced in appropriate amounts to prevent cytotoxic effects within the host. This is an important consideration in designing immunotherapies and is discussed in later sections. *M. tuberculosis* LAM inhibits IFN-γ secretion, an important macrophage activating cytokine (47). In addition to LAM, internalization of *M. tuberculosis* induces an increased production of IL-10, an anti-inflammatory cytokine associated with IFN-γ suppression. O'Leary *et al.* (48) reported that macrophages infected with live *M. tuberculosis* secreted approximately 2X more IL-10 in comparison to macrophages infected with dead/inactivated *M. tuberculosis*. Addition of an anti-IL-10 antibody led to enhanced phagosome maturation highlighting the importance of IL-10 in mycobacterial pathogenicity and survival (48). An increased production of IL-10 leads to the decreased production of IL-12 and reduced recruitment of macrophage activating cytokines (7,48). By successfully inhibiting macrophage activation, *M. tuberculosis* consequently arrests the phagosome maturation process as well as the subsequent degradative pathways.

Cytokine and chemokine production leads to the recruitment of various cell populations to the site of the *M. tuberculosis*-containing macrophages including epithelioid cells, Langhans giant cells, mononuclear phagocytes, fibroblasts, and T and B lymphocytes (23,35). These immune regulatory cells form a highly organised structure known as a granuloma. Necrotic macrophages are generally located within the centre of the granulomatous structure surrounded by interconnected layers of multinucleated giant cells, apoptotic macrophage, foam cells, dendritic cells, and neutrophils (49) . The outermost layer of the structure is surrounded by a layer of T cells, B cells, and natural killer cells known to be major producers of IFN-γ and recognize specific peptides bound to major histocompatibility complexes (49). The granuloma is a hallmark of most *M. tuberculosis* infections and creates an immune microenvironment that enables the host to control the infection, mediated by a fine balance between pro- and anti-inflammatory cytokine production. TNF-α and IFN-γ are considered to be important pro-inflammatory cytokines involved in the functioning and formation of the granuloma with IL-10 being the major negative regulator (35). In 90–95% of infected patients, the subsequent formation of a solid granuloma is a telling sign that the immune system is effectively containing *M. tuberculosis* and the bacilli in general enters the NRP state resulting in a latent, asymptomatic infection. Should the cytokine balance be tipped, the bacilli may reactivate, forming caseous lesions and the development of active symptomatic TB (23). The likelihood of this balance being

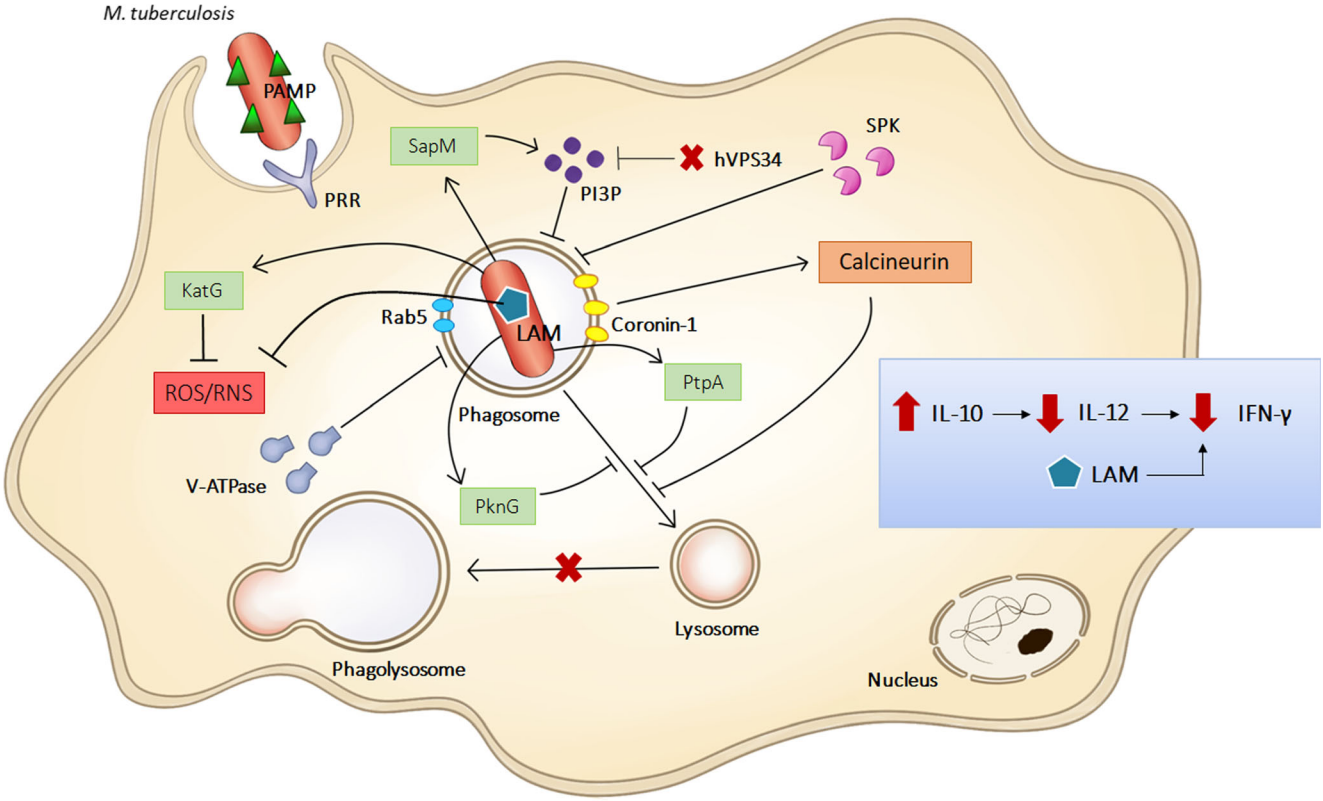

Fig. 2 Overview of the major immune regulatory strategies exploited by *M. tuberculosis* within the macrophage. The bacilli can prolong its survival by actively preventing maturation and fusion of the *M. tuberculosis*-containing phagosome characterised by continuous association with Rab5, absence of PI3P, low V-ATPase levels hence a near neutral pH, the active retention of coronin-1 on the membrane, and a decrease in cytosolic Ca^{2+} levels due to SPK suppression. In addition, *M. tuberculosis* can secrete various proteins including SapM, PtpA, PknG and KatG acting on various stages of the maturation and fusion steps. The mycobacterial polysaccharide, LAM, can directly inhibit IFN-γ secretion. Moreover, *M. tuberculosis*-containing macrophages secrete higher levels of IL-10, a major negative regulator of macrophage activation. IL-10 production inhibits IL-12 secretion and consequently supresses IFN-γ production.

disrupted in favour of active disease development increases for patients with compromised immune systems as in the case of human immunodeficiency virus (HIV) co-infection. Once the balance is tipped, the granuloma becomes increasingly necrotic, characterised by caseation, eventually leading to the active transmission of *M. tuberculosis* bacilli (35).

THE ADAPTIVE IMMUNE RESPONSE TO *M. TUBERCULOSIS* INFECTION

Despite the initial attempt to eliminate the bacilli by cells of the innate immune system, prolonged host protection from active disease requires the generation of adaptive immune response that are initiated and driven by the activated antigen presenting cells (APCs), i.e. macrophages and dendritic cells (DCs) (50,51). The coordination between innate and adaptive immune responses is comprised of cellular, cytokine and chemokine components which are necessary for effective control of bacilli replication and dissemination.

ANTIGEN PRESENTATION

Antigen presentation by APCs is a crucial step that links the innate and adaptive immunity and involves distinctive mechanisms. For major histocompatibility complex (MHC) class II presentation, *M. tuberculosis* peptide antigens are presented by APCs to antigen-specific $CD4^+$ T cells, which is thought to be the most important protective response for control of intracellular infection (52). For MHC-I presentation, all nucleated cells are able to present *M. tuberculosis* peptide antigens to antigen-specific $CD8^+$ T cells. This mechanism allows infected cells with cytosolic peptide antigens to be killed through Fas/Fas-L induced apoptosis or granule-mediated function (53). The stimulation of T cells in the context of MHC molecules activates adaptive immunity and induces IFN-γ secretion and cytolytic CD8 T cell activity. However, as described earlier, *M. tuberculosis* employs several evasion strategies to circumvent the phagosome-lysosome fusion pathway, and thereby prevents antigen processing and presentation to T cells.

Unlike macrophages, DCs are considered professional APCs and act as initiators of specific T cell immunity against *M. tuberculosis* infection (54,55). Depletion of DCs delays onset of *M. tuberculosis* specific $CD4^+$ T cells priming that compromises host immunity in conjunction with uncontrolled bacilli replication (56). After bacilli uptake, DCs present antigens to T cells following migration to the draining lymph node in an IL-12 and TNF-α dependent manner. Upon arrival in lymph nodes, DCs become functionally mature, characterized by upregulation of MHC-II, CD40, CD80, and CD86, to effectively stimulate naïve T cells (57,58). Although a complex regulatory process, TNF-α is known to be an important factor that facilitates DC maturation towards specific T cell priming (59,60). Interestingly, while persistent TNFRp55 expression leads to potential defective T cell responses during chronic *M. tuberculosis* infection (61), sustained TNF-α expression by DCs enhances maturation and generates a much more robust T cell response (62). Thus, DCs are positioned to serve as an important link between innate and adaptive immunity and may act as useful targets for immunotherapy and vaccine development (63,64).

T CELL RESPONSES IN ADAPTIVE IMMUNITY

The adaptive immune response is initiated in the peripheral lymphoid organs, and consists of cell-mediated immunity by T cells and humoral immunity by B cells. Research in the area of immunity to *M. tuberculosis* has largely focused on T cells due to their critical role in the elimination of bacteria during primary infection, followed by their ability to generate *M. tuberculosis* specific and memory responses to protect from subsequent infections. The importance of $CD4^+$ T cells against *M. tuberculosis* is supported by the clinical association of increased susceptibility to TB in HIV infected patients (65,66). This is supported by murine studies with antibody depletion of $CD4^+$ T cells or the use of gene-deficient mice (52,67), which show that the loss of $CD4^+$ T cells significantly increases susceptibility to *M. tuberculosis* infection.

Following priming, synthesis of IL-2 promotes naïve T cell proliferation and differentiation into different subsets of effector T cells, particularly $CD4^+$ Th1 cells and type 1 cytokine responses that are crucial for protection against *M. tuberculosis*. The major effector function of $CD4^+$ T cells is considered to be the production IFN-γ which is pivotal for host protection. The critical role of IFN-γ in *M. tuberculosis* infection was demonstrated in various experimental studies (68,69) and confirmed in children with genetic deficiencies of IFN-γR (70). Moreover, TNF-α from *M. tuberculosis*-specific $CD4^+$ T cells has been explored for its use as a biomarker for diagnosis of active TB disease (50). The contribution of T cell derived TNF-α to immune defense against *M. tuberculosis* has been investigated using T cell-specific TNF-α deficient mice and shown that, while myeloid TNF-α is required for initial control of bacterial replication, T cell-derived TNF-α is essential to sustain protection during chronic TB infection (71). Interestingly, in contrast, TNF-α from T cells was found to be largely redundant in cerebral immunity against TB infection (72).

HUMORAL IMMUNITY BY B CELLS

It is generally accepted that while cell-mediated immune response is the effector branch of adaptive immunity to defend intracellular pathogens, the extracellular counterparts are protected by the humoral immune response, in which B cells are activated to secrete antibodies. Like T cell responses, the antibody responses are antigen specific and have different ways to mediate the clearance of pathogens, such as neutralization and opsonization. Although the understanding of adaptive immune response against *M. tuberculosis* relies predominantly on the studies of cell-mediated immunity, increasing evidence supports the role of B cell and humoral immunity in the defence against *M. tuberculosis* infection (71,73). Serological detection tests indicate that *M. tuberculosis* infection induces humoral immune responses to a wide variety of mycobacterial antigens (74). Studies have also shown that the BCG vaccine can elicit antibody responses to various mycobacterial antigens (75,76), and contribute to immune protection against mycobacteria. Moreover, *in vitro* and animal studies using antibodies to *M. tuberculosis* antigens have shown enhanced protection with increased survival and reduced bacterial burden (77,78). Recent immunization studies with mycobacterial capsular arabinomannan conjugates have demonstrated antibody and T cell responses that contribute to protective immunity against *M. tuberculosis* (79). These findings provide evidence for the potential role of humoral immunity in TB vaccine development strategies.

IMMUNOTHERAPEUTIC NPS AND *M. TUBERCULOSIS* ERADICATION

NPs have been used to stimulate macrophages to achieve eradication of intracellular *M. tuberculosis*. Greco *et al.* (80) developed an immunotherapeutic liposome comprised of phosphatidylserine (PS) on the outer membrane and phosphatidic acid (PA) in the inner membrane (Janus faced liposomes). PS was included in the NP design to inhibit cellular production of pro-inflammatory cytokines and enhance anti-inflammatory cytokine secretion. PA is a lipid IMC involved in phagolysosome maturation (80). Hence the strategy was to achieve *M. tuberculosis* killing using PA and avoid the pathology associated with over production of pro-inflammatory cytokines using PS. From the same research group, Poerio *et al.* (81)

synthesized similar liposomes having PS in the outer layer, however, additional lipids in the inner layer were investigated for ability to lead to mycobacterial killing, i.e. PA, PI3P, phosphatidylinositol 5-phosphate (PI5P), lysobisphosphatidic acid (LBPA), S1P and arachidonic acid (AA). These lipids were selected due to their known ability to promote phagosome maturation (81).

Greco *et al.* demonstrated that the presence of PA on the liposomes enhanced intracellular mycobacterial (*M. tuberculosis* H37Rv) killing in THP-1 macrophages and in human bronchoalveolar lavage cells through promotion of Ca^{2+}-mediated phagolysosome maturation and increased ROS production (80). Further, the desired balance in the immune response was demonstrated. Production of pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α was dampened, while TGF-β production was enhanced. Similarly, Poerio *et al.* demonstrated enhanced phagosome acidification (to a pH of up to 5.5) when PS/PA, PS/PI3P and PS/PI5P NPs were incubated with BCG infected THP-1 macrophages. These NPs also promoted ROS production and ultimately enhanced mycobacterial killing in macrophages (81).

Intranasal administration of the PS/PA liposomes to *M. tuberculosis* infected BALB/C mice was shown to result in a 100-fold reduction in pulmonary bacterial burden after 4 weeks, in comparison to a 2-fold reduction from orally administered INH. Combined administration of the PA liposomes and INH also proved effective. A tenfold reduction in serum levels of TNF-α, IL-1β and IFN-γ was observed with PA liposome treatment alone or in combination with isoniazid (80). These studies demonstrate that cellular induction of phagosome maturation and ROS production, while suppressing pro-inflammatory cytokine secretion is a viable strategy to killing intracellular mycobacterium species. NPs in this instance allowed co-delivery of the IMCs to macrophages, allowing presentation of one type of IMC (i.e. PS) to the macrophage surface receptors and the presentation of multiple IMCs within the macrophage.

An IMC functionalized polymeric NP was developed by Dube *et al.* (82). The polysaccharide IMC, i.e. 1,3-β-glucan, was adsorbed onto the chitosan shell and the NP core comprised the polymer poly(lactide)co-glycolide (PLGA). RIF was also loaded into the NP core, and could be released in a sustained manner. 1,3-β-glucan activates dectin-1 on macrophage surfaces subsequently activating various downstream signal transduction pathways which promote pro-inflammatory gene expression as well as intracellular ROS/RNS production. Pro-inflammatory cytokines known to be produced through Dectin-1 activation include IL-12 (83,84). Apart from gene induction, Dectin-1 signalling also increases intracellular Ca^{2+}, following phosphorylation of various intracellular phospholipases (85). Given these pharmacological effects, Dectin-1 activation by 1,3-β-glucan can potentially reverse the immune suppressive effects of *M. tuberculosis* in MPs. A significant increase of IL-12p70, TNF-α and INF-γ and ROS was reported, following incubation of these NPs with healthy human alveolar-like macrophages. Levels of anti-inflammatory cytokines IL-4 and IL-10 remained unchanged (82). In later work, linear chain 1,3-β-glucan, i.e. curdlan, was chemically conjugated onto PLGA producing IMC functionalized PLGA polymer which could form NPs (86). The curdlan-PLGA NPs could stimulate THP-1 macrophages as evidenced by enhanced phosphorylated ERK production, an upstream mediator of ROS/RNS. These studies demonstrate the stimulation of APCs using β-glucan functionalized NPs, however, further studies in *M. tuberculosis* infected cells are required, as well as studies to determine an acceptable balance between macrophage activation secretion and bacterial killing. Recently, Hwang *et al.* (87) conjugated single stranded β-glucan onto silica NPs. These NPs also encapsulated INH which could be released in a sustained manner. However, these NPs were observed to minimally activate peripheral blood mononuclear cell (PBMCs) as both the silica NPs and INH loaded silica/glucan NPs stimulated the PBMCs at similar levels to control (87).

NP SYSTEMS FOR VACCINATION AGAINST *M. TUBERCULOSIS*

Various NP based vaccine candidates have been evaluated in animal models and show encouraging results. Ballester *et al.* (88) conjugated the DNA vaccine expressing antigen 85B (Ag85B) onto pluronic-stabilized sulphide NPs. The NPs alongside the immuno-stimulatory oligonucleotide CpG were delivered to mice and it was demonstrated that vaccination with NP-Ag85B via the pulmonary route substantially reduced lung bacterial burden (88). These findings therefore suggest that pulmonary immunization with NPs can serve as an effective strategy for the design of a future TB vaccines. There is a sufficient evidence to prove that in comparison to other sites, mucosal vaccination via the respiratory tract provides improved immune protection against pathogenic bacteria (89,90). However, mucosal adjuvants are challenged with respect to ability to generate robust cellular immune responses via this route which has curtailed their progression to the clinic (91). The induction of immune responses by mucosal immunization requires the co-administration of appropriate adjuvants that can initiate and support the effective collaboration between innate and adaptive immunity (92) . BCG-primed mice were shown to have an enhanced immune response following intranasal delivery of Ag85B-HBHA (heparin binding hemagglutinin adhesion protein) by carnauba wax NPs (91). BHA is a mycobacterial protein utilized by *M. tuberculosis* to achieve adherence to alveolar epithelium (77) and this property was exploited by linking to the highly immunogenic and protective Ag85B (93–95). Human clinical trials were

performed demonstrating that Ag85B and early secretory antigen target (ESAT-6) adjuvanted with IC31 could generate long-lasting Th1 cell responses (96,97). Additional work to deliver Ag85B and ESAT-6 using liposomes (CAF01) as adjuvant was performed in humans. CAF01 is a liposome composed of dimethyldioctadecyl-ammonium stabilized with a glycolipid immunomodulator trehalose 6,6-dibehenate which is a synthetic variant of cord factor located in the mycobacterial cell wall. The vaccine system has been demonstrated to be safe and efficacious in humans (98). Clinical testing of this formulation is ongoing.

To enhance the magnitude of the immune response, Yu *et al.* (99) applied Fe3O4-glutamic acid-polyethyleneimine (PEI) NPs as a delivery system to co-deliver Ag85A with ESAT-6 of *M. tuberculosis* and IL-21.The results indicated that the NP based vaccine induced a strong immune response (in comparison to administration of Ag85A-ESAT-6-IL-21 alone) and significantly reduced growth of *M. tuberculosis* in the lungs of mice. The authors attributed the enhanced immune response to improved cellular delivery and consequent transfection by the NPs (99).

One of the more common materials that have been used for vaccine delivery is PLGA (100–102). In one example, Bivas Benita *et al.* (103) loaded the TB antigen Rv1733c onto the surface of PLGA-PEI NPs. Intratracheal intubation of the NPs led to enhanced T cell responses. These NPs were able to stimulate and induce maturation of DCs (evidenced by up-regulation of surface expression of the molecules CD40, CD80, CD83 and CD86 in culture) and IFN-γ induction in mice. Liposomes have also been recognized as efficient immunoadjuvants and delivery systems (10,104). A peptide DNA-liposome conjugated vaccine was investigated by Rosada *et al.* (105). By entrapping DNA-hsp65 vaccine within cationic liposomes, these particles could elicit a strong Th1 pattern of immune response (following intranasal administration) that resulted in bacilli reduction and lung preservation in mice (105). The use of liposomes significantly reduced the amount of DNA-hsp65 vaccine required (16 fold reduction) while maintaining optimum levels of immune response. In the first decade of 2000's, Okada *et al.* reported 100% survival in non-human primate model of TB (Cynomolgus monkey) when administered with BCG plus DNA-hsp65 vaccine encapsulated in liposomes (106). However, there has been no indication of any clinical testing of this vaccine candidate.

Researchers from South Africa have advanced an NP vaccine formulation to clinical trials, i.e. a synthetic nanoemulsion adjuvant, GLA-SE (a synthetic TLR-4 agonist glucopyranosal lipid adjuvant) formulated in an oil-in-water emulsion and further adjuvanted with TB antigen ID93 (107). Based on the reproducible efficacy and enhanced Th1 response exhibited by the ID93/GLA-SE formulation, clinical trials to estimate safety and immunogenicity in humans are underway, and recent reports of the Phase I clinical trial indicate very promising results (107). The reader is also directed to Khoshnood *et al.* (2018) for a comprehensive review of novel vaccine candidates for TB currently at various stages of pre-clinical and clinical evaluation (108).

SUMMARY AND FUTURE DIRECTIONS

It is encouraging that one of the major global funders of TB biomedical research the United States National Institute of Allergy and Infectious Diseases (NIAID) recently released a research plan towards ending the TB epidemic (3,109), which also describes the need to conduct research to improve fundamental knowledge of bacterial biology and host immune mechanisms that eliminate or control the bacterium. These research activities are seen as part of the components of a toolkit for development of host-directed therapies, less toxic drug regimens, new diagnostic tools and vaccines.

To date, limited work has been performed on the application of immunotherapeutic NPs to achieve intracellular eradication of *M. tuberculosis*. Much more work has been performed to apply NPs as delivery systems and adjuvants to achieve effective vaccination, and it is exciting that at least one candidate is currently undergoing clinical evaluation (107). An immunotherapeutic NP can have the design of the IMC encapsulated within the NP core (providing opportunity for compound protection and controlled release), or incorporated within the NP shell (Fig. 3). Janus faced NPs are particularly interesting in this regard, as they enable different IMC presentation to various parts of the cells over time (80). The IMCs could also be chemically conjugated onto the surface of the NP (86) and such conjugation could be achieved through linkers to provide triggered release of the IMC, e.g. under certain pH, enzyme or redox conditions. Conjugation will enable precise insight of the amount of IMC delivered to the immune cells; which may prove beneficial in cases where fine tuning of the immune response is required to mitigate toxicity. Using NPs, combination immunotherapy has been described (80) and multiple IMCs or an IMC and an antibiotic (82) can be delivered to cells with higher efficiency compared to compound delivery in the absence of the NP.

Looking to the future, it is our opinion that studies to evaluate several other types of NPs and IMCs for intracellular TB eradication are required. Future studies should include more mechanistic and immunological assessments in order to gain understanding of NP and immune cell interactions and cell activation processes, NP elimination pathways and kinetics. Rational selection of materials for NP synthesis and IMCs will be required and some of the general NP design considerations presented in Fig. 3, could be taken into account.

The efficacy of NP immunotherapy approaches to eradicate intracellular *M. tuberculosis* within the granulomatous structure remain to be shown. Existing *in vitro* granuloma as well as

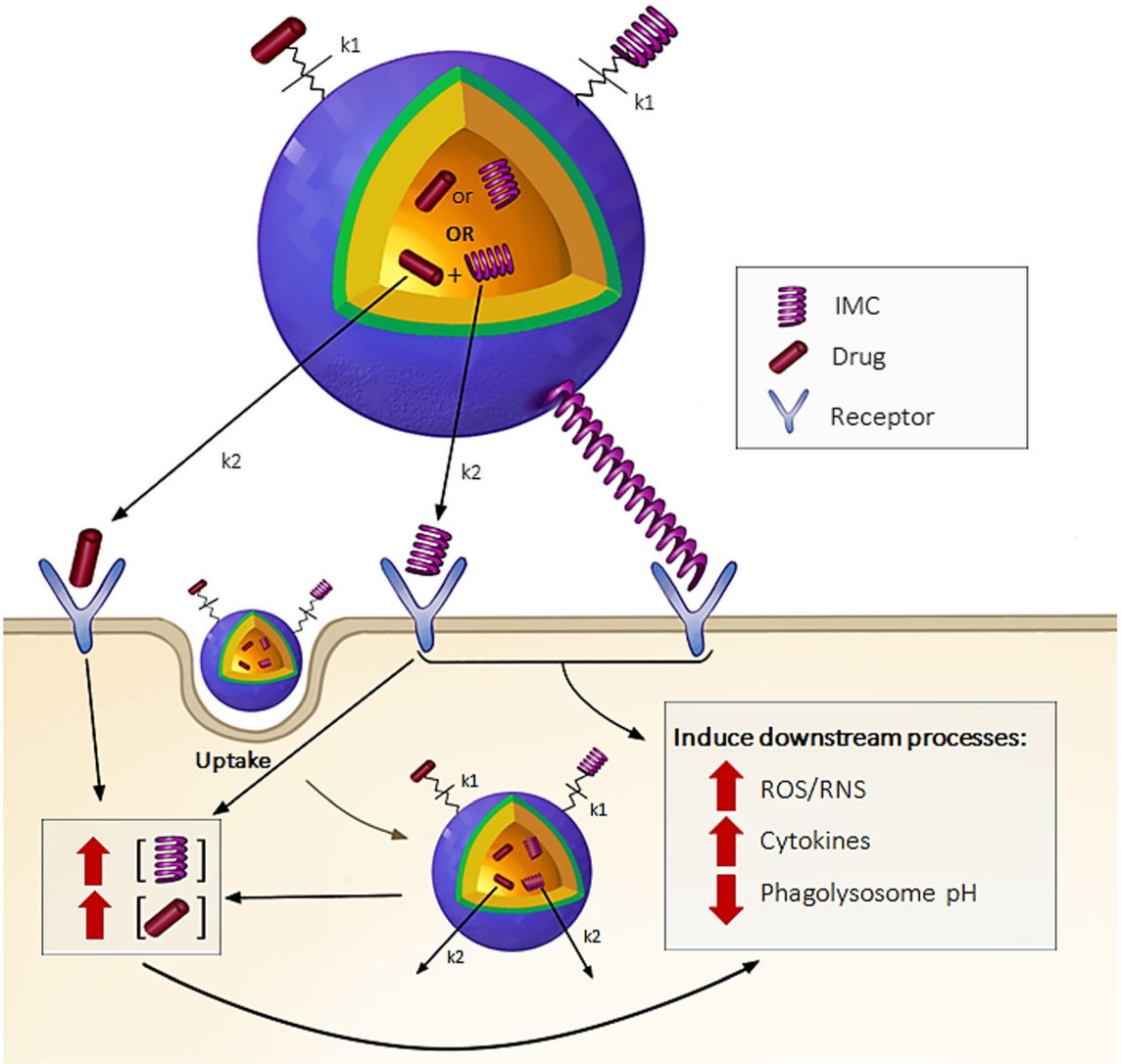

Fig. 3 Schematic representation of typical NP design for cellular targeted immune modulation for TB treatment and vaccination. The IMC may be conjugated onto the particle and remains an intrinsic part of the NP (integration of immune functionality into the NP). The IMC may also be conjugated onto the surface of the NP via linker which allows release under specific conditions. The rate (k_1) of release of the IMC can be controlled through linker selection. The IMC may in both cases bind to specific receptor located on target cell surface. The IMC may also be loaded in the core of the NP and released at a controlled rate (k_2) and bind to surface receptors or enter cell and interact with intracellular receptors or proteins.

animal models could be used to determine efficacy in this clinically relevant scenario. Typically, in immunotherapy, macrophages have been the target, however, DCs could also be targeted, as they are positioned as a link between the innate and adaptive immunity and thus can act as useful targets for both therapy and vaccination. Toxicity is a concern which should be addressed. Some authors have used combination immunotherapy to dampen the pro-inflammatory response while activating other antibacterial responses of the macrophage. This is an interesting approach which should be looked into further with the aim of striking a balance between toxicity and efficacy. TB typically occurs in the context of HIV co-infection. Hence preclinical and clinical studies to evaluate these therapies and vaccination approaches should consider the HIV co-infection scenario. For example, how immune activation may lead to immune reconstitution inflammatory syndrome.

Currently, the field of cancer immunotherapy is an area of intense exciting research offering many possibilities for disease treatment. Researchers in infectious disease immunotherapy could derive lessons from the cancer field to drive this emerging field at a faster rate towards the clinic. Indeed, from early stages, research in this field requires global partnerships of multidisciplinary teams involving at least microbiologists, immunologists, material scientists and pharmaceutical scientists. We believe that NP immunotherapies hold the key to highly effective and safe TB treatments and vaccinations. We hope that research in this field will become intensified in the near future.

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