REVIEW

For reprint orders, please contact: reprints@futuremedicine.com

Future MICROBIOLOGY

Mycobacterium tuberculosis virulence: insights and impact on vaccine development

Giovanni Delogu[1], Roberta Provvedi[2], Michela Sali[1] & Riccardo Manganelli*[,2]

ABSTRACT The existing TB vaccine, the attenuated *Mycobacterium bovis* strain BCG, is effective in protecting infants from severe forms of the disease, while its efficacy in protecting adults from pulmonary TB is poor. In the last two decades, a renewed interest in TB resulted in the development of several candidate vaccines that are now entering clinical trials. However, most of these vaccines are based on a common rationale and aim to induce a strong T-cell response against *Mycobacterium tuberculosis*. Recent advancements in the understanding of *M. tuberculosis* virulence determinants and associated pathogenic strategies are opening a new and broader view of the complex interaction between this remarkable pathogen and the human host, providing insights at molecular level that could lead to a new rationale for the design of novel antitubercular vaccines. A vaccination strategy that simultaneously targets different steps in TB pathogenesis may result in improved protection and reduced TB transmission.

KEYWORDS
• BCG • immunity
• infectious diseases
• pathogenicity
• tuberculosis • vaccine
• virulence

Among the infectious diseases affecting mankind, TB still ranks among the deadliest, posing a major threat to the health of millions people around the globe (Box 1). Southeast Asia, Western Pacific regions and sub-Saharan Africa are the regions with the highest incidence of TB, with countries such as South Africa and Swaziland showing an astonishing one new case every 100 people each year [1]. Globalization and recent trends in migration are changing the epidemiology of TB at a global level and the emergence of multidrug-resistant strains (MDR-TB), extensively resistant (XDR-TB) and totally resistant (TDR-TB) [2] is raising severe concern among health authorities. New drugs, better diagnostics and improved prophylactic measures are urgently needed to control the TB pandemic at global level. In this context, it is widely accepted that the availability of an effective vaccine against TB would provide a powerful tool to reduce TB incidence and mortality.

The more than 90-year-old bacillus Calmette–Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, is the only vaccine available for TB. BCG is effective in preventing the most severe forms of TB in early childhood, and for this reason administration of the vaccine is mandatory in TB endemic countries immediately after birth [3]. The efficacy of BCG against the adult forms of TB has been questioned by several clinical studies and since pulmonary TB in adults is the only transmissible form of the disease, BCG failed to reduce transmission and to provide the public health benefits that are classically associated with vaccination [4,5]. The lack of any immunological correlate of protection for TB and the insufficient understanding of the role and function of the *M. tuberculosis* virulence factors in the mechanism of pathogenesis are major obstacles toward the development of an improved vaccine that up to now have prevented the rational design of new prophylactic regimens. Here, we review *M. tuberculosis* pathogenesis and virulence factors,

[1]Institute of Microbiology, Università Cattolica del Sacro Cuore, Largo Francesco Vito 1, 00168, Rome, Italy
[2]Department of Molecular Medicine, University of Padova, Via Aristide Gabelli 63, 35121, Padova, Italy
*Author for correspondence: Tel.: +39 049 827 2366; Fax: +39 049 827 2355; riccardo.manganelli@unipd.it

Future Medicine part of fsg

Box 1. Tuberculosis burden in 2012.

- 1.3 million deaths
- 8.8 million new cases of active TB
- 450,000 cases of MDR-TB
- 170,000 deaths from MDR-TB

and discuss the potential using this knowledge to develop new vaccines for TB.

TB pathogenesis

TB is an airborne, transmitted disease whereby once bacilli released in small droplets by patients with active pulmonary disease are inhaled by a human subject, can reach the alveolar space **(Figure 1)**. Here, alveolar macrophages ingest and, in most cases, kill *M. tuberculosis*, preventing the establishment of TB infection. It is estimated that in 20–50% of exposed subjects, *M. tuberculosis* can resist these innate antimicrobial defenses and start multiplying in alveolar macrophages, dendritic cells, interstitial macrophages and epithelial cells. Replication during these early stages of infection is active due to the unique ability of *M. tuberculosis* to delay initiation of the adaptive immune responses [6,7]. Within a few weeks, *M. tuberculosis* can reach a high bacterial burden at the primary infection site and can disseminate through the lymphatics and bloodstream to other parts of the body, where it can infect and persist in many types of cells. Once the adaptive host immune response mounts and specific T lymphocytes are available, in 90–95% of cases, *M. tuberculosis* replication is restricted and contained without the appearance of overt signs or symptoms of the disease, leading to latent infection. In 2–8% of cases, the adaptive host immune response fails to control bacterial replication and instead promotes tissue damage, leading to the necrotic immunopathological lesions that are the hallmark of active TB disease.

In the last few years, intensive work on *M. tuberculosis* has provided a new view of the microbiological, immunological and biological features of latent infection, which is characterized by a dynamic equilibrium between the bacilli and the host [8–10], with the simultaneous presence in host tissues of dormant and actively replicating bacilli that continuously stimulate T-cell responses that in turn control bacterial replication preventing the emergence of active disease [11–13]. What is remarkable is the ability of *M. tuberculosis* to resist and evade the strong immune responses that take place during latent infection, while maintaining an active metabolism, and the ability to switch between a dormant and an active state [11,12].

Here we review the main features of *M. tuberculosis* interaction with the host that might be useful for the design of new vaccines.

• Macrophage infection

Uptake

The first step of *M. tuberculosis* infection is the invasion of alveolar macrophages after its arrival in the alveoli. Here, *M. tuberculosis* can use several specific receptors including C-type lectin receptors, GPI-anchored proteins, complement receptors and Toll-like receptors [14,15] **(Table 1)**. Entry through some receptors can be beneficial for the pathogen bypassing the proinflammatory response and thus promoting intracellular survival [15]. In particular, the complement receptor 3 (CR3) and the mannose receptor are considered the most favorable receptors for *M. tuberculosis*. A better understanding of the complex interaction between bacterial surface components and macrophage receptors can lead to the rational design of novel immunization strategies aimed at favoring the uptake of the bacteria through the less permissive pathways as Fc or scavenger receptors finally resulting in an increase of the minimal infective dose. For example, it has been shown that nonopsonic recognition by CR3 involves distinct binding sites whose targets are capsular polysaccharides [16] or specific proteins as PE_PGRS33 [Delogu G and Manganelli R, Unpublished Data] and results in different uptake pathways involving TLR2, RacI and Cdc42 or TLR2, CD14 and PI13K, which might be more favorable for the bacterium [14,15]: a humoral response against capsular polysaccharides or PE_PGRS33 could mask these receptors blocking this favorable uptake pathways. Moreover, uptake through the mannose receptor has been shown to arrest phagosome maturation and to reduce the production of nitric oxide, oxygen radicals and proinflammatory cytokines [15].

Arrest of phagosome maturation

The capacity to replicate inside macrophages is one of the most interesting features of *M. tuberculosis*. In a recent elegant work, Rohde and coworkers used transcriptional profiling to characterize the behavior of *M. tuberculosis* in response to the environmental changes encountered by the bacteria during macrophage infection, underscoring

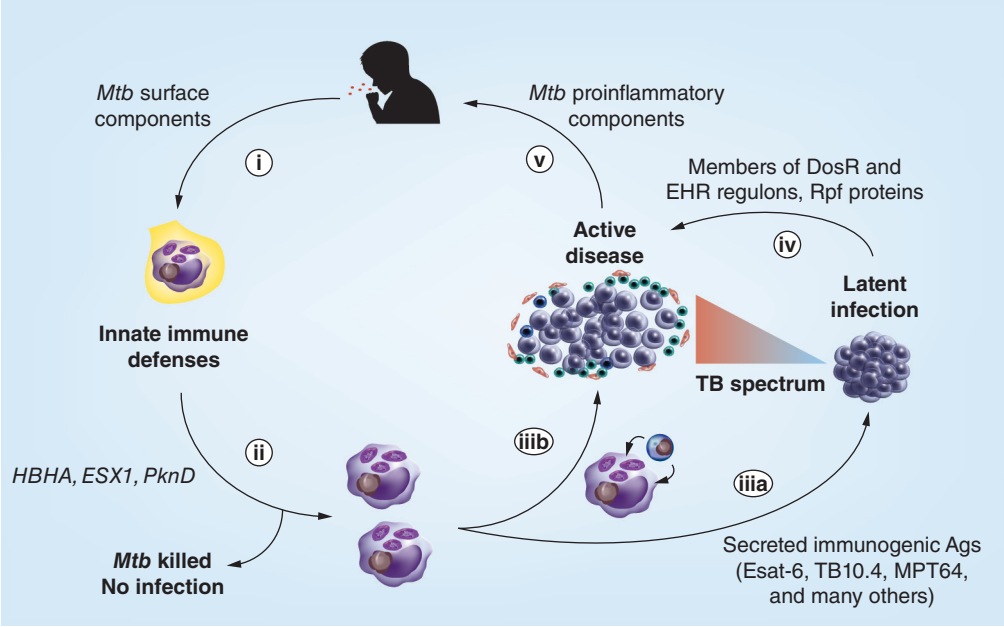

Figure 1. Schematic showing the different steps of *M. tuberculosis* mechanism of pathogenesis. **(A)** *M. tuberculosis* is an airborne pathogen and is transmitted from patients with pulmonary active tuberculosis (TB) through the emission of infected aerosol which, once inhaled by a new host, may reach the alveolar space where bacilli are ingested by alveolar macrophages; **(B)** phagocytosed bacilli can be killed without establishing an infection, or can resist innate antimicrobial defenses and start to multiply intracellularly and the spread to nearby cells; **(C)** during active bacterial replication, that occur mostly in the first 2 weeks following infection, bacilli can spread through the lymphatics and the bloodstream. Once the adaptive host immune response begins, bacterial replication is usually restrained, infection controlled and latent infection ensues **(Ci)**. However, when the adaptive immune response is ineffective in controlling bacterial replication, as it happens in many infants and immunocompromised patients, infection is not controlled by the host and primary progressive disease develops, which may involve the lung or other tissues **(Cii)**. During latent infection, which can last for life, a dynamic equilibrium between the bacilli and the host immune response is established, with no overt signs or symptoms of the disease. Recent findings suggest that *M. tuberculosis* infection encompasses a spectrum of conditions that span from complete control of bacterial replication to active disease; **(D)** in a number of subjects with latent infection (2–8%), reactivation of the disease may occur with the development of the typical signs and symptoms of active TB; **(E)** active disease is more likely to involve the lung tissue, where the extensive immunopathology can result in large cavitation and open lesions, which allow the release of the bacilli into the air.

the complex response and metabolic shift that the bacterium must enact to survive in this otherwise hostile environment [17].

The strategy used by *M. tuberculosis* to survive and replicate inside macrophages is mainly based on its ability to arrest phagosome maturation at the stage of early endosome, blocking vesicle fusion between stages controlled by Rab5 and Rab7 [18]. This allows the bacterium to limit phagosome acidification, gaining access of the nutrients present in the endosomal compartment and to avoid the consequences of delivery to the lysosome. Despite the massive amount of work that has been performed to understand the mechanism used by *M. tuberculosis* to achieve this goal, several aspects still remain obscure [19]. In recent years, a plethora of bacterial factors were shown to be involved with this phenomenon [20], clearly showing that the process resulting in the arrest of *M. tuberculosis* phagosome–lysosome fusion is multifactorial and requires several virulence factors acting at different levels [19]. For some of these, the mechanism of action is well known: protein tyrosine phosphatase PtpA is a secreted protein able to inhibit both phagosome-lysosome fusion and the recruitment of the macrophage vacuolar-H^+-ATPase (V-ATPase) machinery, responsible of

Table 1. Pattern recognition receptors implicated in *M. tuberculosis* entry in macrophages and dendritic cells.

Receptor	Target	Ref.
C-type lectin receptors		
Mannose receptor	ManLAM, PIM	[14,15]
DC-SIGN	ManLAM, PIM	[14,15]
MINCLE	TDM	[14,15]
Dectin 1	?	[14,15]
GPI-anchored proteins		[14,15]
Scavenger receptors	?	[14,15]
CD14	?	[14,15]
Complement receptors		[14,15]
CR1,3,4	Opsonized or nonopsonized mycobacteria (through TLR2 ligands) capsular polysaccarides	[14,15]
Toll-like receptors		[14,15]
TLR1	Diacylated LP	[14,15]
TLR2	Di and triacylated LP, LM, LAM, PIM, 19kDa LP, PE_PGRS33	[14,15]
TLR4	Lipomannan, HPS65	[14,15]
TLR6	Triacylated LP	[14,15]
Other		[14,15]
Fcγ	IgG-opsonized bacteria	[14,15]

phagosome acidification [21]; lipoamide dehydrogenase LpdC is able to bind coronin-1 (TACO) in the presence of cholesterol preventing its loss from the phagosomal membrane [22]; nucleotide diphosphate kinase Ndk is able to dephosphorylate both Rab7-GTP and Rab5-GTP inhibiting their function [23]; secreted acid phosphatase SapA can dephosphorylate phosphotidylinositol 3-phosphate (PI3P) present on phagosomal membranes, a molecule involved in phagosome maturation [24]; finally lipoarabinomannan (LAM) can be incorporated into membrane rafts of the macrophage cell membrane via its glycosylphosphatidylinositol anchor [25], where it is able to inhibit the increase of cytosolic Ca^{2+} concentration in macrophages, which is required for the Ca^{2+}/calmodulin PI3 kinase cascade essential for the recruitment of the Rab5 effector early endosome autoantigen (EEA1) to the phagosome and consequently for phagosome maturation [26]. Other virulence factors involved in the arrest of phagosome maturation for which the mechanism is still not completely understood are PE_PGRS62 [27], PE_PGRS30 [28], the lipoprotein signal peptidase LspA [29], the accessory secretion system SecA2 [30], the protein kinase PknG [31], the alternative sigma factor SigE [32], the secreted Zn^{2+}-dependent metalloprotease Zmp1 [33], and the two component system regulator PhoP [34]. Since a more efficient trafficking might result in better antigen presentation, *M. tuberculosis* mutant strains unable to arrest phagosome maturation are not only strongly attenuated, but might be more immunogenic and represent valid alternatives to BCG for immunization, as confirmed by the performance of some candidate vaccines, such as a *phoP* mutant, which recently entered a Phase I clinical trial [35], and a *sigE* mutant that is still in preclinical development [36]. As further developments occur, we foresee the production of *M. tuberculosis* or BCG multiple mutants in which the arrest of phagosome maturation might be more efficient and result in a better attenuation and in a more effective induction of protective immunity.

Evasion from the phagosome

The classical paradigm of *M. tuberculosis* macrophage infection is that bacilli would reside inside the phagosome for the entire course of infection. However, this paradigm was recently challenged by the finding that *M. tuberculosis* can escape from the phagosome gaining access to the cytoplasm of the infected cell inducing macrophages apoptosis and contributing to cell-to-cell spread in a process that implicates the ESX-1 type VII secretion system [37,38]. The major secretion targets of ESX-1 are EsxA (ESAT-6) and EsxB (CFP-10), two small proteins forming a heterodimer able to destabilize the phagosome membrane [39]. It is worth mentioning that the main mutation involved in *Mycobacterium bovis* BCG attenuation is the deletion of the ESX-1 locus, making this bacterium unable to escape from the phagosome and induce apoptosis *in vitro* [40]. The lack of expression of ESAT-6 (one of the main protective mycobacterial antigens) and the impossibility to gain access to the cytoplasm are considered detrimental for BCG immunogenicity. However, the discovery that the disruption of *espF* and *espG* (two genes encoded in the *esx-1* gene cluster), still results in strong attenuation without affecting ESAT-6 secretion, opening the possibility of designing more effective BCG strains [41].

Apoptosis

The role of apoptosis in *M. tuberculosis* infection has been extensively debated with sustainers of apoptosis as an effective innate-immunity defense mechanism that *M. tuberculosis* is able to partially inhibit [42] and sustainers of apoptosis as a Trojan horse induced by *M. tuberculosis* to spread and colonize the human body [43]. Recent

studies suggested that both hypotheses may be correct: *M. tuberculosis* is indeed able to induce apoptosis with an ESX-1-dependent mechanism, probably involving its access to the cytoplasm of the infected cell and the subsequent interaction between surface components such as the protein HBHA, with the surface of mitochondria [44]. This leads to the formation of *M. tuberculosis*-containing apoptotic bodies that can be engulfed by other macrophages without exposing the bacteria to the external environment thus facilitating their cell-to-cell spread [37]. However, apoptosis can also boost the induction of *M. tuberculosis*-specific immune response, so that excessive apoptosis would be detrimental for the bacterium. Consequently, *M. tuberculosis* evolved at least two antiapoptotic mechanisms enabling it to modulate this cellular process [45]. The first is based on NuoG, a subunit of the type I NADH dehydrogenase complex that is able to neutralize the reactive oxygen intermediates (ROI) produced by NOX-2 resulting in the inhibition of TNF-α-mediated host cell apoptosis. Accordingly, a NuoG mutant was shown to accumulate ROI in the phagosome and to have a proapoptotic phenotype [46], resulting in a more efficient acquisition of bacteria from dendritic cells, earlier trafficking to lymph nodes and, consequently, faster CD4 T-cell priming [47]. The second antiapoptotic mechanism is based on SecA2-dependent secretion of the superoxide dismutase SodA, another enzyme able to reduce ROI concentrations. A SecA2 mutant, unable to secrete SodA in the external milieu, was shown to have a proapoptotic phenotype resulting in an increased priming of antigen-specific $CD8^+$ T cells *in vivo* [48].

These data suggest that proapoptotic mutants might represent promising vaccines for their high immunogenicity, and indeed it was shown that deleting *secA2* in an attenuated auxotrophic mutant of *M. tuberculosis* strongly improved its protection potential when used as a live vaccine [49]. In the future, deletions of *secA2* and/or *nuoG* could be introduced in recombinant BCG strains or in attenuated *M. tuberculosis* strains to improve their protective efficacy.

• Dissemination

Dissemination from the primary site of infection to peripheral lymph nodes and bloodstream is a fundamental step in *M. tuberculosis* pathogenicity. Hematogenous dissemination results in the colonization of previously uninfected lobes of the lung and establishment of extrapulmonary disease and migration to the peripheral lymph nodes is key to the development of acquired immune response [50]. Despite their importance, the mechanisms by which tubercle bacilli can disseminate are still not well characterized and are hypothesized to include: dissemination through professional phagocytes through the migration of infected macrophages to distant secondary sites of infection [51] (although it must be noted that this has been shown using *Mycobacterium marinum* in the zebrafish embryo model); translocation through the alveolar epithelium due to the heparin-binding hemagglutinin HBHA [52]; translocation through endothelial cells, similar to the breaching of the blood–brain barrier implicated in the pathogenesis of TB meningitides [53,54]. Bacterial factors, such as HBHA and the serine-threonine protein kinase, PknD, have been shown to be involved in these processes.

Surface proteins involved in bacterial dissemination may represent promising targets for developing a vaccine that contains the infection at the site of primary infection, as suggested by Pethe *et al.* for HBHA [52] and Skerry *et al.* for PknD [53]. Using an *in vitro* model of blood–brain barrier infection, Jain *et al.* identified 33 genes strongly upregulated during *M. tuberculosis* blood–brain barrier infection [54]. Several of the proteins encoded by these genes are hypothesized to be surface exposed (including 3 PE_PGRS and one PPE), making them promising candidates for vaccine development aimed at limiting pathogen dissemination during infection.

Latency & reactivation

One of the most interesting features of *M. tuberculosis* is its ability to cause latent infection, during which infected individuals have no symptoms and cannot transmit the disease, but maintain a 2–8% probability of developing active disease in their lifetime due to reactivation of the infection even decades after primary infection. The lack of a simple and widely accepted animal model for latent TB has prevented our complete understanding of the mechanism *M. tuberculosis* uses to cause latent infection and characterization of the bacterium physiological state in latently infected individuals. Two main nonmutually exclusive points of view are debated in the field: first, bacteria enter a dormant state to adapt to the environmental stresses to which they are

subjected, while in the second they continue to replicate, reaching an equilibrium with the bactericidal effects of the immune system [11]. Also the nature of the environmental stresses inducing the development of the dormant state has been (and still is) the object of intense debate with the main candidates being inhibition of respiration by nitric oxide or hypoxia, carbon dioxide produced by activated macrophages, or nutritional stress [11,55,56]. The recent dynamic hypothesis of TB infection predicts that most bacteria during latent infection are in a dormant state, but that at different times scouts reactivate to sense the immune status of the host eventually progressing to active disease or forming new infection foci that the immune system will eventually control with the development of new granulomas [9,57]. The physiological shift leading to the development of nonreplicative persistent bacteria is hypothesized to result in the expression of a modified antigen repertoire, as confirmed by human studies showing that the tubercular antigens recognized by healthy individuals with latent TB are different from those recognized by active TB patients [58,59]. This of course has a very important impact in the rational design of a vaccine and antigens hypothesized to be involved in bacterial adaptation and survival during long-term bacteriostasis are considered potentially interesting candidates for the development of an immune response targeting nonreplicating cells. The main proteins hypothesized to be involved in this process are the members of the DosR regulon, a group of 48 proteins whose structural genes respond to inhibition of aerobic respiration and carbon monoxide that would help *M. tuberculosis* to the initial adaptation to hypoxia-induced bacteriostasis [55], and the members of the enduring hypoxic response (EHR) regulon [60], consisting of 230 proteins whose structural genes are induced up to 7 days after exposure to hypoxic environment hypothesized to be involved in bacterial adaptation and survival during long-term bacteriostasis. Consequently, members of the DosR and EHR regulons have been considered as promising antigens to be included in a vaccine [61]. It is worth noting that, even if the DosR regulon is present in BCG, vaccination with this live vaccine does not induce an immune response against the members of its regulon [61].

Finally, dormant bacteria must wake up from dormancy. *M. tuberculosis* genome encodes five resuscitation promoting factors (Rpf), homologous to the well characterized Rpf of *Micrococcus luteus*. Their importance in *M. tuberculosis* resuscitation from a 'nonculturable' state induced by starvation under anoxia and in survival during the persistent phase in mice infection was demonstrated in mutant strains in which multiple *rpf* genes were deleted [62]. Given their role in mycobacterial physiology, these proteins have been hypothesized to represent important antigen targets for an immune response aimed at controlling bacterial reactivation [61]. Taken together these data indicate that the antigen repertoire expressed by *M. tuberculosis* changes over time and space. Beyond the antigens preferentially expressed during active growth, the other set of antigens are those included in the DosR and EHR [55,60], those induced by starvation [63] and those induced during reactivation [64,65]. This antigen variability during infection poses a serious problem in the design of a vaccine able to target the bacteria during the whole course of infection [66,67].

The search for new vaccines against TB

In the last two decades, a renewed interest in TB by the scientific community spurred research aimed at developing a new and improved preventive or therapeutic vaccine and several new experimental candidates, generated using different technological platforms and expressing various antigens, have been tested in preclinical animal models and more than 15 have entered or completed clinical trials [68–72]. **Tables 2 & 3** show a list of some of the new TB vaccines under development.

Ideally, a vaccine superior to BCG will provide enhanced protection from *M. tuberculosis* infection and/or from the development of active TB in the newly infected or reduce the risk of TB reactivation. Most of the new vaccines that are being developed aim to elicit a strong and sustained T-cell response against one or more *M. tuberculosis* antigens, resulting in a rapid mobilization of T lymphocytes immediately after *M. tuberculosis* infection which, by containing bacterial replication and tissue damage, will prevent either infection or disease development [66]. Among the options that are being pursued to make a more effective TB vaccine, is a vaccination strategy based upon priming with BCG at birth followed by a boosting with a subunit or vectored vaccine [3]. The rationale for this approach stems from the hypothesis that immunity induced by BCG wanes over time and that boosting with

Table 2. List of some of the new bacterial-based tuberculosis live vaccine candidates under development.

Type of vaccine	Name	Description	Ref.†
Live attenuated *Mtb*	MTBVAC	Double mutant where the gene encoding the global regulator *phoP* and the phthiocerol dimycocerosates (DIMs)-biosynthetic gene *fadD26* were deleted. Safe and protective in preclinical models, currently undergoing clinical trials	[35]
	Mtb Δ*sigE*	Mutant where the gene for the alternative sigma factor SigE was deleted; it showed improved protection over BCG in mice challenged with a clinical strain	[36]
	Mtb Δ*secA2*	Mutant where the *secA2* gene, encoding a component of a virulence-associated secretion system, was deleted	[49]
Recombinant BCG	VPM 1002	rBCG Δ*ureC*::*hly*, expressing listeriolysin from *L. monocytogenes* and mutant for the urease gene so to promote phagosome lysis and warrant improved antigen processing and presentation; enhanced protection in preclinical animal model and currently in clinical trials	[73]
	rBCG PE MPT64	rBCG overexpressing MPT64 on its surface resulting in enhanced protection in mice; effective in homologous prime-boost immunization strategies	[74]
Recombinant NTM	IKEPLUS	*M. smegmatis* Δ*esx-3* complemented with *Mtb esx-3*; elicited protective bactericidal immunity against *Mtb* in mice	[75]

†One relevant reference for each experimental vaccine was selected.
BCG: Bacillus Calmette–Guérin; NTM: Nontuberculous mycobacteria.

another vaccine capable of inducing strong T-cell responses directed against immunodominant antigens of *M. tuberculosis* would maintain levels of T-cell immunity high enough to prevent TB progression or TB reactivation [3,83]. In line with this approach, it has been proposed that immunization of adolescents and adults may provide beneficial effects in terms of protection and help meet the WHO 2050 goals [71,84]. In this context, research in TB vaccine development has two major goals: to develop a new and improved live attenuated vaccine capable of substituting BCG, either by generating a *M. tuberculosis* mutant or a recombinant BCG strain; and to develop subunit or vectored vaccines expressing one or more *M. tuberculosis* antigens to be administered in one or more boosting doses.

• Live attenuated vaccines

M. tuberculosis attenuated strains

BCG lacks many genes compared with *M. tuberculosis*, some of which encode for highly immunogenic antigens, such as those found in the RD1 region containing the genes coding the ESX-1 secretion system [85,86], or those found in the RD2 region that has been lost during the propagation of BCG between 1921 and 1927 [86] and that includes important antigens as MPT64 and Rv1986 [87,88]. For this reason live-attenuated *M. tuberculosis* strains were seen as valid candidates for the development of an improved vaccine, although they must fulfill stricter safety rules than other vaccines to obtain approval [89] and, in absence of any dramatic improvement, BCG remains the gold standard.

The most promising *M. tuberculosis* attenuated vaccines include several auxotrophic strains that were shown to be highly attenuated and still capable of inducing levels of protective immunity similar to BCG (reviewed in [90]), and strains deleted in specific virulence-related genes resulting in attenuated strains with enhanced immunogenicity and improved protection. Among them a Δ*secA2* mutant was shown to induce apoptosis in infected macrophages as a result of the diminished amount of secreted mycobacterial superoxide dismutase, which warranted enhanced antigen priming compared with BCG and superior protective activity [48], a mutant strain lacking the alternative sigma factor SigE [36] and a *phoP* mutant with changes in the mycobacterial surface composition, showing enhanced protection and safety compared with BCG [91]. This *M. tuberculosis phoP* mutant has been further engineered to delete another gene essential for virulence (*fadD26*), so to strengthen its safety profile and meet the Geneva consensus on the use of live *M. tuberculosis* attenuated vaccines [92] and the new strain (MTBVAC01) is being evaluated in clinical trials [35].

Recombinant BCG

The identification of molecular determinants of pathogenesis in *M. tuberculosis* has been exploited to genetically engineer the current vaccine BCG and improve its protective potential. Overexpression of the immunodominant antigen Ag85B and reintroduction of the RD1 in BCG led to vaccines with superior protective activity than parental BCG [93]. Overexpression

of an immunodominant antigen such as MPT64 on the mycobacterial surface and in association with the highly immunogenic mycobacterial cell wall components resulted in improved protection over the parental BCG strain and other recombinant BCGs expressing the very same antigen in other cellular compartments (secreted, cytosol, inner membrane), indicating that the host immune response against selected antigens can be manipulated by forcing antigen localization in BCG [74]. VPM1002 is the most advanced BCG strain under development and has been engineered so to express lysteriolisin O from *Lysteria monocytogenes* and where the gene coding for a urease (*ureC*) has been deleted [94], resulting in its ability to escape from the phagosome. The VPM1002 strain was able to stimulate broader and different T-cell populations compared with the parental strain that resulted in superior protection [95] and was shown to be safe in clinical trials [73]. The undergoing clinical studies to assess immunogenicity and protection will be very important to assess in humans whether a vaccine superior to BCG can be obtained. The AERAS-422, a recombinant BCG vaccine designed similarly to VPM1002 [96], was stopped in phase I clinical studies because of serious adverse effects observed in two participants, highlighting the potential risks associated with vaccination with a recombinant BCG vaccine [97].

The possibility to generate recombinant nontuberculous mycobacteria has also been exploited to develop new live mycobacterial strains with vaccine potential against TB. Remarkably, intravenous inoculation with *M. smegmatis* caused early death of mice unless the *esx-3* region was deleted [75]. This region encodes for a type VII secretion system involved in iron and zinc uptake essential in *M. tuberculosis* but not in *M. smegmatis* [98,99]. Complementation of the *M. smegmatis* Δ*esx-3* with a cosmid encoding the *M. tuberculosis esx-3* region resulted in a strain that remained susceptible to innate immune killing but was capable of stimulating a bactericidal immunity against *M. tuberculosis*, indicating the possibility to develop novel sterilizing vaccines [75].

• Subunit & vectored vaccines

The immunological characterization of the host immune response against *M. tuberculosis* led to the identification of a panel of antigens that could possibly be used as a candidate antigens for the development of a subunit vaccine [100]. The lack of evidence of the potential role of antibodies in exerting a protective activity against TB, paired with the pivotal role of T-cell immunity during *M. tuberculosis* infection, has led to the development of immunization strategies aimed at eliciting T-cell responses against *M. tuberculosis* antigens and primarily those that are actively secreted, regardless of their role and function in *M. tuberculosis* biology and TB pathogenesis [85]. Among the most promising candidate antigens are those secreted through the ESX-1 secretion system, such as ESAT-6 and CFP-10 [85], other highly immunogenic and secreted proteins as MPT64 and MPT83 and other secreted or cell bound antigens such as Ag85A/B, HSP65, PPE18 [83,85,101]. An active field of study aims at identifying antigens expressed in the different steps of the infectious process and specifically during the latent infection or associated with dormancy, so to generate vaccines able to elicit T-cell responses that would prevent reactivation [85].

Once a candidate antigen is selected to be included in a vaccine, three types of options are usually pursued: protein adjuvanted vaccines, DNA vaccines and live vectored recombinant vaccines.

• Prime-boosting & the BCG MVA strategy

Immunization strategies aimed at eliciting T-cell responses by priming with BCG, followed by a boost with subunit or vectored vaccines, were designed and tested with promising results in animal models. Boosting of BCG immunized mice with the MVAAg85A was associated with the induction of Ag85A-specific T-cell responses and improved protection compared with mice immunized with BCG only [102,103]. Administration of two doses of a DNA vaccine expressing the Mtb72F polyprotein to BCG-immunized guinea pigs resulted in reduced lung lesions and extended median survival time compared with animals only vaccinated with BCG [104]. Boosting with a polyvalent DNA vaccine expressing ESAT-6 and Ag85B was found to enhance the protective activity in BCG immunized mice 1 year after priming with BCG [105] and intranasal administration of an adjuvanted protein subunit vaccine made of Ag85B and ESAT-6 enhanced anti-TB immunity in BCG primed mice [106], further supporting the potential usefulness of boosting strategies for TB. Enhanced protection of the boosting strategies against TB were also demonstrated in

Table 3. List of some of the new acellular vaccine candidates under development.

Type of vaccine	Name	Description	Ref.[†]
Viral vectored	MVA85A	Vaccinia virus Ankara expressing Ag85A; protection in animal models when administered alone or in boosting immunization strategies; completed Phase 2b trial with no improved protection against *M. tuberculosis* infection or development of TB disease in children	[76]
	AdAg85A	Replication-deficient adenovirus expressing Ag85A, enhanced protection in animal models	[77]
	AERAS-402	Replication-deficient adenovirus expressing Ag85A, Ag85B and TB10.4, enhanced protection in animal models	[78]
Protein subunit	H1, H56, H4	Combination of different fusion proteins (Ag85B, Esat-6, TB10.4, Rv2660c) administered with cationic liposomes as adjuvant; enhanced protection in animal models, safe and immunogenic in humans and undergoing clinical trials; used in prophylactic and postexposure immunization strategies	[79]
	Mtb72F	Fusion protein PepA-PPE18, long-term protection in nonhuman primates	[80]
	ID93	Fusion protein Rv2608-Rv3619-Rv3620-Rv1813; increased protection in BCG-vaccinated guinea pigs after boosting	[81]
	HBHA	Mycobacterial Heparin-Binding Hemagglutinin, good protection in mice also in boosting BCG immunized animals	[82]
Therapeutic vaccines	RUTI	Liposomes containing detoxified fragmented *Mtb* cells; used as immunotherapy to reduces treatment time; effective in animal models	[72]
	M. indicus pranii		[72]
	M. vaccae	Killed *M. vaccae*; improve the efficacy of chemotherapy	[72]

[†]One relevant reference for each was selected.
BCG: Bacillus Calmette–Guérin.

BCG immunized cattle [77] and in a nonhuman primate model of TB [107]. The protective activity of prime-boost strategies have also been tested using the improved recombinant BCG strains [108] and recent data in the mouse model indicate that the implementation of homologous prime-boost strategies involving the use of an improved recombinant BCG overexpressing a candidate antigen on the surface [74] and a boosting with a live bacterial delivery system expressing the same *M. tuberculosis* antigen may further enhance anti-TB immunity [109].

The promising results obtained in preclinical animal models and the feasibility of this vaccination approach in humans prompted testing in clinical trials of one of the most promising vaccine against TB. Boosting with MVA-Ag85A was shown to be immunogenic and safe in children and adolescents [110], and a double-blind, randomized, placebo-controlled, Phase IIb trial was performed in a high burden TB country as South Africa [76].

While boosting with MVAAg5A elicited specific CD4-positive T-cell responses, predominantly expressing IFN-γ, TNF-α and IL-2, no enhanced efficacy against TB or *M. tuberculosis* infection was observed in children that received BCG and MVAAg85A compared with those that received BCG and placebo [76]. The results from this landmark clinical study were of great relevance, and highlighted the complexity and the problems associated with developing and testing a new vaccine against TB in humans [66,111,112].

• Vaccines aimed at eliciting T-cell responses may be insufficient in providing protective immunity

The lack of any significant improvement in protection of the BCG-MVAAg85A strategy over BCG in children was important for the TB community, and while the results of other current clinical trials will provide a better assessment of the true validity of the new immunization strategies, a number of reviews and comments have attempted to discuss the implications of these results for the development of a new vaccine against TB [111–114].

The efficacy end point of the trial was to assess incidence TB, based on microbiological, radiological and clinical criteria, in children immunized at birth with BCG and boosted with MVA-Ag85A or placebo and followed up for 37 months [76]. No differences in TB disease incidence were observed in the two groups, indicating that the Ag85A-specific CD4 T cells elicited following boosting did not prevent infection or development of TB disease in infected children. It remains to be seen whether the lack

of significant improved protection is the results of the modest or insufficient immunogenicity induced by the MVAAg85A vaccine in this population or the apparent inability to elicit CD8 T-cell responses, which are known to play an important role in controlling *M. tuberculosis* [115]. However, recent results obtained in the guinea pig model indicate that BCG is highly protective against *M. tuberculosis* clinical strains isolated in South Africa, raising the possibility that the activity elicited by BCG cannot be easily boosted [116]. The lack of enhanced protection in humans of a vaccination strategy that provided consistent improved protection in animal models questions the assumptions that have been so far used to select the new vaccine candidates [76,111,116]. The results that will emerge from the other ongoing clinical trials will be very important to truly understand the significance and reliability of the preclinical animal studies for TB.

The inability to reduce *M. tuberculosis* infection in children by the MVAAg85A boosting was seen as unsurprising since BCG itself is regarded as poorly effective in preventing *M. tuberculosis* infection [76]. This assumption implies that T-cell responses against antigens that are expressed in the early stages of infection, as is the case of Ag85A [117], cannot exert any antimycobacterial activity able to kill bacilli and prevent establishment of the infection, which may develop as latent TB infection (LTBI) or progress, especially in young children, in active disease. In support of this hypothesis are the results obtained in animal models, where none of the vaccines so far evaluated were able to induce sterilizing immunity and prevent *M. tuberculosis* infection. However, a number of studies have demonstrated the possibility of transient infections in humans, observed in household contacts that convert to an IFNγ gamma release assay and that later revert to negative [118,119], indicating the possibility that the human immune response can, under certain yet undefined circumstances, eradicate the bacilli. A better understanding of the early events occurring immediately after infection in humans and the establishment of animal models that can mimic human infection are urgently needed to design vaccination strategies capable of exerting anti-infective activities [66,120].

It is well established that T-cells are required to control *M. tuberculosis* replication *in vivo* and the essential role of IFNγ in orchestrating the host immune response has been demonstrated by several studies [121,122]. Nevertheless, T-cell responses are also involved in the immunopathology and tissue damage that are the hallmarks of active TB [123]. More recent data have shown that human T-cell epitopes in *M. tuberculosis* are more conserved than essential genes, suggesting that the immune responses elicited by these T-cell epitopes, while essential for the survival of the human host, might also be beneficial for the bacteria [124]. The highly immunogenic antigens belonging to the PE and PPE protein families and those comprising and secreted by the *M. tuberculosis* secretory systems are the main targets of the T-cell responses during TB infection [85,125]. In recent years, data from genomic analysis of large collections of *M. tuberculosis* strains paired with *in silico* and phenotypic immunological characterization of human T-cell responses [125,126] are providing a new view of the *M. tuberculosis*–host interaction throughout the evolution, suggesting that *M. tuberculosis* T-cell epitopes greatly contributed to the success of *M. tuberculosis* as human pathogen [124,127] and, as such, these can be considered as noncanonical *M. tuberculosis* virulence factors. For all these reasons, it has been hypothesized that vaccine-induced immunity against immunodominant *M. tuberculosis* antigens may perversely contribute to increase transmission [124]. Hence, while the efficacy of the boosting strategy against TB awaits proper evaluation [112], some concerns of the short- and long-term consequences of this vaccination strategy shall be properly taken into account.

• Targeting the different steps in TB pathogenesis

Since most of the new experimental vaccines in advanced stage of evaluation share the same immunological rationale, there is the need to identify and pursue other avenues that will require, among other things, out-of-the-box approaches and creativity in research [128]. The characterization of the *M. tuberculosis* molecular determinants of virulence and the dissection of their role in the complex mechanism of pathogenesis could open new and unanticipated ways to target key events and related bacterial components through immunization strategies.

Considering the different steps in TB pathogenesis summarized in **Figure 1** and the *M. tuberculosis* determinants involved in these stages, it is possible to envision the design of immunization

strategies specifically targeting these steps, as summarized in **Table 4**. These strategies must rely on the coherent use of specific *M. tuberculosis* proteins and molecular determinants, selected not only because of their immunogenicity but also for their involvement in key events of TB pathogenesis and vaccine delivery systems so to elicit an immune response that contributes to the control of TB infection and/or prevent the development of TB disease or TB infection itself.

Preventing infection immediately after *M. tuberculosis* oasis:entry in the alveolar space, by precluding or reducing the efficiency of its ability to gain access and survive in alveolar macrophages and nearby cells, may result in an improved ability of the host to clear infection. In this context, antibodies targeting surface constituents that are involved in the early events following infection, either promoting entrance into macrophages or by harnessing or subverting host immune responses, may provide level of protection by interfering with these processes. Enhanced *M. tuberculosis* killing was observed following phagocytosis of opsonized bacilli [129,130], indicating the potential role of antibodies in clearing infection. Recent findings, obtained in wild boars immunized with heat inactivated BCG, suggest a role for antibodies in inducing protection against *M. bovis* infection by promoting, in association with the complement component C3, increased opsonophagocytosis that by interfering with CR3-mediated opsonic and nonopsonic phagocytosis of mycobacteria, can promote bacterial clearance [131]. A better characterization of the *M. tuberculosis* surface constituents, with emphasis on proteins involved in the interactions with host cell receptors such as for instance CR3 and TLR2 [132], is needed to design and implement tailored immunization strategies aimed at eliciting antibodies against surface constituents. A recent study demonstrated that immunization with membrane vesicles (MV) from *M. tuberculosis* containing proteins, polysaccharides and lipids that are capable of subverting host immune responses in a TLR2-dependent manner [133], induced humoral and cellular responses directed to membrane and cell wall components that warranted a level of protective activity comparable to that elicited by BCG [134]. Co-administration of MV with live BCG or boosting of BCG immunized mice, enhanced the levels of protective activity against *M. tuberculosis* [134]. Interestingly, MV from *M. tuberculosis* were shown to be more effective than MV obtained from BCG, and this correlated with a differential specificity and enhanced avidity of antibodies against cell wall components in mice immunized with *M. tuberculosis* MV [134]. The antibodies elicited by such vaccines may contribute to clear *M. tuberculosis* since the early steps following infection, by interfering with *M. tuberculosis* oasis:entry and persistence in macrophages and other cells, thereby reducing bacillary load, thus increasing the possibility that the emerging adaptive cellular response could clear infection. Indeed, studies showing that among household contacts conversion to IFN-γ release assay may be followed by reversion after several weeks, led to the concept of acute resolving infection, whereby the emerging *M. tuberculosis*-specific T-cell response may be responsible for the bacilli clearance, an outcome inversely correlated with the dose of infection [118,119]. These experimental evidences highlight the potential that the humoral response against TB may provide level of anti-infective immunity. However, beside the poor characterization of the *M. tuberculosis* surface constituents that may serve as targets of an antibody-based immune response, there are a number of issues that needs to be properly taken into account when designing an anti-infective vaccination strategy against TB [120].

Targeting the dissemination from the site of primary infection is another opportunity for vaccination strategies against TB. Antibodies directed against HBHA, one of the best characterized *M. tuberculosis* surface proteins, were shown to reduce bacterial dissemination from the site of primary infection in a murine model of infection [52] and systemic and local immune responses elicited by an HBHA-based mucosal vaccine impaired extrapulmonary dissemination of BCG [135]. HBHA-based vaccines were shown to elicit significant level of protection in mice when administered alone or following BCG priming [82,136]. Immunization with the extracellular exposed domain of PknD resulted in a specific reduction of bacillary load in the brain of guinea pigs, and antibodies directed against this *M. tuberculosis* surface protein were able to inhibit bacterial invasion in a human blood–brain barrier model of infection, providing experimental evidences for the potential use of vaccines that specifically target dissemination [53].

Most of the vaccines currently under development aim to elicit an immune response that

dampens bacterial replication, contains extension of the lesions and prevent emergence of primary TB disease. These prophylactic vaccines have been designated to elicit T-cell responses against *M. tuberculosis* antigens to induce antimicrobial activity primarily against intracellular bacteria [56].

Another strategy is to target the population of latently-infected subjects so to elicit an immune response aimed at preventing TB reactivation [68,114]. This latter strategy is been actively pursued and relies on the use of subunit vaccines containing latency-associated and secreted and immunogenic antigens as ESAT-6. Promising results were obtained in preclinical animal models [137,138] and some of these postexposure vaccines, have entered clinical trials and it would be of interest to assess their safety and activity in target populations.

A different potential option is to design immunization strategies that may have an impact on TB disease and transmission by reducing cavitation and tissue damage in the lung [139]. It has been hypothesized that antibodies may promote clearance or neutralize the activity of proteins or nonproteinaceous components with immunomodulatory properties, and in doing so may impact disease outcome by limiting tissue damage and cavitation which in turn may reduce transmission [140]. A monoclonal antibody directed against arabinomannan was capable of protecting mice despite lacking bactericidal activity or any ability to inhibit infection or bacterial replication and protection was associated with reduced immunopathology in the lung [141]. The characterization of the *M. tuberculosis* proteome *in vivo* provided a detailed list of the proteins deployed by the tubercle bacilli in necrotic and caseous lesions [142], which showed an unanticipated accumulation, among the most abundant proteins, of PE_PGRS proteins, which may promote inflammatory responses and induce tissue damage [143]. The functional and immunological characterization of the most abundant proteins detected in the caseous lesions may lead to the identification of new antigens that could serve as targets of antibodies aimed at neutralizing the proinflammatory responses. Moreover, the hypothesis that *M. tuberculosis* may persist mainly in the extracellular environment offers the opportunity for antibodies to interfere with surface components that promote necrosis, and therefore disease and transmission [56]. Further studies are needed to assess the role of the humoral response in TB pathogenesis and there are some critical research questions that need to be carefully addressed to define the most appropriate experimental framework able to assess and measure the protective activity of vaccines that elicit antibodies [66]. These include: a better molecular, structural and functional characterization of the *M. tuberculosis* surface constituents; the use, in proof-of-concepts *in vitro* and *in vivo* experiments, of *M. tuberculosis* with preserved surface structure resembling that of bacilli released by a patient with active pulmonary TB rather than bacteria cultured in axenic media and eventually in the presence of detergents; and a new model to assess protective activity that measure infection rather than extent of the disease following infection [66].

Conclusion & future perspective

Among the main obstacles in developing new and effective vaccines against TB are the lack of an understanding of the nature of the protective immunity against infection with *M. tuberculosis* and development of disease in those already infected. The complex mechanism of pathogenesis of the tubercle bacilli, the many and multifactorial virulence factors involved in the different steps of the infectious process, the ability of the infectious agent to resist innate and adaptive immune responses, including the fact that *M. tuberculosis* may have co-evolved with humans so to take advantage from the robust T-cell responses to warrant its own transmission, have so far prevented the rationale design of a vaccine against TB. A better characterization of the molecular determinants of pathogenesis, with a special focus on *M. tuberculosis* surface constituents is needed to identify potential targets of a protective immune response.

Among the many types of vaccines developed and tested, several were able to induce, in preclinical animal models, levels of protection similar to those elicited by BCG, although none was capable of providing sterilizing immunity. Regardless of the different types of vaccines to be administered, it would be important to tailor their antigenic composition and immunogenicity so to target specific events in the TB pathogenetic process and generate a multicomponent immunization strategy that can control *M. tuberculosis* infection and development of disease. In this context, one can envision an immunization strategy relying on the

Table 4. Strategy to design vaccines against different steps of tuberculosis pathogenesis.

Step in TB pathogenesis[†]	Aim	Type of immunity sought for	Type of vaccine(s)	Live vaccines for priming	*Mtb* candidate antigen(s) for boosting
1	To prevent infection of *Mtb* immediately after oasis:entry	Mucosal and systemic neutralizing antibodies; T-cell responses against antigens expressed and presented in the early events following infection	Prophylactic and potentially anti-infective vaccines to be administered in infants or uninfected subjects		*Mtb* surface components involved in direct interaction with host components (PE_PGRS, secreted antigens)
2	To limit *Mtb* dissemination from the site of primary infection and reduce the risk of TB reactivation in the secondary sites (such as in the upper lung)	Mucosal and systemic neutralizing antibodies that prevent effective adhesion and invasion of phagocytic and nonphagocytic cells; T-cell responses against antigens expressed during dissemination	Prophylactic vaccines to be administered in infants or uninfected subjects	BCG or attenuated *Mtb* strains further mutated in *secA2* or *nod2*;	HBHA, ESX-1, PknD
3	To dampen bacterial replication following infection to prevent primary TB disease	T-cell responses directed against *Mtb* antigens to exert antimicrobial activity against intracellular bacilli	Prophylactic vaccines to be administered in infants or uninfected subjects.	BCG or *Mtb* mutants with altered cellular trafficking;	Secreted immunogenic antigens (ESAT-6, TB10.4, MPT64, Ag85A/B, PPE18, α-crystallin, etc.)
4	To prevent TB reactivation in LTBI subjects	T-cell responses and neutralizing antibody against components involved in reactivation	Postexposure vaccines	BCG or attenuated *Mtb* mutants expressing *Mtb* antigens on their surface	Members of the DosR and EHR regulons, Rpf proteins
5	To reduce cavitation and tissue damage in the lung	T-cell responses with anti-inflammatory properties; mucosal and systemic neutralizing antibodies to prevent the action of proinflammatory *Mtb* components	Prophylactic vaccines to be administered in infants or uninfected subjects and post exposure vaccines		*Mtb* proinflammatory components (PE_PGRSs, TDM, LAM, α-glucan)

[†]Numbers refer to the steps in TB pathogenesis as indicated in **Figure 1.**

use of an improved live attenuated vaccine and a boosting with a vectored or subunit vaccine that may vary in antigen composition and immunogenicity depending on the vaccine's status (age, immunological status, TB infection status, etc.) so to strengthen different aspects of the anti-TB immunity (such as anti-infection, containment of infection, anti-inflammatory).

To develop such a vaccination strategy several key issues need to be adequately addressed and the technological advancements, expertise and level of scientific and political commitment at a global level that has been gained in the last two decades may provide the proper environment to tackle these main challenges in the near future, which include:

- The characterization of the *M. tuberculosis* molecular determinants of pathogenesis and the dissection of their role in the different steps of the disease, which will be useful to identify potential targets for a vaccination strategy;
- The identification of the immunological correlates of protection and risk. The many clinical trials that are ongoing or have been completed in several parts of the world are allowing the fine characterization of the host immune response against BCG or other new vaccines. The results obtained with the most advanced technologies directly on human clinical samples will provide a new understanding of the host immunity against *M. tuberculosis*, which could greatly contribute to rationally design the new vaccines;
- The identification and characterization using highly sensitive proteomic approaches of the

main antigens and host factors directly involved in the host tissues where disease ensues, will help identify the most promising antigen candidates and type of host cell responses that a vaccine shall elicit;

- The development of reliable animal models so it is possible to investigate: the early steps of the infectious process and assess the potential of anti-infective vaccines; the biology of latent infection; and the reactivation process with the typical cavitation that mimic transmission.

Financial & competing interests disclosure

G Delogu and R Manganelli are supported by the Italian Ministry of Health (MIUR 2008Y8RZTF). R Manganelli is also supported by the Italian by the European Community Seventh Framework Programme (FP7/2007–2013) under grant agreements 241745 and 260872. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

EXECUTIVE SUMMARY

Aspects of *Mycobacterium tuberculosis* virulence

- *M. tuberculosis* virulence is a multifactorial and very complex phenotype.
- Several aspects of *M. tuberculosis* pathogenicity could be targeted by novel vaccines as infection (primarily macrophage invasion), dissemination from the primary site of infection, control of bacterial replication and emergence of immunopathology and reactivation.
- The antigenic profile of *M. tuberculosis* changes during the different phases of infection.

Development of new vaccines against tuberculosis

- The development of an effective vaccine against tuberculosis (TB) is a major goal to improve global health.
- The strategy traditionally used to design TB vaccines, based on stimulation of T-cell response may be insufficient in providing protective immunity.
- A new efficient vaccine should target different steps in TB pathogenesis and should also rely on the development of a humoral and/or mucosal immunity targeting bacterial molecules involved in macrophage invasion and immune subversion.
- The major goals of a new vaccine, together with conferring a better and longer T-cell response against dividing and dormant *M. tuberculosis* cells might be those: to increase the minimal infectious dose, by interfering with bacteria uptake in phagocytic cells forcing the bacteria to enter from less favorable pathways; to reduce dissemination from the primary infectious site; to interfere with the inflammatory process and cavitation to reduce transmission; and to interfere with reactivation of dormant cells.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 WHO. Global tuberculosis report 2013. www.who.int

2 Muller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Trends Genet.* 29(3), 160–169 (2013).

3 Andersen P, Doherty TM. The success and failure of BCG – implications for a novel tuberculosis vaccine. *Nat. Rev. Microbiol.* 3(8), 656–662 (2005).

4 Younga D, Verreck FA. Creativity in tuberculosis research and discovery. *Tuberculosis* 92(Suppl. 1), S14–S16 (2012).

5 Kaufmann SH. Future vaccination strategies against tuberculosis: thinking outside the box. *Immunity* 33(4), 567–577 (2010).

6 Wolf AJ, Desvignes L, Linas B *et al.* Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. *J. Exp. Med.* 205(1), 105–115 (2008).

7 Gallegos AM, Pamer EG, Glickman MS. Delayed protection by ESAT-6-specific effector CD4$^+$ T cells after airborne *M. tuberculosis* infection. *J. Exp. Med.* 205(10), 2359–2368 (2008).

8 Barry CE 3rd, Boshoff HI, Dartois V *et al.* The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat. Rev. Microbiol.* 7(12), 845–855 (2009).

9 Cardona PJ. A dynamic reinfection hypothesis of latent tuberculosis infection. *Infection* 37(2), 80–86 (2009).

10 Young DB, Gideon HP, Wilkinson RJ. Eliminating latent tuberculosis. *Trends Microbiol.* 17(5), 183–188 (2009).

11 Gengenbacher M, Kaufmann SH. *Mycobacterium tuberculosis*: success through dormancy. *FEMS Microbiol. Rev.* 36(3), 514–532 (2012).

12 Chao MC, Rubin EJ. Letting sleeping dogs lie: does dormancy play a role in tuberculosis? *Annu. Rev. Microbiol.* 64, 293–311 (2010).

13 Delogu G, Goletti D. The spectrum of tuberculosis infection: new perspectives in the era of biologics. *J. Rheumatol. Suppl.* 91, 11–16 (2014).

14 Hossain MM, Norazmi MN. Pattern recognition receptors and cytokines in *Mycobacterium tuberculosis* infection – the double-edged sword? *Biomed. Res. Int.* 2013, 179174 (2013).

• **Complete overview on the mechanisms of immune recognition of *M. tuberculosis* during phagocytosis.**

15 Rajaram MV, Ni B, Dodd CE, Schlesinger LS. Macrophage immunoregulatory pathways in tuberculosis. *Semin. Immunol.* 26(6), 471–485 (2014).

16 Cywes C, Hoppe HC, Daffe M, Ehlers MR. Nonopsonic binding of *Mycobacterium tuberculosis* to complement receptor type 3 is mediated by capsular polysaccharides and is strain dependent. *Infect. Immun.* 65(10), 4258–4266 (1997).

17 Rohde KH, Veiga DF, Caldwell S, Balazsi G, Russell DG. Linking the transcriptional profiles and the physiological states of *Mycobacterium tuberculosis* during an extended intracellular infection. *PLoS Pathog.* 8(6), e1002769 (2012).

•• **Description of the dynamical changes of the *M. tuberculosis* transcriptional profile during the different phases of macrophage infection.**

18 Via LE, Deretic D, Ulmer RJ, Hibler NS, Huber LA, Deretic V. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J. Biol. Chem.* 272(20), 13326–13331 (1997).

19 Jayachandran R, Bosedasgupta S, Pieters J. Surviving the macrophage: tools and tricks employed by *Mycobacterium tuberculosis*. *Curr. Top. Microbiol. Immunol.* 374, 189–209 (2013).

20 Forrellad MA, Klepp LI, Gioffre A *et al.* Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence* 4(1), 3–66 (2013).

21 Wong D, Bach H, Sun J, Hmama Z, Av-Gay Y. *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar-H^+-ATPase to inhibit phagosome acidification. *Proc. Natl Acad. Sci. USA* 108(48), 19371–19376 (2011).

22 Deghmane AE, Soualhine H, Bach H *et al.* Lipoamide dehydrogenase mediates retention of coronin-1 on BCG vacuoles, leading to arrest in phagosome maturation. *J. Cell. Sci.* 120(Pt 16), 2796–2806 (2007).

23 Sun J, Wang X, Lau A, Liao TY, Bucci C, Hmama Z. Mycobacterial nucleoside diphosphate kinase blocks phagosome maturation in murine RAW 264.7 macrophages. *PLoS ONE* 5(1), e8769 (2010).

24 Puri RV, Reddy PV, Tyagi AK. Secreted acid phosphatase (SapM) of *Mycobacterium tuberculosis* is indispensable for arresting phagosomal maturation and growth of the pathogen in guinea pig tissues. *PLoS ONE* 8(7), e70514 (2013).

25 Welin A, Winberg ME, Abdalla H *et al.* Incorporation of *Mycobacterium tuberculosis* lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block. *Infect. Immun.* 76(7), 2882–2887 (2008).

26 Rajni, , Rao N, Meena LS. Biosynthesis and virulent behavior of lipids produced by *Mycobacterium tuberculosis*: LAM and cord factor: an overview. *Biotec. Res. Int.* 2011, 274693 (2011).

27 Thi EP, Hong CJ, Sanghera G, Reiner NE. Identification of the *Mycobacterium tuberculosis* protein PE-PGRS62 as a novel effector that functions to block phagosome maturation and inhibit iNOS expression. *Cell. Microbiol.* 15(5), 795–808 (2013).

28 Iantomasi R, Sali M, Cascioferro A *et al.* PE_PGRS30 is required for the full virulence of *Mycobacterium tuberculosis*. *Cell. Microbiol.* 14(3), 356–367 (2012).

29 Rampini SK, Selchow P, Keller C, Ehlers S, Bottger EC, Sander P. LspA inactivation in *Mycobacterium tuberculosis* results in attenuation without affecting phagosome maturation arrest. *Microbiology* 154(Pt 10), 2991–3001 (2008).

30 Sullivan JT, Young EF, Mccann JR, Braunstein M. The *Mycobacterium tuberculosis* SecA2 system subverts phagosome maturation to promote growth in macrophages. *Infect. Immun.* 80(3), 996–1006 (2012).

31 Walburger A, Koul A, Ferrari G *et al.* Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* 304(5678), 1800–1804 (2004).

32 Casonato S, Provvedi R, Dainese E, Palu G, Manganelli R. *Mycobacterium tuberculosis* requires the ECF sigma factor SigE to arrest phagosome maturation. *PLoS ONE* 9(9), e108893 (2014).

33 Master SS, Rampini SK, Davis AS *et al.* *Mycobacterium tuberculosis* prevents inflammasome activation. *Cell Host Microbe* 3(4), 224–232 (2008).

34 Ferrer NL, Gomez AB, Neyrolles O, Gicquel B, Martin C. Interactions of attenuated *Mycobacterium tuberculosis phoP* mutant with human macrophages. *PLoS ONE* 5(9), e12978 (2010).

35 Arbues A, Aguilo JI, Gonzalo-Asensio J *et al.* Construction, characterization and preclinical evaluation of MTBVAC, the first live-attenuated *M. tuberculosis*-based vaccine to enter clinical trials. *Vaccine* 31(42), 4867–4873 (2013).

36 Hernandez Pando R, Aguilar LD, Smith I, Manganelli R. Immunogenicity and protection induced by a *Mycobacterium tuberculosis sigE* mutant in a BALB/c mouse model of progressive pulmonary tuberculosis. *Infect. Immun.* 78(7), 3168–3176 (2010).

37 Aguilo JI, Alonso H, Uranga S *et al.* ESX-1-induced apoptosis is involved in cell-to-cell spread of *Mycobacterium tuberculosis*. *Cell. Microbiol.* 15(12), 1994–2005 (2013).

38 Houben D, Demangel C, Van Ingen J *et al.* ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. *Cell. Microbiol.* 14(8), 1287–1298 (2012).

39 De Jonge MI, Pehau-Arnaudet G, Fretz MM *et al.* ESAT-6 from *Mycobacterium tuberculosis* dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. *J. Bacteriol.* 189(16), 6028–6034 (2007).

40 Pym AS, Brodin P, Brosch R, Huerre M, Cole ST. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol. Microbiol.* 46(3), 709–717 (2002).

41 Bottai D, Majlessi L, Simeone R *et al.* ESAT-6 secretion-independent impact of ESX-1 genes *espF* and *espG1* on virulence of *Mycobacterium tuberculosis*. *J. Infect. Dis.* 203(8), 1155–1164 (2011).

42 Behar SM, Martin CJ, Booty MG *et al.* Apoptosis is an innate defense function of macrophages against *Mycobacterium tuberculosis*. *Mucosal Immunol.* 4(3), 279–287 (2011).

43 Seimon TA, Kim MJ, Blumenthal A *et al.* Induction of ER stress in macrophages of tuberculosis granulomas. *PLoS ONE* 5(9), e12772 (2010).

44 Sohn H, Kim JS, Shin SJ *et al.* Targeting of *Mycobacterium tuberculosis* heparin-binding hemagglutinin to mitochondria in macrophages. *PLoS Pathog.* 7(12), e1002435 (2011).

45 Aguilo N, Marinova D, Martin C, Pardo J. ESX-1-induced apoptosis during mycobacterial infection: to be or not to be,

that is the question. *Front. Cell. Infect. Microbiol.* 3, 88 (2013).

46 Miller JL, Velmurugan K, Cowan MJ, Briken V. The type I NADH dehydrogenase of *Mycobacterium tuberculosis* counters phagosomal NOX2 activity to inhibit TNF-alpha-mediated host cell apoptosis. *PLoS Pathog.* 6(4), e1000864 (2010).

47 Blomgran R, Desvignes L, Briken V, Ernst JD. *Mycobacterium tuberculosis* inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. *Cell Host Microbe* 11(1), 81–90 (2012).

48 Hinchey J, Lee S, Jeon BY *et al.* Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. *J. Clin. Invest.* 117(8), 2279–2288 (2007).

49 Hinchey J, Jeon BY, Alley H *et al.* Lysine auxotrophy combined with deletion of the SecA2 gene results in a safe and highly immunogenic candidate live attenuated vaccine for tuberculosis. *PLoS ONE* 6(1), e15857 (2011).

50 Krishnan N, Robertson BD, Thwaites G. The mechanisms and consequences of the extra-pulmonary dissemination of *Mycobacterium tuberculosis*. *Tuberculosis* 90(6), 361–366 (2010).

51 Davis JM, Ramakrishnan L. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 136(1), 37–49 (2009).

52 Pethe K, Alonso S, Biet F *et al.* The heparin-binding haemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. *Nature* 412(6843), 190–194 (2001).

53 Skerry C, Pokkali S, Pinn M *et al.* Vaccination with recombinant *Mycobacterium tuberculosis* PknD attenuates bacterial dissemination to the brain in guinea pigs. *PLoS ONE* 8(6), e66310 (2013).

54 Jain SK, Paul-Satyaseela M, Lamichhane G, Kim KS, Bishai WR. *Mycobacterium tuberculosis* invasion and traversal across an *in vitro* human blood-brain barrier as a pathogenic mechanism for central nervous system tuberculosis. *J. Infect. Dis.* 193(9), 1287–1295 (2006).

55 Magombedze G, Dowdy D, Mulder N. Latent tuberculosis: models, computational efforts and the pathogen's regulatory mechanisms during dormancy. *Front. Bioeng. Biotechnol.* 1, 4 (2013).

56 Orme IM. A new unifying theory of the pathogenesis of tuberculosis. *Tuberculosis* 94(1), 8–14 (2014).

• **This very original paper presents a new interesting theory of *M. tuberculosis* pathogenesis that could help rethinking the way vaccines against tuberculosis are rationally designed.**

57 Buerger S, Spoering A, Gavrish E, Leslin C, Ling L, Epstein SS. Microbial scout hypothesis, stochastic exit from dormancy, and the nature of slow growers. *Appl. Environ. Microbiol.* 78(9), 3221–3228 (2012).

58 Schuck SD, Mueller H, Kunitz F *et al.* Identification of T-cell antigens specific for latent *Mycobacterium tuberculosis* infection. *PLoS ONE* 4(5), e5590 (2009).

59 Govender L, Abel B, Hughes EJ *et al.* Higher human CD4 T cell response to novel *Mycobacterium tuberculosis* latency associated antigens Rv2660 and Rv2659 in latent infection compared with tuberculosis disease. *Vaccine* 29(1), 51–57 (2010).

60 Rustad TR, Harrell MI, Liao R, Sherman DR. The enduring hypoxic response of *Mycobacterium tuberculosis*. *PLoS ONE* 3(1), e1502 (2008).

61 Geluk A, Van Meijgaarden KE, Joosten SA, Commandeur S, Ottenhoff TH. Innovative strategies to identify *M. tuberculosis* antigens and epitopes using genome-wide analyses. *Front. Immunol.* 5, 256 (2014).

62 Kaprelyants AS, Mukamolova GV, Ruggiero A *et al.* Resuscitation-promoting factors (Rpf): in search of inhibitors. *Protein Pept. Lett.* 19(10), 1026–1034 (2012).

63 Betts JC, Lukey PT, Robb LC, Mcadam RA, Duncan K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* 43(3), 717–731 (2002).

64 Commandeur S, Van Meijgaarden KE, Lin MY *et al.* Identification of human T-cell responses to *Mycobacterium tuberculosis* resuscitation-promoting factors in long-term latently infected individuals. *Clin. Vacc. Immunol.* 18(4), 676–683 (2011).

65 Serra-Vidal MM, Latorre I, Franken KL *et al.* Immunogenicity of 60 novel latency-related antigens of. *Front. Microbiol.* 5, 517 (2014).

66 Delogu G, Manganelli R, Brennan MJ. Critical research concepts in tuberculosis vaccine development. *Clin. Microbiol. Infect.* 20(Suppl. 5), 59–65 (2014).

67 Andersen P, Kaufmann SH. Novel vaccination strategies against tuberculosis. *Cold Spring Harb. Perspect. Med.* 4(6). pii: a018523 (2014).

68 Ottenhoff TH, Kaufmann SH. Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog.* 8(5), e1002607 (2012).

69 Brennan MJ, Stone MR, Evans T. A rational vaccine pipeline for tuberculosis. *Int. J. Tuberc. Lung Dis.* 16(12), 1566–1573 (2012).

70 Kaufmann SH. Fact and fiction in tuberculosis vaccine research: 10 years later. *Lancet Infect. Dis.* 11(8), 633–640 (2011).

71 Evans TG, Brennan MJ, Barker L, Thole J. Preventive vaccines for tuberculosis. *Vaccine* 31(Suppl. 2), B223–B226 (2013).

72 Groschel MI, Prabowo SA, Cardona PJ, Stanford JL, Van Der Werf TS. Therapeutic vaccines for tuberculosis – a systematic review. *Vaccine* 32(26), 3162–3168 (2014).

73 Grode L, Ganoza CA, Brohm C, Weiner J 3rd, Eisele B, Kaufmann SH. Safety and immunogenicity of the recombinant BCG vaccine VPM1002 in a Phase 1 open-label randomized clinical trial. *Vaccine* 31(9), 1340–1348 (2013).

74 Sali M, Di Sante G, Cascioferro A *et al.* Surface expression of MPT64 as a fusion with the PE domain of PE_PGRS33 enhances *Mycobacterium bovis* BCG protective activity against *Mycobacterium tuberculosis* in mice. *Infect. Immun.* 78(12), 5202–5213 (2010).

75 Sweeney KA, Dao DN, Goldberg MF *et al.* A recombinant *Mycobacterium smegmatis* induces potent bactericidal immunity against *Mycobacterium tuberculosis*. *Nat. Med.* 17(10), 1261–1268 (2011).

76 Tameris MD, Hatherill M, Landry BS *et al.* Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled Phase 2b trial. *Lancet* 381(9871), 1021–1028 (2013).

• **This paper presents the results of the first Phase IIb clinical trial for the evaluation of a new vaccination strategy against TB in infants in a high-burden country.**

77 Dean G, Whelan A, Clifford D *et al.* Comparison of the immunogenicity and protection against bovine tuberculosis following immunization by BCG-priming and boosting with adenovirus or protein based vaccines. *Vaccine* 32(11), 1304–1310 (2014).

78 Hoft DF, Blazevic A, Stanley J *et al.* A recombinant adenovirus expressing immunodominant TB antigens can significantly enhance BCG-induced human immunity. *Vaccine* 30(12), 2098–2108 (2012).

79 Van Dissel JT, Soonawala D, Joosten SA *et al.* Ag85B-ESAT-6 adjuvanted with IC31(R)

promotes strong and long-lived *Mycobacterium tuberculosis* specific T-cell responses in volunteers with previous BCG vaccination or tuberculosis infection. *Vaccine* 29(11), 2100–2109 (2011).

80 Leroux-Roels I, Leroux-Roels G, Ofori-Anyinam O *et al.* Evaluation of the safety and immunogenicity of two antigen concentrations of the Mtb72F/AS02(A) candidate tuberculosis vaccine in purified protein derivative-negative adults. *Clin. Vaccine Immunol.* 17(11), 1763–1771 (2010).

81 Bertholet S, Ireton GC, Ordway DJ *et al.* A defined tuberculosis vaccine candidate boosts BCG and protects against multidrug-resistant *Mycobacterium tuberculosis*. *Sci. Transl. Med.* 2(53), 53ra74 (2010).

82 Parra M, Pickett T, Delogu G *et al.* The mycobacterial heparin-binding hemagglutinin is a protective antigen in the mouse aerosol challenge model of tuberculosis. *Infect. Immun.* 72(12), 6799–6805 (2004).

83 Delogu G, Fadda G. The quest for a new vaccine against tuberculosis. *J. Infect. Dev. Ctries* 3(1), 5–15 (2009).

84 Abu-Raddad LJ, Sabatelli L, Achterberg JT *et al.* Epidemiological benefits of more-effective tuberculosis vaccines, drugs, and diagnostics. *Proc. Natl Acad. Sci. USA* 106(33), 13980–13985 (2009).

85 Vordermeier HM, Hewinson RG, Wilkinson RJ *et al.* Conserved immune recognition hierarchy of mycobacterial PE/PPE proteins during infection in natural hosts. *PLoS ONE* 7(8), e40890 (2012).

86 Brosch R, Gordon SV, Garnier T *et al.* Genome plasticity of BCG and impact on vaccine efficacy. *Proc. Natl Acad. Sci. USA* 104(13), 5596–5601 (2007).

87 Kozak RA, Alexander DC, Liao R, Sherman DR, Behr MA. Region of difference 2 contributes to virulence of *Mycobacterium tuberculosis*. *Infect. Immun.* 79(1), 59–66 (2011).

88 Gideon HP, Wilkinson KA, Rustad TR *et al.* Hypoxia induces an immunodominant target of tuberculosis specific T cells absent from common BCG vaccines. *PLoS Pathog.* 6(12), e1001237 (2010).

89 Kamath AT, Fruth U, Brennan MJ *et al.* New live mycobacterial vaccines: the Geneva consensus on essential steps towards clinical development. *Vaccine* 23(29), 3753–3761 (2005).

90 Sambandamurthy VK, Jacobs WR Jr. Live attenuated mutants of *Mycobacterium tuberculosis* as candidate vaccines against tuberculosis. *Microbes Infect.* 7(5–6), 955–961 (2005).

91 Martin C, Williams A, Hernandez-Pando R *et al.* The live *Mycobacterium tuberculosis phoP* mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. *Vaccine* 24(17), 3408–3419 (2006).

92 Walker KB, Brennan MJ, Ho MM *et al.* The second Geneva Consensus: Recommendations for novel live TB vaccines. *Vaccine* 28(11), 2259–2270 (2010).

93 Pym AS, Brodin P, Majlessi L *et al.* Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat. Med.* 9(5), 533–539 (2003).

94 Grode L, Seiler P, Baumann S *et al.* Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. *J. Clin. Invest.* 115(9), 2472–2479 (2005).

95 Grode L, Kursar M, Fensterle J, Kaufmann SH, Hess J. Cell-mediated immunity induced by recombinant *Mycobacterium bovis* Bacille Calmette-Guerin strains against an intracellular bacterial pathogen: importance of antigen secretion or membrane-targeted antigen display as lipoprotein for vaccine efficacy. *J. Immunol.* 168(4), 1869–1876 (2002).

96 Sun R, Skeiky YA, Izzo A *et al.* Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with *Mycobacterium tuberculosis*. *Vaccine* 27(33), 4412–4423 (2009).

97 Kupferschmidt K. Infectious disease. Taking a new shot at a TB vaccine. *Science* 334(6062), 1488–1490 (2011).

98 Serafini A, Boldrin F, Palu G, Manganelli R. Characterization of a *Mycobacterium tuberculosis* ESX-3 conditional mutant: essentiality and rescue by iron and zinc. *J. Bacteriol.* 191(20), 6340–6344 (2009).

99 Serafini A, Pisu D, Palu G, Rodriguez GM, Manganelli R. The ESX-3 secretion system is necessary for iron and zinc homeostasis in *Mycobacterium tuberculosis*. *PLoS ONE* 8(10), e78351 (2013).

100 Andersen P, Doherty TM. TB subunit vaccines–putting the pieces together. *Microbes Infect.* 7(5–6), 911–921 (2005).

101 Morandi M, Sali M, Manganelli R, Delogu G. Exploiting the mycobacterial cell wall to design improved vaccines against tuberculosis. *J. Infect. Dev. Ctries* 7(3), 169–181 (2013).

102 Goonetilleke NP, Mcshane H, Hannan CM, Anderson RJ, Brookes RH, Hill AV. Enhanced immunogenicity and protective efficacy against *Mycobacterium tuberculosis* of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J. Immunol.* 171(3), 1602–1609 (2003).

103 Verreck FA, Vervenne RA, Kondova I *et al.* MVA.85A boosting of BCG and an attenuated, *phoP* deficient *M. tuberculosis* vaccine both show protective efficacy against tuberculosis in rhesus macaques. *PLoS ONE* 4(4), e5264 (2009).

104 Brandt L, Skeiky YA, Alderson MR *et al.* The protective effect of the *Mycobacterium bovis* BCG vaccine is increased by coadministration with the *Mycobacterium tuberculosis* 72-kilodalton fusion polyprotein Mtb72F in *M. tuberculosis*-infected guinea pigs. *Infect. Immun.* 72(11), 6622–6632 (2004).

105 Derrick SC, Yang AL, Morris SL. A polyvalent DNA vaccine expressing an ESAT6–Ag85B fusion protein protects mice against a primary infection with *Mycobacterium tuberculosis* and boosts BCG-induced protective immunity. *Vaccine* 23(6), 780–788 (2004).

106 Andersen CS, Dietrich J, Agger EM, Lycke NY, Lovgren K, Andersen P. The combined CTA1-DD/ISCOMs vector is an effective intranasal adjuvant for boosting prior *Mycobacterium bovis* BCG immunity to *Mycobacterium tuberculosis*. *Infect. Immun.* 75(1), 408–416 (2007).

107 Lin PL, Dietrich J, Tan E *et al.* The multistage vaccine H56 boosts the effects of BCG to protect cynomolgus macaques against active tuberculosis and reactivation of latent *Mycobacterium tuberculosis* infection. *J. Clin. Invest.* 122(1), 303–314 (2012).

108 Tchilian EZ, Desel C, Forbes EK *et al.* Immunogenicity and protective efficacy of prime-boost regimens with recombinant Δ*ureC hly+ Mycobacterium bovis* BCG and modified vaccinia virus Ankara expressing *M. tuberculosis* antigen 85A against murine tuberculosis. *Infect. Immun.* 77(2), 622–631 (2009).

109 Sali M, Dainese E, Morandi M *et al.* Homologous prime boosting based on intranasal delivery of non-pathogenic invasive *Escherichia coli* expressing MPT64, decreases *Mycobacterium tuberculosis* dissemination. *Vaccine* 32(32), 4051–4058 (2014).

110 Scriba TJ, Tameris M, Mansoor N *et al.* Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional $CD4^+$ T cells. *Eur J. Immunol.* 40(1), 279–290 (2010).

111 Mcshane H, Williams A. A review of preclinical animal models utilised for TB vaccine evaluation in the context of recent human efficacy data. *Tuberculosis* 94(2), 105–110 (2014).

112 Dye C, Fine PE. A major event for new tuberculosis vaccines. *Lancet* 381(9871), 972–974 (2013).

113 Kaufmann SH. Tuberculosis vaccine development at a divide. *Curr. Opin. Pulm. Med.* 20(3), 294–300 (2014).

114 Andersen P, Woodworth JS. Tuberculosis vaccines – rethinking the current paradigm. *Trends Immunol.* 35(8), 387–395 (2014).

115 Woodworth JS, Behar SM. *Mycobacterium tuberculosis*-specific $CD8^+$ T cells and their role in immunity. *Crit. Rev. Immunol.* 26(4), 317–352 (2006).

116 Shanley CA, Streicher EM, Warren RM, Victor TC, Orme IM. Characterization of W-Beijing isolates of *Mycobacterium tuberculosis* from the Western Cape. *Vaccine* 31(50), 5934–5939 (2013).

117 Shi L, North R, Gennaro ML. Effect of growth state on transcription levels of genes encoding major secreted antigens of *Mycobacterium tuberculosis* in the mouse lung. *Infect. Immun.* 72(4), 2420–2424 (2004).

118 Ewer K, Millington KA, Deeks JJ, Alvarez L, Bryant G, Lalvani A. Dynamic antigen-specific T-cell responses after point-source exposure to *Mycobacterium tuberculosis*. *Am. J. Respir. Crit. Care Med.* 174(7), 831–839 (2006).

119 Pai M, Joshi R, Dogra S *et al.* T-cell assay conversions and reversions among household contacts of tuberculosis patients in rural India. *Int. J. Tuberc. Lung Dis.* 13(1), 84–92 (2009).

120 Orme IM. Vaccines to prevent tuberculosis infection rather than disease: Physiological and immunological aspects. *Tuberculosis* doi:10.1016/j.tube.2014.10.008 (2014) (Epub ahead of print).

121 Etna MP, Giacomini E, Severa M, Coccia EM. Pro- and anti-inflammatory cytokines in tuberculosis: a two-edged sword in TB pathogenesis. *Semin. Immunol.* 26(6), 543–551 (2014).

122 O'garra A, Redford PS, Mcnab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. *Annu. Rev. Immunol.* 31, 475–527 (2013).

123 Dorhoi A, Kaufmann SH. Perspectives on host adaptation in response to *Mycobacterium tuberculosis*: modulation of inflammation. *Semin. Immunol.* 26(6), 533–542 (2014).

124 Comas I, Chakravartti J, Small PM *et al.* Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. *Nat. Genet.* 42(6), 498–503 (2010).

•• **This is a very important paper that demonstrates that genes and gene sequences encoding human T-cell epitopes in *M. tuberculosis* are under strong purifying selection, suggesting that *M. tuberculosis* benefits from recognition by human T cells.**

125 Lindestam Arlehamn CS, Gerasimova A, Mele F *et al.* Memory T cells in latent *Mycobacterium tuberculosis* infection are directed against three antigenic islands and largely contained in a CXCR3+CCR6+ Th1 subset. *PLoS Pathog.* 9(1), e1003130 (2013).

126 Copin R, Coscolla M, Seiffert SN *et al.* Sequence diversity in the *pe_pgrs* genes of *Mycobacterium tuberculosis* is independent of human T cell recognition. *mBio* 5(1), e00960–00913 (2014).

127 Gagneux S. Host-pathogen coevolution in human tuberculosis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367(1590), 850–859 (2012).

128 Brennan MJ, Thole J. Tuberculosis vaccines: a strategic blueprint for the next decade. *Tuberculosis* 92(Suppl. 1), S6–S13 (2012).

129 Armstrong JA, Hart PD. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. *J. Exp. Med.* 142(1), 1–16 (1975).

130 Malik ZA, Denning GM, Kusner DJ. Inhibition of $Ca^{(2+)}$ signaling by *Mycobacterium tuberculosis* is associated with reduced phagosome–lysosome fusion and increased survival within human macrophages. *J. Exp. Med.* 191(2), 287–302 (2000).

131 Beltran-Beck B, De La Fuente J, Garrido JM *et al.* Oral vaccination with heat inactivated *Mycobacterium bovis* activates the complement system to protect against tuberculosis. *PLoS ONE* 9(5), e98048 (2014).

132 Fenton MJ, Riley LW, Schlesinger LS. Receptor-mediated recognition of *Mycobacterium tuberculosis* by host cells. In: *Tuberculosis and the Tubercle Bacillus*. Cole ST (Ed.). ASM Press, Washington, DC, USA, 405–426 (2008).

133 Prados-Rosales R, Baena A, Martinez LR *et al.* Mycobacteria release active membrane vesicles that modulate immune responses in a TLR2-dependent manner in mice. *J. Clin. Invest.* 121(4), 1471–1483 (2011).

134 Prados-Rosales R, Carreno LJ, Batista-Gonzalez A *et al.* Mycobacterial membrane vesicles administered systemically in mice induce a protective immune response to surface compartments of *Mycobacterium tuberculosis*. *mBio* 5(5), e01921–01914 (2014).

135 Kohama H, Umemura M, Okamoto Y *et al.* Mucosal immunization with recombinant heparin-binding haemagglutinin adhesin suppresses extrapulmonary dissemination of *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) in infected mice. *Vaccine* 26(7), 924–932 (2008).

136 Stylianou E, Diogo GR, Pepponi I *et al.* Mucosal delivery of antigen-coated nanoparticles to lungs confers protective immunity against tuberculosis infection in mice. *Eur J. Immunol.* 44(2), 440–449 (2014).

137 Aagaard C, Hoang T, Dietrich J *et al.* A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat. Med.* 17(2), 189–194 (2011).

138 Hoang T, Aagaard C, Dietrich J *et al.* ESAT-6 (EsxA) and TB10.4 (EsxH) based vaccines for pre- and post-exposure tuberculosis vaccination. *PLoS ONE* 8(12), e80579 (2013).

139 Elkington PT. Tuberculosis: time for a new perspective? *J. Infect.* 66(4), 299–302 (2013).

140 Achkar JM, Casadevall A. Antibody-mediated immunity against tuberculosis: implications for vaccine development. *Cell Host Microbe* 13(3), 250–262 (2013).

• **This is a very important paper that summarizes the experimental evidences for the potential role of antibodies in immunity against *M. tuberculosis* and provides a scientific framework that supports the development of vaccines capable of eliciting antibodies against TB.**

141 Teitelbaum R, Glatman-Freedman A, Chen B *et al.* A mAb recognizing a surface antigen of *Mycobacterium tuberculosis* enhances host survival. *Proc. Natl Acad. Sci. USA* 95(26), 15688–15693 (1998).

142 Kruh NA, Troudt J, Izzo A, Prenni J, Dobos KM. Portrait of a pathogen: the *Mycobacterium tuberculosis* proteome *in vivo*. *PLoS ONE* 5(11), e13938 (2010).

143 Zumbo A, Palucci I, Cascioferro A *et al.* Functional dissection of protein domains involved in the immunomodulatory properties of PE_PGRS33 of *Mycobacterium tuberculosis*. *Pathog. Dis.* 69(3), 232–239 (2013).