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Updates on antibody functions in *Mycobacterium tuberculosis* infection and their relevance for developing a vaccine against tuberculosis

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Abstract

A more effective vaccine to control tuberculosis (TB), a major global public health problem, is urgently needed. Current vaccine candidates focus predominantly on eliciting cell-mediated immunity but other arms of the immune system also contribute to protection against TB. We review here recent studies that enhance our current knowledge of antibody-mediated functions against *M. tuberculosis*. These findings, which contribute to the increasing evidence that antibodies have a protective role against TB, include demonstrations that i) distinct human antibody Fc glycosylation patterns, found in latent *M. tuberculosis* infection but not in active TB, influence the efficacy of the host to control *M. tuberculosis* infection, ii) antibody isotype influences human antibody functions, and iii) that antibodies targeting *M. tuberculosis* surface antigens are protective. We discuss these findings in the context of TB vaccine development and highlight the need for further research on antibody-mediated immunity in *M. tuberculosis* infection.

Graphical abstract

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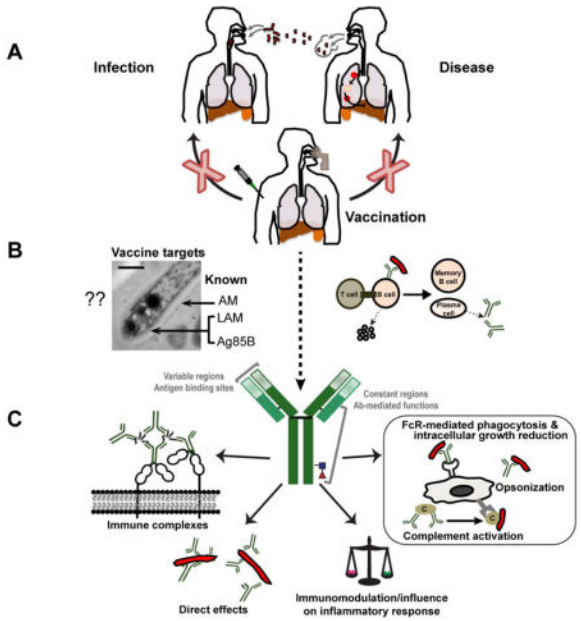

Introduction

Active tuberculosis (TB) is a transmissible respiratory disease that is caused by uncontrolled *Mycobacterium tuberculosis* (Mtb) infection. It is one of the top 10 causes of death globally, and the leading cause of death from a single pathogen worldwide, surpassing HIV [1]. To control this major global public health problem, more effective vaccines are urgently needed [2]. Recent estimates suggest that a quarter of the world's population, approximately 1.7 billion people, has asymptomatic controlled latent Mtb infection (LTBI) [3]. However, only ~10% of ostensibly healthy people develop TB during their lifetime [1]. The immune components preventing and controlling Mtb infection remain incompletely understood [4,5]. It has long been known that cell-mediated immunity (CMI) plays a pivotal role (reviewed in [6]), but there is increasing evidence that the innate immunity (reviewed in [7-9]), and other arms of the adaptive immune response (reviewed in [10,11]) contribute to protection against the disease. While it becomes increasingly apparent that all arms of the immune response and their interplay are important in the effective prevention of TB development, their detailed discussion is beyond the scope of this review. We focus here on the humoral immune response, and review recent studies providing further evidence for a role for antibodies (Abs) in protecting against Mtb infection. We discuss the relevance of these findings for TB vaccine development, highlight the need for further research on Ab-mediated immunity in Mtb infection, and discuss the challenges involved in such investigations.

The ideal TB vaccine would both prevent Mtb infection, and, in the already infected, the development of the disease (Figure 1A). While the Bacillus Calmette–Guerin (BCG) vaccine, the only TB vaccine in clinical use, prevents disseminated TB in young children, it has limited efficacy in preventing transmissible disease in adolescents and adults in its present version (reviewed in [12,13]). Current TB vaccine candidates predominantly target

the enhancement of CMI [4,5,12-15]. However, the only recent large human TB vaccine trial targeting CMI (MVA85A) showed no enhanced protection [16]. This trial was run in infants and the efficacy of several MVA85A trails, currently being performed in adolescents or adults, may differ. The observation that elevated IgG titers to Ag85A were associated with a decreased risk for TB development in a post hoc analysis [17], and other data from the TB vaccine and pathogenesis fields discussed below, argue for a more unbiased approach to TB vaccine development [4,5] (Box 1 and 2).

Knowledge of the epitopes and Ab constant region (Fc) structure–function relationships most relevant for protection against Mtb infection in humans would have important implications in TB vaccine development. However such information remains quite limited (reviewed in [18] and [19-24]; Figure 1B). The reasons are manifold and some have been discussed in detail in prior reviews [10,25]. They include: i) the conviction that extracellular Ab is less relevant for immunity to a predominantly intracellular pathogen like Mtb; ii) inconsistent results of passive transfer studies (many decades to a century ago) using horse and other animal immune sera in various Mtb infected animal models including rabbits and guinea pigs animals, as well as in humans with TB (reviewed in [26]); and iii) the highly heterogeneous and tremendously diverse Ab responses to Mtb antigens in humans and non-human primates [20,27-29]. The latter issue is influenced by immune competency, age and Mtb infection states spanning the continuum from primary to controlled and uncontrolled infection [28,30-33] (Box 1). Other contributing factors are likely the infecting organism and host genetics.

We know that Abs contribute to the defense against many intracellular pathogens, including Mtb, through various functions (reviewed in [10,34]). These include interactions with Fc-gamma receptors (FcyR; reviewed in [35]), the modulation of innate and adaptive immune responses (reviewed in [35,36]), and the more recently demonstrated direct effects on the physiology of intracellular pathogens while residing both outside and inside host cells [24,37,38] (Figure 1C). Furthermore, recent studies in humans and non-human primates suggest the importance of direct B cell involvement in the defense against Mtb infection, at both the systemic and local granuloma level [39-41]. Collectively, these data highlight the need for a thorough, detailed, and unbiased profiling and characterization of both systemic and mucosal Ab responses in Mtb infection states spanning the continuum from primary infection to controlled and uncontrolled infection (Box 1).

Human anti-mycobacterial Ab functions

Passive transfer studies, performed by independent groups mostly decades ago with murine monoclonal Abs (mAbs) against a handful of mycobacterial antigens, have shown variable protective efficacy in Mtb infected mice (reviewed [25]). However, little is known about the specific functions of these murine mAbs or about the protective efficacy and functions of antigen-specific human Abs. Recently published data provide compelling evidence for a functional role of human Abs to Mtb [19-23]. Importantly, Mtb infection-state-specific differences in IgG functions have been observed [21,23]. Using an unbiased approach for Ab profiling, Lu *et al.* provided evidence for protective *in vitro* functions of human polyclonal IgG in subjects with LTBI but not those with TB [21]. They demonstrated that the protective

LTBI IgG functions, including Ab-dependent cellular phagocytosis and cytotoxicity, were associated with distinct glycosylation profiles in the IgG Fc region. Such observations are important because IgG Fc–Fc R interactions have implications for vaccine development strategies (reviewed in [35]). However, the study was limited by analyzing total IgG and IgG to purified protein derivative (PPD), which contains many denatured Mtb proteins (>100–200). Different preparations of PPD vary in the concentrations of these proteins and contain moderate and varying amounts of the mycobacterial cell wall glycolipid lipoarabinomannan (LAM) [42], thereby limiting any conclusions on antigen-specific Ab structures. On the other hand, Zimmermann *et al.* showed that anti-heparin-binding hemagglutinin (HBHA) IgA, but not IgG mAbs, generated from B cells isolated from healthy individuals exposed to Mtb, inhibit mycobacterial infection of epithelial cells *in vitro* [22]. This finding suggests that isotype might be another key variable in influencing Ab efficacy for specific host cells. These results could have relevance in airway mucosa but should be taken with caution because the beneficial properties of naturally occurring and induced human systemic anti-Mtb IgGs have been demonstrated with human macrophages [19-21].

In passive intraperitoneal transfer experiments with mice infected with aerosolized Mtb, Li *et al.* demonstrated *in vivo* protective efficacy of total human serum IgG [23]. Serum from some LTBI and Mtb-exposed asymptomatic healthcare workers (7/48) were protective; however, that from 12 TB patients was not. The protective effects were reversed by pre-absorbing IgG against heat-killed Mtb but not against soluble Mtb antigens, thereby suggesting that the protective Abs targeted the Mtb surface. Similar to Lu *et al.* [21], this study investigated total serum IgG, thus limiting conclusions about specific protective antigens or relevant antigen-specific Ab structures. Nevertheless, both studies demonstrate differences in functions and efficacy between Abs from Mtb exposed but uninfected and/or individuals with controlled LTBI compared to Abs from individuals with uncontrolled infection (TB). In line with infection state-specific differences, Joosten *et al.* recently described the impairment of general human B-cell functions (e.g., proliferation, cytokine production and activation) during TB disease and recent Mtb infection, which resolved following TB treatment [39]. They further found that the B-cell dysfunction also compromised cellular immunity [39]. Collectively, these findings provide evidence of the diversity of functions of Mtb-specific Abs and B cells found in different infection contexts. These include patients with advanced disease despite high levels of Abs to many Mtb antigens and individuals who successfully resist or control Mtb infection with lower levels of Abs.

Relevance of Abs targeting the mycobacterial surface

Abs to capsular and other surface polysaccharides (PS) are protective against several microbial pathogens, including those with intracellular location [10,34]. Some of our most successful vaccines are based on inducing Abs to capsular PS [43,44]. Mycobacteria have a capsule, an important virulence factor consisting largely of PS, proteins, and, to a smaller extent, glycolipids [45,46]. The major capsular PS are α-glucan and arabinomannan (AM), accounting for 70–80% and 10–20% of PS content, respectively [47,48]. The lipidated counterpart of AM (LAM) is a component of the mycobacterial cell wall and membrane, but not of the capsule [45,49]. The Mtb capsule has antiphagocytic properties [46] but surface

glycans can also mediate adhesion and promote Mtb uptake and intracellular survival via direct interaction with mannose host cell receptors [45,50,51]. Thus, targeting mycobacterial surface glycans with Abs could interfere with Mtb virulence by preventing macrophage uptake through mannose receptors, and instead promoting Fc R-mediated phagocytosis and intracellular growth inhibition.

Little is known about the relevance of specific potentially protective Mtb antigens. Our groups are especially interested in Abs targeting AM. Although α-glucan is the major mycobacterial capsular PS, it has not generated as much interest as AM. Early studies showed that Mtb produces α-glucan during experimental infection in mice, in which Abs to this PS can be elicited [52]. However, humans infected with Mtb have low levels of Abs to α-glucan [53]. This is presumably because α-glucan is very similar in structure to glycogen and starch [52], staples of the human diet. Passive murine transfer studies have demonstrated that some, but not all, murine mAbs to AM and LAM have protective efficacy in Mtb infected mice [54,55]. Similar effects have not been seen with Abs recognizing the α-glucan. This, together with the low levels anti-a-glucan Abs in humans, could explain the lack research on protective effects of Abs recognizing this PS. On the other hand, Mtb-infected humans have high titres of anti-AM Abs; immunization with AM/LAM-protein conjugates has been shown to improve the outcome of Mtb infected mice ([24,56-58]; discussed in more detail below). These studies provide important *in vivo* evidence. However, they are limited in capturing the tremendous complexity and heterogeneity of protective Mtb epitopes, as well as infection-specific Ab functions and structure–function Ab-antigen relationships encountered in humans [18]. We, and others, have demonstrated that Abs to AM and LAM are elicited in human Mtb infection and through BCG vaccination [19,20,53,59-61], and that reactivity to both correlates strongly ($p<0.001$) [20,53]. Importantly, IgG titers to AM/LAM were significantly associated with enhanced mycobacterial opsonophagocytosis and intracellular macrophage growth inhibition [19,20]. Using glycan microarrays, we found highly heterogeneous IgG responses to AM OS fragments and a significant correlation of IgG reactivity to specific AM OS with enhanced mycobacterial phagocytosis ([20] and unpublished data). These data provide important insights because the field of glycoconjugate vaccine development, in part informed by the study of human sera, is moving towards OS-conjugates. Such vaccines have proven to be effective for several extracellular and intracellular pathogens, such as *Streptococcus pneumoniae, Candida albicans*, and *Shigella* [62-68].

Evidence of Ab efficacy in TB vaccine studies

In the past twenty years, vaccine development for many intracellular pathogens has been limited by the dogma that T cell-mediated immunity is the main contributor to the protective response. Vaccination with protein antigens will generate both Ab and T-cell specific responses. Consequently, evaluation of protective responses after vaccination with such antigens would necessarily need to include the evaluation of how both arms of the adaptive immune response contribute to the overall measured protective response. Passive transfer of vaccine-immune serum and adoptive transfer of T cells would complement such studies. However, negative results could lead to incorrect interpretations because of the potential interdependence of the two arms. This is particularly evident in TB vaccine research where

most studies do not incorporate measurement of Ab responses after vaccination; those that do lack functional investigations of Abs, such as FcyR-mediated effects. Surprisingly, studies testing vaccine efficacy of TB subunit vaccines typically neither assess nor mention Ab responses. This includes those using some of the most immunogenic Mtb antigens capable of inducing marked Th1 responses, such as the Ag85b and the RD1-encoded antigens ESAT-6 and CFP-10 [5]. In a more general perspective of vaccine development against intracellular pathogens, recent studies on PS-conjugate vaccines demonstrate that both cellular and humoral immunity can act synergistically to orchestrate a more efficient protective response than those based on inducing responses to either arm alone [24,69].

Most of the knowledge generated in the context of Ab-mediated protection against Mtb through vaccination has been restricted to targeting Mtb surface PS antigens (Box 2). This is because PS are classical B cell antigens with the inability to stimulate T-cell responses. To generate B cell memory, PS antigens need to be conjugated to T-cell dependent antigens (i.e., proteins), triggering the required T-cell help leading to B cell maturation and the production of high affinity Abs [70]. In this context, both cell wall-associated LAM and capsular AM have attracted most of the attention. Conjugates including secreted AM [58] or delipidated LAM [56] have been linked to Ag85b or other non-mycobacterial proteins as carriers. These studies demonstrated significant Ab-based protection against Mtb. More recently, other conjugate formulations including synthetic LAM OS [71] or a baculovirus-conjugated mimotope vaccine [72] have also demonstrated substantial immunogenicity. Similarly, ssDNA aptamers were developed to suppress the immunomodulatory properties of LAM leading to control of bacterial replication in animal models [73,74]. Other Mtb cell surface components, such as phenolic glycolipids, have also been targeted with conjugate vaccines [75]. However, in none of these studies was evidence of a direct contribution of vaccine-induced Abs provided.

We have recently reported PS-conjugate vaccines prepared using Ag85b and capsular AM to induce Ab responses to the mycobacterial cell surface [24]. Our studies showed protection at the level of BCG. More importantly, this work demonstrated, through passive transfer studies of immune sera, the significant contribution of both AM- and Ag85b-binding Abs to controlling bacterial dissemination in mice. The enhanced capacity of AM-Ag85b-immunized mice to reduce bacterial dissemination to the spleen relative to mice immunized with Ag85b alone supports the beneficial additive effect of AM-binding Abs to the overall protective response. This study also demonstrated, for the first time, that one of the most immunogenic Mtb protein antigens, Ag85b, induces protective Abs. Adoptive T-cell transfer from both Ag85b- and AM-Ag85b-immunized mice led to a reduction in bacterial burden in both the lung and spleen, indicating that both arms of the adaptive immune response contribute to the protective properties of Ag85b [24] (Figure 1B). We further demonstrated that several Ab mechanisms can explain the contribution of vaccine-induced Abs to the overall protective response. Changes in the Mtb transcriptional response upon binding of AM-specific Abs was also demonstrated. This novel observation indicates that Abs could have a direct effect on Mtb by compromising its physiology [24]. Similar Ab effects on the intracellular pathogen *Cryptococcus neoformans* have been reported [38]. We also demonstrated that pretreatment of Mtb with AM-specific polyclonal murine serum enhanced opsonophagocytosis by macrophages [24], an observation consistent with results from our

human studies with high anti-AM IgG titer polyclonal sera and human macrophages [20]. Importantly, we and others have shown that the opsonic entry of Mtb into phagocytic cells triggers a macrophage response leading to reduced bacterial survival via increased phagosome–lysosome fusion [20,76].

The role of vaccine-induced Abs against Mtb during an ongoing infection has been also explored. Generation of such data is important because the WHO estimates that the largest immediate impact of a TB vaccine would be in the prevention of disease in already infected individuals thus preventing transmission [2]. Two independent studies have shown the benefit of the therapeutic administration of immune sera in Mtb-infected SCID [77] or B-cell knockout mice [78]. The results of the former study should, however, be taken with caution as they were developed in the context of TB relapse after antibiotic treatment using a DBA/2 mouse strain that is known to be impaired in Ab development after chemotherapy. The later study clearly showed the amelioration of lung inflammation associated with reduced neutrophil infiltration and Th17 levels after passive transfer of murine BCG-immune serum. No mechanism of regulation of neutrophilic response was provided. However, this study encourages further explorations between humoral and innate immune responses to gain more insight into additional potential Ab-mediated mechanisms and the interactions between the various arms of the immune response. Such investigations would provide critical information for TB vaccine development.

Future perspectives

While the studies discussed here support protective efficacy of human polyclonal IgG *in vitro* and *in vivo*, they also indicate substantial complexity in structure–function relationships of Abs targeting Mtb. This complexity contributes to the challenges of proving protective Ab efficacy, particularly in humans with highly heterogeneous Ab responses to Mtb [18]. To decipher this complexity, and to determine structure–function relationships relevant for protection, more single Ag-specific studies with both human polyclonal and mAbs are needed (Box 1). The studies described above should also encourage the rethinking of TB vaccine design strategies, specifically the consideration that neither Ab- nor T cell-mediated immunity alone might suffice to generate an optimal protective response against Mtb infection. Based on what we have learned from conjugate vaccine studies, future strategies aiming to exploit this interaction should consider the optimization and development of second generation conjugate vaccines that incorporate specific glycan fragments and protein epitopes. We anticipate that vaccines harnessing both aspects of the immune response could have the highest impact in the prevention of TB. Another important area that has potentially tremendous implications for vaccine efficacy, especially for Ab-based vaccines targeting the Mtb surface, is the phenomenon of microbial antigenic variability of Mtb. This is expected to be particularly important for developing vaccines that target different strains in various TB endemic regions.

In conclusion, current evidence now indicates that it is critical for TB vaccine development to consider the humoral response in the experimental design of novel candidates. In addition, for those vaccine candidates in clinical trials with reagents capable of eliciting an Ab response, it is essential for the field to learn whether part of the protective response is due to

Abs. The complexity of the Ab response to Mtb is currently being increasingly uncovered. However, there is sufficient evidence to demonstrate that Abs could make a difference in developing vaccines that protect against this tremendously successful human pathogen.

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Box 1

The need for an unbiased and holistic evaluation of the humoral immune responses to Mtb

The tremendous heterogeneity of the humoral immune response to Mtb, even among individuals with apparently similar immune competency and Mtb infection states creates major challenges in delineating beneficial Ab responses in humans. Several factors, most of all the state of Mtb infection (controlled in LTBI versus uncontrolled in TB), immune competency (e.g. HIV uninfected versus HIV co-infected), and age (young children versus adults) have a major impact on Ab responses to Mtb. Other factors – prior exposure to environmental non-tuberculous mycobacteria, the infecting Mtb strain, and host genetics, to name just a few – are also likely influencing the repertoire of mycobacterial antigens eliciting Abs in humans. Mtb Ab responses must be analyzed in the context of these factors to avoid drawing misleading conclusions. Recent Positron Emission Tomography/Computed Tomography (PET/CT) studies demonstrate the diversity of Mtb infection even within a single individual (reviewed in [32]). Investigations of this type could provide new insights into additional causes for the heterogeneity of Ab responses. Nevertheless, the extrinsic and intrinsic factors affecting the tremendous heterogeneity of the humoral immune response to Mtb remain incompletely understood. Even less is known about the involvement and timing of immune responses at the local airway level. Studies with non-human primates suggest that early immunological events at the airway level could impact the outcome of Mtb infection [33,79]. Therefore, an unbiased and comprehensive profiling of mucosal airway Abs is essential to delineate Ab isotypes and antigens involved in controlling Mtb infection at the local level. This knowledge could inform mucosal airway vaccines. Overall, profiling of both systemic and mucosal airway Ab responses to Mtb in humans and non-human primates, along the continuum from uninfected but Mtb exposed to asymptomatic controlled latent infection to symptomatic uncontrolled disease, could lead to rationally designed studies with antigen-specific mAbs. Such studies are facilitated by the development of new tools that allow precise mapping of the specificity of Abs that bind to mycobacterial antigens [20,28,29,80], as well as by studies that allow the detailed analysis of Ab Fc structures and mediated FcyR functions [35]. Ultimately, the use of chemically defined reagents in form of mAbs would allow the rigorous evaluation of the specific Ab structure– function relationships and associated mechanisms of action that are needed to inform TB vaccine development strategies.

Box 2

Improving TB vaccine efficacy

One of the longstanding problems in the TB field is defining what a protective response against Mtb is. Contrary to most other pathogens, an initial infection does not provide protection against reactivation and/or reinfection. Therefore, what we can learn from natural infection might not be enough to generate an effective vaccine against TB. It is clear that T-cell mediated immunity is critical to generate a protective response against TB, but, it is also clear that it is insufficient for high vaccine efficacy. Nevertheless, TB vaccine candidates currently in clinical development are immunologically similar in that they are mostly based on CMI and do not exploit other arms of the adaptive immune response. This is apparent by the failure to perform detailed investigations on Ab responses and functions after vaccination. Past and new studies, mostly with PS-conjugate vaccines, have demonstrated that targeting the mycobacterial cell surface with Abs induced through vaccination can be complementary to and synergistic with the induced cellular response (Figure 1B). These data highlight that the understanding of what a protective immune response against TB requires should incorporate the interplay of the different components of the immune system.

Highlights

- Human antibody functions against *Mycobacterium tuberculosis* differ among states of *M. tuberculosis* infection, from asymptomatic controlled latent infection (LTBI) to symptomatic uncontrolled infection (active tuberculosis)
- Antibody functions against *M. tuberculosis* are influenced by isotypes and IgG Fc glycosylation structures
- Induction of *M. tuberculosis* surface antigen-specific antibody responses can influence TB vaccine efficacy
- Antibodies induced through vaccination may be complementary to and synergistic with the induced cellular response against *M. tuberculosis*

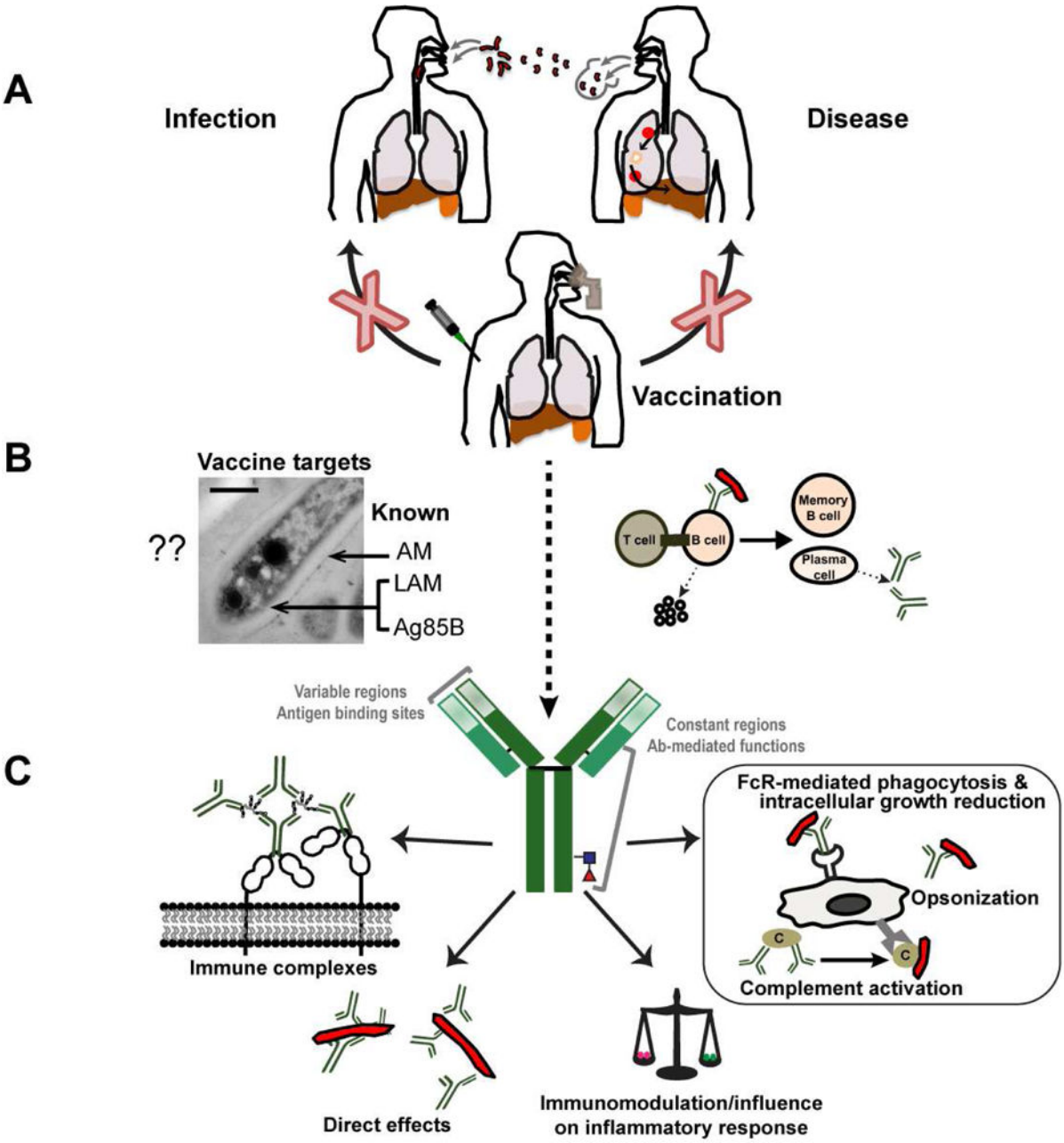

Figure 1.

Conceptual view of Ab-mediated protection induced by a TB vaccine. **(A)** The ideal vaccine would prevent *M. tuberculosis* infection in the uninfected and development of disease in the already infected individual through mucosal airway and/or systemic vaccination. **(B)** Induction of protective *M. tuberculosis* antigen-specific antibody responses with potential enhancement of cell-mediated responses. **(C)** Illustration of several antibody-mediated functions against *M. tuberculosis*, including opsonization, FcR-mediated phagocytosis and intracellular growth reduction, influence on the host's inflammatory response, direct effects

on the *M. tuberculosis* physiology, and influence of immune complexes on the host and host cells.