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The immunology of tuberculosis: From bench to bedside

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Abstract

Tuberculosis (TB) is an international public health priority and kills almost two million people annually. TB is out of control in Africa due to increasing poverty and HIV coinfection, and drug-resistant TB threatens to destabilize TB control efforts in several regions of the world. Existing diagnostic tools and therapeutic interventions for TB are suboptimal. Thus, new vaccines, immunotherapeutic interventions and diagnostic tools are urgently required to facilitate TB control efforts. An improved understanding of the immunopathogenesis of TB can facilitate the identification of correlates of immune protection, the design of effective vaccines, the rational selection of immunotherapeutic agents, the evaluation of new drug candidates, and drive the development of new immunodiagnostic tools. Here we review the immunology of TB with a focus on aspects that are clinically and therapeutically relevant. An immunologically orientated approach to tackling TB can only succeed with concurrent efforts to alleviate poverty and reduce the global burden of HIV.

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INTRODUCTION

Tuberculosis (TB) is one of the commonest serious infectious disease worldwide, is one of the commonest causes of serious respiratory disability and afflicts almost 10 million people annually.[1] The disease, fuelled by HIV infection and poverty, is out of control in Africa. Drug-resistant TB threatens to destabilize TB control in several other regions of the world.[2] Although several factors including HIV infection and socioeconomic deprivation facilitate the development of TB, contributing factors include suboptimal diagnostic tools, lack of cheap and effective new therapeutic interventions and the lack of a widely available effective vaccine. However, a better understanding of the immunopathogenesis of TB can drive the development of new immunodiagnostic tools, accelerate and facilitate the evaluation of new therapeutic interventions and is indispensible to the development of an effective vaccine. There have been several excellent published reviews on the immunology of TB.[3–7] Here, however, we will update the reader on the latest new developments in the field and, in particular, focus on clinically relevant and translational aspects of TB immunology.

THE LIFE CYCLE OF *MYCOBACTERIUM TUBERCULOSIS* AND CLINICAL SPECTRUM OF TUBERCULOSIS INFECTION

There is no gold standard for the diagnosis of latent TB infection (LTBI; asymptomatic state with no clinical or radiological evidence of active disease but with viable *M. tuberculosis* organisms within tissues). However, the discovery of new immunodiagnostic tools such as the interferon-γ (IFN-γ) release assays (IGRA) has improved our understanding about human TB.[8–11] Although well documented, it is not widely appreciated that a substantial proportion (perhaps up to 50%) of close contacts of microbiologically confirmed index cases, even in many (although not all) high burden settings, have no immunodiagnostic (positive tuberculin skin test (TST)) evidence of LTBI.[12] The same phenomenon is documented in repeatedly exposed health-care workers working in hospital TB wards. Thus, in many exposed individuals sterilizing innate immunity may presumably prevent the induction of adaptive immune responses and explain the persistently negative post-exposure TST and IGRA test results (Fig. 1). Specific genetic loci may be associated with the lack of TST reactivity.[13] That the available tools (IGRA or TST) lack adequate sensitivity to detect LTBI remains a possibility.

Of the remaining exposed individuals who have a detectable adaptive immune response and presumably develop LTBI, only a small number (~5%) go on to develop active TB within 2–5 years of becoming infected (Fig. 1). The remainder (~95%) of those who become infected remain disease-free for the duration of their lifetime unless they become immunocompromised by intervening illness, HIV infection or immunosuppressive drugs, etc. *M. tuberculosis* antigen-specific Th1 responses in the lungs,[14] and expanded CD8+ T cells with capacity to control growth of *M. tuberculosis*[15] *in vitro* can be found in household

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contacts. It is also recognized that some individuals with presumed LTBI may undergo reversion of the TST or IGRA,[16,17] and may convert their reactions if they become reinfected.[16,18] It is reasonable to speculate therefore that some individuals may clear their infection several weeks or months after becoming infected; some investigators have termed this an 'acute resolving infection'.[17,18] Alternatively, whether this, in a proportion of individuals, merely represents transition into dormancy or suboptimal detection (poor test sensitivity), rather than sterilization, is unclear. The various disease and immunodiagnostic phenotypes and their clinical, radiological and microbiological correlates are outlined in Table 1. Collectively, these data support a dynamic model of TB infection[10,11,19] incorporating innate immunity against *M. tuberculosis* infection, acquisition of LTBI, conversions (TST or IGRA positivity after exposure in previously negative individuals), reversions (TST or IGRA negativity in previously positive and exposed individuals), immune escape (LTBI undetected by immune surveillance tools such as IGRA and TST), acute resolving infection and reinfection. Thus, there is a poor correlation between immunodiagnostic test results and disease phenotype, and this may explain the generally poor positive predictive value of the TST and IGRA (less than ~5%) for active TB.[8] It also suggests that serial testing may be required to delineate disease phenotypes.[20] Consensus is required about the standardization of terminology (the spectrum of tuberculosis infection and disease), and further studies are needed to clarify several aspects of *M. tuberculosis* infection. Therefore, the development of reliable tools, including new imaging modalities (e.g. antibody-bound isotope markers), to detect LTBI is a research priority.

OVERVIEW OF THE IMMUNOPATHOGENESIS OF TUBERCULOSIS

The likelihood and intensity of aerogenic transmission of *M. tuberculosis* from a TB index case to a contact person in a shared air space depends on (i) exposure duration; (ii) intensity of exposure; (iii) cough and sputum-related host factors; and (iv) *M. tuberculosis* strain-related virulence characteristics. Infectious droplet nuclei are deposited in the alveolar spaces where *M. tuberculosis* can be phagocytosed by alveolar macrophages,[21–24] epithelial cells, dendritic cells (DC)[25,26] and neutrophils. Alveolar macrophages and DC are then believed to transport *M. tuberculosis* to local lymph nodes where T cells are primed and clonally expanded.

During active TB disease there is an exuberant local pulmonary immune response characterized by an alveolitis of activated α/β T-cell receptor-positive lymphocytes, recently recruited immature macrophages,[27,28] and strongly enhanced *M. tuberculosis* antigen-specific Th1 responses,[29–31] with large amounts of locally secreted IFN-γ. Increased numbers of alveolar neutrophils can also be found in subgroups of patients.[14] *M. tuberculosis*-induced immune evasion mechanisms[32–34] with production of suppressive cytokines and effector molecules[35–38] may counteract protective immune responses and abrogate bactericidal immune mechanisms.[39] The role of regulatory T (Treg) cells in suppressing local immune responses[40,41] and probably permitting mycobacterial growth is not yet well understood.

Susceptibility to TB is associated with HIV-1 infection (decreased numbers and function of CD4+ and CD8+ T cells[42–46]), therapeutic tumour necrosis factor-α (TNF-α)

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blockage,[47–49] and hereditary IFN-γ and IL-12 receptor abnormalities.[50–54] These conditions have provided insights into immune requirements for protection against *M. tuberculosis*. However, because the potentially protective Th1 responses (IFN-γ and TNF-α) are undermined *in vivo* by suppressive immune mechanisms, clear correlates of protection are still lacking for the assessment of new tuberculous vaccines and adjuvant immune therapies.

A role for protective immune response also underlies the persistence of LTBI (a state assumed to exist in a third of humans worldwide) during which *M. tuberculosis* growth is suppressed. These non-replicating bacilli are characterized by intracellular lipid bodies[55] and foamy lipid-laden macrophages may be associated with TB-related tissue pathology.[56]

In the parenchymal tissue, *M. tuberculosis* may induce the formation of pathognomonic granulomas (inflammatory reaction characterized by a ball-like collection of immune cells). Necrotizing granuloma centres[57] provide ideal culture conditions for *M. tuberculosis* that multiply extracellularly to large numbers, gain access to the airways and thus eventually disseminate the disease within the lung and to close contacts.

INNATE IMMUNITY TO *MYCOBACTERIUM TUBERCULOSIS*

Upon entry into the host lungs by aerosol inhalation, *M. tuberculosis* interacts with various receptors such as pattern recognition receptors such as toll-like receptors (TLR),[58] complement receptor 3,[21] mannose receptor,[59] scavenger receptor,[59] DC-specific intercellular-adhesion-molecule-3-grabbing non-integrin, on the surface of macrophages and DC (Fig. 2). These receptors recognize components of *M. tuberculosis* such as lipoprotein, CpG-containing DNA, mannose-capped lipoarabinomannan and phosphatidylinositol mannoside, respectively. Lung surfactant protein D binds *M. tuberculosis* surface lipoarabinomannan and limits the intracellular growth of *M. tuberculosis* by increasing phagosome lysosome fusion.[60] In addition, cytosolic nucleotide-binding and oligomerization domain-like receptors such as NOD2 that recognizes muramyl dipeptide[61] and also C-type lectin dectin-1 that interacts with *M. tuberculosis* cooperate with TLR-2 to activate NF-κB and mediate pro-inflammatory cytokine and antimicrobial responses. Toll NF-κB pathway activation promotes nucleus translocation of NF-κB and activates vitamin D pathway: (i) the activation of NF-κB results in production and secretion of many pro-inflammatory mediators including cytokines TNF-α, IL-1, IL-12, IL-18 and chemokines, which attract neu-trophils, natural killer (NK) cells, T cells, and more DC and macrophages to the infection site,[62,63] and nitric oxide. It is of interest to note that *M. tuberculosis*-secreted protein ESAT-6 could inhibit activation of NF-κB through preventing interaction between MyD88 and downstream kinase IRAK4;[64] (ii) TLR activation also upregulates expression of the vitamin D receptor (VDR) and the vitamin D-1-hydroxylase genes, which converts pro-vitamin D into the active form 1,25(OH)2D3 and leads to induction of the antimicrobial peptides cathelicidin and β-defensin to kill intracellular mycobacteria.[65–68] NADPH oxidase 2, which mediates phagocytic killing of ingested pathogens like *M. tuberculosis* via reactive oxygen species, interacts with TLR-2 and affects VDR-mediated antimicrobial peptide production.[69] Knockdown of NADPH oxidase 2 inhibited 1,25D(3)-mediated antimicrobial activity against *M. tuberculosis* through the modulation of cathelicidin expression in human

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macrophages.[69] The importance of the host VDR and TLR in controlling TB is shown in polymorphisms in VDR and TLR-2 that are associated with increased susceptibility to TB infection.[70,71] In contrast, DC-specific intercellular-adhesion-molecule-3-grabbing non-integrin signal pathway activation leads to production of IL-10 and transforming growth factor-β (TGF-β), which suppress the immune response.[62] Macrophages are heterogeneous and have different roles during TB infection, that is, IL-23-producing type 1 macrophages that promote Th1 immunity to mycobacteria, and IL-10-producing type 2 macrophages that suppress immunity to mycobacteria.[72] The type 2 macrophages have been shown to induce CD4+ T cells to adopt a Treg CD25+FoxP3+ mTGFβ-1+ suppressor phenotype[73] (see section below on Treg).

Interferon-γ, secreted from activated T cells and NK cells have the capability to activate macrophages and promote bacterial killing by permitting phagosomal maturation and production of antimicrobial reactive nitrogen intermediates and reactive oxygen intermediates.[74,75] Recent research also found that IFN-γ and TLR signalling pathways induce autophagy in macrophages,[76] which enhances the delivery of ubiquitin conjugates to the lysosome and increases the bactericidal capacity of the lysosomal soluble fraction.[77] Th1 cytokine IFN-γ facilitates phagosome lysosome fusion (autophagy) through cell signalling pathway IRGm1[78] (LRG-47)[79] and PI3K,[76] whereas Th2 cytokines IL-4 and IL-13 abrogate autophagy and autophagy-mediated killing of *M. tuberculosis* through Akt signalling pathway.[80] Besides IFN-γ, TNF-α also plays an important role in killing intracellular *M. tuberculosis* through reactive nitrogen intermediates together with IFN-γ and is involved in granuloma formation.[81] TNF is needed for controlling LTBI as anti-TNF antibody infliximab increases the risk of activating latent TB[47] through direct neutralization of TNF and also depletion of granulysin-expressing CD45RA+ subset of effector memory CD8+ T cells that contributes to the killing of intracellular *M. tuberculosis*.[82]

The CD1d-restricted NKT cells have recently been shown to mediate protection against *M.tuberculosis* in the mouse model.[83,84] NK cells also contribute to protective immunity through killing CD4+CD25+FoxP3+ Treg cells.[62] In addition, invariant NKT have recently been found to inhibit development of Th17 cells,[85] which may impact the pathology mediated by *M. tuberculosis* infection.

The role of neutrophils in host defence against *M. tuberculosis* is conflicting. Polymorphonuclear neutrophils (PMN) are the first cells recruited to sites of microbial entry and express a range of receptors and a vast arsenal of antimicrobial effector molecules as in macrophages.[62] Most experiments on human PMN suggest that PMN could be activated in response to *M. tuberculosis*, and have the ability to restrict mycobacterial growth *in vitro*.[62] For example, PMN produce human neutrophil peptides 1–3 and cathelicidin LL-37 and lipocalin 2, which have the ability to kill or restrict *M. tuberculosis* growth.[86] Furthermore, PMN could activate macrophages through releasing granule proteins[87] and heat shock protein 72 from apoptotic neutrophils.[88] However, on the other hand, PMN accumulated in *M. tuberculosis*-infected sites seem to have no obvious effect on mycobacterial growth and play a pathological rather than protective role in active disease. The results from different animal models are also conflicting. Some studies suggest neutrophils have a protective role during early stage of infection, while others suggest PMN do not play a role in control of

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TB.[62,86,89] Mast cells seem to play a role in controlling *M. tuberculosis* infection as activation of mast cells via TLR-2 could compensate the defect in controlling *M. tuberculosis* infection in TLR-2 knockout mice.[90]

V-γ-9V-δ-2-encoded T cells are the main γ-δT cells in humans, which recognize non-peptidic phosphoantigens without known requirement for MHC molecules. Mature V-γ-9V-δ-2T cells are rapidly recruited to the *M. tuberculosis* infection site, displaying a potent NK-like cytotoxic activity, expressing chemokine receptors, secreting a plethora of cytokines including IFN-γ and TNF-α, and killing *M.tuberculosis*-infected cells.[91] Like γ-δ T cells, activated CD1 molecules-restricted T cells that present mycobacterial glycolipid antigens produce IFN-γ and express cytolytic activity.[74] These antigen-specific γ-δ T cells and CD1-restricted T cells have adaptive immune responses, and can have immune memory and mount rapid, strong recall expansion after *M. tuberculosis* reinfection.[92] Furthermore, parts of γ-δ T cells produce IL-17 and express TLR-1 and TLR-2, as well as dectin-1, and could directly interact with certain pathogens to orchestrate an inflammatory response after bacterial invasion.[93]

Collectively, these data indicate that several pathways and cell types interact to mediate innate immunity against *M. tuberculosis*, and provide likely mechanisms for the observation that many individuals fail to display any immunodiagnostic evidence of TB infection despite significant and prolonged exposure to *M. tuberculosis*.

ADAPTIVE IMMUNITY TO *MYCOBACTERIUM TUBERCULOSIS*

Mycobacteria-infected macrophages and DC of the innate immunity present antigens to T cells and B cells that belong to adaptive immunity. Cytokine IL-12p40 plays a fundamental role in the pathogen-induced activation of pulmonary DC.[94] Macrophage apoptosis that releases apoptotic vesicles to carry mycobacterial antigens to uninfected DC can lead to more effective antigen presentation. Inhibition of macrophage apoptosis could reduce transfer of antigens to bystander cells and affect activation of CD8 T cells.[95]

MHC class CD1-restricted CD4 T cells and MHC class CD1-restricted CD8 T cells that recognize peptide antigens and the γ-δ T lymphocytes as well as the CD1-restricted specific T lymphocytes produce IFN-γ and constitute the protective immunity (Fig. 3). The CD4+ Th1 cells mount a much stronger IFN-γ response than CD8+ T cells after mycobacterial infection[96] and are thought to play a prominent role in protection.[97] The lack of CD4 T cells may result in delayed distribution of activated CD8 T cells from draining lymph nodes to distant organs and consequently cause a delayed acquisition of immune protection.[98] The CD8 cytolytic T lymphocytes (CTL) secrete granulysin, granzymes and perforins to kill mycobacteria-infected cells and are capable of immune protection against secondary mycobacterial challenge in the absence of CD4+ T cells.[96]

The CD4 T helper cells can be differentiated into Th1, Th2, Th17 and Treg cells. The Th1 cells produce cytokines, notably IFN-γ, TNF-α, IL-2, lymphotoxin and granulocyte-macrophage colony-stimulating factor (GM-CSF), which prompt stimulation of Th1 cells, CTL, and maturation and activation of macrophages as well as granulocytes. The Th2 cells

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produce B cell-stimulation factors such as IL-4, IL-5, IL-10 and IL-13, which promote antibody production but suppress the Th1 type immune response. The Th17 cells, a distinct subset of helper T cells, produce unique cytokines of IL-17, IL-17F, IL-21 and IL-22, which stimulate defensin production and recruit neutrophils and monocytes to the site of inflammation, and are involved in the early phase of host defence. The Th1, Th2 and Th17 subsets may be modulated by Treg cells, of which there are several types and the list is growing. The CD4+CD25+FoxP3+ natural Treg cells are characterized by TGF-β and IL-10 production,[99,100] while the Treg cells also co-produce IFN-γ.[101] In addition to CD4+ Treg cell subsets, CD8+ Treg cells are also described, which could inhibit T-cell proliferation and cytokine production.[102] The FoxP3-expressing Treg cells are expanded during TB infection,[103] and inhibit human memory γ-δ T cells to produce IFN-γ in response to *M. tuberculosis* antigens.[104] However, phosphoantigen-activated Vγ2Vδ2 T cells could antagonize IL-2-induced CD4+CD25+Foxp3+ Treg cells in mycobacterial infection.[105] CD4(+)CD25(+)FoxP3(+) Treg cells also produce TGF-β to downregulate CD4+ T-cell response,[39] and suppress the effector-immune response and induce bacillary dissemination and disease manifestation.[106,107] High per cent of Treg cells characterized as CD4(+)CD25(high)CD39(+) was also identified in active TB patients.[108] Attenuation of Treg cells has a positive impact on the protective capacity of vaccine against *M. tuberculosis* infection.[109,110] Nevertheless, given the extensive immunopathology and lung damage that characterize human TB, the precise role of Treg cell (deleterious, beneficial or bystander) in the immunopathogenesis of TB remains unclear.

Different cytokines can decide the differentiation of T-cell sets (Fig. 3). IL-12, IL-18 and IFN-γ promote Th1 cell development, while IL-4, IL-5 and IL-13 induce Th2 cell development. IL-23, Il-6, IL-21 and low concentration of TGF-β could induce Th17 differentiation, while IL-2 and high concentration of TGF-β could induce Treg differentiation.[99,100] IL-6 inhibits the generation of Treg cells induced by TGF-β, but together with TGF-β induces the differentiation of Th17 cells.[111]

Traditionally, B cells are not generally thought to play a significant role in protection against TB. However, recently it has been demonstrated that B cells[112] are required for optimal protection in *M. tuberculosis*-infected mice through interactions with the cellular immune response and activation of complement.[113] *M. bovis Bacille Calmette Guérin* (BCG) directly activates the classical, lectin and alternative pathways, resulting in fixation of C3b onto macromolecules of the mycobacterial surface,[114] which will contribute to mycobacterial killing.

Memory T (T_M) cells form after *M. tuberculosis* exposure or infection. T_M cells can be developed into effector ($CCR7^{lo}$, $CD62L^{lo}$, $CD69^{hi}$ in humans) and central T_M cells ($CCR7^{hi}$, $CD62L^{hi}$, $CD69^{lo}$ in humans).[97] T_M cells proliferate promptly after encounter with antigens, and produce multiple cytokines such as IFN-γ, IL-2, TNF-α, lymphotoxin and/or GM-CSF.[99] Nevertheless, the identification of specific biomarker signature of protective immunity remains elusive. Multifunctional CD4+ T-cell responses, like those raised to the TB subunit vaccine, Ag85B-ESAT-6/CAF01-induced,[115] may be a better marker of protective immunity.

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IMMUNOTHERAPY FOR TUBERCULOSIS

It has been suggested that the development of TB is due to failure of immune regulation or inappropriate immune regulation.[116–118] Moreover, much of the lung damage associated with TB is host-mediated immunopathology rather than due to *M. tuberculosis*-derived virulence factors. The disturbance in immune regulation may speculatively involve the subversion of a protective Th1 response, including the generation of CD8+ CTL, by several mechanisms including Th2-like cytokines,[118] TGF-β, Treg or other regulatory cells,[116] and hitherto undescribed mechanisms afflicting the downstream protective Th1 pathways. The observation that those with active TB require 6 months of treatment despite almost 95% of the bacterial sterilization occurring within the first 2 weeks of therapy has never satisfactorily been explained. Thus, more effective treatment may require modulation of the immune system and a switch away from an immunopathologic phenotype to a protective one. Restoring this immunoregulatory balance may take several months.[37]

Attempts to restore mycobactericidal immunity with IL-2[119] and IFN-γ[120,121] have been disappointing. Agents such as steroids,[122] thalidomide[123] and TNF-α antagonists have also been studied.[124] It is hypothesized that immunomodulatory agents (outlined in Table 2) may drive an appropriate Th1 response while concurrently turning on or off the appropriate regulatory cells. For example, *M. vaccae* may drive a Th1 response and CD8+ CTL but at the same turn on $CD25CD45Rb^{low}$ Treg cells.[127] However, clinical trials of *M. vaccae* have been disappointing.[128–130] It has been suggested that this failure may be related to the administration of a single rather than multiple doses, which is used with success in China.[131] A recent study of multiple-dose *M. vaccae* in HIV-infected African participants, and published in abstract form, demonstrated a reduction in mycobacteraemia in the intervention group.[132] In a murine model IVIG was shown to dramatically improve mycobacterial sterilization but there are no human data.[133] There are several intriguing preliminary reports, involving small numbers of patients, that a proprietary extract of several plants from the Ukraine (Immunoxel) may be associated with improved outcomes in drug-sensitive and drug-resistant TB[134–136]. Several other potential immunomodulatory agents including Mycobacterium w,[137] DNA vaccine-encoding HSP65 of *M. leprae*,[138] HE2000,[139] RUTI,[140] SCV-07 SciCLone,[125] anti-IL-4[125] and GM-CSF[125] are described in Table 2, and reviewed in detail in Churchyard *et al*.[125] The value of these immunomodulatory agents remain unclear and well-conducted prospective clinical trials are required to clarify their utility for routine use. The emergence of extensively drug-resistant TB had intensified the urgency for these studies to be conducted.

MONITORING TUBERCULOSIS TREATMENT AND PREDICTORS OF RELAPSE

It is extraordinary that no new TB drugs have been introduced into clinical management since the discovery of rifampicin in the 1960s. With the advent of multi-drug-resistant TB and extensively drug-resistant TB the concerted global effort to find new drugs has been re-energized. The development of any new drug is a prolonged and costly process. The definition of 'drug efficacy' includes successful completion of treatment and up to 2 years of

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follow up to identify post-treatment relapses. Given that treatment regimens for TB may last for up to 18 months, and recruitment of large cohorts may span several years, the time frame for evaluating new drugs in phase III trials may be as long as 5–8 years.

Use of early bactericidal activity[141,142] and 2-month culture conversion[143] allow for assessment of drug efficacy but the latter correlates crudely with disease relapse. Data from several studies reveal that the median (interquartile range) 2-month sputum conversion rate in patients with pulmonary TB was 81% (69.5; 87.5) and this was associated with a relapse rate of 6% (3.8; 12).[144] Immunological biomarkers/profiles hold promise as proxy markers to monitor treatment responses and predict future relapse.[144–146] Although a multitude of potential markers exist (recently reviewed[144,145]) none has been validated in large prospective studies. Cytokines such as IFN-γ and the IL4/IL4δ2 ratio have been shown to vary with treatment of LTBI and active disease;[147–150] these changes, however, are not always consistent[151,152] and may be confounded by the natural variation in stimulated cytokine responses over time.[16,153] Numbers and ratios of Treg cells and NK cells differ in TB and non-TB patients[154,155] and surface receptors such as soluble intercellular adhesion molecule 1 and soluble TNF receptor 1 show promise in distinguishing active from inactive TB and evaluating disease severity.[156,157] Inflammatory markers such as CRP and neopterin both decrease with TB treatment but are nonspecific.[158,159] Recent studies suggest that a profile using several cytokines, rather than one biomarker, may be more useful,[146,160–162] and it is likely that the best approach might entail a comprehensive 'relapse prediction score' using clinical variables, for example, weight, laboratory parameters and immunological markers. The biggest challenge will be to fund large cohorts with enough power to identify useful markers that predict relapse. Immunological biomarkers thus show promise and in combination with other parameters may be useful indicators of disease response and efficacy of therapy, thereby accelerating the development of much needed new anti-tuberculous treatments.

TUBERCULOSIS IMMUNOLOGY AND THE DESIGN OF EFFECTIVE VACCINES

Cytokine-specific immune responses to antigens from *M. tuberculosis* (such as ESAT-6, CFP-10, Ag85) have been found in latently infected persons and in healthy contacts of persons with TB.[163–165] The same antigens have been shown in animal models (murine and guinea pigs, some non-human primates) to provide protection against experimental *M. tuberculosis* infection. The expression of these (such as ESAT-6, CFP-10, Ag85) and additional *M. tuberculosis* antigens either in BCG or through viral vectors is a unifying characteristic of modern genetically engineered vaccine candidates against TB.

Bacille Calmette Guérin, derived from *M. bovis* in 1902, is the only currently approved anti-tuberculous vaccine and widely used globally.[166] It has a very good safety profile in immunocompetent persons.[167,168] Recently identified genetic differences between BCG strains and *M. tuberculosis*[169] are encoded within the region of difference 1, which is absent in BCG. The protective efficacy of BCG as a vaccine has been evaluated in many clinical studies to assess protection against many forms of TB in various clinical subgroups.[168] BCG

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vaccine-induced protective efficacy appears to be influenced by multiple factors[170–172] including age of the vaccine recipient, background infection rates with ubiquitous mycobacteria (mycobacteria other than *M. tuberculosis*, MOTT), virulence of the infecting clinical *M. tuberculosis* strains, coinfection with helminths,[173–175] acquisition of T-cell immunity against helminths *in utero*[176] and other conditions that alter immune responses (e.g. malnutrition). Although protection against adult pulmonary TB varies from none to some effect in many studies (summarized in Rieder[170]), there is good evidence that BCG protects against disseminated TB (summarized in Rieder[170]). Newer studies in household contacts support the notion that BCG may also protect against adult *M. tuberculosis* infection.[177,178] Concerns about potential adverse vaccination events from the live BCG vaccine (such as disseminated mycobacteriosis and mycobacterial meningitis) in HIV-1-infected persons have led to the development of new BCG vaccination guidelines by the World Health Organization.[168,179] Similar safety precautions impact on the efficacy testing and future use of new experimental recombinant BCG (rBCG) vaccines in populations with high rates of HIV-1 infection. Despite of these considerations, current planning for future prime-boost vaccination strategies incorporates conventional BCG and/or new rBCG-derived vaccines as the priming backbone followed by booster vaccines that are not based on BCG. Some of the rBCG vaccines improve antimycobacterial T-cell responses through engagement of an alternative antigen presentation pathway (endosome escape).[180]

Thus, current TB vaccine development efforts aim at preventing primary *M. tuberculosis* infection through preinfection vaccination, or at interrupting the transition from latent infection (LTBI) to active disease through post-BCG booster vaccination approaches. The number of promising tuberculous vaccine candidates has increased in the last decade (Table 3). Currently there are eight vaccine candidates in human phase I and phase IIa trials.[188]

As vaccines need to be developed for neonates, adolescents and adults, each of these vaccine recipient groups requires specific considerations related to the maturity of the immune system, and pre-existing antimycobacterial immunity (boosting after primary vaccination), including that conferred by mothers in the case of neonates. Field trials to assess the protective efficacy of vaccines against *M. tuberculosis* infection are risky, costly, and require a sophisticated clinical set-up in areas highly endemic for *M. tuberculosis*. Alterations of the underlying immune status affect the vaccination safety, vaccine-induced protection and the possibility to assess vaccine-induced immunity. As high incidence areas for TB most often are also areas with high HIV-1 infection rates, HIV-1 infection in vaccine recipients[189] must be considered in the choice of vaccines for future vaccination campaigns. Helminth coinfection that cause a Th2 cytokine bias must also be considered as they can alter TB immunity and responses to TB vaccines.[173,174,190]

The urgency to identify and use correlates of protective immunity is particularly obvious in the vaccine development field in which the study of immunological correlates is a crucial component of vaccine efficacy assessments, prior to embarking on large-scale efficacy field trials. It is increasingly clear that the induction of no single cytokine (IFN-γ, TNF, IL-2) or effector molecule (granzyme, nitric oxide, granulysin) can predict immunological protection. Polyfunctional immune responses[191,192] have recently been considered useful correlates of protection. Attempts to determine functional assays that would allow measurement of

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vaccine-induced immunological protection in *in vitro* growth inhibition assays are ongoing. In such assays whole blood or peripheral blood mononuclear cells (PBMC) from vaccinees (before and after vaccination) will be incubated with isolates of virulent *M. tuberculosis* and the vaccine induced inhibitory effect on *M. tuberculosis* growth assessed *in vitro*.

Novel tuberculous vaccination approaches may also involve the lungs. As human infection with *M. tuberculosis* most often occurs via inhalation, and TB pathology most often affects the lungs, it is logical to induce pulmonary protective immunity. An aerosolized TB vaccine candidate is currently undergoing safety and immunogenicity studies in non-human primates. Preliminary studies have shown strongly induced local pulmonary antigen-specific T-cell responses that may have characteristics of protective immunity (Aeras Global TB Vaccine Foundation, Annual Report 2008, http://www.aeras.org./newscenter/downloads/presskit/Aeras%202008%20Annual%20Report_Final_9.29.08.pdf).

RAPID IMMUNODIAGNOSIS OF TUBERCULOSIS

Mycobacterium tuberculosis infection has traditionally been ascertained using the TST, which measures skin induration caused by a delayed type hypersensitivity reaction and the sequential cutaneous infiltration of neutrophils, macrophages and several lymphocyte subsets[10,193] including Treg cells.[194] More recently quantitative and relatively specific TB antigen-driven (ESAT-6, CFP-10, TB 7.7) T-cell responses using PBMC have been investigated for their utility in diagnosing TB infection[8,195] (IGRA; Fig. 4). These commercially available assays enumerate the frequency of CD4+ antigen-specific effector T cells in peripheral lood.[8,195] Both these assays (IGRA and the TST) are measures of T-cell priming and in themselves do not necessarily indicate the presence of potentially viable *M. tuberculosis* organisms in the tissues of asymptomatic individuals. The IGRA are more specific, and in contrast to the TST that may be induced by BCG vaccination or non-tuberculous mycobacterium, correlate well with the magnitude of exposure to *M. tuberculosis*.[8,9,195] However, exposure *per se* does not necessarily imply the tissue persistence of organisms in a non-replicating state. There is good evidence for the TST, but not the IGRA, that treating test-positive individuals reduces the subsequent risk of developing active TB.[208,209] Although not precise, the IGRA and TST are the only available proxy markers of TB infection. The performance outcomes of these assays have been reviewed in detail elsewhere.[8] Several international guidelines recommend the use of IGRA, when using peripheral whole blood or PBMC, for the diagnosis of LTBI, either alone[210] (USA), or in conjunction with the TST[8] (UK, Canada, France, Italy, etc.).

Can the IGRA be used to diagnose active disease? Peripheral antigen-specific responses do not distinguish between active TB disease and LTBI. However, it is reasonable to hypothesize that patients with non-TB diagnoses, for example, cancer or bacterial infection, should not have a high frequency of antigen-specific T cells at the site of disease. Thus, it should be possible to diagnose active TB if there is a high frequency of *M. tuberculosis*-specific T cells within a specific disease compartment. Indeed, quantitative T-cell responses (T-SPOT.TB (Oxford Immunotec, Oxford, UK)) are accurate and clinically useful for the diagnosis of pulmonary TB when using BAL cells in both low-[198] and high-burden settings[199] (Fig. 4). Our recent work suggests that although IGRA can be used for the

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immunodiagnosis of active TB using cells from induced sputum this approach is not feasible in clinical practice with the current assay format (T. Cashmore and K. Dheda, 2010, unpubl. data). Can the same approach be used in other compartments? Although preliminary studies were encouraging we[201] and others[211,212] have recently shown that the IGRA are not clinically useful for the diagnosis of pleural TB at least in high-burden settings (this may be different in low-burden settings[213]). This is likely due to the suboptimal specificity as a consequence of migration of the *M. tuberculosis*-specific T cells into the pleural compartment of patients with non-TB diagnoses, for example, cancer, bacterial infection, etc.[201] Preliminary reports of the utility of IGRA for the diagnosis of TB meningitis are encouraging.[202] We recently found, in 140 consecutively recruited TB suspects, that T-SPOT.TB is a clinically useful and accurate diagnostic tool for TB meningitis.[214] The utility of a similar approach to diagnose abdominal TB may be feasible.[207] Thus, the accuracy of quantitative T-cell responses for the diagnosis of active TB is compartment-specific.

In summary, IGRA are useful for the diagnosis of presumed LTBI in low-burden settings.[8] Although they cannot be used to distinguish LTBI from active disease using peripheral PBMC or whole blood, they are useful tools for the diagnosis of active TB when using cells from the site of disease (lung and cerebrospinal fluid). Thus, an immunologically oriented approach for the diagnosis of TB is feasible and useful. The main advantage of this approach is the rapid and user-friendly diagnosis of TB. Further studies are now required to evaluate alternative testing platforms, alternative antigens (e.g. heparin-binding haemagglutinin),[215] and different biomarker readouts (e.g. IFN-γ-induced protein (IP-10), monocyte chemotactic protein 1 (MCP-1) and IL-1 receptor antagonist (IL-1RA))[216] for the rapid diagnosis of TB (Fig. 4).

CONCLUSIONS

It is becoming evident that *M. tuberculosis* infection is a dynamic state with a wide spectrum of pathology. Further research using cells from the lung and in individuals who are probably innately immune to TB are likely to yield important insights into correlates of immune protection. Although the immunology of TB is complex and poorly understood an improved understanding of the immunopathogenesis of TB can facilitate the design of effective vaccines and evaluation of their efficacy, the rational selection of immunotherapeutic agents, identification of correlates of immune protection, the evaluation of new drug candidates, and drive the development of new immunodiagnostic tools. Nevertheless, these newer tools are only likely to translate into improved outcomes if adequate resources are devoted to their evaluation, and if poverty, political instability and HIV infection are concurrently tackled on an international scale.

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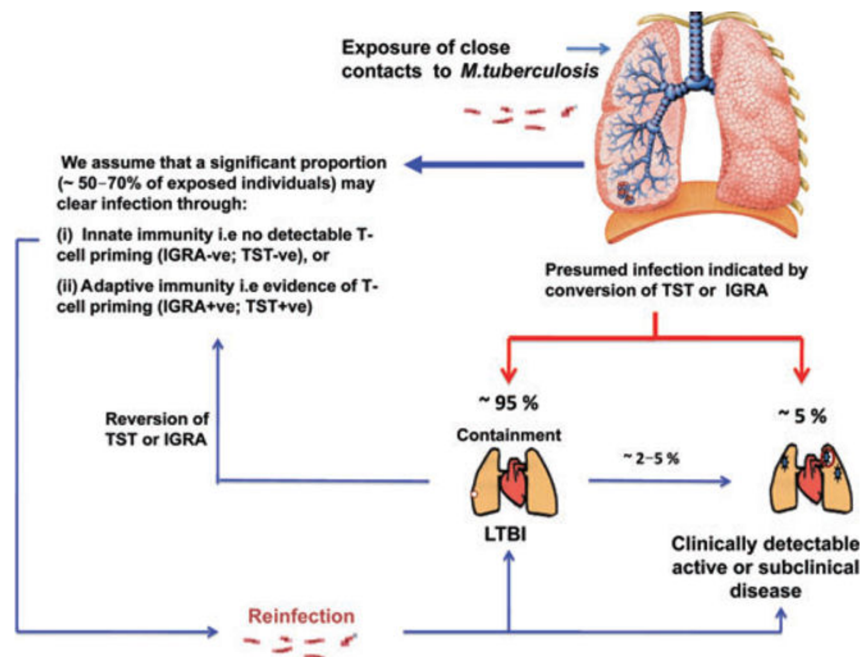

Figure 1.

The spectrum of *Mycobacterium tuberculosis* infection and the life cycle of *M. tuberculosis*. Many exposed individuals (~30–50%) have no immunodiagnostic evidence of *M. tuberculosis* infection or T-cell priming despite heavy exposure to *M. tuberculosis*. These individuals, although unproven, may be innately resistant to *M. tuberculosis* infection. Of those who have immunodiagnostic evidence of T-cell priming (+ve TST or IGRA) it is likely that a substantial proportion have LTBI (clinically asymptomatic *M. tuberculosis* infection during which organisms are in a state of non-replicating persistence). Some individuals have transiently positive responses, and may subsequently 'revert' their reactions—they may have 'acute resolving infection' or clear their infection. These individuals may become reinfected and progress to active tuberculosis. A proportion of patients with LTBI may also progress to active disease. Given the lack of a gold diagnostic standard for LTBI some of these postulates are based on circumstantial evidence and remain unproven. A multitude of clinical conditions (HIV-1 infection, diabetes mellitus, malnutrition, tobacco smoking, TNF-α inhibitor therapy, helminth coinfection) may permit tipping of the immunological balance and promote transition from latent infection to active disease. IGRA, interferon-γ release assay; LTBI, latent tuberculosis infection; TNF-α, tumour necrosis factor-α; TST, tuberculin skin test.

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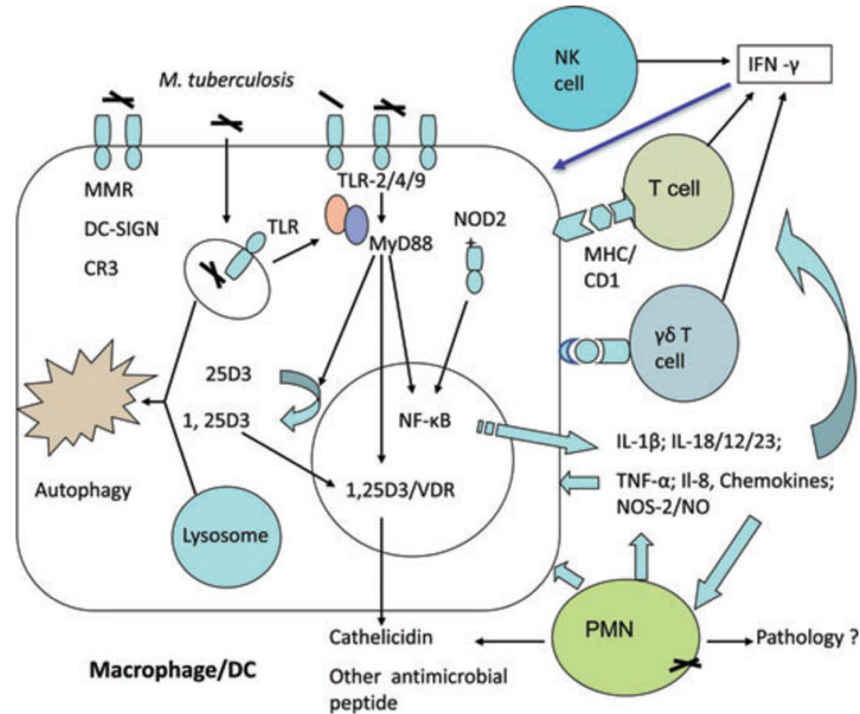

Figure 2.

Innate immunity to tuberculosis infection. *Mycobacterium tuberculosis* is phagocytosed by macrophages and dendritic cells through membrane-bound receptors such as CR3, scavenger receptor, MMR, TLR, NOD2 and DC-SIGN. These lead to activation of macrophage signalling pathways (NF-κB), causing secretion of pro-inflammatory cytokines, chemokines, and antimicrobial molecules, and activation of VDR, which induces the expression of the antimicrobial peptides cathelicidin and β-defensin. In addition, induction of autophagy mediates antimicrobial activity. PMN cells recognize and engulf *M. tuberculosis* and secrete antimicrobial peptides to kill bacteria. NK cells, γδ T cells and CD1-restricted T cells are also be activated by specific ligands and cytokines, release cytotoxic factors and secrete IFN-γ, which activates macrophages. CR3, complement receptor 3; DC-SIGN, dendritic cell-specific intercellular-adhesion-molecule-3-grabbing non-integrin; INF, interferon; MMR, macrophage mannose receptor; NK, natural killer; PMN, polymorphonuclear neutrophils; TLR, toll-like receptors; TNF, tumour necrosis factor; VDR, vitamin D receptor.

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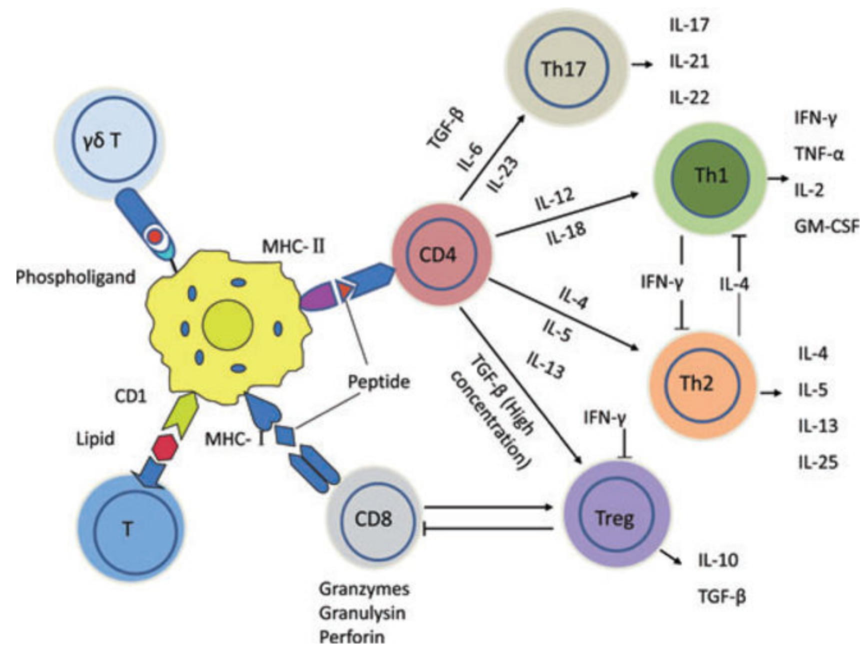

Figure 3.

Adaptive immunity to tuberculosis infection. The infected macrophages and dendritic cells secrete cytokines that include IL-12, IL-23, IL-7, IL-15 and TNF-α, and present antigens to several T-cell populations including CD4+ T cells (MHC class II), CD8+ T cells (MHC class I), CD1-restricted T cells (glycolipid antigens) and γδ T cells (phospholigands). These T cells produce the effector cytokine IFN-γ, which activates macrophages in conjunction with TNF-α to effect killing of intracellular mycobacteria through reactive oxygen and nitrogen intermediates. In addition, CD8+ cytotoxic T cells can kill intracellular mycobacteria through granulysin and perforin-mediated pathways. However, CD4+ Th2 cells produce immunosuppressive cytokines such as IL-4, and CD4+CD25+FoxP3+ regulatory T (Treg) cells produce IL-10 and TGF-β that may suppress mycobactericidal effector mechanisms.[41] A new subset of T helper cells called Th17 cells that are produced in the presence of IL-23, and are characterized by production of IL-17, is important modulator of inflammation and recall memory responses. Th17 cells can recruit neutrophils and monocytes, and IFN-γ-producing CD4+ T cells, and stimulate chemokine expression. However, IFN-γ in turn can suppress the IL-17 producing Th17 cells. Thus, there appears to be a more complex cross-regulation of Th1, Th2, Th17 and Treg cell responses, than previously recognized and the precise role of individual responses in protective immunity remain controversial. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; TGF, transforming growth factor; TNF, tumour necrosis factor.

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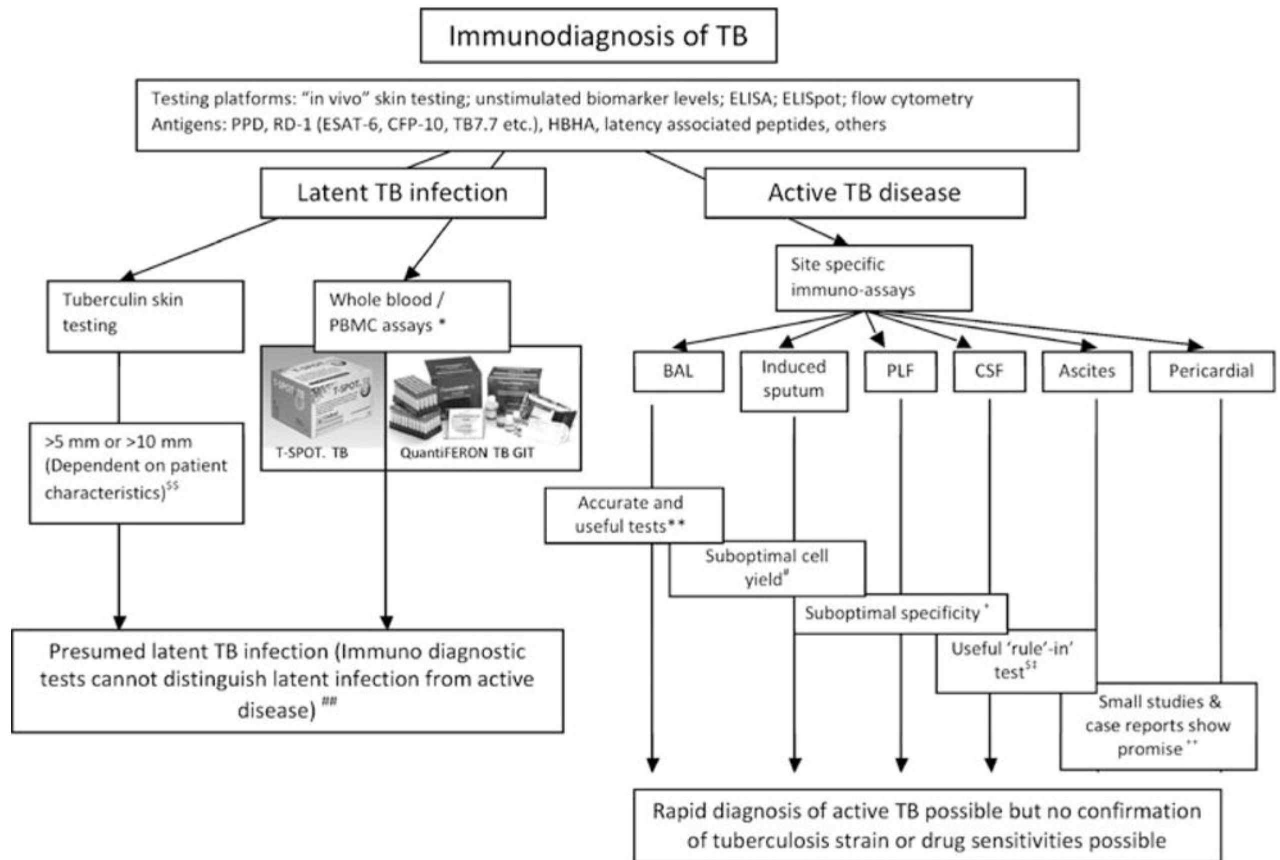

Figure 4.

Rapid immunodiagnosis of TB. An outline of the different antigens, testing platforms, biological markers and cells from different compartments used for the rapid quantitative immunodiagnosis of active TB and LTBI. *Flow cytometric platforms have been evaluated (Cosmi *et al.*).[196] $$Cut-points are defined by international guidelines and vary according to geographical location, BCG vaccination and HIV/immune status. ##Preliminary evidence that HBHA may help distinguish latent from active disease.[197] **Jafari *et al.*,[198] Dheda *et al.*[199] #Cashmore *et al.*[200]. +Dheda *et al.*[201] $Thomas *et al.*,[202] Kosters *et al.*,[203] Kim *et al.*[204] ‡van Zyl-Smit *et al.*[205] ++Kim *et al.*,[206] Tinelli *et al.*[207] BCG, *Bacille Calmette Guérin*; CSF, cerebrospinal fluid; LTBI, latent TB infection; HBHA, heparin-binding haemagglutinin; PBMC, peripheral blood mononuclear cells; PLF, pleural fluid; TB, tuberculosis.

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Table 1

Spectrum of TB infection and disease in exposed individuals stratified by symptoms, test results, CXR changes and bacterial burden

Immunological/disease phenotype	Presence of symptoms	TST status	IGRA status	Presence of CXR changes	Bacterial burden[†]	Persistence of non-replicating *Mycobacterium tuberculosis*
1. Innately immune	No	−ve	−ve	No	None	No
2. T-cell priming but clearance of infection[‡]	No	Transiently +ve/then −ve	Transiently +ve/then −ve	No[§]	None	No
3. T-cell priming but LTBI	No	+ve[¶] (may be −ve)	+ve[††] (may be −ve)	No[§]	+	Yes
4. Subclinical TB[‡‡] (quiescent disease)	No	+ve[¶] (may be −ve)	+ve[††] (may be −ve)	Yes	++	N/A
5. Active TB disease	Yes	+ve[¶] (may be −ve)	+ve[††] (may be −ve)	Yes	++++	N/A
6. Treated TB with disease resolution	No	+ve[¶] (may be −ve)	+ve[††] (may be −ve)	Yes	+/−	Possible
7. Old X-ray changes—no history of active disease	No	+ve[¶] (may be −ve)	+ve[††] (may be −ve)	Yes	+/−	Possible

[†] Bacterial burden refers to the quantitative load of TB bacilli (lowest in LTBI and highest in active TB). In treated TB patients there may be residual foci of non-replicating but persisting organisms. 3 may progress to 4, and then to 5 and 6 after treatment. In 1 and 2 there may be clearance of infection but these individuals may in future become reinfected and then progress to 3, and/or 4 and 5.

[‡] Clearance of the infection without T-cell priming assumes bacterial sterilisation by innate immune mechanisms, and with no immunodiagnostic evidence of T-cell priming.

[§] No features of active TB but calcified granulomas may be present.

[¶††] In these cases the TST or IGRA is often positive but may be negative, for example, even in active TB ~30% of patients have a negative TST/IGRA, TST and IGRA −ve exposed individuals may progress to develop active TB, and T-cell priming may occur but the infection is cleared and thus there is a poor correlation between disease phenotype and immunodiagnostic test results.

[‡‡] Subclinical TB refers to active disease where *M. tuberculosis* can often be microbiologically cultured from biological samples, and radiological changes may be present, but the patient is asymptomatic.

IGRA, interferon-γ release assay; LTBI, latent TB infection; N/A, not applicable; TB, tuberculosis; TST, tuberculin skin test.

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Table 2

Potential immunotherapeutic agents that have been studied in murine or human models for the treatment of TB

Agent	Mechanism	Comment
Immunoregulatory agents		
Mycobacterium vaccae	Drives Th1 and CD8+ CTL but downregulates Th2 through CD4+CD45+Rb^{low} regulatory cells	Single-dose *M. vaccae* not effective in clinical trials but full results of multiple-dose studies are awaited (reduced TB incidence shown in HIV-positive subjects in the DARDAR study). Used in China to treat MDR-TB
Mycobacterium w	Drives a Th1 response in mice but mechanism of action is unknown. Licensed in India as a immunomodulator for use in leprosy	Preliminary data suggest a beneficial effect in a murine model and one small human study
High-dose IVIG	Unknown but may involve pathways implicating sialic acid residues on IgG	Effective in a murine model. No human trials undertaken
HE2000	Modified form of DHEA (an adrenal steroid). Mode of action is unknown	Therapeutic in a murine model of TB. Reduced coinfection with TB shown in a cohort of HIV-infected individuals
DNA vaccine encoding HSP65 from *M. leprae*	Enhances CD8+ CTL activity and downregulates Th2	Effective in a mouse model but there are conflicting data. Phase 1 studies are planned
Fragmented lipid-depleted *M. tuberculosis* delivered in liposomes (RUTI)	Liposomal preparation of the *M. tuberculosis* cell wall skeleton. Suggested as an adjunct to eradicate long-term persisters	Accelerates bacterial clearance in a mouse model. Phase 1 studies underway
Immunoxel (Dzherelo)	Combination of plant extracts used in the Ukraine. Mechanism of action is unknown	Striking effects in small uncontrolled studies of M and XDR-TB patients. Larger controlled studies are warranted
SCV-07 SciCLone	Unknown	Results from one murine study
Anti-IL-4	Reduced macrophage apoptosis, enhanced macrophage function, reduced TGF-β, drives expansion of CD8+ CTL etc.	Therapeutic effect in mouse models. Humanized monoclonal IL-4 has been produced by Glaxo Smith Kline but no human studies have been performed
Supplemental effector cytokines		
Recombinant IFN-γ	Theoretically upregulate Th1-mediated macrophage killing	Two controlled trials showed minimal effects and their results have never been published
Recombinant IL-2	Promotes T-cell proliferation and granuloma formation	No beneficial effects found in an RCT (in fact reduced culture conversion was observed in the IL-2 arm)
IL-12	Drives a Th1 response	Beneficial in a murine model
Recombinant GM-CSF	Reduced *M. tuberculosis* replication in macrophages and DC	Trend to faster sputum conversion in one human study
Immunosuppressive agents		
Thalidomide, newer thalidomide analogues and TNF blockers e.g. etanercept	Disruption of immunopathological environment in which *M. tuberculosis* resides with replication upon exposure of bacteria to drugs. Newer analogues are PDE4 inhibitors	Murine and human studies have been undertaken. Newer thalidomide analogues show enhanced killing in murine models. Etanercept showed enhanced culture conversion in a clinical trial
High-dose prednisolone	Attenuates TNF-α production	Large controlled trial showed a dramatic effect on enhanced sputum culture conversion. Two other prospective studies showed a beneficial effect on sputum conversion

These agents, their mechanisms of actions and evidence for their utility have recently been reviewed in detail elsewhere[116,125,126] and this table should be read in conjunction with Churchyard *et al.*[125]

CTL, cytolytic T lymphocyte; DARDAR, Dartmouth and Dar es Salaam; DC, dendritic cell; DHEA, Dehydroepiandrosterone; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; MDR-TB, multi-drug-resistant tuberculosis; RCT, randomized controlled trial; TB, tuberculosis; TGF, transforming growth factor; TNF, tumour necrosis factor; XDR-TB; extensively drug-resistant tuberculosis.

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Table 3

Promising anti-tuberculous vaccine candidates in human phase I and phase IIa trials

Vaccine	Description	Clinical development stage	Source/producer
BCG30	rBCG with plasmid over expressing *Mycobacterium tuberculosis* Ag85A[181,182]	Phase I	UCLA/Aeras
VPM-X	rBCG with chromosomal expression of listeriolysin (for endosome escape)[180,183]	Phase I	Max Planck/VPM/TBVI
rBCG (AERAS-407)	Recombinant BCG with chromosomal overexpression of perfringolysin (for endosome escape) and *M. tuberculosis* antigens 85A, 85B, 10.4, Rv3427, rpfA, rpfB, rpfC40 and DosR-regulated proteins[184]	Phase I	Aeras
Crucell Ad35 (AERAS-402)	Recombinant adenovirus 35 expressing *M. tuberculosis* antigens 85A, 85B and 10.4, boosts BCG or rBCG	Phase I + IIa	Crucell/Aeras
MVA85A Oxford	Modified vaccinia Ankara 85A vector expressing *M. tuberculosis* antigen 85A, boosts BCG or rBCG[185,186]	Phase IIa	Oxford/Isis/Aeras/Emergent
GSK M72	Fusion molecule comprised of a *M. tuberculosis* protein from the PPE family (Rv1196), combined with an inactive serine protease Rv0125, boosts BCG	Phase I + IIa	GSK/Aeras
Hybrid I	Fusion molecule of proteins from the *M. tuberculosis* PPE family, inactive serine protease Rv1025 and AS01 adjuvant	Phase I + IIa	SSI/Intercell/TBVI
HyVac 4	Recombinant *M. tuberculosis* antigens 85B and 10.4 combined with adjuvant IC31, boosts BCG[187]	Phase I	SSI/Intercell/Sanofi Pasteur

Sources: http://www.aeras.org/our-approach/vaccine-development.php?discovery-overview and Barker *et al.*[188] BCG, *Bacille Calmette Guérin*; rBCG, recombinant *Bacille Calmette Guérin*.