Immunological biomarkers of tuberculosis

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Abstract | Currently there are no sufficiently validated biomarkers to aid the evaluation of new tuberculosis vaccine candidates, the improvement of tuberculosis diagnostics or the development of more effective and shorter treatment regimens. To date, the detection of *Mycobacterium tuberculosis* or its products has not been able to adequately address these needs. Understanding the interplay between the host immune system and *M. tuberculosis* may provide a platform for the identification of suitable biomarkers, through both unbiased and targeted hypothesis-driven approaches. Here, we review immunological markers, their relation to *M. tuberculosis* infection stages and their potential use in the fight against tuberculosis.

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Tuberculosis is caused by infection with *Mycobacterium tuberculosis*, which is transmitted through inhalation of aerosolized droplets. Tuberculosis constitutes a serious global health problem with nearly 10 million new cases of tuberculosis and 1.7 million deaths every year[1]. Furthermore, it is estimated that two billion people live with latent *M. tuberculosis* infection and represent a potential source of future active tuberculosis[2]. Global control of tuberculosis can only be achieved through the development of effective vaccines, improved diagnostics, and novel and shortened therapy regimens[3].

The need for tuberculosis biomarkers arises, in part, from the lack of suitable tests to detect *M. tuberculosis* or its products in host samples. The most widely used diagnostic test is microscopic detection of acid-fast bacilli in sputum (referred to here as the sputum smear test), which has a sensitivity of 34–80%[4]. Although the sputum culture test is more sensitive than the sputum smear test, it can take weeks to obtain results from the sputum culture test and laboratory facilities for this test are often not available in high incidence areas. A recently developed *ex vivo M. tuberculosis* gene amplification test (GeneXpert MTB/RIF) can be used to diagnose tuberculosis and can also detect resistance to rifampicin, one of the main antibiotics used in tuberculosis treatment, which serves as a marker for multidrug resistance. This test allows automated sample processing and produces results within two hours with excellent sensitivity[5]. However, its use is restricted to the identification of active pulmonary tuberculosis, as it cannot detect latent disease.

Host biomarkers are therefore needed to help to diagnose tuberculosis, to provide correlates of risk of tuberculosis and correlates of protection against active disease, and to determine the response to therapy. This Review discusses potential host immunological biomarkers for *M. tuberculosis* exposure, infection, disease and treatment and correlates these with stages of host–pathogen interaction.

Pathogenesis of tuberculosis

The interactions of *M. tuberculosis* with its host are complex, and our understanding of pathogenesis and of the protective immune responses during infection is constantly changing as technology advances. The recent observation of marked heterogeneity of the host immune responses[6], and possibly even of bacterial metabolism, within the same individual[7] has important implications for biomarker identification. The early conclusion from these studies is that latent and active tuberculosis do not represent two separate and distinct states, but that a continuum of host–pathogen interactions exists[7].

FIGURE 1 summarizes some current concepts of the clinical phenotypes, pathogenesis and host immune responses in tuberculosis and the resulting opportunities for biomarker discovery. Not all individuals who are exposed to *M. tuberculosis* become infected, suggesting that some may clear the bacteria through innate immune mechanisms; however, this has not been proven. In those individuals who become infected, the pathogen may be cleared through adaptive immune mechanisms,

Latent *M. tuberculosis* infection
Latent infection with *M. tuberculosis* indicates the presence of live *M. tuberculosis* organisms in a human host who is asymptomatic. It is detected by demonstrating immune responsiveness of the host to *M. tuberculosis* antigens (using the tuberculin skin test or interferon-γ release assays). Latent infection can last a lifetime.

Active tuberculosis
The symptomatic disease caused by *M. tuberculosis* infection. Approximately 10% of infected individuals develop active disease in their lifetime owing to a loss of immune control over the pathogen. The disease manifests mainly in the lungs but can be extrapulmonary or disseminated.

Tuberculosis biomarker
An ideal tuberculosis biomarker should: differentiate between patients with active tuberculosis and individuals with latent *M. tuberculosis* infection; return to normal levels during treatment; reproducibly predict clinical outcomes (for example, cure, relapse risk or eradication of *M. tuberculosis* infection) in diverse patient populations; and predict vaccine efficacy and provide end points for clinical trials.

Sputum smear test
Quantification of mycobacteria in stained sputum preparations by microscopic examination. Traditionally, this test is used for diagnosis and after the 2-month intensive phase of tuberculosis treatment to assess treatment response.

Sputum culture test
Assessment of the growth of *M. tuberculosis* from sputum in (currently mostly liquid) culture medium. Sputum culture conversion is used to assess treatment success. Successful treatment is determined by a lack of *M. tuberculosis* growth in a sample from an individual whose previous sputum culture test was positive.

Correlates of risk
Markers whose presence is associated with a low risk of disease, or whose absence is associated with a high risk of disease.

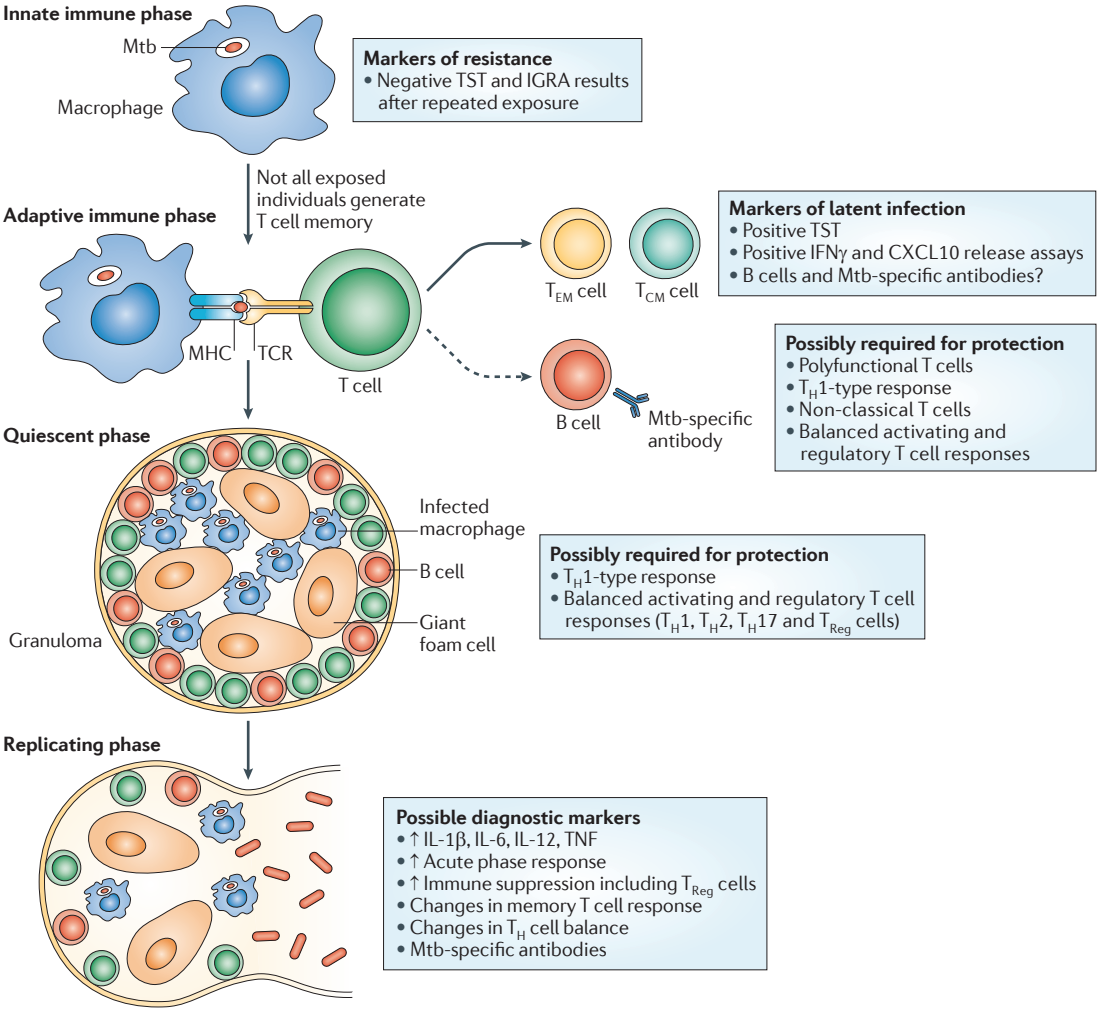

Figure 1 | **Immune responses and potential host biomarkers of *Mycobacterium tuberculosis* exposure and infection.** Exposure to *Mycobacterium tuberculosis* (Mtb) can result in different clinical outcomes, which include the absence of any clinical or laboratory evidence of infection, infection without clinically active disease and active disease. The stage of infection is determined by the ability of the host innate and adaptive immune systems to eradicate or control *M. tuberculosis*. A wide range of specific and nonspecific host immune responses contribute to the differential outcomes of exposure and infection, although there is a lack of detailed understanding of the underlying mechanisms. The host response at the different infection stages represents opportunities to measure individual markers or combinations of markers (biosignatures) that have diagnostic or prognostic potential, although they may have to be interpreted in a specific clinical context. The first three phases of *M. tuberculosis* infection are asymptomatic. Only some patients with tuberculosis develop immunological signs of infection; others can eliminate the bacteria during the innate immune phase, without generating T cell memory (and thus have negative tuberculin skin test (TST) and interferon-γ release assay (IGRA) results). In the adaptive immune phase, T cells are engaged by antigen-presenting cells, and this generates effector and memory T cells (both effector memory T (T_{EM}) and central memory T (T_{CM}) cells). B cells are also activated and *M. tuberculosis*-specific antibodies are produced. The infection may be cleared at this stage. However, most exposed individuals will enter the quiescent phase, which may persist for life. In this phase, the bacteria are contained inside granulomas, which consist of a central area, containing infected macrophages and giant foam cells, surrounded by T_{CM} and B cells. Although the host fails to eradicate the pathogen, replication and dissemination of the bacteria are prevented. An optimal T helper (T_H) cell balance is required to control *M. tuberculosis* while limiting immunopathology. This balanced reaction includes pro-inflammatory T_H1-type responses (characterized by interferon-γ (IFNγ), tumour necrosis factor (TNF) and interleukin-12 (IL-12) production) and T_H17-type responses (characterized by IL-17 production). However, it also involves T_H2-type responses (associated with IL-4 production) and regulatory T (T_{Reg}) cell phenotypes that limit immunopathology. The replicating phase is symptomatic and at this stage the bacteria have escaped immune control. Granulomas are disrupted, the acute-phase response is activated and the levels of pro-inflammatory markers increase. Enhanced immunosuppression becomes evident as T_H cell balance is disturbed, and memory T cell populations and antibody production may change. HIV co-infection and associated $CD4^+$ T cell depletion can trigger this immune escape by *M. tuberculosis*. CXCL10, CXC-chemokine ligand 10; TCR, T cell receptor.

involving different T cell populations. These populations include effector memory T cells (which may be transiently present in the blood if bacteria are cleared) and central memory T cells (which may remain for life but may not provide protection in all individuals). Failure of the immune system to eradicate the bacteria may result in a spectrum of host–pathogen interactions. The bacteria may exist in a quiescent state for prolonged periods (referred to as latent tuberculosis); however, the bacteria may ultimately start replicating and escape immune control, resulting in clinically active tuberculosis.

Only up to 10% of humans infected with *M. tuberculosis* will progress to active tuberculosis disease during their lifetime[8]. *M. tuberculosis* has evolved elaborate survival mechanisms in humans, allowing the bacterium to remain in a clinically inactive state, although it constantly engages with the human immune system. In this state, the immune response prevents active replication but fails to eradicate the bacteria. Any subsequent weakening of the host immune system may result in clinical disease. The mechanisms for these differential outcomes to *M. tuberculosis* exposure remain unclear.

Live *M. tuberculosis* may also persist after an initially successful treatment of active tuberculosis, and dynamic interchanges between host and pathogen determine whether relapse to active disease will occur (FIG. 2). These interchanges may be particularly relevant in immunocompromised individuals; in sub-Saharan Africa a high proportion of HIV-infected patients are co-infected with *M. tuberculosis*, and the HIV-induced immunodeficiency contributes to the increasing numbers of tuberculosis cases and deaths[9].

So, the traditional views of *M. tuberculosis* infection as either a latent or active disease have been replaced by a model where a continuum of host–pathogen interactions exists, resulting in a spectrum of immune responses[7].

Immunology of tuberculosis

Protective immunity against *M. tuberculosis* is not completely understood but depends on a wide range of innate and adaptive immune mechanisms. T cell-mediated immune responses are important in the host control of *M. tuberculosis* infection. The ability of $CD4^+$ T cells to produce interferon-γ (IFNγ), which activates phagocytes to contain the intracellular pathogen, is central in protection. Indeed, T helper 1 (T_H1) cells and the IFNγ that they produce are crucial for protection against disease. This is evident from the increased risk of tuberculosis in individuals with deficiencies in their IFNγ and interleukin-12 (IL-12; which promotes T_H1 cell differentiation) signalling pathways[10], and from the association between $CD4^+$ T cell depletion and elevated susceptibility to tuberculosis in HIV-infected individuals[11].

Many other $CD4^+$ T cell subsets, in addition to IFNγ-producing T_H1 cells, may also have a role; for example, IL-17-producing $CD4^+$ T cells were shown to mediate the recruitment of protective T_H1 cells to the lung upon *M. tuberculosis* challenge[12]. Induction of IL-17 expression following vaccination of cattle using a prime–boost vaccine strategy also correlated with protection against *Mycobacterium bovis* tuberculosis[13]. Furthermore, increased frequencies of regulatory $CD4^+$ T (T_{Reg}) cells during active disease may ensure that the T_H1 cell response is not excessive, and this would help minimise lung damage in tuberculosis[14]. This control of overactive inflammatory responses may also be achieved by inhibitory molecules, such as programmed cell death 1 (PD1), that are expressed by the effector T cells themselves, and mice that lack expression of PD1 develop more severe disease following *M. tuberculosis* challenge[15]. However, too much T_{Reg} cell activity may suppress protective inflammatory responses. Therefore, the critical determinant of protection may be a balanced and well-regulated immune response.

The $CD8^+$ T cell response to *M. tuberculosis* is normally of a lower magnitude than the $CD4^+$ T cell response; however, $CD8^+$ T cells may modulate phagocyte activity or produce molecules such as granulysin that may be directly cytotoxic to the mycobacteria[16,17]. Similarly, other cytokines, in addition to IFNγ, may also be crucial; for example, tumour necrosis factor (TNF) is important for establishing the granuloma[16], which is a well-organized collection of innate and adaptive cells that forms to contain the pathogen.

Furthermore, the importance of innate immunity cannot be overemphasized. The dendritic cells or macrophages that engulf *M. tuberculosis* in the lung can interact with the pathogen through numerous receptors, which can induce distinct innate immune responses[18]. These responses may even eradicate the bacterium before the establishment of adaptive immunity. However, bacterial evasion of innate cellular responses, leading to delayed induction of the adaptive immune system[19], appears to be a more characteristic outcome of *M. tuberculosis* infection and allows the pathogen to become established at the site of disease. Natural killer (NK) cells and granulocytes are also thought to have a role in protection.

Our knowledge of protective immunity against tuberculosis remains incomplete; a classical example is that the role of B cells remains undefined, even though these cells are found in substantial numbers in granulomas[20]. A list of immunological markers that differentiate between different outcomes of *M. tuberculosis* infection is shown in TABLE 1.

Correlates of risk and protection

Knowledge about the correlates of tuberculosis risk and protection will facilitate rapid screening of new tuberculosis vaccine candidates and targeted intervention to prevent tuberculosis disease. A non-placebo-controlled clinical trial (for example, following *M. bovis* bacillus Calmette–Guérin (BCG) vaccination or *M. tuberculosis* infection) allows for the delineation of correlates of risk of tuberculosis disease, by comparing the immune responses of those who ultimately develop tuberculosis with those who do not. By contrast, correlates of protection can be identified only in a randomized controlled clinical trial of an effective vaccine[21], in which immune responses are compared between protected individuals in the vaccine and placebo groups. This is due to the fact that there is no evidence that latent infection induces protection against the development of active

Correlates of protection
Several terms are used for this concept, including surrogates of protection. These markers reliably predict the level of protective efficacy induced by a vaccine on the basis of differences in the immunological measurements of vaccinated and unvaccinated groups.

Effector memory T cell
A terminally differentiated T cell that lacks lymph node-homing receptors but expresses receptors that enable it to home to inflamed tissues. Effector memory T cells can exert immediate effector functions without the need for further differentiation.

Central memory T cell
An antigen-experienced T cell that expresses cell-surface receptors for homing to secondary lymphoid organs. These cells are generally thought to be long-lived and can serve as the precursors for effector T cells in recall responses.

Relapse
A recurrent episode of tuberculosis after initial cure, resulting from incomplete clearance of the original infection. The same bacterial strain is involved at both episodes.

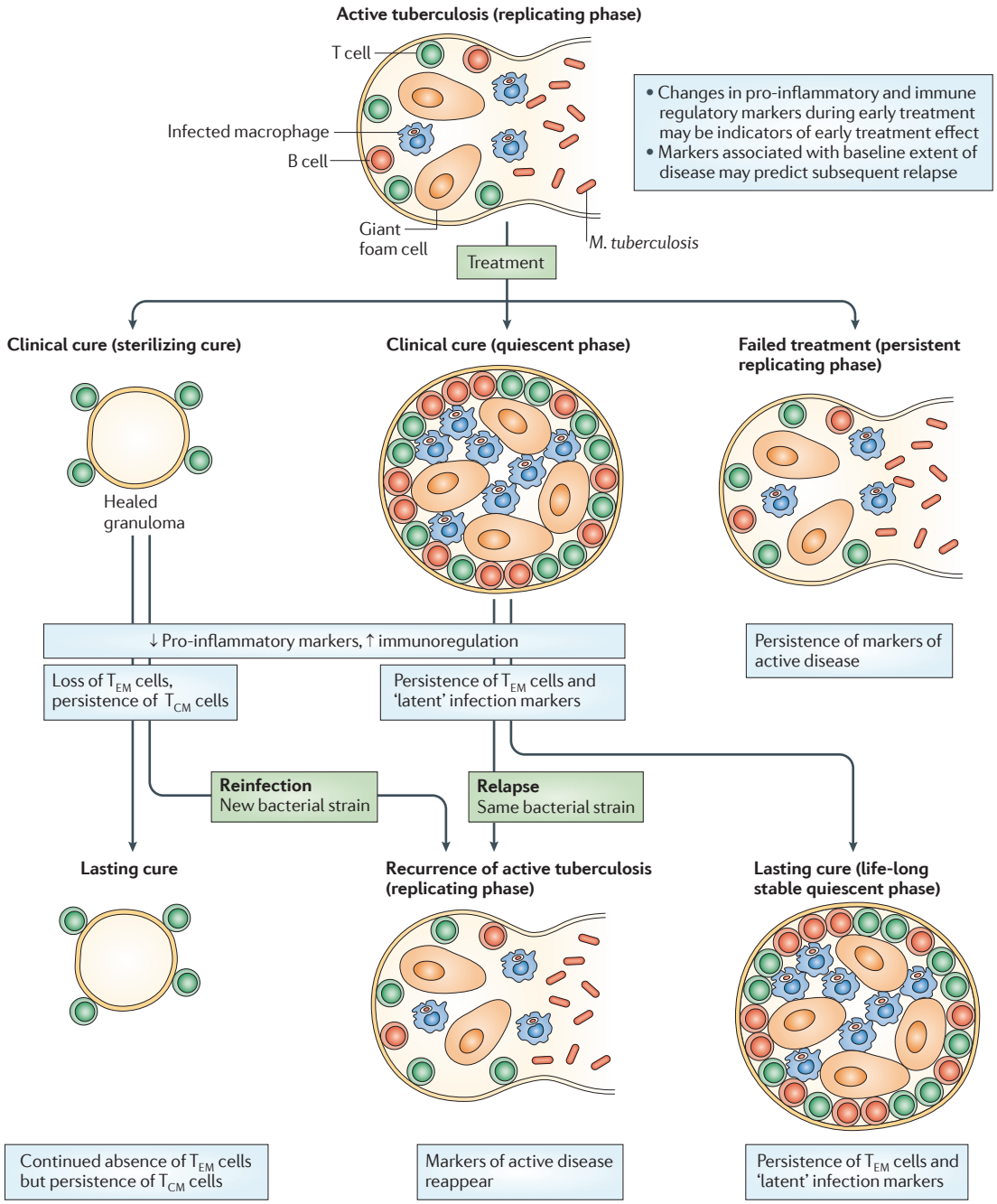

Figure 2 | **Differential outcomes of tuberculosis treatment are associated with different infection phases.** Treatment of active tuberculosis results in clinical cure in the majority of patients, although drug-resistant organisms and poor treatment adherence can result in treatment failure. Clinical cure is characterized by negative bacteriological examination for *Mycobacterium tuberculosis* and by resolution or improvement of symptoms and changes in chest X-ray examinations. The term is used to refer to both sterilizing cure and a return to the quiescent phase (non-sterilizing cure). However, sterilizing cure cannot be reliably differentiated from non-sterilizing cure, although the persistence of effector memory T (T_{EM}) cells may point towards the continued presence of live, non-replicating bacteria. T_{EM} cells home to peripheral tissue, can differentiate directly into effector cells and are detectable by measuring interferon-γ (IFNγ) production in short-term assays using whole blood or peripheral blood mononuclear cells stimulated with *M. tuberculosis* antigens. Central memory T (T_{CM}) cells are long-lived, even in the absence of persistent antigen, and home to secondary lymphoid organs, require longer term stimulation assays and signify previous immunological sensitization to the pathogen. Active tuberculosis can recur, either through reinfection with a new bacterial strain (in patients whose tuberculosis treatment resulted in sterilizing cure) or through relapse with the original bacterial strain (in patients whose infection returned to a quiescent phase after treatment). Measurements of memory T cell subpopulations and other biomarkers for pathogen persistence have so far not been adequately investigated for their ability to predict treatment outcome, and the field relies on clinical evidence of mycobacterial activity.

tuberculosis, rather it places such people at risk of tuberculosis. Therefore, the immune responses in latently infected individuals who never develop tuberculosis (who account for >90% of latently infected individuals) are not useful as correlates of protection. It has been proposed that correlates of risk of tuberculosis disease could guide future exploration of correlates of protection. Unfortunately, no validated clinical correlate of either risk or protection currently exists.

The tuberculin skin test reaction in individuals who have received the BCG vaccine correlates poorly with subsequent development of active disease[22]. Therefore, in clinical trials of new tuberculosis vaccines, the vaccine response (immunogenicity) is now commonly determined by measuring specific $CD4^+$ T cell responses, particularly the production of T_H1 cell-associated cytokines such as IFNγ. However, Kagina *et al.* recently reported that specific $CD4^+$ T cell responses 10 weeks after BCG vaccination of newborns do not correlate with ultimate risk of tuberculosis disease[23]. Importantly, $CD4^+$ T cell expression of IFNγ, co-expression of IFNγ, TNF and IL-2 (by polyfunctional $CD4^+$ T cells) or $CD8^+$ T cell responses did not correlate with risk of disease during the first 2 years of life. By contrast, antigen-specific polyfunctional T cells in animal models have been correlated with protection against intracellular pathogens such as *Leishmania major*[24], and studies in macaques have suggested an importance for $CD8^+$ T cells in the control of *M. tuberculosis*[25].

These results suggest that the immune responses that are crucial for protection against clinically active *M. tuberculosis* infection may not necessarily translate into correlates of protection or risk in humans. Furthermore, the failure to identify such correlates may highlight the limitations of sampling peripheral blood instead of the site of infection (the lung); however, the procedures used to obtain lung samples can only be performed in highly specialized facilities. $CD4^+$ T cell responses at this mucosal site, including those measured in local lymph nodes, have shown the best correlation with protection in mice[26]. There are also multiple examples of antigen-specific $CD4^+$ T cell responses in the spleen of mice that have correlated with protection[27], and these studies also highlighted the importance of measuring responses at an optimal post-vaccination time point. It is noteworthy that an association between $CD4^+$ T cell response and protection against tuberculosis in mice has not been a universal finding; other studies suggest that IFNγ levels may simply be a measure of inflammatory status[28]. This is also supported by human studies of tuberculosis disease, and it emphasizes that protection cannot be defined by peripheral blood T_H1 cell responses alone.

Assays to determine the inhibition of *M. tuberculosis* growth evaluate both T cell and innate immune cell functions. Bacterial growth is inhibited when *M. tuberculosis* is incubated with peripheral blood mononuclear cells (PBMCs) or whole blood from BCG-vaccinated adults, but not during incubation with cells from non-vaccinated individuals[29]. As mentioned above, multiple animal studies have suggested important roles for various subsets of T cells, such as T_H17 cells, in addition to T_H1 cells. Moreover, a well-orchestrated, balanced immune response, rather than simply an effector or memory T_H1 cell response, may be vital for protection. For example, cynomolgus monkeys that developed tuberculosis disease following low-dose aerosol infection had lower numbers of $CD4^+$ T_{Reg} cells in the bronchoalveolar lavage fluid than monkeys that did not progress to disease[30]. Furthermore, γδ T cells[31] and T cells restricted by non-classical molecules — such as CD1, HLA-E and MR1 (MHC class I-related protein) — also have a role in protection and should be included in hypothesis-driven approaches for describing immune correlates of protection.

Human immune responses to *M. tuberculosis* and tuberculosis vaccines, on both individual and population levels, are characterized by marked heterogeneity. For example, Malawian BCG recipients produce significantly less *M. tuberculosis*-specific IFNγ than British vaccinees[6], suggesting a role for genetic and/or environmental factors (such as environmental mycobacterial exposure) in the immune response to BCG. This raises the possibility that identified biomarkers may not apply to all individuals within a population or across different populations. Given this hurdle, and the limitations of working primarily with peripheral blood rather than with material from the site of disease, we propose that new approaches to identify correlates of risk and protection should account for host heterogeneity and not be limited to hypotheses that focus primarily on T cells. Ongoing analyses of large clinical cohorts that aim to find correlates of risk for tuberculosis disease have therefore focused on data-driven methods, such as global screens with 'omics' approaches, including transcriptomics (see below).

Taken together, these studies suggest that the immune responses that have so far been considered to be crucial for protection against tuberculosis, including IFNγ production and $CD4^+$ T cell responses, are not sufficient for protection and do not represent usable correlates of risk or protection in the context of vaccine trials.

Host markers for active tuberculosis

Early accurate diagnosis of active tuberculosis disease is important to stop transmission, but current diagnostic tests are inadequate[32]. We continue to rely on the century-old sputum smear test in most high-tuberculosis-endemic countries. Furthermore, specific challenges are associated with the diagnosis of extrapulmonary tuberculosis, sputum smear-negative tuberculosis (active pulmonary tuberculosis with less than 10,000 bacilli per ml of sputum) and childhood tuberculosis.

Antigen-induced IFNγ screening for active and latent tuberculosis. For the past century, the tuberculin skin test has been the only available screening test for latent tuberculosis infection, but this method cannot differentiate between *M. tuberculosis* and environmental mycobacterial infection. A decade ago, T cell-based IFNγ release assays (IGRAs) were developed, which assess IFNγ production after *in vitro* stimulation of

Tuberculin skin test reaction
A delayed-type hypersensitivity reaction following intradermal injection of purified *M. tuberculosis*-derived proteins. The tuberculin skin test is also known as the Mantoux test and is used as a diagnostic tool for latent *M. tuberculosis* infection.

γδ T cells
T cells that express the γδ T cell receptor. These cells are present in the skin, vagina and intestinal epithelium as intraepithelial lymphocytes. Although the exact function of γδ T cells is unknown, it has been suggested that mucosal γδ T cells are involved in innate immune responses.

Table 1 | **Differentiating markers for tuberculosis**

Biomarkers	Diagnosis	Correlate of risk or of protection*	Treatment outcome‡	References
Cytokines and chemokines				
IFNγ	Latent or active TB	Vaccine efficacy or disease progression	Treatment response	70–73
CXCL10, IL-10	Active TB	Increased after BCG	Under evaluation	74–77
IL-6	Active TB	Increased after BCG	Treatment response	72,77
IL-4	Active TB	Progression	Under evaluation	78,79
CXCL8, CCL8 and IL-12	Active TB	ND	Under evaluation	75,76,80,81
IL-4δ2/IL-4 ratio	Extent of disease	ND	Treatment response	54,82
IFNγ/IL-4 ratio	Latent or active TB	ND	Treatment response	54
IL-17 and TNF	Latent or active TB	Increased after BCG	Under evaluation	81
Receptors and soluble receptors				
Soluble urokinase PAR	Extent of disease	ND	Treatment response	83,84
Soluble ICAM1	Extent of disease	ND	Treatment response	83,85,86
Soluble E-selectin receptor	Mtb infection status	ND	ND	86
Soluble IL-2R, soluble TNFR1, soluble TNFR2	Extent of disease	ND	Treatment response	83,87–89
CD11c	Extent of disease	ND	Treatment response	90
LAG3	Extent of disease	ND	Treatment response	83
CXCR4, CCR5	Extent of disease	ND	ND	91
Other inflammation markers				
Neopterin	Extent of disease	ND	Treatment response relapse	92
Procalcitonin	Extent of disease	ND	ND	93
CRP	Extent of disease	ND	Treatment response or treatment failure	83,93
Granzyme B	Extent of disease	ND	Month 2 sputum conversion	83
Adenosine deaminase	Extent of disease	ND	Treatment response	94
Immune cells and their markers				
Polyfunctional T cells	Mtb infection status	Vaccine efficacy (inconsistent data)	Treatment response	44,45, 55,77,95
Single-positive TNF-expressing $CD4^+$ T cells	Mtb infection status	ND	ND	47
MHC class I and II tetramer-specific T cells	Under evaluation	ND	ND	96,97
$CD3^{low}CD56^+$ NKT cells	Mtb infection status	ND	Treatment response	98
FOXP3, CXCL8, IL-12β	Mtb infection status	ND	ND	42
TNF/TNFR1 ratio	Extent of disease	ND	ND	88
Total white blood cell and monocyte or neutrophil numbers	Extent of disease	ND	Treatment response	97
Antibodies to Mtb antigens and autoantibodies				
Antibodies specific for 38 kDa antigen, ESAT6 and LAM	Mtb infection status, extent of disease	ND	Treatment response	99
Antibodies specific for Rv3369 and CFP10	Mtb infection status	ND	ND	57
Antibodies specific for 38 kDa antigen, MPT64, TRXC and HSPX	Extent of disease	ND	ND	63
Antibodies specific for alanine dehydrogenase and malate synthetase	No differences between TB and control	ND	Treatment failure	99
BPI-specific ANCA	ND	ND	Treatment response	100

Table 1 (cont.) | **Differentiating markers for tuberculosis**

Biomarkers	Diagnosis	Correlate of risk or of protection*	Treatment outcome‡	References
Differential gene or protein expression profiles				
CIS, SOCS3, IL-2RA, JAK3, PIM1	Diagnosis active TB, latent TB	ND	ND	101
Lactotransferrin, CD64 and RAB33A	Mtb infection status, extent of disease	ND	ND	59
FcγRIB	Mtb infection status, extent of disease	ND	ND	61
RIN3, LY6G6D, TEX264, MP68, SOCS3, KIAA2013, ASNA1, ATP5G1, NOLA3	Mtb infection status	ND	Treatment response	60
SAA, transthyretin, neoptertin, CRP	Mtb infection status	ND	Treatment response	66
Neutrophil driven transcript signature of IFNγ and type I IFN signalling	Mtb infection status, extent of disease	ND	Treatment response	58

ANCA, anti-neutrophil cytoplasmic autoantibodies; ASNA1, arsenite-stimulated ATPase; ATP5G1, mitochondrial ATP synthase lipid-binding protein; BCG, *Mycobacterium bovis* bacillus Calmette–Guérin; BPI, bactericidal permeability-increasing protein; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CFP10, 10 kDa culture filtrate antigen; CIS, cytokine-inducible SH2-containing protein; CRP, C-reactive protein; CXCL, CXC-chemokine ligand; CXCR, CXC-chemokine receptor; ESAT6, 6 kDa early secretory antigenic target; FcγRIB, high-affinity IgG Fc receptor IB; FOXP3, forkhead box P3; HSPX, heat shock protein X, ICAM1, intercellular adhesion molecule 1; IFN, interferon; IL, interleukin; IL-2RA, IL-2 receptor antagonist; JAK3, Janus kinase 3; LAG3, lymphocyte activation gene 3; LAM, lipoarabinomannan; MP68, 6.8 kDa mitochondrial proteolipid (also known as C14orf2); Mtb, *Mycobacterium tuberculosis;* ND, not determined; NKT, natural killer T; NOLA3, nucleolar protein family A, member 3; PAR, plasminogen activator receptor; PIM1, proto-oncogene serine/threonine-protein kinase PIM1; RIN3, RAS and RAB interactor 3; SAA, serum amyloid A protein; SOCS3, suppressor of cytokine signalling 3; TB, tuberculosis; TEX264, testis-expressed gene 264; TNF, tumour necrosis factor; TNFR, TNF receptor; TRXC, thioredoxin. *Correlates of risk of tuberculosis are markers that are associated with low risk of disease development or the absence of markers associated with high risk of disease, whereas correlates of protection against tuberculosis reliably predict the level of protective efficacy induced by a vaccine on the basis of differences in the immunological measurements of vaccinated and unvaccinated groups. ‡Treatment outcome includes early treatment effect as measured by conversion of sputum smear or culture tests from positive to negative, cure or failure to achieve cure after treatment, and relapse or relapse-free status after initial cure.

whole blood or PBMCs with *M. tuberculosis*-specific immunodominant antigens, such as 6 kDa early secretory antigenic target (ESAT6), 10 kDa culture filtrate antigen (CFP10) and TB7.7 (REFS 33,34). These assays have now become the gold standard for the identification of sensitization to *M. tuberculosis*[35]. However, evaluation of IGRA results for the detection of latent tuberculosis has been difficult owing to the absence of a gold standard for latency.

A meta-analysis of these studies showed that IGRAs are as sensitive as and more specific than the tuberculin skin test[36]. However, a multi-centre Phase III clinical study demonstrated that IGRAs are unsuitable for diagnosing active disease, particularly in high-tuberculosis-endemic areas[37]. Nevertheless, some studies suggest that the response detected by IGRAs, when quantified, is stronger in patients with active tuberculosis than in those with latent tuberculosis[38]. Furthermore, IGRAs performed on T cells isolated from the site of tuberculosis disease (for example, pleural effusions or cerebrospinal fluid) were found to be highly sensitive and specific[39,40]. In addition, multi-cytokine biosignatures may differentiate between active or latent tuberculosis[41]; the expression levels of mRNA transcripts encoding CXC-chemokine ligand 8 (CXCL8; also known as IL-8), the T_{Reg} cell-associated transcription factor forkhead box P3 (FOXP3) and IL-12β following ESAT6 stimulation of PBMCs hold promise in this regard[42]. Similarly, Djoba *et al.* have shown that cytokine expression levels in the blood can differentiate between pulmonary and pleural tuberculosis[43].

It has also been suggested that distinct cytokine expression profiles of $CD4^+$ T cells are associated with the bacterial loads of different infection states[44]. Latent infection is dominated by the presence of T cells secreting IL-2 only or IFNγ and IL-2, whereas T cells secreting only IFNγ are more frequent during active disease[45]. More recently, *M. tuberculosis*-specific T cells secreting only TNF have been found to be more frequent in individuals with active tuberculosis, and this may have an application as a new diagnostic test for active disease versus latent infection[46].

Serological biomarkers for active tuberculosis. Serological tests based on the detection of circulating antibodies against *M. tuberculosis*-specific antigens have several advantages, as they are simple, cheap and feasible for point-of-care diagnostics. However, a comparative study of 19 commercially available tests found sensitivities ranging from 0.09% to 59.7%[47]. Specificities ranged from 53% to 98.7%, and the tests with high specificities frequently exhibited poor sensitivity. Potential *M. tuberculosis* antigen targets were reviewed in a recent meta-analysis by Steingart *et al.*[48]. A total of 254 studies were identified, encompassing 9 native and 27 recombinant proteins, 15 lipid-derived antigens and an additional 30 combined antigen targets, but low sensitivity and specificity limits the use of these serological tests.

A recent study used a systems immunology approach involving high-throughput screening to detect antibodies to the entire *M. tuberculosis* proteome in sera from 500 patients. The results showed that antibody responses correlated with bacterial burden and that a small pool of antigens is recognized during active tuberculosis, suggesting a diagnostic potential for these antibodies[49]. Therefore, combinations of host markers, including serological markers, need to be evaluated further to identify biosignatures with diagnostic potential for active disease.

Meta-analysis
A statistical approach that combines results from multiple related studies to define a composite effect. When applied to genome-wide association studies, more modest association effects can be identified.

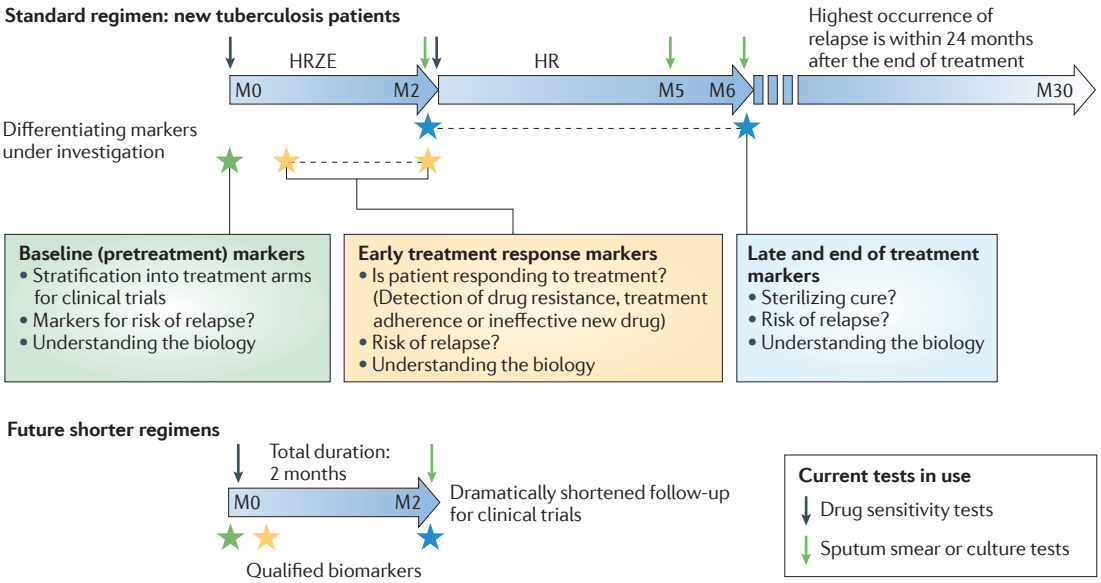

Figure 3 | **Tuberculosis treatment and the potential role of biomarkers in clinical decision making and clinical trials.** Patients diagnosed with a first episode of tuberculosis are treated for 2 months with four drugs — isoniazid, rifampicin, pyrazinamide and ethambutol; collectively known as HRZE — and subsequently with isoniazid and rifampicin (HR) for 4 months[69]. Patients with recurrent tuberculosis have an increased risk for drug-resistant tuberculosis and are treated for even longer periods, and drug-resistant tuberculosis is treated for 20 months or longer (not shown). The aim is to eventually develop 2-month treatment regimens, but the current drug combinations have high relapse rates if treatment duration is shorter than 6 months. Baseline testing for drug susceptibility is recommended and this is also performed after 2 months of treatment if sputum smear or culture tests remain positive. However, the results of these tests at month 2 have poor individual predictive ability for ultimate treatment outcome. Differentiating markers are currently being investigated at baseline, early during treatment (in the first 2 months), later during treatment (up to month 6) and after treatment completion. Qualified biomarkers are needed to accelerate the development of new, shorter treatment regimens, to guide clinical decision making for individual patients and to increase our understanding of the biology of host–pathogen interactions. Baseline markers may allow the stratification of patients into different treatment arms, based on the extent of their disease and the risk for poor treatment response. Early treatment response markers will give an earlier indication of response to chemotherapy than sputum tests and will alert clinicians to the potential presence of drug resistance, poor treatment adherence or ineffective drugs in clinical trials. Late and end of treatment markers are needed to indicate sterilizing cure or persistence of live, albeit non-replicating, bacteria, which may subsequently lead to relapse. The development and subsequent implementation of future shorter treatment regimens will be greatly aided by qualified biomarkers at baseline, during early treatment and at the end of treatment.

Biomarkers for treatment response

The different outcomes of antituberculous chemotherapy and the underlying continuum of infection phases are summarized in FIG. 2. Biomarkers for the outcome of tuberculosis treatment are most urgently needed both for clinical decision making and to facilitate the shortening of clinical trials of new antituberculosis drugs or regimens. Current tuberculosis treatment strategies and the role of treatment response markers in developing new regimens are summarized in FIG. 3.

Pretreatment markers with predictive ability. Baseline biomarkers could identify the requirements of individual patients for specific treatment regimens. Individuals with high bacterial burden and extensive inflammation may require longer treatment regimens than individuals with minimal disease. For clinical trials, such biomarkers could ensure standardization across treatment groups and thereby reduce the required study size. High baseline bacterial load, measured by time-to-detection in liquid culture, low body mass index and more extensive disease on chest X-rays at diagnosis have been shown to predict relapse following treatment[50,51]. However, results for baseline bacterial load tests take several days or even weeks, and X-rays are not universally available and their assessment is difficult to standardize.

The decision to shorten treatment time cannot just be based on the presence or absence of cavities on chest X-rays at the time of diagnosis and on early treatment response. Indeed, Johnson *et al.* found an increased relapse rate when tuberculosis treatment was reduced from 6 to 4 months in patients without chest X-ray cavities and whose sputum culture test was negative 2 months after treatment initiation[51]. Although none of the participants in this study had cavities, the individuals who suffered a relapse had more extensive lung damage and a higher bacterial burden on initial diagnosis than the patients who were cured. This suggests that more sophisticated measures of disease severity at diagnosis and of treatment response may be required to guide treatment regimens. Therefore, host immunological markers that correlate with extent of disease and may indicate a risk for relapse at baseline are currently being investigated.

Baseline biomarkers
Markers that can be measured at diagnosis of tuberculosis disease before the commencement of treatment.

Time-to-detection in liquid culture
The number of days until growth of *M. tuberculosis* is detected in liquid culture medium.

Host markers during early treatment. Currently, the earliest measures of the effect of treatment are sputum smear or sputum culture conversion from positive at baseline to negative 2 months after starting therapy. This time interval of 2 months is a prohibitive delay for clinical management and for clinical trials of new drugs. Ineffective treatment allows unchecked bacterial replication and ongoing tissue destruction, spread of bacteria to other tissues and even the development of drug resistance in bacteria exposed to suboptimal drug combinations. Combinations of host immunological and clinical markers that are better indicators of early response to treatment and that reflect restoration of the balance of pro- and anti-inflammatory responses may therefore be useful, possibly in conjunction with markers of the extent of disease (TABLE 1).

Although host pro-inflammatory signatures may not be specific for tuberculosis, they may still be very useful if interpretation is performed within a specific clinical context or in conjunction with tuberculosis-specific markers.

IGRAs have not proven useful for monitoring treatment success[52], although the levels of IFNγ in PBMC stimulation assays using recombinant *M. bovis* BCG 32 kDa protein (also known as Ag85A) increase during treatment of tuberculosis. By contrast, IL-10 levels decrease, and the IFNγ/IL-10 ratio correlates with treatment success and can also distinguish between active and latent tuberculosis[53]. Similarly, the ratio of IL-4 and its antagonistic splice variant IL-4δ2 increases during tuberculosis treatment, and changes in the IL-4/IL-4δ2 ratio occur early during tuberculosis treatment and may predict subsequent outcome[54].

End of treatment markers for relapse. Shorter treatment regimens are a major goal of new drug development, but relapse will be the main risk of such new therapies. As relapse usually occurs within 2 years of treatment completion, any study evaluating shortened regimens would have to follow up cured tuberculosis patients for more than two years, and this would substantially increase the complexity and cost of the study. Therefore, a marker of sterilizing cure would answer an important clinical and drug trial need.

After tuberculosis treatment, the frequencies of certain T cell populations — T cells secreting IFNγ only, those secreting both IFNγ and IL-2 (REF. 55) and IFNγ-, IL-2- and TNF-secreting T cells[44] — change to the frequencies found in latently infected individuals. The presence of *M. tuberculosis*-specific effector memory T cell responses after a previous episode of spontaneously cured active tuberculosis (that occurred in the pre-antibiotic era, for example) may indicate the persistence of live bacteria and a return to a stable, quiescent phase of infection. By contrast, some individuals retain only central memory T cells, suggesting that the bacteria have been eradicated[56]. No data are available for the persistence of memory T cells as predictors of relapse. However, as a quiescent phase may subsequently revert to a phase of bacterial replication and relapse with active tuberculosis the implications of memory T cell phenotypes at the end of treatment should be further investigated.

Cured tuberculosis patient
A patient whose sputum smear tests (and sputum culture tests, if available) are negative in both the last month of treatment (conventionally month 6) and on at least one previous occasion. This does not necessarily equate to sterilizing cure.

In sum, certain immune markers hold promise as potential treatment biomarkers. These could replace the current baseline measures of the extent of disease, as well as the earliest treatment response markers at month 2, and could also help to identify markers for relapse.

A new direction for biomarker discovery

Owing to our limited knowledge of host immune responses to *M. tuberculosis* infection and the resultant lack of adequate biomarkers for the different phases of infection, the use of 'omics' approaches may be needed for biomarker discovery. Notably, several recent studies of this type have reported encouraging results[57–60].

Transcriptomic signatures. Transcriptomic studies from high endemic areas of tuberculosis have identified signatures involving host gene expression profiles that differentiate between different *M. tuberculosis* infection states. The first study used unique transcriptional features to identify nine RNA transcripts from whole blood, and these were used to successfully differentiate between patients with active, cured and recurrent tuberculosis, as well as latently infected individuals[60] (TABLE 1). Similarly, Maertzdorf *et al.*[61] identified unique transcriptional profiles that could distinguish between active tuberculosis, latent infection and uninfected donors. High-affinity IgG Fc receptor IB (FcγRIB) was the most differentially expressed gene and, together with four other transcripts, could discriminate between active disease and latent infection. Genes related to the innate immune response were overexpressed in active tuberculosis, whereas genes related to apoptosis and NK cell activity were upregulated during latent tuberculosis. Another study reported that a minimal group of genes (lactotransferrin, *FCGR1A* (also known as *CD64*) and *RAB33A*) was sufficient for classification of uninfected and latently infected individuals and patients with active tuberculosis. RAS and RAB interactor 3 (RIN3) was also identified as important when comparing active, recurrent, cured and latent tuberculosis[60].

Molecular profiling in heterogeneous tissues, such as blood, is confounded by the relative proportions of different cell types in such tissues. Separation of tissues into pure cell populations by antibody-based methods or by microdissection would be appropriate but is not always feasible. An *in silico* deconfounding approach may offer an alternative to cell-specific molecular profiling. In this approach, normalization of gene expression data takes into account the number of cell types, the relative proportions of different cell types and cell type-specific gene expression profiles in heterogeneous tissues[62]. Using whole blood, a recent study showed that transcriptional profiles correlate with the extent of tuberculosis disease and change during treatment[63]. In addition, a specific transcript signature that was characterized by neutrophil-dependent IFNγ and type I IFN signalling could distinguish between active tuberculosis and other inflammatory diseases.

Other recent technological advances may also feature largely in future biomarker research. For example, deep sequencing allows for the detection of epigenetic changes, including alterations in DNA methylation and in the

MicroRNAs
Small RNA molecules that regulate the expression of genes by binding to the 3′-untranslated regions (3′-UTRs) of specific mRNAs.

transcription of microRNAs. MicroRNAs do not encode proteins but possess regulatory functions and can alter gene expression. They have been shown to have significant roles in tumour biology, as well as in cardiovascular and rheumatic diseases. MicroRNAs are also involved in regulating inflammation and possibly infectious disease[64] and may constitute useful biomarkers for tuberculosis.

Proteomic and metabolomic profiling. Tuberculosis may be differentiated from other infectious and inflammatory conditions based on proteomic fingerprinting of serum using surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry and ProteinChip array technology[65]. This single analytical method can detect a very large number of peptides, although it is relatively insensitive. Using this technology, Agranoff and colleagues found that serum amyloid A protein and transthyretin were among the most promising markers to differentiate tuberculosis from other infectious and inflammatory conditions[66].

Another approach focuses on the 'metabolome', which is the set of small molecules that encompasses metabolic intermediates, hormones and other signalling molecules, and secondary metabolites[67]. The potential advantage of metabolomics is the reduced number of potential markers in a single biological specimen. The main disadvantage is that multiple analytical methods seem to be necessary to complete the characterization of these markers. However, no metabolomic data have been reported for tuberculosis to date.

So, unbiased 'omics' approaches, which are largely hypothesis generating and may guide more focused hypothesis-driven approaches, could culminate in qualified biomarkers for use in patient care and in clinical trials.

Conclusions and future perspectives

The challenges posed by *M. tuberculosis* infection, through its interaction with the immune system and its mechanisms for evasion, require many more breakthroughs from basic science research if we are to make a significant impact on the worldwide tuberculosis problem. Currently, appropriate samples still need to be collected from individuals with clinically characterized protection and susceptibility phenotypes in different populations. The recent advances in technology provide the capacity to search for biomarkers in an unbiased manner using complementary technological platforms, although this requires high-level bioinformatics support.

Host molecules that are present at different levels in clinical phenotypes do not necessarily constitute biomarkers. Most markers discussed in this Review represent differentiating markers, which are markers that are differentially expressed in people with specific outcomes of infection or treatment. These markers are identified on the basis of general exploratory data and have not reached the status of qualified biomarkers.

A very significant effort is required to conduct biomarker validation and biomarker qualification before a differentiating marker can become a qualified biomarker, which is one that has undergone multistep and comprehensive evaluation to confirm precision and accuracy with diagnostic or prognostic value[68]. New signatures should be validated in a second cohort and may then be used to guide further exploration. This process has to be carefully conducted, as many initially promising markers eventually prove disappointing and many valuable markers are probably never evaluated sufficiently. Our current understanding of host–pathogen interactions and their dynamic nature must be carefully considered in clinical study design to ensure that appropriate and well-differentiated clinical phenotypes are selected for these expensive technologies.

If accurate differentiation between infection and disease states can be achieved, then it could eventually become possible to develop simple point-of-care tests. These could include hand-held devices based on lateral-flow technology that would detect multi-marker signatures (which are much more likely to be successful than single molecules) in patient samples such as serum by immunochromatography (in a similar way to pregnancy tests that detect human chorionic gonadotropin in urine), or hand-held devices for the detection of multi-gene expression signatures.

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Competing interests statement
The authors declare no competing financial interests.