Tuberculosis biomarkers: from diagnosis to protection

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Abstract

New approaches to control tuberculosis (TB) worldwide are needed. In particular, new tools for diagnosis and new biomarkers are required to evaluate both pathogen and host key elements of the response to infection. Non-sputum based diagnostic tests, biomarkers predictive of adequate responsiveness to treatment, and biomarkers of risk of developing active TB disease are major goals. Here, we review the current state of the field. Although reports on new candidate biomarkers are numerous, validation and independent confirmation are rare. Efforts are needed to reduce the gap between the exploratory *up-stream* identification of candidate biomarkers, and the validation of biomarkers against clear clinical endpoints in different populations. This will need a major commitment from both scientists and funding bodies.

Introduction

Tuberculosis (TB) is a communicable infectious disease, spread almost exclusively by coughed aerosols carrying pathogens from the Mycobacterium tuberculosis (Mtb) complex. TB is characterized pathologically by necrotizing granulomatous inflammation usually in the lung, although almost any extra-pulmonary site can be involved. TB remains one of the most significant infectious causes of mortality and morbidity worldwide. As reported by the World Health Organization (WHO) it causes disease among 9.6 million people each year and ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide.[1] In 2014, 1.5 million TB deaths were reported and among them approximately 140,000 were children. The number of TB deaths is unacceptably high because with a well-timed diagnosis and appropriate treatment, almost all people with TB can be cured. Therefore all efforts to fight TB must be intensified. Additionally, it is estimated that one-third of the world's population is latently (asymptomatically) infected with Mtb, and approximately 3 to 10% of these infected individuals are likely to progress to active disease during their life. The risk of reactivation and subsequent disease and mortality is significantly increased in individuals with HIV coinfection[2,3] and therapy with TNFa inhibitors.[4,5] Approaches to decrease TB morbidity and mortality, along with Mtb transmission, rely on effective treatment, correct diagnosis, and prevention of infection and disease.

Effective therapy is central to any strategy for controlling TB and biomarkers that indicate initiation of successful treatment could facilitate development of alternative treatment strategies. There is a need for shorter treatment regimens to increase compliance. Unfortunately, recent studies have not shown definitive results.[6-8] In fact, in the REMoxTB Clinical Trials experience, despite early effectiveness (superior early bactericidal activity and 2-month culture conversion rates in patients treated with moxifloxacin compared to the standard regimen), the 4-month-regimen was less effective than the standard 6-month regimen in preventing TB recurrence.[6,8] Moreover, correct and efficacious treatment is also needed to avoid multidrug-resistant (MDR)-TB. Globally, an estimated 3.3% of new TB cases and 20% of previously treated cases had MDR-TB in 2014.[1] This translates into an estimated 480,000 people having developed MDR-TB in 2014 with a treatment success rate of only 48%.[1] Patients with MDR-TB urgently require treatments that quickly eradicate active infection while preventing emergence of additional resistance, which otherwise causes treatment failure and death.

Accurate diagnosis is also a cornerstone of TB control. Active TB diagnosis is based on the detection of Mtb in sputum, which depends on the presence of necrotic infection foci in proximity to the airways. The diagnosis then is based on sputum smear and culture,[9-12] and more recently positive GeneXpert MTB/RIF tests.[13] Microscopy is largely available and highly specific, but lacks sensitivity, missing the diagnosis in over one third of patients seeking care.[9,14] Mycobacterial culture remains the gold standard for TB diagnosis, but provides results only after considerable delay (3-4 weeks). All these diagnostic tests require a Mtb-positive sputum while many active TB patients, including HIV-coinfected individuals, diabetes patients, and children, often do not present with Mtb positive sputum.[15,16] In pulmonary TB a positive microbiological diagnosis inevitably means the presence of Mtb in the airway secretions, such that in all likelihood Mtb has been already transmitted to others. By definition, sputum diagnostics are not useful in extra-pulmonary disease, the diagnosis of which relies on samples (tissue or biological fluids as pleural-, cerebral-, synovial-fluids) collected by invasive procedures. For all of these reasons, there is need for the development of highly sensitive and specific diagnostic tests for TB to rapidly identify – or rule out – the presence of active disease. These tests need to perform in endemic settings with limited laboratory facilities, at low cost, using easily accessible non-sputum based samples such as blood, urine, or breath. These are the four high priority target product profiles (TPPs) recently published by the WHO as a result of a consensus meeting on new TB diagnostics.[17] Therefore the urgent need to search for bio-

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markers (defined as measurable characteristics that indicate normal or pathogenic biological processes, or pharmacological responses to therapeutic intervention)[18] needs to be highlighted: biomarkers can serve as surrogate endpoints in clinical trials, and can be used to improve treatment outcome by informing therapeutic decisions for individual patients.[19] Sputum culture conversion using solid medium is the best-characterized TB biomarker for successful treatment, having been examined in many studies either as a simple measure (*e.g*. month 2 culture status) or in more complex forms requiring subsequent negative cultures (*e.g. stable* culture conversion). However, as reported above, in the REMoxTB trial patients developed recurrent TB despite negative sputum cultures at month 2. Thus better biomarkers predictive of TB treatment outcome are needed.[20,21] This is a priority for the TB research field and has the potential to impact not only research but also clinical practice globally.[22-24]

In this paper we will review most of the recent advances in research into TB biomarkers for the diagnosis of active TB, latent TB infection (LTBI) and prevention of TB disease.

Biomarkers for diagnosing active tuberculosis

We may distinguish biomarkers related to the pathogen and to the host (Figure 1). From the pathogen perspective, Mtb products could be detected directly in blood, sputum or urine. Mtb DNA can be detected in blood and urine of pulmonary TB patients with a better sensitivity than Mtb culture from the same biological fluid.[25-27] The Mtb cell wall component lipoarabinomannan (LAM) has been proposed as TB biomarker; however the available commercial test on urine has a poor sensitivity.[28] This can be partly enhanced by other LAM assays.[29-31] Although unsatisfactory as yet, in HIV-infected patients the Mtb DNA and LAM detection in urine may be an important tool to consider especially for those advanced cases with low CD4 T-cell counts.[32-34] The Mtb Ag85 complex is a 30-32 kD family of three proteins (Ag85A, Ag85B, and Ag85C) with enzymatic mycolyl transferase activity involved in the coupling of mycolic acids to the arabinogalactan of the cell wall and in the biogenesis of the cord factor.[35,36] The detection of Ag85 in blood and urine, however, shows highly variable performance in different studies.[29,37,38]

Among the host biomarkers, there are various non-sputum based-assays for active TB diagnosis, relying on serum, plasma, urine or stimulated or unstimulated blood. Considering serum or plasma products, Mtb specific antibody detection is not a promising diagnostic approach due to heterogeneity of the response to Mtb.[11,39] Moreover WHO negatively advised on the use of such tests for diagnosing active TB disease.[40] The evaluation of serum microRNAs has shown different levels of accuracy for diagnosing active TB in drug-sensitive and drug resistant TB.[41-44]

A broad range of potential transcriptional TB biomarkers has been reported. Modular and pathway analysis revealed that the neutrophil driven interferon (IFN)-inducible gene profile, consisting of both Type 2 (IFNγ) and Type I (IFNαβ) IFN signaling represented a significant TB signature detectable in the peripheral blood from pulmonary TB patients.[45] These findings have been also validated in other populations,[21,46-50] and in several studies could differentiate TB from other respiratory infections and inflammatory diseases.[24,45,49,51] Moreover it has been shown that disease activity increased the signature whereas treatment decreased it.[21,22,49] Integrated analysis of gene expression signatures obtained in eight independent studies revealed additional pathways that are likely to contribute to discrimination of TB disease from other diseases.[52] Diagnostic signatures to distinguish TB from other diseases and from LTBI were also found in children from South Africa, Malawi and Kenya.[53] However one of the major challenges in the evaluation of new childhood TB diagnostic is the lack of a reference, due to the difficulty of microbiological diagnosis of active disease. Taking all these studies together it is important to mention that the minimum TPP requirements are not yet satisfied in terms of sensitivity and specificity. The complexity of the analysis and the expensive molecular techniques related to the transcriptional profiles make it currently difficult to be used as routine diagnostic tests unless easier technologies are developed.[52] However, all studies reported above are important for our comprehension of TB pathogenesis.

The interferon (IFN)γ inducible protein 10 (IP10) was found to be increased in the unstimulated plasma of children and adults with active TB,[54-58] and has been evaluated by different methodologies including also innovative technologies based on lateral flow assays using the interference-free, fluorescent up converting phosphor (UCP) labels in multicenter studies conducted in Africa.[59-63] Interestingly, IP10 can be also detected in the urine of adult patients,[64] Ugandan children with active TB,[58] and IP10 levels decreased after efficacious therapy.[64] In comparison with blood, urine biomarkers offer the advantage of non-invasive sample collection, especially in children, and also pose lower bio safety risks for health care workers.

Flow-cytometry has been proposed as a potential tool to help improving TB diagnosis. Advancement in multiparametric flow cytometry allows the simultaneous evaluation of several immune functions in single cells such as cytokine production and memory status. Polyfunctional T-cells, cells able to produce more than one cytokine simultaneously, have been described as part of immune response to different pathogens such as viruses, bacteria and worms.[65-68] Moreover T-cells coproducing

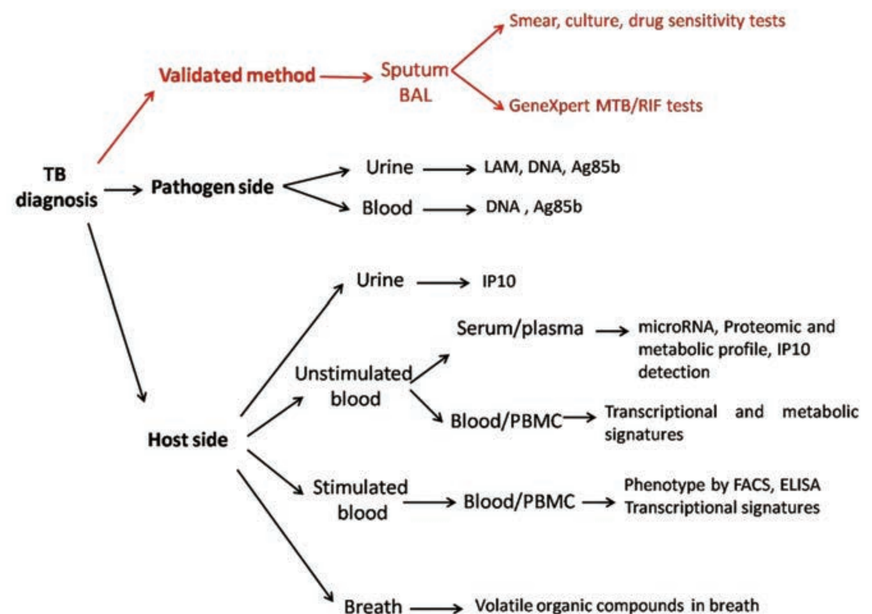

Figure 1. Flow chart of the biomarkers for active tuberculosis diagnosis. TB: tuberculosis; Ag: antigen; LAM: lipoarabinomannan; BAL: broncholavage; IP: Interferon-γ inducible protein; FACS: Fluorescence-activated cell sorting.

IFNγ, TNFα and IL2 have been associated with protective T-cell immune responses in HIV non-progressors subjects.[67] Studies evaluating the role of polyfunctional T-cells in TB did not show consistent results. Active TB has been associated with either monofunctional TNFα+CD4+ T-cells,[69] or double functional IFNγ+TNFa+ CD4+ T-cells,[70-73] or triple functional IFNγ+TNFα+IL2+ CD4+ T-cells.[74] By contrast, studies on activation and memory status of Mtb-specific T-cells seem to be more consistent, even when comparing patient populations enrolled at different sites or using different experimental settings. Effector T-cells expand during active Mtb replication, whereas memory cells associate with control and eradication of Mtb infection.[71,72,75-78] In particular, it has been shown that active TB is associated with a decrease in CD27 surface expression on circulating Mtb-antigen stimulated CD4+ T-cells.[70,77,79-81] Recently, a novel T-cell activation marker-TB (TAM-TB) assay was described for diagnosis of active TB in children.[80] The TAM-TB assay has been validated in an adult population in Tanzania and is based on the ratio of the median fluorescence intensity of all CD4+CD27+ T-cells over the median fluorescence intensity of Mtb-specific CD4+CD27+ T-cells (CD27 MFI ratio). This approach has also been tested in an adult population from a low TB endemic country and confirmed discrimination between different stages of TB infection.[77]

Another interesting blood-based study showed that the expression of immune activation markers CD38 and HLA-DR and proliferation marker Ki-67 on Mtb-specific CD4+ T-cells associated with Mtb load. The modulation of these markers accurately distinguishes active from LTBI with 100% specificity and over 96% sensitivity. These markers also correctly classified individuals who had successfully completed TB therapy, indicating a correlation with the decrease in mycobacterial load following treatment.[82] Interestingly, recently the T-cell activation has been described also as an immune correlate of risk for TB development in BCG-vaccinated infants.[83]

Among the untargeted discovery approaches to identify new markers for TB patients' stratification, the transcriptomic, proteomic, or metabolomic approaches have been used.[43,84] In particular Tientchieu *et al.* evaluated the transcriptomic and metabolic profiles of subjects infected with two different lineages of Mtb, the *M. africanum* (Maf) and Mtb before and after anti-TB therapy to access the differences in host factors and/or biological processes associated with disease pathology and response to treatment. Peripheral blood gene expression profiles were not different between Maf- and Mtb-infected patients pre-treatment but differed significantly post-treatment, and these were mainly associated with immune responses and metabolic diseases. Notably, the upstream regulator hepatocyte nuclear factor 4-α regulated about 15% of the genes differentially expressed between the groups post-treatment. The serum metabolic profiles were similar between Maf- and Mtb-infected patients both pre- and post-treatment, but significantly different between pre- and post-treatment, particularly in Mtb- than in Maf-infected groups. Using different approaches, as the mass spectrometry or protein chip technology, it is possible to have a proteomic profiling of many peptides when comparing TB patients and healthy subjects. Analysis of sera for host markers showed that transthyretin, C-reactive protein and neopterin might discriminate TB patients from subjects with other infectious and inflammatory conditions with high accuracy.[85] Similarly, sputum may also be used to analyze proteomic profiles, as data on smear-negative *vs*. smear-positive TB patients were significantly different from those found in control subjects.[86]

Volatile organic compounds (VOCs) in breath may contain biomarkers of active pulmonary TB derived directly from the infectious organism (*e.g.* metabolites of Mtb) and/or from the infected host (*e.g.* products of oxidative stress). A breath test based on the detection and quantification of VOCs identified potential biomarkers of active pulmonary TB with 85% accuracy in symptomatic high-risk subjects.[87] However, detection of VOCs is technically difficult because most breath VOCs is excreted in picomolar concentrations (parts per trillion), and most analytical instruments currently used cannot detect VOCs at such low concentrations.

Biomarkers to monitor tuberculosis therapy efficacy

The absence of satisfactory tools for monitoring TB therapy efficacy impedes optimal clinical management of patients, especially for extra-pulmonary TB where it is not possible to detect Mtb in sputum,[88,89] precluding the possibility to make a link between sputum culture and clinical outcome. The majority of publications investigating treatment response biomarkers failed to articulate the intended use and underlying TPP. Furthermore, most studies compared changes in proposed biomarkers over time during treatment without testing, or being powered to test, the correlation with patient outcome, *i.e.,* relapse-free cure (Figure 2). Study comparisons of Xpert MTB/RIF, smear microscopy and culture using both solid and liquid media have shown that the Xpert MTB/RIF assay has high sensitivity (97%) but poor specificity (49%) to identify culture positive specimens when Xpert is used as a binary readout. The quantitative measurement from the Xpert MTB/RIF assay, showed that the change in quantitative sputum bacterial load correlated with smear grades, solid culture grades, and time to liquid culture positivity.[90]

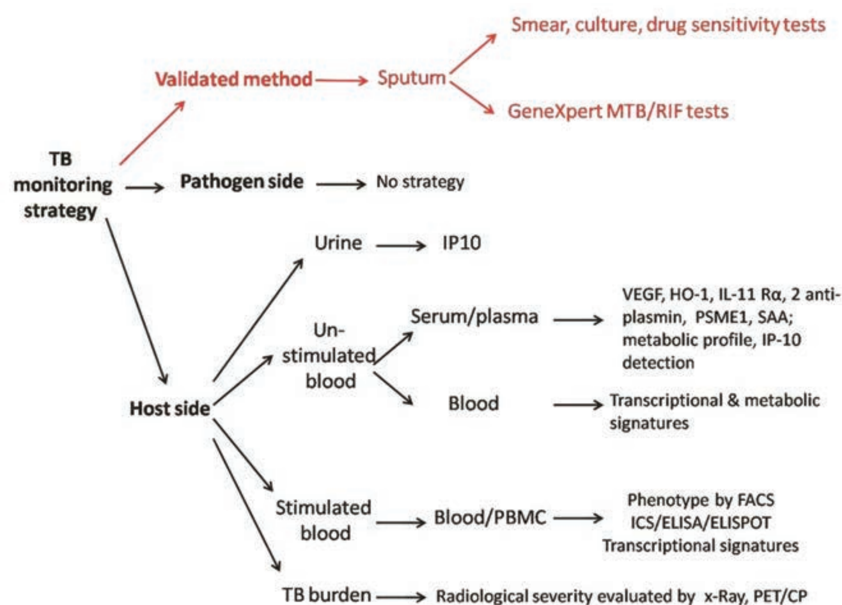

Figure 2. Flow chart of the biomarkers to monitor TB therapy efficacy. TB: tuberculosis; Ag: antigen; LAM: lipoarabinomannan; VEGF: vascular endothelial growth factor; BAL: broncholavage; IP: Interferon-γ inducible protein; FACS: Fluorescence-activated cell sorting; IL: interleukin; PSME1: proteasome activator complex subunit 1; SAA: serum amyloid A; PET/CP: positron emission tomography/computed tomography.

This quantitative data may be used in the future to predict clinical outcome of patients. Considering that the Xpert MTB/RIF test detects DNA from dead as well as live Mtb, a recent study proposed to perform the quenching of DNA detection from dead mycobacteria by adding propidium monoazide (which quenches PCR-mediated detection of DNA from dead mycobacteria) to specifically detect only viable bacilli.[91] This test seems promising since a positive correlation with time to positivity of Mtb cultures on liquid media was reported. Concerning the host side, the development of biomarkers able to monitor Mtb load is still far from reality. Recently, Cliff *et al.* reported that cytotoxic cell gene expression signatures, expressed at diagnosis might predict disease relapse after initial successful cure (sputum conversion), indicating that host factors are important indicators of treatment success.[22] The first profiles of a response to TB therapy were reported by Joosten *et al*.[23] These promising data need to be confirmed in large-scale studies. Many efforts have evaluated different proteins in *e.g*. serum or plasma samples. It has been shown that plasma vascular endothelial growth factor (VEGF)[92] concentrations at 2 weeks of therapy correlated positively with time to sputum conversion. Similarly, hemeoxygenase-1 (HO-1) and matrix metalloproteinases (MMPs) levels correlated with clinical outcome of pulmonary TB, although contrasting findings were reported.[93] This is likely due to the inhibition of MMP by CO, a product of Mtb-induced HO-1 activity, observed in vitro after infection with Mtb in human macrophages.[94] Other factors as IL11 receptor antagonist, 2-antiplasmin, proteasome activator complex subunit 1, and serum amyloid A predicted sputum conversion with an estimated 80% sensitivity and specificity.[95]

The chemokine IP10 that has increased in the unstimulated plasma of children and adults with active TB has also been measured in dry plasma spots as biomarker for therapy response.[54-58,96] In addition, IP10 kinetics in the first week of TB therapy has been proposed as a tool to confirm a clinical diagnosis and guide specific therapy.[97]

Radiological severity has been classified with different scores to predict treatment outcome in adults with pulmonary TB using different tools.[98,99] High-resolution 3-dimensional imaging helps evaluating the pulmonary TB burden during therapy.[100] In the lungs, a quantitative volumetric change in the uptake of 2-deoxy-2-[18F]-fluro-D-glucose (FDG) after 2 and 6 months of TB therapy has been detected by positron emission tomography/computed tomography (PET/CT) quantification and this modulation could be correlated with treatment outcome. However, due to machine equipment, complexity, cost and radiation exposure, the use of PET/CT approach is still restricted to clinical trials. As reported above modulation of CD27 evaluated by flow cytometric studies may be a novel marker not only for active TB diagnosis but also therapy monitoring.[70,77,80,81] The same may hold true for the modulation of CD38, HLA-DR and proliferation marker Ki-67.[82]

In analogy to cancer, an expansion of myeloid derived suppressor cells (MDSCs), which have a remarkable ability to suppress T-cell responses,[101,102] has been observed in the lung and blood of patients with active TB whereas a contraction is reported after efficacious anti-TB therapy.[102]

TB therapy significantly decreased the *in vitro* IFNγ response induced by peptides selected from ESAT-6 and CFP-10 in patients with active TB in studies conducted in Europe,[103,104] Uganda,[105] and India,[106] suggesting that this response can be a tool to monitor anti-TB treatment efficacy. The results have been confirmed using IP10 instead of IFNγ.[106,107]

Biomarkers for latent tuberculosis infection identification

Using a clinically pragmatic approach, LTBI is defined by the presence of a specific immune response detected by an IFNγ release assay (IGRA) or the tuberculin skin test (TST) (Figure 3) in the absence of lung lesions of active TB in xRay images, in individuals from whom it is not possible to isolate Mtb.[108] IGRA [QuantiFERON TB Gold in tubes (Qiagen, Venio, the Netherlands; QFT-GIT) and T-SPOT.TB (Oxford Immunotec, Marlborough, MA, USA)] measure *in vitro* IFNγ production by whole blood ELISA[109] or an enzyme-linked immunospot (ELISPOT)[110] assay on peripheral blood mononuclear cells (PBMC), respectively.[109,110] Blood is stimulated with Mtb-specific antigens,[111] which are deleted from the genome of *M. bovis* BCG and are not present in most environmental mycobacteria.[112-115] TST is based on skin infiltration caused by intradermal injection of purified protein derivative (PPD), which is a crude mixture of antigens many of which are shared by Mtb, *M. bovis*, BCG and several species of environmental mycobacteria. A particular benefit of *in vitro* testing is that there is a laboratory test with negative and positive controls, and that one visit suffices. In contrast to the TST, these *in vitro* tests may discriminate true negative responses from energy.[116] Recently an updated version of the QFT-GIT has been launched (https://www.qiagen.com/it/about-us/press-releases/pressreleaseview?id=%7Bc861949e-df50-475b-8148-b4c70034c49e%7D&lang=en).

Results from ongoing studies will show if the test has a better accuracy compared to the old QFT-GIT.[117] It should be noted that both TST and IGRAs share limitations: a low accuracy in immune-compromised patients, impossibility to distinguish between LTBI and active disease, which is a major issue in TB endemic areas, and low predicting values for active TB diagnosis.[118-119]

Several efforts have been undertaken to distinguish LTBI from active TB, with no clear success probably due to the fact that LTBI is characterized by a high heterogeneity of TB lesions that may depict as a broad spectrum of conditions that overlap in part with those seen in active disease.[120] Some subjects show only the remnant of a waning infection, while others show a slowly progressing form of disease,

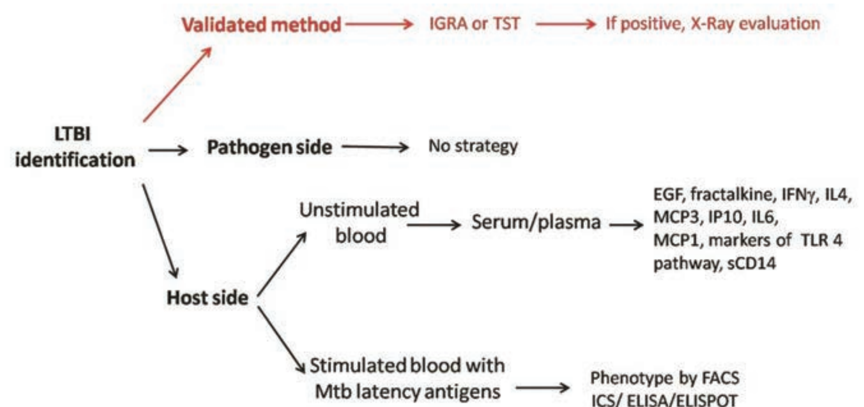

Figure 3. Flow chart of the biomarkers for LTBI identification: LTBI: latent tuberculosis infection; TST: tuberculin skin test; IGRA: IFNγ release assay; Interferon-γ inducible protein; IL: interleukin; PSME1: proteasome activator complex subunit 1; EGF: endothelial growth factor; MCP: monocyte chemoattractant protein; TLR: toll like recepetors; sCD14: soluble CD14; FACS: Fluorescence-activated cell sorting; ICS: intracellular staining.

or a chronic non-progressing infection.[120]

In the QFT-GIT format the chemokine IP10 has been suggested as an alternative marker for IFNγ,[106,121-123] with high accuracy in the HIV-infected patients.[122,124,125]

Besides IGRA, several approaches for LTBI identification have been proposed: evaluation of plasma concentrations of epidermal growth factor fractalkine, IFNγ, IL4, monocyte chemoattractant protein (MCP)3, IP10;[126] evaluation of serum pro-inflammatory cytokines IL6, IP10, MCP1;[127] detection of plasma levels of markers involved in the Toll-like receptor 4 pathway, like soluble CD14 and myeloid differentiation-2.[128] A recent study on LTBI subjects demonstrated that Mtb-specific CD4+ T-cells have a characteristic chemokine expression signature ($CCR6^+CXCR3^+CCR4^-$), and that the frequency of these cells is increased in LTBI subjects compared with healthy donors (129). This study suggested a possible role of specific subsets of $CD4^+$ T-cells in the containment of Mtb and raises interesting questions on the possible role of these cells. In particular the transcriptional profile of $CCR6^+CXCR3^+CCR4^-$ $CD4^+$ T revealed characteristics important for TB containment, since gene expression profiles correlated with TB susceptibility genes, enhanced T-cell activation, cell survival and cytotoxic response.[129] Stimulation with the so called Mtb latency antigens, such as Rv1733c, Rv2029c, Rv2628 and HBHA,[130-136] seem promising tools to identify LTBI subjects and distinguish recent LTBI from remote LTBI.[130,137,138] If confirmed in larger studies, these results may have important implications for risk stratification when deciding to initiate preventive therapy.[130,138,139]

Biomarkers for prevention: vaccine studies

As discussed above, among the T-cell based biomarkers, polyfunctional T-cells have been explored as potential biomarkers by multiparametric flow technology. In animal models, *i.e.* mice, vaccine-induced protection against Mtb infection strongly correlated with a high frequency of polyfunctional $CD4^+$ T-cells.[140,141] However the correlation of this polyfunctional cytokine profile with protective efficacy of BCG vaccination was absent in humans, as reported in a cohort of BCG-immunized infants monitored for 2 years.[142] Similar results were obtained in a TB vaccine study based on MVA85A (modified vaccinia virus Ankara expressing antigen 85A.[143] In addition, polyfunctional T-cells have been reported at increased frequencies in active TB.[74] These studies suggest that polyfunctional T-cells play a role in vaccine induced protection against TB in animal models, but do not represent a correlate of BCG-induced or natural protection in humans, as they are also present in active TB.[74,144] Th17 cells are capable of providing protection in immunization and cellular transfer mouse models.[145-147] Th17 cells are long lived and can become memory cells, despite expressing markers characteristic of terminally differentiated cells,[148] and have self-renewal capacities.[149] Th17 cells preserve the molecular signature that is characteristic of T stem cell memory (TSCM).[150-152] IL17 seems to play an important role in Mtb protection. It has been shown that mice lacking IL17A receptor, despite being able to control acute infection, are unable to stably maintain long-term control of Mtb infection.[153] This is due to decreased early neutrophil recruitment, more than IFNg deficiency. Recently it has been shown that the requirement for IL17 in host protection against Mtb in the mouse model is Mtb strain dependent. IL17 was dispensable for protective immunity against the lab-adapted strain H37Rv while necessary for protection against Mtb HN878, a hypervirulent Mtb strain.[154] IL17 is important in vaccine-mediated protection in TB. Following BCG and ESAT-6 peptide immunization,[155,156] antigen-specific Th17 cells localized in the lungs and were critical for the recruitment of Th1 cells to the lung after Mtb challenge. Innate immune responses are conventionally thought to provide immediate protection before the adaptive immune response is generated, thus contributing towards early containment of the pathogen. However, a growing number of studies suggests their involvement in the recall response and protection during secondary challenge, as shown by the generation and long-term maintenance of NK cells in response to viral infections such as those with cytomegalovirus (CMV) and hepatitis C virus (HCV).[157] There are studies ongoing to evaluate the role of NK memory cells in Mtb protection. γδT-cells recognize a variety of unrestricted, unprocessed and small phosphate antigens.[158] In the mouse model, during the early phase of infection with Mtb, γδT-cells secreting IFNγ and IL17 with cytotoxic effector functions are recruited to the lungs.[159] Expansion of γδT-cells in response to BCG vaccination and their presence in Mtb-specific recall response are also reported in the nonhuman primate macaque model.[160] In addition, γδT-cells reduce the viability of intracellular Mtb via mechanisms dependent on perforin or granulysin.[161,162] These data, together, indicate not only that γδT-cells are present during Mtb infection and following BCG vaccination but that, in humans, they are capable of restricting Mtb growth. Also many other components of the innate immune system participate in the control of Mtb infection, but this is beyond the scope of this brief review.[144]

Conclusions

There is a pressing need for new biomarkers in TB at all different levels discussed above.[144,163] Though studies on new candidate biomarkers are numerous, validation and independent confirmation are rare, unfortunately. Efforts are needed to reduce the gap between the exploratory *up-stream* identification of candidate biomarkers, the validation of biomarkers against clear clinical endpoints in different populations, and the development of simple point of care tests for use in low resourced settings.[20] This needs important commitment from both researchers and economic funders.

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