Clinical Immunology and Multiplex Biomarkers of Human Tuberculosis

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The discovery of tuberculosis (TB) biomarkers is an important goal in current TB research, because the availability of such markers would have significant impact on TB prevention and treatment. Correlates of protection would greatly facilitate vaccine development and evaluation, whereas correlates of TB disease risk would facilitate early diagnosis and help installing early or preventive treatment. Currently, no such markers are available. This review describes several strategies that are currently being pursued to identify TB biomarkers and places these in a clinical context. The approaches discussed include both targeted and untargeted hypothesis-free strategies. Among the first are the measurements of specific biomarkers in antigen-stimulated peripheral blood, in serum or plasma, and detailed immune cell phenotyping. Among the latter are proteomic, genomic, and transcriptomic (mRNA, miRNA) approaches. Recent and promising developments are described.

KEY CLINICAL IMMUNOLOGICAL ASPECTS OF TUBERCULOSIS

Three to eight weeks after initial infection with *Mycobacterium tuberculosis* (*Mtb*), cell-mediated immunity develops. This is characterized by a delayed type hypersensitivity reaction, which forms the basis for a positive reaction to the tuberculin skin test. The majority of infected individuals contain the infection (latent tuberculosis), but in ~10% active tuberculosis (TB) disease develops. It is thought that latent and active tuberculosis are not two separate states,

but rather a continuum of host and pathogen interactions with a spectrum of immune responses (Barry et al. 2009).

The underlying immunology of protection against TB is not fully understood and certainly involves both innate and adaptive responses; however, T helper 1 (Th1) cell-mediated immune responses appear to play a major role (Ottenhoff 2012a; Ottenhoff and Kaufmann 2012). The initial infection is dominated by a Th1-type immune response; however, if the infection is not contained, a gradual shift toward T helper 2 responses occurs (Fletcher

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2007). Th1 cells are activated and proliferate in response to interleukin-12 (IL-12) produced by *Mtb*-infected macrophages, as well as other cytokines released by antigen-presenting cells such as IL-18 and IL-23 (Raja 2004). Activated Th1 cells are the major source of interferon- γ (IFN- γ), which is crucial for macrophage activation and containment of the infection. At this stage, elimination of *Mtb* before establishment of adaptive immunity is possible; however, *Mtb* has evolved immune evasion mechanisms that allow the bacteria to survive in the host for prolonged periods (Flynn and Chan 2003; Ottenhoff 2012b).

The importance of the IL-12/IL-23/IFN- γ axis for protective immunity against mycobacteria is discussed in the section Primary Immune Deficiency and Mendelian Susceptibility to Mycobacterial Diseases. However, despite the importance of IFN- γ , this cytokine alone is not sufficient for protection. Tumor necrosis factor- α (TNF- α), which induces chemokine secretion from macrophages, is equally important in controlling primary infection by supporting the formation of granulomas. Individuals treated with TNF- α receptor antagonists for rheumatologic diseases frequently suffer from reactivation TB, highlighting the importance of this cytokine in containment of mycobacteria (Dietrich and Doherty 2009). Although TNF- α is required for protection, high IL-4 levels together with TNF- α appear to promote immunopathology (Dietrich and Doherty 2009). In fact, IL-4 levels directly correlate with severity of disease. Similarly, IFN- γ and IL-2R α are present at higher levels in bronchoalveolar lavages (BALs) from TB patients with severe disease than in those from moderate TB disease cases (Jo et al. 2003). Cytokines can give an indication not only to the severity of pathology, but also to the type of active TB. Patients with pleural TB generally have elevated levels of markers of systemic inflammation such as IL-6 and IL-8, whereas patients with classical pulmonary TB tend to have increased levels of cytokines associated with cell-mediated immunity (IL-12p40, sCD40L) (Djoba Siawaya et al. 2009b).

Other T-cell subsets playing an important role during *Mtb* infection are Th17 cells, which

facilitate the recruitment of Th1 cells to the site of disease (Khader et al. 2007) and regulatory T cells (Tregs), which control excessive immunopathology (Joosten and Ottenhoff 2008; Green et al. 2010). Our understanding of immune responses against TB, however, remains incomplete, because, for example, the importance of B cells is not well-defined, despite their presence in granulomas. Apart from B and T cells, other cell subsets also play a role during *Mtb* infection including myeloid-derived cells. Myeloid-derived suppressor cells (MDSCs) suppress T-cell function in cancer biology (Gorgun et al. 2013) and infectious diseases (Qin et al. 2013), including TB (du Plessis et al. 2013). MDSCs are present in higher frequencies and suppress T-cell activation and trafficking in TB patients and in individuals recently infected with *Mtb* (du Plessis et al. 2013).

PRIMARY IMMUNE DEFICIENCY AND MENDELIAN SUSCEPTIBILITY TO MYCOBACTERIAL DISEASES

Although TB occurs most commonly in immunocompetent individuals in high-transmission settings and in acquired immunodeficient people due to HIV coinfection (or less commonly in people with diabetes mellitus or other systemic disorders), primary immune deficiency may present with disease because of less virulent mycobacteria or with unusual presentations of infection with *Mtb*. A group of inherited deficiencies in the immune system that are associated with increased susceptibility to mycobacterial diseases is collectively known as primary immune deficiency (PID) diseases. Among these, a high level of genetic heterogeneity exists in syndromes referred to as Mendelian susceptibility to mycobacterial diseases (MSMD) (Ottenhoff et al. 2002; reviewed in Al-Muhsen and Casanova 2008). MSMD is a rare mostly congenital syndrome, which confers a predisposition to mycobacterial infections caused by weakly virulent mycobacteria such as BCG (*Bacillus Calmette-Guérin*) vaccines and nontuberculous environmental mycobacteria (NTMs) as well as virulent mycobacterial species such as *Mtb*. Mutations in six MSMD-causing genes have been identified,

and these genes include an X-linked gene (nuclear factor- κ B-essential modulator [*NEMO*]) and five autosomal genes (IFN- γ receptor 1 and 2 [*IFNGR1*, *IFNGR2*], signal transducer and activator of transcription 1 [*STAT1*], IL-12p40 subunit [*IL12B*], and IL-12 receptor β -subunit [*IL12RB1*]) (reviewed in Al-Muhsen and Casanova 2008). Mutations in these genes either prevent or result in the dysfunctional expression of the encoded proteins. Patients with these mutations often present with a severe clinical phenotype, causing early and often fatal mycobacterial infections. IFN γ R1 deficiency results histologically in lepromatous-type granulomas, which are poorly differentiated. These granulomas contain relatively few giant cells and lymphocytes, but they do contain abundant macrophages with high bacillary loads. Affected patients with partial IFN γ R1 deficiency display less severe clinical disease phenotypes than patients completely deficient for IFN γ R1 (Ottenhoff et al. 2002; reviewed in Al-Muhsen and Casanova 2008). *NEMO* and *STAT1* mutations are associated with several infectious diseases, with a spectrum extending beyond mycobacteria, including fungal, bacterial, and viral infections (reviewed in Al-Muhsen and Casanova 2008; Lee et al. 2011). Monocytes from patients with X-linked recessive *NEMO* deficiency have an intrinsic defect in T-cell-dependent IL-12 production, which leads to impaired IFN- γ production by T cells. In the X-linked MSMD syndrome chronic granulomatous disease (CGD) mutations in the *CYBB* gene result in a diminished respiratory burst (release of reactive oxygen species mediated by NADPH oxidase) in monocyte-derived macrophages, but not in monocytes or granulocytes (Bustamante et al. 2011). Other PID diseases enhancing susceptibility to severe mycobacterial infections can be caused by autosomal dominant mutations in the interferon regulatory factor 8 (*IRF8*) as well as the excessive production of neutralizing autoantibodies to IFN- γ . These disorders result in the depletion of circulating monocytes and dendritic cells and block downstream mediators of IFN- γ , respectively, highlighting the importance of these cells and mediators in antimycobacterial immunity (Lee et al. 2011).

Mycobacterium bovis BCG can cause life-threatening diseases in children with PIDs like MSMD and severe combined immunodeficiencies (SCID). The most common initial presentation is the development of persistent ulcerations at the BCG inoculation site followed by systemic dissemination that can present as osteomyelitis, pneumonia, bone marrow involvement, and hepatosplenic abscesses (Ottenhoff et al. 2002; reviewed in Lee and Lau 2013). BCG-related complications soon after birth might therefore provide early warning of possible underlying immunodeficiencies before more serious infections take root.

Two steps are important for the successful treatment of MSMD. First, the underlying gene mutations responsible for the impaired immunity need to be ascertained and, second, the causative microorganism needs to be identified. Cytokine neutralization assays may point to the presence of anticytokine autoantibodies, and flow cytometry and gene sequencing (polymerase chain reaction [PCR]) can detect defects in candidate markers or receptors (van de Vosse et al. 2004; Wang et al. 2012). Oligonucleotide arrays could serve as rapid and useful alternative methods to identify mycobacteria because characterization can be completed within hours, in contrast to mycobacterial culture which could take up to several weeks (Wang et al. 2012).

IMPORTANCE OF DEVELOPING IMMUNOLOGIC TB BIOMARKERS FOR EARLY (DEVELOPING) DISEASE

Up to now, sputum smear and culture, and more recently the GeneXpert MTB/RIF (mycobacterium TB/rifampicin) tests, are the most frequently used sputum-based diagnostic tests to detect active TB disease. Sputum smear requires 10,000 bacteria/mL and has a relatively low sensitivity of 24% (Davies and Pai 2008). Although sputum culture is more sensitive, with a sensitivity of 40%, and can detect as little as 150 bacteria/mL, it can take up to 2 mo to obtain culture results (Horne et al. 2010). The PCR-based GeneXpert MTB/RIF test can be performed within 2 h with a sensitivity of >98% in smear-positive pulmonary TB and 68% in smear-

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negative TB, and at the same time, it detects rifampicin resistance (Boehme et al. 2010; Steingart et al. 2013). However, all of these diagnostic tests require an *Mtb*-positive sputum, and many active TB patients, including but not exclusively HIV-positive individuals, diabetes patients, and children, often do not present with *Mtb*-positive sputum (Bacakoglu et al. 2001; Walzl et al. 2011). Similarly, extrapulmonary disease is characterized by *Mtb*-negative sputum.

In addition to diagnosing TB through the detection of mycobacteria or their products in body fluids, immune responses of the host to mycobacterial antigens have been exploited for diagnostic tests. The tuberculin skin test (TST) measures a delayed-type hypersensitivity reaction at the injection site and IFN- γ release assays (IGRAs) measure IFN- γ secretion of peripheral blood cells to mycobacterial antigens. However, detection of immune reactivity against *Mtb* may also be the result of vaccination, latent infection with TB, or infection with nontuberculous mycobacteria and does not prove TB disease.

For this reason, identification of biomarkers that can reliably distinguish between latent TB infection and active TB disease (as well as vaccination or NTM infection) is of utmost importance, particularly because early detection of active TB is crucial to stop transmission of *Mtb* and thus reduce TB incidence rates. Therefore, there is currently an actively ongoing search for other types of TB biomarkers. Biomarkers of disease progression would provide insights in which latently infected individuals are likely to progress to active TB. Once identified, these individuals can be closely monitored or offered prophylactic therapy. In addition, there is a search for biomarkers of treatment response, which would allow stratification of patients into different treatment options, including shortened, standard, or prolonged courses of antibiotic therapy. Biomarkers of treatment response would thus contribute to reducing drug resistance due to nonadherence and also improve the validation of novel TB drugs and shorten clinical trials. Markers of treatment failure and relapse would accelerate recognition of poor outcomes, regardless of whether this is due to poor adherence, drug resistance, ineffective drugs in a

clinical trial, or host factors that increase susceptibility. Therefore, not only diagnostic markers but also biomarkers of disease progression, infection status, treatment response, and treatment outcome would provide critical tools in the global fight against TB. Finally, biomarkers of protection against TB disease would be very useful in distinguishing protected from potential disease susceptible individuals, as well as be of tremendous value in TB vaccine trials, because such correlates of protection could predict in an early stage whether or not the administered vaccine would be protective. This could dramatically shorten TB vaccine trials. Ideally, such correlates should work also in the immunocompromised (HIV-coinfected individuals) or in comorbidities such as type 2 diabetes.

Below, we discuss various strategies that are currently followed in the global TB research community to discover and refine TB biomarkers for the key phenotypes discussed above.

PROTEIN-BASED APPROACHES TO TB BIOMARKER DISCOVERY

Targeted Approaches

Biomarkers in Antigen-Stimulated Peripheral Blood

IGRAs determine the release of IFN- γ after stimulation of peripheral whole blood or peripheral blood mononuclear cells (PBMCs) in response to *Mtb*-specific antigens (ESAT6, CFP10, TB7.7) (Lalvani et al. 2001; Mori et al. 2004). These assays have become the gold standard for assessing infection with *Mtb* rather than the less specific TST (tuberculin skin test) (Janssens 2007). IGRAs are not able to distinguish between latent and active TB; however, although interestingly, a substantial increase of IFN- γ production has been reported in latently infected individuals shortly before diagnosis of TB (Diel et al. 2008; Higuchi et al. 2008). Antigen-stimulated multicytokine signatures, however, can differentiate between latent and active TB in adults. In *Mtb* infection phase-dependent antigen-stimulated whole-blood cultures, multiple cytokines other than IFN- γ showed diagnostic potential (Chegou et al. 2012). Similarly in

QuantiFERON tube supernatants, levels of epidermal growth factor (EGF), soluble CD40 ligand (sCD40L), macrophage inflammatory protein-1 β (MIP-1 β), vascular endothelial growth factor (VEGF), transforming growth factor- α (TGF- α), and IL-1 α could differentiate between latent and active TB (Chegou et al. 2009). Even in HIV-positive and -negative children, a diagnostically challenging population group, the levels of IFN- α 2, IL-1Ra, sCD40L, IP-10 (interferon-induced protein-10), and VEGF in QuantiFERON supernatant proved useful in differentiating active and latent TB (Chegou et al. 2013).

Biomarkers in Serum and Plasma

Biomarkers measured directly in serum or plasma without additional antigen stimulation would reduce the time to test results and thus offers a distinct advantage. Multicytokine signatures in serum have indeed been found to be useful for the differentiation between latent and active TB. Plasma concentrations of EGF, fractalkine, IFN- γ , IL-4, MCP-3 (monocyte chemoattractant protein-3), and IP-10 were found to be significantly different between TB patients and healthy household contacts (Mihret et al. 2013). An earlier study showed that serum levels of proinflammatory cytokines IL-6 and IP-10 were significantly lower in latently infected individuals than in TB patients, whereas MCP-1 levels were higher (Djoba Siawaya et al. 2009a). Cytokine profiles during early TB treatment were also able to predict month-2 culture conversion and hold promise in predicting treatment outcome and future relapse (Walzl et al. 2011; K Ronacher, unpubl.). Plasma granulysin levels also correlate with treatment response (plasma granulysin levels and cellular IFN- γ production correlate with curative host responses in TB, whereas plasma IFN- γ levels correlate with TB disease activity in adults) (Sahiratmadja et al. 2007).

Plasma levels of markers involved in the Toll-like receptor 4 pathway, like soluble CD14 (sCD14) and myeloid differentiation-2 (MD-2), have furthermore been shown to differentiate between latently infected individuals and TB patients (Feruglio et al. 2013).

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Immune Cell Phenotyping

Polychromatic flow cytometry is often used to measure different cell surface markers and intracellular cytokine production, allowing for the characterization of different types of immune cells. Phenotyping of immune cells using this approach has shown that different T-cell subsets can differentiate between latent and active TB. *Mtb*-specific CD4⁺ and CD8⁺ T-cell subsets secreting TNF- α only, IFN- γ only, and dual TNF- α /IFN- γ appear in higher frequencies in TB patients compared with latently infected individuals (Pollock et al. 2013). CD4⁺ T cells from individuals latently infected are polyfunctional and express TNF- α , IFN- γ , and IL-2 compared with the dominant TNF- α -only response of TB patients (Harari et al. 2011). During active disease, *Mtb*-specific CD4⁺ T cells also switch from central to effector memory T cells, which are accompanied by a decrease in IL-7 receptor α expression (Pollock et al. 2013). PPD-specific TNF- α -only secreting CD4⁺ T cells with an effector memory phenotype might therefore discriminate active disease and latent TB infection (LTBI) with promising accuracy.

Untargeted Approaches and New Technologies

Untargeted approaches can include transcriptomic, proteomic, or metabolomic approaches. Transcriptomic approaches will be discussed in detail in the section Transcriptomic Approaches to TB Biomarker Discovery, whereas the current section will focus on protein-based untargeted approaches. Proteomic profiling by mass spectrometry or protein chip technology allows for nontargeted screening of large numbers of peptides between TB patients and controls. Proteomic fingerprinting of serum for host markers identified three markers (transthyretin, C-reactive protein [CRP], and neopterin) that could discriminate with high accuracy between TB patients and controls with other infectious and inflammatory conditions (Agranoff et al. 2006). Similarly, serum proteomic profiles could differentiate sputum smear-negative and smear-positive TB patients from controls with reasonable accuracy (Liu et al. 2010). High-

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throughput screening was also applied to detect antibodies in sera against antigens from culture filtrate (Sartain et al. 2006) and later to the entire *Mtb* proteome (Kunnath-Velayudhan et al. 2010) and identified a small pool of antibodies with diagnostic potential.

A novel highly multiplexed proteomic technique has recently been applied in the search for TB treatment response markers. De Groote et al. (2013) used an unbiased proteomic analysis approach using SOMAmers (slow off-rate modified aptamers) to identify protein markers associated with active TB and to determine alterations in their expression levels in response to TB treatment. Serum concentrations of serum amyloid A (SAA) and CRP were shown to decrease from baseline to week 8 of anti-TB treatment, whereas serum albumin levels increased. Some serum markers—CRP, SAA, and NPS-PLA2—were able to distinguish TB patients based on their disease severity before or shortly after initiation of treatment.

Nontargeted metabolomic approaches have led to the discovery of metabolic profiles that correlate with different TB disease states and anti-TB treatment outcome. Although metabolomic approaches will be discussed in detail in a separate article, it is nevertheless important to note that biosignatures of small-molecule molecular features (MFs) have been identified in urine sample of TB patients using mass spectrometry (Mahapatra et al. 2014). In these samples, levels of 45 MFs changed significantly by month 1 of anti-TB treatment, with levels of 23 MFs consistently changing in abundance until the end of treatment (Mahapatra et al. 2014). This approach of identifying urine-based anti-TB treatment response biosignatures serves as proof of concept for further metabolomics investigations.

TRANSCRIPTOMIC APPROACHES TO TB BIOMARKER DISCOVERY

A Meta-Like Analysis of Genome-Wide Approaches Used to Identify TB Biomarkers of Disease and Early Disease Progression

Early detection of active TB infection is key toward better control of the TB pandemic,

because it would allow initiation of curative treatment before TB transmission can occur. However, the early identification of “asymptomatic” TB progressors remains a major challenge. Current diagnostic tools rely on detecting live *Mtb* (bacterial cultures, *Mtb* DNA) in samples from individuals that present with already active disease, which in all likelihood have already transmitted infection. Approaches are needed to identify biomarkers that predict, or detect at a very early stage, the risk of developing active TB disease.

A number of recent studies have attempted to identify biomarkers of TB disease by performing unbiased genome-wide human gene expression studies. These studies mostly relied on peripheral blood samples from patients with active (pulmonary) TB disease directly after diagnosis and contrasted gene expression profiles to those of healthy (infected) controls or patients with other inflammatory/pulmonary disorders (Jacobsen et al. 2007; Mistry et al. 2007; Berry et al. 2010; Maertzdorf et al. 2011a,b, 2012; Ottenhoff et al. 2012; Bloom et al. 2013; Cliff et al. 2013; Kaforou et al. 2013). Each individual study performed extensive data analysis to identify key signatures that could discriminate TB disease from the respective control group(s). The studies used either unseparated peripheral blood or isolated leucocytes thereof to study TB disease–related gene expression profiles, although the blood is not the primary site of TB disease. Despite the rather limited sample sizes of most individual studies, the studies were able to identify active TB disease–associated signatures and could link these to known biological pathways and processes. IFN- α/β signaling was found to be a key process in TB disease; it was first identified by Berry et al. (2010) and subsequently confirmed in several other studies. IFN- α/β signaling had been known mostly for its association with intracellular viral infections. The activation of similar signaling pathways by *Mtb* and viruses reveals the activation of overlapping intracellular response pathways triggered by quite diverse microbial danger signals. The upstream events triggering IFN- α/β signaling in TB are incompletely defined as yet.

We recently performed a combined analysis of all human genes identified by the above-cited published genome-wide gene expression studies (except the studies by Kaforou et al. 2013 and Anderson et al. 2014, which were not available at the time of our analysis), aiming to assess potentially novel pathways involved in TB pathogenesis (Joosten et al. 2013). The combination of all genes identified to be associated with TB disease by the individual studies resulted in a gene set of 409 genes. Subsequent combined modular analysis (Chaussabel et al. 2008), ingenuity-based pathway analysis, and gene set enrichment analysis (GSEA) resulted in the identification of several novel pathways associated with TB pathogenesis. Somewhat surprisingly, analysis of the combined data did not yield IFN- α/β signaling as the strongest process in TB disease. Instead, a strong myeloid-derived inflammatory signature was found to be of particular importance. Myeloid-related processes that were strongly represented in the data set included signaling through pattern recognition receptors, FcR activation, and enhanced communication between innate and adaptive immune cells. In addition, we identified the TREM-1 (triggering receptor expressed on myeloid cells) signaling pathway as a potential new target pathway involved in TB. Increased secretion of TREM-1 has been described in the lungs of patients with TB, although this was not unique for infection with *Mtb* (Richeldi et al. 2004; Chan et al. 2007; Tintinger et al. 2012). In addition, B-cell activation was identified to be important for TB disease pathogenesis, and indeed B cells have been identified in human TB lesions but their function in TB pathogenesis and protective immunity remains largely unknown (Ulrichs et al. 2004). As a next step, in-depth studies are needed to unravel the potential role of TREM-1 signaling, FcR signaling, and B cells in TB. Functional and gene/protein expression studies will help in determining the role of these cells and molecules in infection and decipher new pathways involved in TB disease pathogenesis. These insights should provide a more rational basis for identifying biomarkers of TB disease risk and early progression.

MicroRNA-Based Profiling as a New Nontargeted Approach

Nontargeted approaches such as RNA sequencing, including the identification of host microRNA (miRNA) signatures, can contribute to the discovery of biomarkers for pulmonary tuberculosis. An miRNA signature in serum, consisting of 15 miRNAs, could potentially discriminate patients with pulmonary TB from individuals with latent TB and was able to categorize them with a diagnostic accuracy of 82% (Miotto et al. 2013). Zhang et al. (2013) furthermore identified six serum miRNAs that had a sensitivity and specificity of 95% and 92%, respectively, to diagnose TB. miRNAs shown to contribute to biomarker signatures in TB patients include, among others, let-7e, miR-29c, miR-146a, miR-148a, miR-178, miR-192, miR-193a-5p, miR-365, miR-378, and miR-483 (Miotto et al. 2013; Zhang et al. 2013).

Focused Transcriptomic Approaches to Identify TB Biomarkers of Disease and Early Disease Progression

Quantitative changes in RNA expression levels to identify TB biomarkers are currently primarily being analyzed using hypothesis-free genome-wide (microarray/RNA-Seq) screening methods at the transcriptomic level (see subsection A Meta-Like Analysis of Genome-Wide Approaches Used to Identify TB Biomarkers of Disease and Early Disease Progression). However, because TB biomarker signatures identifying those infected individuals that are at risk of progressing to active TB (vs. those whose immune system will effectively contain the infection) will most likely encompass a limited set of multiple gene transcripts, neither genome-wide (microarray/RNA-Seq) nor single-gene (real-time PCR) approaches are ideally suited to develop practically useful TB biomarker assays. Furthermore, microarray analysis, RNA-Seq, and real-time PCR are technically challenging and too costly to be applied on a routine basis in resource-poor settings.

To identify and monitor multicomponent host biomarker signatures in large human co-

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hort studies, techniques have been developed that combine sets of markers at the transcriptomic level. Fluidigm, for example, provides high-throughput real-time quantitative PCR based on proprietary microfluidic chips (Rahimov et al. 2012). These gene expression assays are commercially available, but they are still costly and require specialized equipment not routinely available in laboratories in most TB-endemic areas. In contrast, dual-color reverse-transcriptase multiplex ligation-dependent probe amplification (dcRT-MLPA) is an inexpensive and robust technique permitting rapid and accurate RNA expression profiling of as many as 90 transcripts in a single reaction. As the assay is high-throughput, it is exceptionally suited to determine biomarker signatures on a population scale. We have used dcRT-MLPA to characterize the human immune response in the peripheral blood during TB infection as well as in the response to curative treatment in several cohort studies in genetically and geographically diverse populations (Sutherland et al. 2011, 2013; Joosten et al. 2012). Biomarker signatures were identified and validated with excellent classifying values between TB cases versus healthy infected or uninfected controls. Biomarkers within the signatures included apoptosis-related genes and T-cell/B-cell markers, suggesting important contributions of adaptive immunity to TB pathogenesis (Joosten et al. 2012). In a follow-up study with 523 participants across four sub-Saharan countries (Ethiopia, Malawi, South Africa, and The Gambia), differing also in TB and HIV status, we found a number of genes to be expressed at lower levels in active diseased compared with latently infected individuals, which normalized during treatment. A remarkably consistent classifier of active disease appeared to be the high-affinity IgG Fc receptor 1 FCGR1A (CD64), which was expressed at significantly higher levels in active TB regardless of HIV status or genetic background (Sutherland et al. 2013).

The classifying value of the identified biomarker signatures may be further enhanced in future studies by including markers derived from recent microarray studies highlighting a potential role for type I IFN- $\alpha\beta$ signaling path-

ways (Berry et al. 2010) and/or myeloid-derived inflammatory pathways (Joosten et al. 2013). Moreover, probing immunological differences between TB progressors and nonprogressors at early time points after contact with a TB case identified several markers that may predict the onset of active TB at a very early stage after infection (Sutherland et al. 2011). Once these markers have been validated in larger studies, they will provide an initial platform to prospectively identify people at risk of developing TB.

BRIDGING FOCUSED ("BIASED") AND GENOME-WIDE ("UNBIASED") APPROACHES: TOWARD TB DISEASE-SPECIFIC BIOMARKER SIGNATURES

From the genome-wide host gene expression studies discussed above and in Maertzdorf et al. (2014), it is evident that a predominantly inflammatory signature is detectable during active TB disease. This signature is detectable systemically, probably reflecting dynamic leukocyte trafficking through the inflamed lung tissue. Indeed, TB granulomas are highly dynamic structures with significant cellular influx and efflux. Although the robust inflammatory signature in TB is rather unsurprising, an important issue is that this signature is shared with many other inflammatory disorders, including sarcoidosis (which poses a differential diagnostic dilemma for TB and nontuberculous mycobacteria [NTM] pulmonary disease) as well as melioidosis, which is caused by the intracellular bacterial pathogen *Burkholderia pseudomallei* (Koh et al. 2013). The 86-gene signature, which was initially believed to be specific for TB because it was absent from individuals with *Staphylococcus aureus* or *Streptococcus pyogenes* infections or those affected by autoimmune disease (SLE [systemic lupus erythematosus] and Still's disease) (Berry et al. 2010), was later found to be present also in melioidosis and sarcoidosis patients (Maertzdorf et al. 2012; Koh et al. 2013). Thus, the IFN- α/β signaling signature in itself is unlikely to be of clinical diagnostic relevance in TB, although it might be of value as part of

a diagnostic signature identifying individuals as early TB progressors. Kaforou et al. (2013) identified a diagnostic, active TB disease-specific signature based on a limited number of host genes, mostly distinct from the IFN- α / β signaling cascade (see below).

The different nature of the diseases with a prominent IFN- α / β signaling signature (viral and bacterial vs. infection; bacterial infection vs. autoimmune/inflammatory disease) would suggest that more discriminatory signatures should be identifiable. Such more specific signatures might be obscured by the overwhelmingly present inflammatory phenotype. One way to uncover these more specific signatures is through antigen-specific stimulation approaches, such as discussed in the sections Importance of Developing Immunologic TB Biomarkers for Early (Developing) Disease and Protein-Based Approaches to TB Biomarker Discovery. The stimulation of blood cells by *Mtb* lysates, BCG, recombinant protein or lipid antigens, or peptides could strongly enrich for antigen-specific disease-related immune reactivity. These responses can be profiled at the level of the expression of genes, proteins, or metabolic products or changes in cell phenotype and function. Sources could encompass unseparated blood cells, offering the advantage that all cell types are represented, including those with less-defined but possibly important roles in anti-TB immunity. These include, besides T cells, also B cells, natural killer (NK) cells, natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, innate lymphoid cells (ILCs), different myeloid cell subsets (monocytes, dendritic cells, neutrophils), and other less conventional circulating cells. Alternatively, but not mutually exclusive, isolated purified cell subsets can be analyzed to dissect more precisely *Mtb*-stimulation- or infection-induced events in key cell types. When biomarkers are identified through such approaches, new algorithms can be developed to mine data from genome-wide whole-blood transcriptomic data sets to analyze selectively immunity-related specific key genes or pathways.

Another, complementary focused strategy to identify TB biomarkers is to select markers

involved in specific pathways known to play important roles in TB control. These encompass both adaptive immunity-related markers, including Th1, Th2, Th17, and Treg subsets (e.g., transcriptional regulators, hallmark effector cytokines), as well as innate signaling and autophagy pathway markers (see, e.g., the subsections Targeted Approaches and Biomarkers in Serum and Plasma). Although not studied directly in an antigen-specific setting, the fact that these cells and pathways play important roles in TB infection and pathogenesis enhances their plausibility of delivering new biomarkers and significant signatures.

CONCLUDING REMARKS AND PERSPECTIVE

New approaches to TB biomarker discovery seem to start yielding promising results. A major and challenging next step now is to translate basic biomarker discovery into clinically useful algorithms that can be applied in the field, using relatively simple tests. Second, such algorithms should be able to distinguish between (early) TB, latent TB, and other diseases that pose a diagnostic dilemma because they present as entities with TB in the differential diagnosis. A third and major challenge is that these algorithms should also work in the immunocompromised, as many TB cases are HIV-coinfected. In a recent study, which was conducted in South Africa and Malawi, important progress was made in each of these areas (Kaforou et al. 2013). Blood transcriptional signatures were identified that were able to distinguish TB from other diseases prevalent in both HIV-uninfected and HIV-infected African adults. These other diseases included patients with other (mostly pulmonary infectious) disorders in which TB was considered in the differential diagnosis but then excluded. The diagnostic signatures were robust and were validated in other data sets, including those of Berry et al. (2010). Subsequently, minimal sets of genes were selected that could robustly differentiate between TB, LTBI, and other diseases in both HIV-infected and HIV-uninfected individuals. Using a set of 27 genes, it was possible to accurately identify

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TB versus LTBI, and a set of 44 genes was able to differentiate TB from other diseases. A disease risk score was developed that can now be developed into a simple test format suitable for use in HIV-infected TB populations. A more recent study extended this work also to TB-infected infants, with similar results in terms of the identification of useful diagnostic TB disease mRNA biosignature (Anderson et al. 2014). Prospective studies using such tests are now needed to validate their actual performance. Nevertheless, these transcriptomic signatures in any case are highly promising and have the potential to translate new TB biomarkers to clinically relevant settings where these tests (e.g., Joosten et al. 2012) are urgently needed.

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