Advances and Challenges in Tuberculosis Biomarker and Vaccine Development

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1 Introduction

Tuberculosis continues to be a major global health problem, causing an estimated 8.8 million new cases and 1.45 million deaths annually despite the availability of a vaccine and inexpensive, effective, and reasonably well-tolerated therapy (WHO, 2011). The current vaccine strain \textit{M. bovis} BacilleCalmette-Guérin (BCG) protect against severe progressive TB in children but are inconsistent in protecting against the predominant adolescent or adult form of TB, and the vaccine is unsafe in HIV-infected infants and its use in this population is no longer recommended by WHO (Kaufmann, 2011). A number of factors have been considered responsible for the variable efficacy of BCG which includes the environmental mycobacteria, strain, dose and protocol for administration of the vaccine and co-infections with helminths and viral infection (Murphy \textit{et al.}, 2008; Ottenhoff & Kaufmann, 2012). It is true that the current available tools to diagnose, treat and protect are ineffective to combat TB. Therefore, novel intervention measures comprising rapid and reliable diagnostics, new vaccines for uninfected or latently infected people and new drugs for patients with active TB disease need to be developed to make substantial progress towards the goal of TB elimination.

Identification of infection-stage specific biomarkers, characterization of the immune response, a clear understanding of the dynamics and interplay of different arms of the immune response are critical to allow the development of new vaccine and better tools for combating TB. This is particularly true for developing countries where the incidence of active and latent TB is high and where smear microscopy is the only technique widely used for active case detection. A reliable test for latent infection would be valuable to guide interventions in those most likely to progress to active TB, including HIV infected people and young children.

In the last decade or so, a lot of research has been conducted to identify biomarker(s) which may be translated into clinical and public health programs as simple and inexpensive point-of-care tests to distinguish the different outcomes of infection and anti TB treatment (Parida & Kaufmann, 2010; Walzl \textit{et al.}, 2011; Wallis \textit{et al.}, 2009). Similarly, in the last decade a dozen of pre and post exposure vaccines entered into different phases of clinical trial. There are two groups of vaccines. The first group aimed to replace BCG. This group of vaccines hoped to be more effective and safer than BCG. The second group of vaccines are booster vaccines designed to boost the immunity given by BCG (or an improved priming vaccine), including viral vectors expressing key antigens of \textit{M. tuberculosis} and fusion proteins in adjuvant (Kaufmann, 2011).

2 Biomarkers of Tuberculosis

The vigorous application of the existing strategies and the coordinated efforts in the past years to control TB have slowed the rate of disease incidence, but far from to make meaningful progress towards elimination. Lack of effective vaccines, drugs, unavailability of simple diagnostic methods, spread of HIV/AIDS in TB-endemic regions and emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR-TB) TB strains, makes all these efforts unfruitful.

This experience has led to do more research on innovating simple and rapid diagnostic tests, new drugs and vaccines. The current widely used diagnostic technique; therapeutic drugs and vaccine are very long time since licensed and are inefficient in detecting, treating or protecting TB. The lack of reliable
simple biomarkers to indicate or predict the different clinical outcomes of *M. tuberculosis* has been given as a key reason for the failure of developing new diagnostic tools, drugs and vaccines against TB.

A biological marker, or biomarker, is a characteristic that is objectively measured and evaluated as an indicator of a normal physiological or pathological process or pharmacological response(s) to a therapeutic intervention (Group, 2001). Host or pathogen specific TB biomarkers may one day provide prognostic information, either for individual patients or study cohorts, about future health status and can advance knowledge of disease pathogenesis in predicting reactivation and cure, and indicating vaccine-induced protection. Biomarker studies use urine, saliva, breath and sputum samples to identify molecules for indicating or predicting the different clinical outcome of *M. tuberculosis* infection, however, peripheral blood remains an attractive sample type due to the ease with which this sample can be obtained. Genes, transcripts, proteins, lipids and metabolites can all be measured in blood for biomarker studies (Parida *et al*., 2010).

A century old sputum smear microscopy is the most widely used test in high endemic countries and this test is inadequate to diagnose early active TB disease and unable to diagnose extrapulmonary TB, sputum smear-negative TB (active pulmonary TB with less than 10,000 bacilli per ml of sputum) and childhood TB. While a number of biomarkers have been found to be associated with TB protection or TB disease, there are no qualified biomarkers to indicate protection by new vaccines against TB. Protective immunity against TB is associated with Th1 dominated cytokines; particularly IFN-γ, however, different studies have indicated that the level of IFN-γ alone does not indicate correlate of protection although its presence is important (Dockrell, 2007; Lalvani & Millington, 2008). Therefore, additional biomarkers which clearly indicate protection or susceptibility against TB are highly needed.

Stimulation of PBMC with *M. tuberculosis* antigens gives rise to high levels of Th1 cytokines and the IL-4 antagonist IL-4d2 in protected individuals in comparison with non protected individuals (Demissie *et al*., 2004) and mRNA level of individuals with a high IL-4d2/IL-4 ratio controlled *M. tuberculosis* infection (Wassie *et al*., 2008). Recent studies reported that IL-2, IFN-γ (Casey *et al*., 2010) and TNF-α expression profiles of CD4+ T cells (Harari *et al*., 2011) hold promise in detecting active TB disease, however; Kagina *et al*. recently reported that the specific CD4 T cell responses 10 weeks after BCG vaccination in new borns do not correlate with ultimate risk of TB disease. Moreover, risk of disease during the first 2 years of life in new born was not associated with the frequency of mono or polyfunctional CD4 or CD8 T cells (Kagina *et al*., 2010). Polycytokine signatures including EGF, sCD40L, MIP-1β, VEGF, TGF-α or IL-1α were reported to be able to differentiate active TB disease and latent TB (Chegou *et al*., 2009). Proinflammatory cytokines such as TNF, IL-12 (p40) and IL-17 are increased in TB cases and can discriminate between active TB disease and latent infection (Sutherland *et al*., 2010) and serum or plasma level of TB cases have high levels of IL-8, IP-10, MCP-1, and MIP-1 b in comparison with non TB cases (Juffermans *et al*., 1999; Azzurri *et al*., 2005; Siawaya *et al*., 2009).

In addition, the RNA expression level of CXCL-8, FoxP3 and IL-12β differentiates latent TB infection from disease (Wu *et al*., 2007). Detection of circulating antibodies for diagnostic of prognostic potential are suggested in studies indicating correlation of *M. tuberculosis*-specific antibodies with bacterial burden (Kunnath-Velayudhan *et al*., 2010) and granulysin expression by CD4+ memory T cells reported as candidate immune marker for TB infection in childhood and adults (Mueller *et al*., 2011). Recent studies have indicated that Fc gamma receptor 1B (FCgR1B) (Maertzdorf *et al*., 2011), combined expression patterns of FCgR1A (CD64), RAB33A and LTF (lactoferrin) (Jacobsen *et al*., 2007) and CD3e, CD8a, IL7R, BLR1, CD19, FCGR1A, CXCL10, CD4, TNF, BCL2, MMP-9, Foxp3, CASP8, CCL-4,
TNFRSF1A, CASP8, Bcl2 and TNF (Joosten et al., 2012) show discriminating power between TB and LTBI. Expression of RIN3, LY6G6D, TEX264, and C14orf2 genes discriminate active, cured, recurrent or latent TB (Mistry et al., 2007). The IFN-inducible transcripts which includes OAS1, IFI6, IFI44, IFI44L, OAS3, IRF7, IFIH1, IFI16, IFIT2, OAS2, IFITM3, IFITM1, GBP1, GBP5, STAT1, GBP2, TAP1, STAT1, STAT2, IFI35, TAP2, CD274, SOCS1, CXCL10, IFIT5 were overexpressed in purified blood neutrophils from patients with active TB, compared with healthy controls (Berry et al., 2010).

Sputum culture or smear microscopy status after 2 months of therapy has been used as a surrogate marker for predictor of non-relapsing cure. The increased level of Mycobacterium tuberculosis antigen 85 and 85B RNA in sputum protein during the first week of treatment predicted relapse or failure (Wallis et al., 1998). Levels of IFN-γ (Chee et al., 2010), IL-10, the ratio of IFN-γ/IL-10 (Sai Priya et al., 2010) and IL-4/IL-4δ (Wassie et al., 2008) have been reported to have association with treatment outcome. There are also other molecules which decreases after treatment like soluble intercellular adhesion molecule (sICAM)-1 (Walzl et al., 2008; Lai et al., 1993; Mukae et al., 2003), C-reactive protein (Plit et al., 1998) soluble urokinase plasminogen activator receptor (Eugen-Olsen et al., 2002) and procalcitonin (Baylan et al., 2006; Prat et al., 2006) and these molecules believed to be useful biomarkers in indicating treatment outcome.

3 Vaccines against Tuberculosis

The current vaccine against TB, Bacillus Calmette-Guérin (BCG) vaccine, which was developed between 1906 and 1919 without any immunological correlation by attenuation of the virulent Mycobacterium bovis has been given 4 billion times over the last 90 years (Kaufmann, 2010). BCG is given as part of the expanded program of immunization and has an excellent safety record, is inexpensive and has proven protective efficacy against severe childhood forms and meningitis and miliary TB and to a lesser extent against lung TB in infants. It is, however, not effective against pulmonary TB in adults which is the most prevalent form of the disease. Moreover, in HIV infected infants the risk of disseminated BCG disease is significantly higher (Bricks, 2004). This failure and risk of BCG underscore the need for novel improved vaccination concepts towards safe vaccines against newborn and adult TB in HIV negative or positive individuals. However, the different efforts in developing different vaccines is hampered by lack of biomarkers that helps for early selection of immunodominant antigens, dose, route and efficacy of candidate in protection against developing active TB disease.

Over the past decade, researchers have made significant progress in TB vaccine development, and a dozen TB vaccine candidates are now being evaluated in clinical trials. TB vaccination strategies follows two different approaches: pre-exposure vaccination in order to prevent disease in individuals that have so far not encountered Mycobacterium tuberculosis and post-exposure vaccination that aims at inhibiting disease outbreak in individuals that are already infected (Kaufmann et al., 2010; Brennan & Thole, 2012). Current vaccines under clinical trials are summarized in Table 1.
<table>
<thead>
<tr>
<th>Products</th>
<th>Product Description</th>
<th>Type of Vaccine</th>
<th>Indication</th>
<th>Application</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AERAS–422</td>
<td>Recombinant BCG expressing mutated PfoA and over expressing antigens 85A, 85B, and Rv3407</td>
<td>Recombinant</td>
<td>Prime</td>
<td>Prophylactic</td>
<td>Phase I (terminated due to side effects)</td>
</tr>
<tr>
<td>AdAg85A</td>
<td>Replication–deficient adenovirus 5 vector Expressing Mtb antigen 85A</td>
<td>Viral</td>
<td>Prime-Boost</td>
<td>Prophylactic</td>
<td>Phase I</td>
</tr>
<tr>
<td>HyVac 4 / AERAS–404 + IC31</td>
<td>Adjuvanted recombinant protein composed of a fusion of Mtb antigens 85B and TB10.4</td>
<td>Recombinant</td>
<td>Prime-Boost</td>
<td>Prophylactic</td>
<td>Phase I</td>
</tr>
<tr>
<td>Hybrid – I + CAF01</td>
<td>Adjuvanted recombinant protein composed of Mtb antigens 85B and ESAT–6</td>
<td>Recombinant</td>
<td>Prime-Boost</td>
<td>Prophylactic</td>
<td>Phase I</td>
</tr>
<tr>
<td>Hybrid – 56 + IC31</td>
<td>Adjuvanted recombinant protein composed of Mtb antigens 85B, ESAT–6 and Rv2660</td>
<td>Recombinant</td>
<td>Prime-Boost</td>
<td>Prophylactic</td>
<td>Phase I</td>
</tr>
<tr>
<td>VPM 1002</td>
<td>rBCG Prague strain expressing listeriolysin and carries a urease deletion mutation</td>
<td>Recombinant</td>
<td>Prime</td>
<td>Prophylactic</td>
<td>Phase IIa</td>
</tr>
<tr>
<td>M72 + AS01</td>
<td>Recombinant protein composed of a fusion of Mtb antigens Rv1196 and Rv0125 &amp; adjuvant AS01</td>
<td>Recombinant</td>
<td>Prime-Boost</td>
<td>Prophylactic</td>
<td>Phase II</td>
</tr>
<tr>
<td>Hybrid – I + IC31</td>
<td>Adjuvanted recombinant protein composed of Mtb antigens 85B and ESAT–6</td>
<td>Recombinant</td>
<td>Prime-Boost</td>
<td>Prophylactic</td>
<td>Phase II</td>
</tr>
<tr>
<td>RUTI</td>
<td>Fragmented Mtb cells</td>
<td>Whole cell,</td>
<td>Prime-Boost</td>
<td>Prophylactic and Therapeutic</td>
<td>Phase II</td>
</tr>
<tr>
<td>AERAS–402 / Crucell</td>
<td>Ad35 Replication deficient adenovirus 35 vector expressing Mtb antigens 85A, 85B, TB10.4</td>
<td>Viral</td>
<td>Prime-Boost</td>
<td>Prophylactic</td>
<td>Phase IIb</td>
</tr>
<tr>
<td>MVA85A / AERAS–485</td>
<td>Modified vaccinia Ankara vector expressing Mtb antigen 85A</td>
<td>Viral</td>
<td>Prime-Boost</td>
<td>Prophylactic</td>
<td>Phase IIb</td>
</tr>
<tr>
<td>Mw [M. indicus pranii (MIP)]</td>
<td>Whole cell saprophytic non-TB mycobacterium</td>
<td>Whole cell,</td>
<td>Whole cell, Inactivated or Disrupted</td>
<td>Immuno-therapy Therapeutic</td>
<td>Phase III</td>
</tr>
</tbody>
</table>

Table 1. Tuberculosis vaccine candidates in clinical trials (Brennan & Thole, 2012)
The first category follows the approach to improve the current BCG vaccine through recombinant (r) BCG strains constructs with improved vaccine efficacy that are intended to replace BCG. The two major representatives of this group are rBCG30, which is a BCG strain over expressing the immunodominant *M. tuberculosis* antigen 85B, and rBCGΔUreC:Hly (VPM1002), which is a recombinant strain that is deficient in urease and expresses listeriolysin produced by *Listeria monocytogenes* (Grode et al., 2005; Tullius et al., 2008). This vaccine facilitates the presentation of antigen to CD8 in the context of class I by translocating *M. tuberculosis* antigen into the cytoplasm via perforating the phagosomal membrane with the help of the acidic phagosomal PH due to deficiency of urease. Another viable vaccine candidate engineered with a similar approach introduces pore-forming capacities into BCG with perfringolysin (pfo) from Clostridium perfringens and expresses antigens Ag85A, Ag85B, and TB10.4 (Sun et al., 2009). The other viable vaccine candidates in the R&D pipeline at preclinical development stage include MtbDRD1DpanCD, Mtb ΔlysA ΔpanCD ΔsecA 2 and MtbDPhoPDfad. These vaccine candidates are auxotroph mutants of human *M. tuberculosis* strain H37Rv in which mycobacterial genes important for replication and persistence were deleted or modified to attenuate replication and several genes important for the evasion of host immune responses were deleted (Kaufmann, 2010). Recently three different recombinant vaccines (MtbDnuoGDpanCDDRΔ1 pSIV Gag and MtbDleuCDDpanCDDsecA2 pSIV Gag and MtbDlysADpanCD) were preclinically tested. These vaccines are auxotroph mutants of human *M. tuberculosis* strain H37Rv in which mycobacterial genes important for replication and persistence and evasion of host immune responses were deleted or modified. The preclinical study showed the vaccines are safe in immunosuppressed Simian Immunodeficiency Virus infected infant macaques and suggesting that a combination recombinant attenuated *M. tuberculosis*-HIV vaccine could be a safe alternative to BCG for the pediatric population as a whole and, more importantly, for the extreme at-risk group of HIV-infected infants (Jensen et al., 2012).

In contrast, the second category of vaccine candidates is considered more for heterologous prime-boost strategies, with BCG or rBCG as the prime. The first subgroup includes viral vectors that express immunodominant *M. tuberculosis* antigens for the initiation of strong Th1-dominated immune response to the expressed heterologous antigen and induce CD8 T-cell response. Currently two viral vectors are exploited for TB vaccines: modified vaccinia virus Ankara (MVA) developed by Oxford University and replication-deficient adenovirus (Ad) of serotype 5 or 35 (Ad5 created by McMaster University and Ad35 created by Crucell and Aeras) with the advantage of a strong lung tropism that leads to an increased expression of immunodominant antigen at the site of mycobacterial entry. The MVA and Ad5 virus carriers both express Ag85A whereas the Ad35 co-expresses the antigens Ag85A, Ag85B, and TB10.4. Pre-existing antibodies to adenovirus from frequent natural infections could impair Ad-based vaccine efficacy (Kaufmann, 2010).

Fusion proteins of immunodominant antigens with the aim of mounting strong immune responses against immunologically important *M. tuberculosis* antigens are also used as a heterologous prime-boost strategy. To ensure immunogenicity, recombinant protein vaccines need an adjuvant that promotes Th1 immune responses. Three different types of adjuvants are currently used for protein vaccines. Hybrid1 (H1) which includes the antigens 85B and ESAT-6 or combined with HyVac4/AERAS-404, which is a fusion protein of Ag85B and TB10.4 (Dietrich et al., 2005; van Dissel et al., 2010) has been used in combination with an adjuvant IC31 developed by Inter-cell. In the M72 vaccine, the antigens Rv1196 and Rv0125 are supplemented with adjuvants AS01 or AS02 (von Eschen et al., 2009). The inactivated *M. vaccae*, an environmental mycobacterium, and the semi-purified *M. tuberculosis* fragments RUTI are
considered as therapeutic vaccinations that could potentially synergize with chemotherapy (Vilaplana et al., 2010; von Reyn et al., 2010). M. vaccae is a whole-cell vaccine thought to mount a protective immune response by providing cross-reactive antigens. RUTI comprises detoxified and fragmented M. tuberculosis components carried in liposomes.

On top of the aforementioned vaccines which are already in clinical trials, there are also other future strategies which aim to induce long-lasting memory T cell responses comprising mostly of CD4 Th1 cell that resists exhaustion, suppression, and deviation. This strategy targets CD4 cells to remain in a stage of alertness, whereby immune mechanisms can be promptly mobilized after encounter with M. tuberculosis or vaccines that achieve sterile eradication of the pathogen in latently infected individuals or protect naive individuals by rapid elimination of M. tuberculosis after infection (Kaufmann, 2010).

4 Challenges of Tuberculosis Biomarkers and Vaccine Development

Currently studies are on-going in many laboratories using serum, plasma, stimulated whole blood supernatant, PBMC, saliva and other samples to identify molecules which can be used as a biomarker for indicating or predicting the different clinical outcome of M. tuberculosis infection and a dozen of pre and post exposure vaccines are under clinical trial. However, there are challenges which need to be addressed in order to come up with a reliable biomarker for diagnosis, predicting the different outcomes of M. tuberculosis infection and to indicate protection by new vaccines against TB.

The first challenge is lack of a clear understanding of the protective immune response against TB. The immune response against tuberculosis is very complex comprising different cells and molecules of the innate and adaptive immune response. Ottenhof et al. (2012) in his recent review indicated that although different cells and molecules are identified as crucial for protection against TB, there is lack of a comprehensive understanding of exactly constitutes protective immunity. The second challenge is the impact of the host genetics, environmental factors like HIV (Bellamy, 2003) and the genetic diversity of M. tuberculosis in determining the clinical consequences of M. tuberculosis infection (Nicol & Wilkinson, 2008; Coscolla, 2010). Several studies including adoption studies, twin studies, genome-wide linkage and population-based case–control association studies showed there is a significant genetic difference in TB susceptibility (Bellamy, 2006). A recent study showed a significant difference in the type and magnitude of immune responses between UK and Malawi children against BCG where Th1 related cytokines were present at higher levels in the UK infants whereas the innate proinflammatory cytokines, regulatory cytokines, interleukin 17, Th2 cytokines, chemokines, and growth factors were higher in the Malawi infants, which could be due to genetic but also environmental factors like exposure to environmental mycobacterium (Lalor et al., 2011).

Moreover, studies using laboratory and clinical strains have shown differences amongst M. tuberculosis isolates in their immune response. For example, strain NH878 has been associated with a low inflammatory immune response and increased virulence in macrophages and animal models compared to H37Rv, H37Ra, Erdman and CDC1551 (Manca et al., 1999; Tsenova et al., 2005; Manca et al., 2001; Manca et al., 2005). A recent study also showed a wide variation in the immune response after measurement of cytokines from infected human peripheral blood monocyte derived macrophages where modern lineages induced lower inflammatory responses in comparison with ancient lineage. This lower immune
response might promote more rapid disease progression and increase transmission in case of modern lineages (Portevin et al., 2011).

The fourth challenge is selection of sample type to be used for biomarker study and harmonization of the different laboratory techniques. In different laboratories different sample types and different laboratory methods are used to study biomarkers. Therefore, the sample type and laboratory methods need to be harmonized at different laboratories.

The fifth challenge is lack of biomarkers to assess whether a vaccine can induce protection against developing active TB at an early stage and the need for using different sets of optimized biomarkers for different vaccines depending on the vaccine context. While a number of biomarkers have been found to be associated with TB protection or TB disease, there are no qualified biomarkers to indicate protection by new vaccines against TB.

The sixth challenge is shortage of high quality clinical trial sites for testing vaccines and validating biomarkers in different population at different areas. Currently there are very few clinical trial sites which can run clinical trials particularly phase III trials. Therefore, it would be very important to develop high quality clinical trial sites at different sites.

5 Conclusion

TB continues to be a major global health problem and we are still using very old diagnostic techniques particularly in developing countries where the disease burden is high, old drugs with 6 month of therapy which makes adherence questionable and a vaccine which is not at all protective in some part of the world.

In the last decade winds of change are being felt in TB vaccine and biomarker development and the situation has now changed significantly with multiple vaccines in clinical trial. One of the big challenges and a bottleneck in evaluating new vaccines is lack of high-quality clinical trial field sites and absence of any reliable biomarkers of TB protection. Currently several biomarker studies are also undergoing at different laboratories and appropriate samples still need to be collected from individuals with clinically characterized protection and susceptibility phenotypes in different populations at different geographical locations and qualify the different biomarkers. From the different studies it seems that no single biomarker stands out as promising biomarkers on their own but that combinations of such markers hold the greatest promise. The exact composition of such bio-signatures seems to allow some flexibility and depends on the specific clinical situation, on bacterial strains that are prevalent and may also be affected by host genetic and environmental factors. 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.

Therefore, in order to achieve the very ambitious targets of the united nations and stop TB partnership targeted to decrease TB prevalence by half in 2015 as compared to 1990 and to lower the incidence of new cases to less than 1 per million in 2050 (Dye & Williams, 2008), explorative studies on biomarkers and immunologic profiling which could provide clues in developing new diagnostic tools, therapeutics and vaccines are more critical than ever before and will have a significant impact on the global TB problem.
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