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CONFERENCE REPORT

TB biomarkers, TB correlates and human challenge models: New tools for improving assessment of new TB vaccines

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S U M M A R Y

The 4th Global Forum on TB Vaccines, convened in Shanghai, China, from 21 – 24 April 2015, brought together a wide and diverse community involved in tuberculosis vaccine research and development to discuss the current status of, and future directions for this critical effort. This paper summarizes the sessions on Biomarkers and Correlates, and Human Challenge Models. Summaries of all sessions from the 4th Global Forum are compiled in a special supplement of *Tuberculosis*. **[August 2016, Vol 99, Supp S1, S1–S30]**.

1. Introduction

The canonical approach to tuberculosis (TB) vaccine development progresses from an idea based on experimental data to preclinical studies [1]. If protection is consistently observed in all relevant preclinical studies and has passed all essential gating criteria, the vaccine can be selected for entering the clinical trial pipeline. In phase 1 this focuses on obtaining safety and early immunogenicity data while in phase 2 this includes dosing, advanced immunogenicity and early efficacy studies. The failure of a novel TB vaccine candidate to demonstrate protection in a recent phase 2b efficacy trial [2,3] has raised questions about this approach, particularly concerning the stringency of the go/no-go decisions utilized during this process [4]. Application of a more rigorous gating strategy optimally would lead either to termination of development efforts for a vaccine candidate prior to an expensive and resource-intensive phase 2b or phase 3 efficacy trial or to an iterative approach resulting in opportunities to improve the vaccine candidate prior to late-stage testing. The identification of biomarkers of protective immunity to *Mycobacterium tuberculosis* (Mtb) infection or TB disease would represent a major step forward in these efforts to more efficiently select vaccine candidates for advancement to late-stage efficacy trials. While the validation of biomarkers generally requires an efficacy trial of a successful vaccine, and could rely on either or both immunologic (antigen-specific responses) and global gene expression (and other biomic) profiles, it may be possible to identify candidate immune correlates of protection to guide more efficient vaccine up-selection via successful non-human primate (NHP) challenge studies, with subsequent immune bridging between NHPs and humans. Additionally, biomarkers predictive of risk of TB disease, obtained from longitudinal studies on Mtb-infected humans who do or do not develop active TB, would be extraordinarily helpful in designing clinical studies [5,6]. The development of a human Mtb challenge model, which would allow early vaccine efficacy assessments in a small group of volunteers, also would represent a critical advance in the ability to more efficiently assess TB vaccine candidates at an early stage for promotion to late-stage efficacy studies.

Approaches to identify biomarkers of protection are well underway, and efforts to establish a human Mtb challenge model are

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still in an early stage of development. In addition to these approaches, information from vaccination studies of animals that naturally acquire TB, such as cattle, also would be helpful in guiding TB vaccine development efforts. The sessions and presentations on Biomarkers and Correlates and on the Human Challenge Model reviewed aspects of these approaches and their potential importance to future TB vaccine development.

2. Immunodiagnosis of TB disease

TB biomarkers are urgently needed to facilitate and improve accurate, rapid TB diagnosis at point-of-care settings. Currently, much diagnostic delay is due to imperfect diagnostic tools for the early diagnosis of active TB. Prof. Gerhard Walzl (Stellenbosch University, South Africa) presented his recent work on discovering diagnostic TB biomarkers, using both antigen-specific (7-day or overnight-cultured whole-blood assays) and serum or plasma responses to differentiate active TB disease from diseases other than TB. Walzl and colleagues screened 118 different Mtb antigens for an association with TB in confirmed active pulmonary TB patients and in healthy household contacts, by assessing their ability to stimulate interferon-gamma (IFN-γ) secretion into the supernatant of antigen-stimulated cultures analyzed by ELISA. This investigation demonstrated that TB diagnosis was more accurate when three to four Mtb antigens were combined (manuscript submitted). Subsequently, the combination of best performing antigens was determined in 322 participants with presumptive TB, and the supernatants of overnight-stimulated cells analyzed for 42 different cytokines to identify additional markers able to differentiate between active-TB and no-TB. The result of this effort was a 6-marker model with 77% sensitivity and 82% specificity for diagnosing active TB, which was insufficiently informative to pursue further. Analysis by Quantiferon (QFT) tests of cytokines in supernatants also proved insufficient for clinical use.

Walzl described ongoing work to identify a panel of circulating serum markers, since markers of this kind theoretically could be used directly *ex-vivo* in a point-of-care setting. From an ambitious screening of 75 markers obtained from samples from different African clinical sites, 10 markers were found to be associated significantly with TB disease. Using a 6-marker combinatorial approach, it was possible to reach 88.7% sensitivity and 76.0% specificity in a validation cohort. Work is ongoing to establish a 6-marker lateral flow platform utilizing this strategy for field-friendly use.

Walzl concluded his presentation by describing similar studies on cerebrospinal fluid (CSF) from children with TB meningitis, a highly fatal form of TB. CSF and serum samples from 146 children with presumptive TB meningitis were evaluated for 27 preselected markers. A diagnostic model that included three CSF markers (IL-13, VEGF and cathelicidin LL-37) resulted from this study. Although the model had only 52% sensitivity and 95% specificity, Walzl noted that these results represented a significant improvement over the diagnostic approaches currently available, therefore representing a potentially valuable test for the diagnosis of TB meningitis [7].

3. TB candidate vaccine development based on genome-wide high-throughput screening for immunogens

Prof. Lijun Bi (Institute of Biophysics, Chinese Academy of Sciences, China) presented a systematic approach to identifying new Mtb antigens with vaccine and diagnostic potential using a genome-wide, high-throughput screening strategy for proteinaceous immunogens. Bi stated that, because TB subunit vaccine development is currently based on a few antigens, a more complete picture of the mycobacterial proteome is needed. To achieve this, Bi and colleagues developed a high-throughput system to express and analyze Mtb proteins. Using the "ORFome approach" they expressed 95% of the Mtb proteome of H37Rv and CDC1551 (4262 Mtb proteins) in yeast (*Saccharomyces cerevisiae*) as they found that a yeast-based expression system yielded better soluble proteins than the *E. coli* system. Approximately 90% of Mtb proteins were successfully expressed in a soluble form and were used to construct a proteome microarray. The applications of this experimental platform include global studies of protein–protein interactions, small molecule–protein interactions and TB biomarker discovery using serum samples. Using their microarray, Bi and colleagues have selected a panel of 14 candidate biomarkers which is able to effectively discriminate between patients with active TB and recovered individuals based on serum antibodies [8]. They have also screened more than 1000 Mtb proteins as cellular immunogens using IFN-γ release assays. Bi is currently testing 20 candidate vaccine antigens in a guinea-pig model. Candidates that show protective immune responses will be tested further and will enter into clinical trials for vaccine development.

4. Seeking immune correlates of protection against TB disease

Dr. Helen Fletcher (London School of Hygiene and Tropical Medicine-LSTHM, UK) described studies analyzing immune correlates of TB vaccination in relation to protection. She focused on datasets exploring immune correlates of BCG-mediated protection in infants in the South African MVA85A trial [2]. A previous study [9] had demonstrated the lack of predictive potential of classical Th1/Th17 key markers (IFN-γ, TNF-α, IL-2 and IL-17). In this earlier study, transcriptomics was used to investigate differences between case and control infants. Although no overarching specific gene signature was found, infants were found to cluster into two distinct immune phenotypes. Cluster 1 was dominated by a strong IFN-γ, IL-2 and TNF phenotype while cluster 2 was dominated by myeloid and inflammatory gene expression patterns with less IFN-γ, IL-2 and TNF. The numbers of TB cases, however, were equally divided between the two clusters. Despite the opposite trend of T cell signatures in these two immune phenotype clusters, when the clusters were stratified by monocyte/lymphocyte ratios this correlated with risk of TB disease: cases were found to be associated with either high or low but not intermediate ratio determinations.

Transcriptomic analysis of samples from infants from the MVA85A efficacy study is on-going to determine if this distinct clustering of infants can be replicated. A series of assays, including IFN-γ ELIspot and cell-surface flow cytometry, have been performed to assess the BCG antigen-specific response underlying host immunity using samples from the MVA85A trial to characterize the immune phenotypes of children that developed TB. The results further underscore the importance of conducting human clinical trials given their impact on searching for biomarkers of vaccine failure and efficacy – a critical aspect of designing improved future clinical trials.

5. Potential of HLA-E as a novel presentation molecule for vaccine design against TB

Prof. Tom Ottenhoff (Leiden University Medical Center, the Netherlands) described a novel type of immune response he and his colleagues recently discovered in individuals infected with mycobacteria, including Mtb. This pathway involves presentation of Mtb antigens to $CD8^+$ T cells via non-classical HLA-E rather than classical HLA-A,B,C molecules. Compared to HLA-A,B,C, HLA-E

molecules are virtually non-polymorphic (i.e., there are only two coding variants), posing a significant advantage if utilized in vaccine design. Moreover, HLA-E is expressed in the mycobacterial phagosome, potentially allowing it to play a significant role in the presentation of antigenic Mtb peptides to T-cells.

In contrast to HLA-A, HLA-E is not down-regulated in HIV infection, such that antigen presentation via HLA-E is likely to be unaffected by HIV co-infection. Building upon previous work [10], functional and genetic analyses of HLA-E responses were performed on monoclonal $CD8^+$ T cells. The data showed that HLA-E-restricted Mtb peptide-specific $CD8^+$ T-cells displayed unexpected and unorthodox multi-functionality. $CD8^+$ T-cells stimulated in this manner were able to kill human target cells infected with BCG or Mtb and to inhibit intracellular Mtb growth. Yet, they expressed a characteristic type 2 cytokine profile (IL-4, IL-5, IL-13) and an ability to help B-cells. Using HLA-E tetramers, Mtb-specific HLA-E-restricted $CD8^+$ T-cells were visualized in the circulation of TB patients [11,12]. Peptide stimulation of these cells again induced typical type-2 cytokine production. Intriguingly, numbers of HLA-E tetramer-positive cells decreased in response to TB treatment, in contrast with the opposite pattern seen for HLA-A2, suggesting that HLA-E-restricted $CD8^+$ T-cells may make differential contributions to the immunopathogenesis of, and/or protection against TB disease compared to canonical HLA-A2-restricted $CD8^+$ T-cells. Thus, the human immune response to Mtb is more diverse than previously thought, and targeting of the unconventional HLA-E pathway by new TB vaccines may be a complementary strategy for TB vaccination.

6. Developing a human challenge model for vaccine testing

Development and utilization of a human challenge (HC) model has significantly accelerated vaccine development for a variety of diseases, including malaria. Prof. Sarah Fortune (Harvard University, USA) introduced the consortium of researchers, supported by Aeras and the Bill & Melinda Gates Foundation, who have initiated efforts to develop an HC model for TB vaccine development. The first goal of the TB HC development program is to create attenuated mycobacterial strains that meet pre-clinical safety requirements acceptable for HC studies. As the objective of HC studies will be to determine whether a test vaccine augments immune-mediated killing of Mtb, the consortium anticipates that a vaccine-induced immune effect will be more robust if the HC strain is capable of replicating, at least briefly, *in vivo*, independent of immune control. To build a safe strain capable of only a limited period of replication, the group is testing several strategies including establishing nutrient reservoirs in auxotrophs to allow a period of limited prototrophy, tightly regulated expression of essential enzymes achieved either through unnatural amino acid-based regulatory systems or regulated promoters and regulated expression of self-kill switches.

The HC strain would also have to be detectable *in vivo*. Fortune discussed that a pulmonary challenge strain would be more relevant to a natural Mtb challenge but systemic detection of bacterial burden would be difficult. Strategies for strain detection included constructing a strain that either secreted a detectable small molecule or which could be detected through endogenous metabolic activity. Creating a strain for a skin challenge was discussed as an alternative to a pulmonary challenge strain. Although skin administration might be less biologically relevant, detection is quite feasible, and safety concerns would be reduced. Clearly, these efforts are relatively recent and at the next Global Forum further developments are likely to receive significant attention.

7. Using the cattle model of bovine tuberculosis to define biomarkers of vaccine efficacy or disease progression

Cattle provide an interesting model for studying natural transmission and assessing TB vaccine candidates. Prof. Martin Vordermeier (Animal and Plant Health Agency, UK) outlined some of the challenges in cattle TB research, including difficulties keeping these large animals in BSL3 facilities, the lengthy nature of infection experiments and the absence of predictive biomarkers. Bovine TB, caused predominantly by *Mycobacterium bovis*, remains a major problem and the BCG vaccine has variable efficacy against this organism. New vaccine candidates are available for testing but there is a shortage of BSL3 facilities to conduct large, costly trials in large animals. He presented examples of vaccine efficacy studies in cattle after *M. bovis* challenge [13,14]. The combination of BCG prime with Ad85A boost significantly reduced overall pathology compared to BCG alone [13,14]. Vaccinated animals from a typical experiment fell into two groups: vaccinated/protected and vaccinated/nonprotected. To identify markers correlated with protection, the outcome of the challenge (by post-mortem pathological analysis) was correlated with the type of immune responses pre-challenge. Examples of hypothesis- and data-driven biomarker discovery research in this model were presented. An example of the hypothesis-driven approach was an assessment of vaccine-driven central memory T-cell generation as a predictor of the outcome of heterologous prime-boost vaccine strategies, requiring the application of a cultured IFN-γ ELISpot assay [13,15]. This stands in contrast to the use of the standard, *ex vivo* ELISpot assay, which does not specifically assess central memory T-cell responses. To characterize the phenotype of bovine memory cells responsible for these cultured ELISpot responses, T-cell memory was evaluated by measuring IFN-γ-producing cells from both effector memory T-cells ($CD45RO^+$, $CD62L^{lo}$) and central memory T-cells ($CD45RO^+$, $CD62L^{hi}$). These cells were isolated from PBMC and cultured for 12–14 days with antigen and regular addition of IL-2, after which the expanded cells were used in the ELISpot. The results showed that in the short term, *ex-vivo* ELISpot central and effector memory/effector T-cells contributed almost equally to the production of IFN-γ while in the cultured ELISpot, approximately 70% of IFN-γ-producing cells expressed a central memory phenotype. The cultured ELISpot assay at the time of challenge thus correlated with vaccine performance. An example of a data-driven approach was transcriptomics, where data were shown suggesting correlation of IL-17 and IL-22 expression with vaccine efficacy [16]. The main source of IL-17 and IL-22 was from $CD4^+$ and γδ T-cells (in cattle, γδ T-cells represent a more prominent T lymphocyte population than in humans). The cattle model can productively complement similar research efforts in humans and other experimental animal models.

8. Conclusion

The summarized presentations addressed a wide range of issues critical to the development of new TB vaccine candidates. Development of more sensitive and specific diagnostics for Mtb infection and TB disease may open up opportunities for new pathways to vaccine evaluation. High throughput, genomic screening for TB immunogens, and exploration of non-classical pathways of TB antigen presentation, such as the HLA-E pathway, offer the potential for the rational development of new vaccine candidates that could stand alone or complement vaccines currently being studied. Identification of immunological correlates of immune protection would prove invaluable to TB vaccine testing regimens, as would the development of an HC model of TB, as witnessed by the

contribution of a malaria HC model to vaccine development efforts for this disease [17]. Developing such tools would provide essential information for early, rational go/no-go vaccine development decisions, and guide the way forward towards designing more effective TB vaccines, including the activation of novel unconventional immunity. Ultimately, biological samples from ongoing vaccine trials will be needed and information from their analyses must be made available to the broader vaccine community to facilitate iterative, rational and efficient vaccine design; vaccine selection based on comparative performance evaluation; and commitment towards further clinical development [4].

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Ethical approval

Not required.

Conflicts of interest

SHEK is coinventor of the VPM1002 vaccine candidate licensed to Vakzine Projekt Management (Hannover, Germany), and sublicensed to Serum Institute of India (Pune, India). THMO is a member of the Senior Management Team of TBVI, the TuBerculosis Vaccine Initiative. SF, LS, IP and MR have no conflicts of interest to declare.

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