Guidelines and Guidance

Immunological Outcomes of New Tuberculosis Vaccine Trials: WHO Panel Recommendations

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The mechanisms of immune protection against human TB, a disease that causes 2 million deaths world-wide each year, are not fully known. T cell immunity is critical for protection [1,2]; therefore, the current TB vaccine, bacille Calmette-Guérin (BCG), and most new vaccines under development aim to induce this immunity. Most of these developmental vaccines [1–4] are designed to boost pre-existing immunity induced by BCG; however, some candidates aim to ultimately replace BCG as the priming vaccine. Following phase I/IIa trials of the vaccines, safety and immunogenicity results will be critical to decide which vaccine candidates should move into efficacy trials. For this choice, the ability to compare immunogenicity would be an important asset. Potential comparisons are confounded by variation in individual laboratory approaches, logistics, and the diverse populations studied in vaccine trials. Some comparison may be achieved by harmonisation of assays (see below); however, even then, antigen components of vaccines and therefore antigens in assays may differ. Further, the desired character of induced immunity may differ according to vaccine candidate, making choice of an assay to be harmonised difficult.

To tackle this problem, the WHO Initiative for Vaccine Research sponsored three meetings of experts representing current TB vaccine development efforts to discuss the requirements for and challenges in harmonising assays for new TB vaccine trials. The primary focus was on phase I and IIa trials; other principles may apply to phase IIb and III trials because of their larger sample sizes and because resources in settings of these trials may differ. In this article, we describe advantages and disadvantages of multiple assay approaches and make recommendations for using specific assay approaches in phase I/IIa trials.

T Cell Assays that May Be Used in New TB Vaccine Trials

Some T cell assays use whole blood, while others use isolated peripheral blood mononuclear cells (PBMCs). PBMCs may either be used fresh, or after cryopreservation. Assays may have relatively short (less than 24 hours), intermediate (one to three days), or longer (five to seven days) periods of incubation. Each assay approach may have distinct advantages, as summarised in Table S1.

The Guidelines and Guidance section contains advice on conducting and reporting medical research.

Summary Points

- Many new and diverse tuberculosis vaccines are currently under development.

- The ability to compare clinical immunogenicity between different candidates would be an important asset.

- The World Health Organization (WHO) Initiative for Vaccine Research sponsored three meetings of experts to discuss assay harmonization for new tuberculosis vaccine trials.

- We describe advantages and disadvantages of multiple T cell assay approaches and make specific recommendations for phase I/IIa trials. These include introducing a single and simple harmonised assay for all trials.

The choice of assay system may be guided by the aspect of T cell immunity to be measured; for example, as described in Table S1, longer-term assays may be better for measuring central memory T cell responses thought to be critical for long-term protection induced by vaccines [5,6,7]. Choice is

Abbreviations: BCG, bacille Calmette-Guérin; ELISPOT, enzyme-linked immunosorbent spot; IFN, interferon; PBMC, peripheral blood mononuclear cell; TB, tuberculosis; WHO, World Health Organization

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often dictated by what is practical in the setting; for example, if incubation of whole blood or PBMC isolation early after blood collection cannot be accomplished readily, it may be wiser to perform longer-term diluted whole-blood or PBMC assays, to minimise a potentially significant effect of processing delay on assay outcome (see below). Blood volume restrictions in infants, compared with adults, may further dictate assay choice (see below).

T cell assays that are widely used and that appear practical in phase I/IIa trials include five to seven-day whole-blood or PBMC assays of cytokine production and proliferation, overnight or 48-hour enzyme-linked immunosorbent spot (ELISPOT) assays (see protocol in Text S1), and intracellular cytokine assays after six to 18-hour incubation of whole blood or PBMC with specific antigens (see protocol in Text S2). A discussion of these assays, including specific advantages and disadvantages of each and experience in TB vaccinology, may be found in Text S3. Text S3 also includes a discussion of less frequently used assays, such as cytotoxic cell degranulation assays, mycobacterial inhibition assays, and tetramer assays. At this stage, comparisons of assay outcomes among different new TB vaccine trials are not possible, mainly due to the diversity of technical approaches, even if the same basic assay system is used.

Time to Incubation of Whole Blood, or to PBMC Isolation

Delaying incubation of whole blood, or delaying isolation of PBMCs after blood collection, may affect assay outcomes (see Text S3 for more detail). Overall, available evidence suggests that sub-optimal outcomes of shorter-term assays are likely when delays occur from the time of blood collection to incubation, or to PBMC isolation and cryopreservation for later incubation. The panel therefore recommended that until further evidence becomes available, PBMCs for later ELISPOT and short-term intracellular cytokine assays be isolated as soon as possible after blood collection and never more than eight hours after collection, preferably at the same time point after collection in all participants of a specific study. The same principles apply to short-term, undiluted whole-blood intracellular cytokine assays; incubation later than two hours after collection should not be considered. In contrast, longer-term assays, such as a six to seven-day whole-blood assay, appear to be less affected by delays in incubation (H. M. Dockrell, personal communication); we hypothesise that these assays measure expansion of specific T cells, and are therefore less affected than shorter-term assays that measure direct ex vivo function quantitatively.

Recent Advances in PBMC Cryopreservation and Thawing

Multiple variables in the PBMC isolation, cryopreservation, and thawing process affect ultimate recovery of viable, functional cells ([8]; Smith and Dockrell, unpublished data). Although most labs use very similar procedures, conflicting results regarding fine details such as freezing media composition have emerged (T. Kollmann, personal communication; [8]). However, most researchers now agree that assay results of increased quality may be obtained when PBMCs are "rested" for at least four hours after thawing, prior to adding antigens for functional assays. In shorter-term

assays, this procedure may decrease assay background and increase functional response [9,10].

Harmonisation, Standardisation, and Validation of Assays

"Harmonisation" refers to a consensus in assay standard operating procedures for multiple testing sites. "Standardisation" comprises all measures necessary to obtain comparable results, in terms of both time and place. Optimal standardisation will result in comparable results when a test is performed at different times and by different technicians in different laboratories. To achieve such results, standardised materials, reagents, and equipment are important. "Validation" refers to a detailed characterisation of assay performance. Typical validation characteristics include accuracy, repeatability, specificity, detection limit, quantitation limit, linearity, and range. Regulatory authorities require that investigators introduce a validated assay as the primary immunological outcome in new vaccine trials, if the data are intended to be used for licensure. "Qualification" is a term sometimes used to describe partial validation, and refers to an experimental protocol that demonstrates that an accepted method will provide meaningful data, given specific conditions and samples.

Currently, a number of different harmonised standard operating procedures have been prepared within different multi-national consortia or projects such as the European Union–supported TB-VAC and MUVAPRED (Mucosal Vaccines for Poverty Related Diseases) initiatives, Bill & Melinda Gates Foundation Grand Challenges in Global Health projects such as GC6-74 ("Biomarkers for TB"), and EUROVAC. All the assays discussed here are used to some extent by these large consortia, and in some cases the differences between protocols are relatively minor. It should therefore be relatively simple to get stakeholders together and produce harmonised protocols, particularly as small differences in protocols may make a major difference in the outcome of certain assays such as the ELISPOT. The panel therefore recommended that efforts at harmonisation and standardisation of assays should be supported. We recommend starting the discussion process with harmonisation and standardisation of the short-term ELISPOT assay and the longer-term whole-blood interferon (IFN)-γ assay. Further, standardisation of positive controls and detection methods should be attempted. (See Text S3 for more information.)

A Single, Common Assay in New Vaccine Trials

It is likely that investigators and sponsors will continue to introduce their "favourite" assays in new TB vaccine trials. However, a single, harmonised assay common to all vaccine trials would be ideal to allow comparison of immunogenicity results between different vaccine candidates, and the use of such an assay is strongly recommended by members of this expert panel. Ideally, such an assay should be widely implementable, even at remote field sites, while delivering informative results. The panel judged that the seven-day whole-blood IFN-γ assay best meets these criteria and recommended that it be introduced into all new TB vaccine trials. Excellent performance of this assay has been demonstrated in multiple large clinical studies. Additionally, GC6-74 ("Biomarkers for

Box 1. Recommendations of the Panel Regarding Assay Selection for Tuberculosis Vaccine Trials

- Particular emphasis should be placed on standardising *time from blood collection to incubation* or to PBMC isolation in assays.
- *PBMCs should be collected and stored,* for later assessment of whether biomarkers of vaccination-induced protection against tuberculosis that are described are indeed induced by the vaccine, or when critical comparative questions need to be resolved.
- *Harmonisation and standardisation* of these assays, across vaccination sites, should be promoted.
- A *combination* of both shorter-term and longer-term assays would be optimal to measure vaccination-induced immunity, be these PBMC or whole blood–based. The choice of specific assays should be left to individual investigators; it was noted that all current vaccine trials already use a combination of shorter-term and longer-term assays.
- A *single and simple harmonised assay* should be introduced in all trials.

TB") has standardised this method to screen new TB antigens at field sites. A harmonised protocol has been developed (Text S4). It will also be important to standardise reagents and the equipment that measures cytokine levels.

"Vaccine Take" and Immune Correlates/Surrogates of Protection against TB

All current assays described here use the magnitude and, to some extent, the qualitative character of the immune response to measure "vaccine take". Without a complete knowledge of immune correlates of vaccination-induced protection against TB, all assays may be described as vaccine take assays. Regardless, the current assays focus on T cell immunity, particularly IFN-γ production, which is thought to be important for protection. Because emerging evidence suggests that IFN-γ production alone is not necessarily an immune correlate of vaccination-induced protection against TB (W. A. Hanekom, unpublished observations; [11]) it is important to define these correlates in complementary projects. Multiple ongoing projects aim to define immune correlates of protection, which may ultimately be validated as surrogates of protection in phase IIb/III trials of effective TB vaccines. Until these correlates/surrogates are available, it would be extremely useful to also store blood products in a manner that is efficient and that would allow an excellent functional yield of cells or products when thawed at a later stage. These blood products would then be available to measure newly described immune correlates/surrogates of protection, in retrospective studies or for application of newer technologies.

The panel therefore recommended that PBMCs should be stored for possible future use in new TB vaccine trials. Various protocols for PBMC isolation, cryopreservation, and thawing are available [8,10]. It should be noted that the shortest possible interval from blood collection until cryopreservation is desirable (see above), but because this may not be practical, it may be best to standardise the time from blood collection to PBMC isolation. ∎

References

1. Flynn JL (2004) Immunology of tuberculosis and implications in vaccine development. Tuberculosis (Edinb) 84: 93-101.
2. Kaufmann SH, Baumann S, Nasser Eddine A (2006) Exploiting immunology and molecular genetics for rational vaccine design against tuberculosis. Int J Tuberc Lung Dis 10: 1068-1079.
3. Martin C (2006) Tuberculosis vaccines: Past, present and future. Curr Opin Pulm Med 12: 186-191.
4. Skeiky YA, Sadoff JC (2006) Advances in tuberculosis vaccine strategies. Nat Rev Microbiol 4: 469-476.
5. Lauvau G, Pamer EG (2001) CD8 T cell detection of bacterial infection: Sniffing for formyl peptides derived from *Mycobacterium tuberculosis*. J Exp Med 193: F35-F39.
6. Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, et al. (2002) HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nat Immunol 3: 1061-1068.

7. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401: 708-712.
8. Disis ML, dela Rosa C, Goodell V, Kuan LY, Chang JC, et al. (2006) Maximizing the retention of antigen specific lymphocyte function after cryopreservation. J Immunol Methods 308: 13-18.
9. Horton H, Thomas EP, Stucky JA, Frank I, Moodie Z, et al. (2007) Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination. J Immunol Methods 323: 39-54.
10. Maecker HT, Moon J, Bhatia S, Ghanekar SA, Maino VC, et al. (2005) Impact of cryopreservation on tetramer, cytokine flow cytometry, and ELISPOT. BMC Immunol 6: 17.
11. Mittrucker HW, Steinhoff U, Kohler A, Krause M, Lazar D, et al. (2007) Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. Proc Natl Acad Sci U S A 104: 12434-12439.