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COMMENTARY

Toward Tuberculosis Vaccine Development: Recommendations for Nonhuman Primate Study Design

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ABSTRACT Clinical trials of novel tuberculosis (TB) vaccines are expensive, while global resources for TB vaccine development are limited. Therefore, there is a need for robust and predictive preclinical data to support advancement of candidate vaccines into clinical trials. Here, we provide a rationale for using the nonhuman primate as an essential component of these efforts, as well as guidance to the TB community for standardizing experimental design and aligning endpoints to facilitate development of new TB vaccines.

KEYWORDS nonhuman primate, tuberculosis, vaccines

In this issue of *Infection and Immunity*, Maiello and colleagues (1) describe differential susceptibility and disease manifestations of rhesus macaques, Chinese cynomolgus macaques, and Mauritian cynomolgus macaques following *Mycobacterium tuberculosis* infection. This report is a critical component of broader efforts in the tuberculosis (TB) vaccine field to better understand and utilize the nonhuman primate (NHP) model for vaccine development.

The Nonhuman Primate Research Community is one of multiple research communities under the umbrella of the Collaboration for TB Vaccine Discovery (CTVD; www.ctvd.co), an initiative of the Bill & Melinda Gates Foundation to foster innovation, collaboration, and cooperation in TB vaccine discovery. The mandate of each community, comprised of 5 to 20 subject experts, is to define research priorities that must be addressed for developing a successful TB vaccine. The CTVD Nonhuman Primate Research Community includes investigators who specialize in NHP models of infectious diseases, evaluation of immune responses induced by vaccines and infection, and vaccine research and development (R&D). The overall goals are to understand the role of the NHP in TB vaccine development and to standardize and harmonize its application for this purpose. Efforts also focus on delineating mechanisms and correlates of vaccine-mediated protection, as these mechanisms and correlates will inform clinical testing of vaccines and guide discovery of next-generation candidates.

TB disease manifestations in NHPs are very similar to those observed in humans, and this model is therefore regarded as the most reliable for testing TB vaccines in product development. However, final validation of the NHP model is possible only after successful efficacy testing of a novel TB vaccine in humans, when results can be back-translated to the NHP. All animal models have limitations, which must be understood for fit-for-purpose application; therefore, definite and distinctive roles also exist for

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mice, guinea pigs, and other species in TB vaccine development. Regardless, similarities of the NHPs to humans make this model the most translatable. Different NHP species develop latent infection, pneumonia, lymph node involvement, and dissemination; this is similar to heterogeneity observed in humans. Importantly, pathological manifestations, particularly different granuloma phenotypes, are comparable to those in humans; e.g., NHP granulomas also progress to caseation and cavitation. Further, anatomical, genetic, and immunological features that closely resemble the features in humans often allow use of widely available human imaging and assay tools in experimental settings.

The CTVD Nonhuman Primate Research Community has made significant progress toward its goals over the last 2 years. Here, we describe advances in understanding different macaque species and approaches to challenging NHPs with TB and measuring outcomes.

Choice of nonhuman primate species. Rhesus and cynomolgus macaques are the most commonly used NHP species in TB vaccine and pathogenesis research. The two species differ in *M. tuberculosis* infection susceptibility and disease manifestations. In naive cynomolgus macaques, low-dose infection (10 to 25 CFU of *M. tuberculosis* Erdman), delivered intrabronchially, results in disease in approximately 50%, while the rest display disease-free latent infection (2). In contrast, in naive rhesus macaques, the same challenge dose commonly results in disease in all animals. As described by Maiello et al. (1), rhesus macaques display higher rates of disease progression and greater involvement of lung parenchyma and intrathoracic lymph nodes, more extrapulmonary dissemination, and higher bacterial burdens than cynomolgus macaques. Similar differences between rhesus and cynomolgus macaques have been previously reported following high- and ultralow-dose aerosol challenge (3, 4).

The highly differential outcomes of cynomolgus macaques, including latent infection and differential disease progression, with lower prominence of intrathoracic lymph nodes and dissemination, may mimic adult pulmonary TB. However, because of the high proportion of animals that develop latent disease, demonstrating a TB vaccine effect would require relatively large numbers of animals. The prominence of intrathoracic lymph nodes and dissemination in macaques is reminiscent of childhood TB, although severe pulmonary disease and higher bacterial burdens are not common in childhood. Still, the more rapid course of disease evolution in this species allows greater stringency in testing vaccine efficacy using smaller numbers of animals. Importantly, differential fit-for-purpose use of species will allow assessment of specific scientific questions. For example, the cynomolgus model may be more useful for experiments studying latent disease or vaccine efficacy in latently infected individuals.

Global vaccine development efforts primarily focus on targeting adolescents and young adults; therefore, we recommend the use of macaques greater than 3 years of age at challenge. Secondary efforts focus on developing new vaccination strategies for infants; to model this, newborn animals should be used.

Prior mycobacterial immunization or infection in vaccine candidate populations. Most adolescents and young adults in countries with a high TB burden have been vaccinated with *Mycobacterium bovis* BCG at birth and subsequently exposed to environmental mycobacteria and/or infected with *M. tuberculosis* (latent infection). This exposure to environmental mycobacteria and other microorganisms varies geographically and has been proposed to modulate immunity to BCG vaccination both in humans (5) and in NHPs, where genetically similar NHPs from different breeding colonies displayed unequal protection after BCG vaccination (6).

While many NHPs raised in outdoor facilities encounter mycobacterial antigens either from natural exposure or from TB surveillance testing, differences in exposure between colonies have not been systematically addressed. However, for vaccine testing, NHPs can be excluded or randomized into groups based on animal origin as well as preexisting cellular immune responses to mycobacterial antigens (e.g., purified protein derivative from *Mycobacterium avium* and *M. bovis*).

Mycobacterial exposure in humans can be modeled in the NHP, although these approaches are resource demanding. BCG vaccination of NHPs at birth could be followed by novel vaccination at 3 to 4 years of age. However, a more practical approach to modeling prior BCG immunization is priming NHPs with the standard human dose of BCG intradermally at least 6 months before administering a novel TB vaccine, understanding that this cannot fully reflect what happens in the clinical setting. Studies comparing the immunogenicity and efficacy of a booster vaccination given to cohort-matched NHPs primed with BCG at birth or at 4 years of age are under way (S. Sharpe, personal communication) and will inform our use of BCG in this model.

To model human vaccination in the context of *M. tuberculosis* infection—up to 80% of adolescents and adults in countries where TB is endemic may have latent infection—the cynomolgus macaque model should be used, as 50% develop latent infection after low-dose *M. tuberculosis* challenge. Animals should be monitored for at least 6 months to reproducibly discriminate between active disease and latency. Novel vaccine "take" can be measured immunologically in comparison to uninfected, vaccinated control animals, but prevention of disease in the setting of latency may require either reinfection or immune modulation (e.g., steroids, anti-tumor necrosis factor [anti-TNF], or infection with simian immunodeficiency virus [SIV]). The conditions for modeling vaccination during latency are not yet optimized but will be resource intensive, requiring large numbers of animals monitored for long periods of time.

Novel TB vaccines will be used in populations with high incidence of human immunodeficiency virus (HIV) infection, diabetes mellitus, and nutritional deficiency. While early vaccine investigation generally occurs in individuals without such comorbidities, HIV infection can be modeled in the NHP at later stages of product development. The SIV-infected NHP model is well characterized, and SIV infection of latently infected cynomolgus macaques results in reactivation disease (7). Therefore, this coinfection model can contribute to the safety and efficacy testing of novel vaccines in the context of prior *M. tuberculosis* infection.

NHP challenge with *M. tuberculosis*. Many diverse clinical and laboratory strains of *M. tuberculosis* exist and can result in significantly different outcomes following infection (8). It is not practical to perform expensive NHP challenge experiments with multiple strains (though this may happen later in product development); therefore, for benchmarking, we have selected the highly pathogenic *M. tuberculosis* Erdman strain. To avoid variability introduced by different sources and to maximize standardization, a large stock of the Erdman strain has been made for the CTVD NHP Research Community and wider research use, and will be made available through BEI Resources. This strain contains digital barcodes (9), enabling quantification of the number of bacteria that establish infection. This allows for the confirmation of the challenge dose and can be used as a surrogate for prevention of infection; barcodes also allow for assessment of infection dynamics and bottlenecks (as some granulomas are more permissive for mycobacterial growth than others). The barcoded strains would also be useful for low-dose repeat challenge studies, although this approach is not used routinely in NHP studies of novel vaccines. Importantly, a CTVD-funded core facility will be established to aid in standardized analysis of barcoded infection data.

Outcome measures of *M. tuberculosis* challenge studies. We are recommending tiered outcomes, depending on available resources in different laboratory settings, and relative depth of evaluation sought after in different experiments.

We have chosen and are recommending two minimum endpoints to align among CTVD NHP Research Community laboratories: serial positron emission tomography and computed tomography (PET-CT) imaging and gross pathology on necropsy. PET-CT imaging assesses structural abnormalities as well as active inflammatory foci with an [^{18}F]fluorodeoxyglucose (FDG) probe. The modality has been optimized for *M. tuberculosis*-infected NHPs and can be used to evaluate disease longitudinally in a sensitive, noninvasive, and quantitative manner. Total lung "PET HOT" activity has been shown to correlate with bacterial burden and gross pathology of *M. tuberculosis*-infected NHPs, as well as to predict

disease outcome (active or latent) and risk of reactivation in latently infected cynomolgus macaques (10). Longitudinal scanning also enables the study of infection dynamics and monitoring the progression (or regression) of individual granulomas (including identification of "cold" granulomas from which bacteria cannot be cultured). Three core CTVD primate facilities will have the same custom-configured PET-CT imagers (Mediso Ltd.), and the data from each imager will be analyzed at a core facility at the University of Pittsburgh. Guidelines for imaging and image analysis have recently been published (11). PET-CT of NHPs can be aligned with PET-CT imaging of *M. tuberculosis*-infected humans. In laboratories that do not have PET-CT capacity, CT scanning alone is a useful, although less sensitive, alternative.

The second criterion is pathology. Gross pathology scores assess the number and size of granulomas, presence of complex pathologies, and extent of dissemination. It is a useful method for assessing the overall extent of disease in an animal. The scoring system that is recommended is based on a study by Lin et al. for the cynomolgus macaque (2), and has been expanded to capture the greater extent of disease observed in rhesus macaques (and included as supplemental material in the article by Maiello et al. [1]). Most members of the CTVD NHP Research Community feel that necropsy should be completed at 12 weeks after challenge; however, we recognize that there may be specific settings where longer follow-up would be required—particularly in the cynomolgus model.

Bacterial burden is also an important outcome parameter in evaluating novel vaccines; however, this endpoint has not been standardized. In Maiello et al. (1), quantitative bacterial burdens were obtained through detailed necropsy and assessment of each individual lesion. Alternative and less-resource-intensive strategies include stereologic sampling of the lung to extrapolate total bacterial burden (12) or whole-lung homogenization for the direct assessment of bacterial burden (following the assessment of gross pathology).

Ultimately, outcome measures in the NHP would have to be back-validated following a successful efficacy study in humans.

Every NHP experiment in TB vaccine development requires statistical consultation for design to ensure that outcomes are able to measure differences between study arms or at least to understand prior to the experiment what assessment is possible. For example, if PET-CT imaging is used to determine the primary outcome, we find that $<$10 animals per group rarely produces results that are interpretable. However, sample size and other outcome parameters depend on many factors and always require expert analysis.

Immune monitoring in studies of novel vaccines. The CTVD NHP Research Community recommends aligning assays that assess the innate and adaptive immune responses after vaccination and challenge to facilitate comparisons of vaccine immunogenicity across institutions. Additionally, every effort should be made in product development planning to align NHP and human immunoassays. Host immune assessment should focus not only on vaccine take but also on delineating mechanisms and correlates of vaccination-induced protection.

Blood is the most accessible source for studies; however, in both NHPs and humans, lung immune responses may be assessed in bronchoalveolar lavage (BAL) fluid specimens. Lung tissue-resident immunity is strategically positioned for immediate effector function after challenge and thus is a critical compartment to measure immune function.

For immune analysis, multiparameter flow cytometry can be used to assess the phenotype and function from lung and blood using standardized staining panels. Flow cytometry panels have been developed to characterize the phenotypes of conventional CD4 (e.g., Th1, Th2, Th17, Th1*, Tfh, and Trm) and CD8 (e.g., major histocompatibility complex class I [MHC-I]- or HLA-E-restricted) T cells, donor unrestricted T cells (e.g., gamma delta, mucosa-associated T cells [MAITs], invariant natural killer cells [iNKT]), memory subsets, activation markers, proliferation, chemokine receptor expression, and

functional cytokine production in vaccinated and/or TB-infected NHPs. Recent advances in rhesus tetramer reagents should be incorporated to define so-called donor-unrestricted T cell populations, such as MAITs and CD1d-restricted T cell populations, which may contribute to protection against TB. As development of these staining panels is time intensive and costly, researchers are encouraged to disclose panel details in primary research or OMIP (optimized multicolor immunofluorescence panel) publications where panels can be shared and referenced by others.

A major advantage of using NHPs for studies is the ability to assess immune responses in tissue following necropsy or lymph node biopsy specimens. Indeed, an important question is whether immune responses in BAL fluid samples are similar to lung parenchymal responses and, if so, whether these can be correlated with protection. Finally, confirmation that T cell responses are derived from tissue, rather than from contaminating blood, has recently become feasible in NHPs using intravascular staining of lymphocytes by injection of a CD45 antibody (13). Collectively, these techniques and analyses allow for systemic assessment of immune responses and should better define immune correlates and mechanisms of protection.

For assessment of immune responses in blood following vaccination and challenge, we recommend batched longitudinal analysis on cryopreserved peripheral blood mononuclear cells (PBMC) to minimize day-to-day variation in flow cytometric analysis. However, BAL fluid samples are typically analyzed fresh due to low cell yield and concerns of decreased viability after freeze-thawing. In addition, some minimal analysis during the vaccination phase should be performed to confirm vaccine take. PBMC samples for immune analysis should be collected before vaccination (preimmune), after vaccination (at peak and at the time of challenge), and after challenge (to monitor anamnestic responses). Some experts feel that BAL fluid collection postchallenge should be avoided, out of concern of exacerbating disease.

Finally, the field of host immune assessment is expanding rapidly, with availability of many novel approaches. Careful planning to preserve samples for future RNA or T cell receptor (TCR) sequencing, or plasma analysis, should be considered in all cases.

Facilitating global NHP experimentation in TB vaccine research. Central to the CTVD NHP Research Community's goal of facilitating the development of a TB vaccine is to provide support to the TB research community at large. We have extensive collections of rhesus and cynomolgus macaque samples, both pre- and postchallenge (including peripheral blood mononuclear cells, whole-blood RNA, plasma, or serum, and fixed tissues), that are available to investigators interested in testing their concepts in the NHP model. We also offer guidance in designing NHP experiments to those considering the use of this model in our laboratories or elsewhere.

CONCLUSION

While small-animal models will justifiably continue to play an important role in TB vaccine research and scientific discovery, NHPs serve as a useful model to define immunological mechanisms and correlates of protection and to allow stage gating of vaccine candidates for clinical development. Until a new partially or even fully protective vaccine becomes available, the predictive value of any animal model remains unclear, but by aligning NHP studies with human clinical trials, we hope to validate the NHP model and maximize the utility of the model and associated tools in TB vaccine research and development.

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