MINIREVIEW

The current state of animal models and genomic approaches towards identifying and validating molecular determinants of *Mycobacterium tuberculosis* infection and tuberculosis disease

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One sentence summary: This review discusses the current state of the important animal models and techniques being used to study *Mycobacterium tuberculosis* infection and tuberculosis disease as it occurs in humans.

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ABSTRACT

Animal models are important in understanding both the pathogenesis of and immunity to tuberculosis (TB). Unfortunately, we are beginning to understand that no animal model perfectly recapitulates the human TB syndrome, which encompasses numerous different stages. Furthermore, *Mycobacterium tuberculosis* infection is a very heterogeneous event at both the levels of pathogenesis and immunity. This review seeks to establish the current understanding of TB pathogenesis and immunity, as validated in the animal models of TB in active use today. We especially focus on the use of modern genomic approaches in these models to determine the mechanism and the role of specific molecular pathways. Animal models have significantly enhanced our understanding of TB. Incorporation of contemporary technologies such as single cell transcriptomics, high-parameter flow cytometric immune profiling, proteomics, proteomic flow cytometry and immunocytometry into the animal models in use will further enhance our understanding of TB and facilitate the development of treatment and vaccination strategies.

Keywords: *Mycobacterium tuberculosis*; animal models; translation

INTRODUCTION

Tuberculosis (TB) is a widespread infectious disease, latently infecting up to one third of the world's population and causing ~1.3 million deaths in 2016 (WHO 2017). Many of the world's regions with high disease prevalence are rife with malnutrition, have limited access to medical resources and are hotbeds for non-compliance with medical interventions, leading to multidrug resistant and extensively drug-resistant strains (Gandhi *et al.* 2006; Migliori *et al.* 2012).

Mycobacterium tuberculosis (Mtb) is a pathogen that is spread by aerosol droplets to uninfected individuals when infected patients cough (Figure 1). In the lungs, *Mtb* comes into contact with innate immune cells that attempt to eliminate the pathogen. When *Mtb* comes into contact with alveolar macrophages, it can infect the cells and persist following phagocytosis as an intracellular pathogen. During the innate immune phase, inflammatory cells, including dendritic cells, neutrophils and macrophages, traffic to the lungs and attempt to control the infection (Nunes-Alves *et al.* 2014). Dissemination of *Mtb* to the lymph nodes occurs, allowing dendritic cells to present bacterial antigens to T cells and prime them (Chackerian *et al.* 2002; Wolf *et al.* 2008). Priming of ESAT6 in particular occurs ~10 days post-infection in the mediastinal lymph nodes and is followed later by generation of effector T cells (Reiley *et al.* 2008). The adaptive immune response that follows T cell priming, particularly the generation of effector T cells and Th1 CD4$^+$ T cells, leads to the formation of granulomas. Granulomas are organized structures where T cells and B cells encapsulate innate immune cells, such as activated neutrophils and macrophages, with a fibrotic capsule to create a gradient of hypoxia, with the greatest concentration of hypoxia formed in its center to isolate and prevent the growth of *Mtb* (Kumar *et al.* 2011). Granulomas caseate and fail to contain *Mtb* when host-specific responses are insufficient to prevent bacterial replication, *Mtb*-infected macrophages necrotize and bacilli escape the hypoxic core to disseminate into uninfected lung tissues or secondary sites of infection including the liver, spleen and brain. *Mtb* has evolved numerous mechanisms of host resistance including its ability to alter T cell cytokines in infected cells, such as interleukin-1β produced by dendritic cells, to induce Th2 immune responses and inhibit protective Th1 host responses (Dwivedi *et al.* 2012).

When patients are infected with *Mtb,* there are multiple clinical outcomes that they can experience. Many patients will control the infection and are considered latently infected (LTBI). These individuals typically possess just a few intact granulomas and are positive on the Mantoux tuberculin skin test (TST). About 10% of all patients with TB will progress to active infection over the course of their lifetimes, some with early progression (Kwan and Ernst 2011). Comorbidities can also induce progression to active disease in latently infected patients, termed reactivation, including, but not limited to, type 2 diabetes (T2D) and human immunodeficiency virus (HIV) (Kwan and Ernst 2011; Ai *et al.* 2016). HIV alone increases the rate of activation from 10% over a patient's lifetime to 10% per year (Kwan and Ernst 2011). Due to the wide variety of potential states and outcomes, we currently lack the knowledge and tools to distinguish which individuals have cleared or will control *Mtb* and which will progress to active TB disease. The lack of information on responses that are associated with subsequent progression to active TB impedes identification of individuals at the highest risk of progression to active TB and who would benefit most from preventative therapy in resource-poor, high TB-prevalent regions. Additionally, the lack of information on favorable immune

responses that lead to containment of infection, as well as their distinctions from those associated with progression to active disease, is a significant impediment to the design and evaluation of efficacious TB vaccines. Although it is generally understood that Th1 responses, such as IFN-γ that are generated in response to mycobacterial antigens, are protective, Th1 responses during active disease are unable to sterilize the host, complicating our ability to measure successful vaccination (Nunes-Alves *et al.* 2014).

As many mechanisms of pathogenesis and host response to infection are poorly understood, it is imperative to utilize animal models in order to investigate them. Animal models of TB infection and TB/HIV co-infection have the potential to yield insights about the host's responses to different strains, to different therapeutics and vaccines. They also have the ability to faithfully replicate comorbidities, particularly in the use of non-human primates (NHPs) with TB that are co-infected with simian immunodeficiency virus (SIV). The model used for studies, however, should always be carefully considered to maximize homology to human disease or symptomatology. For example, zebrafish infected with *M. marinum* have been a vital host to study caseous pathology within granulomas (Swaim *et al.* 2006; Cronan and Tobin 2014) despite being a non-mammalian organism. Balancing the ethical costs of the model, as well as the knowledge gap under investigation, are essential when choosing the proper animal model for studying TB infection. Here we discuss the most prominently used animal models of *Mtb* infection and their specific role in the identification of molecular determinants of TB pathology and immunity. We also describe the use of the various contemporary genome-wide approaches in the animal models of TB.

ANIMAL MODELS OF TB

Numerous animal models have been utilized for mechanistic and vaccine studies for TB. These range from more accurate, but more expensive, NHPs, to small and non-mammalian organisms like zebrafish. While NHPs best capitulate the human immune response and susceptibility to TB, often smaller animal models like mice, rats, guinea pigs, rabbits and zebrafish are better utilized for investigating more narrow aspects of the immune response to mycobacteria, such as granuloma formation, susceptibility to different strains, or pre-clinical vaccine responses. A summary of the characteristics of each model is shown in Table 1.

NHPS

NHPs have been actively used primarily in the national primate research centers. The seven national primate research centers possess specialized personnel, large facilities and a wealth of expertise and experience that allows complex NHP research to be conducted safely and effectively in a BSL3 setting. In addition, several other research centers and universities in the USA and around the world have developed facilities to leverage NHP models in addition to smaller animal models such as mice, rabbits, guinea pigs and rats. The use of NHPs, particularly macaques, as models of human disease has significantly increased recently. Of the >15 000 publications in PubMed that utilized macaque models of human disease, 60% were published in the last 10 years. These systems have also been significantly utilized in the modeling of infectious diseases, particularly HIV (~4500 publications) (Hulskotte, Geretti and Osterhaus 1998; Veazey *et al.*
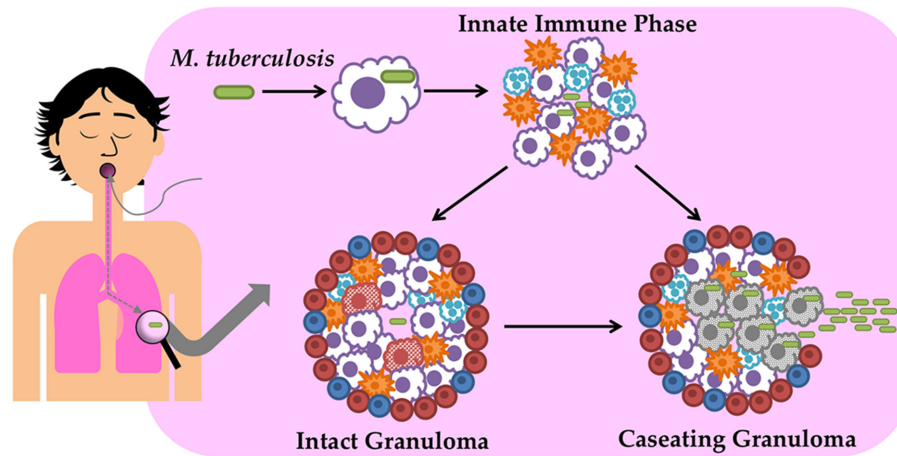
Figure 1 *Mycobacterium tuberculosis (Mtb)* is spread via aerosols and is harbored primarily in the lungs. When macrophages encounter and engulf the pathogens, it may be eliminated or may persist due to compensatory mechanisms. During the innate immune phase, macrophages, neutrophils and dendritic cells are recruited to the site of infection. After severeal week, T and B cells migrate to the site of infection and form granulomas that encapsulate the innate immune cells and bacilli in a fibrotic capsule that creates a gradient of hypoxia. If the immune cells are not able to kill the bacilli and prevent its growth, granulomas caseate and *Mtb* bacilli disseminate to other organs including the spleen, liver and brain.

Table 1. Comparison of biosafety safety level 2 (BSL-2) and BSL-3 animal models of *Mtb* infection.

	Non-human primates	Mouse	Rabbit	Guinea pig	Rat	Zebrafish
Susceptible to human clinical strains of *Mtb*	Yes	Yes	Yes	Yes	Yes	No
Potential for PK studies	Yes, but significant cost	No, differs from humans	Yes	No, differs from humans	Yes	No
BSL-2 model	No	No	No	No	No	Yes
BSL-3 model	Yes	Yes	Yes	Yes	Yes	No
Easy to house	No	Yes	No	No	Yes	Yes
Pulmonary pathology	Yes	Yes	Yes	Yes	Yes	No
Caseous pathology	Yes	No, except C3HeB/FeJ mouse	Yes	Yes	Yes	Yes
Cavitary lesions	Yes	No	Yes	No	No	Yes
Dissemination	Yes	Yes	Yes	Yes	Yes	Yes
Latency	Yes	No	Yes	No	Yes	Yes
Ready availability of immunological reagents	Yes, human cross-reactivity	Yes	No	No	No	No
CD1-cross reactivity	CD1a, CD1b, CD1c, CD1d	CD1d only	CD1a, CD1b, CD1d	CD1b, CD1c	CD1d only	No

1998; Douek *et al.* 2002; Franchini *et al.* 2002; Brenchley *et al.* 2004; Hatziioannou *et al.* 2009; Weed *et al.* 2012; Kimata 2014), HSV (Peretti *et al.* 2005; Crostarosa *et al.* 2009; Calenda *et al.* 2017), influenza (Brown *et al.* 2010; Kitano *et al.* 2010; McDermott *et al.* 2011), *Streptococcus pneumoniae* (Philipp *et al.* 2012), *Neisseria gonorrhoeae* (Weyand *et al.* 2013), non-tuberculous mycobacteria (Winthrop *et al.* 2010; Henkle and Winthrop 2015) and malaria (Lombardini *et al.* 2015; Phares *et al.* 2017). These models have not only investigated the pathogenesis of infection, but have also related tissue pathology, vaccine and drug development and, of late, host-directed or immunotherapeutics (Kaushal *et al.* 2015).

Macaques have been extensively used in TB research (~150 publications) (Kaushal and Mehra 2012; Kaushal *et al.* 2012; Foreman *et al.* 2017). In fact, macaques were extensively used for studying experimental *Mtb* infections in the early and middle parts of the 20th century, but as the advent of antibiotics and the widespread use of the Bacillus Calmette-Guérin (BCG) vaccine resulted in reduced TB rates, interest waned. Barclay led key

studies five decades ago where Indian rhesus macaques were infected via the aerosol route with *Mtb*.(Barclay *et al.* 1970). Barclay *et al.* tested both BCG and *Mtb* cell wall as effective vaccines against *Mtb* challenge in this species (Ribi *et al.* 1971; Anacker *et al.* 1972), even demonstrating the feasibility of utilizing aerosol BCG vaccination to protect against TB challenge (Barclay *et al.* 1973). As TB disease resurged in the developed world in the 1980s and 1990s, this led to renewed interest in its research and it was at once clear that the model systems available to study TB at that time were insufficient. Walsh led the study of *Mtb* infection in the modern era in cynomolgus macaques and showed that it was possible to generate human-like LTBI in that species (Walsh *et al.* 1996). The cynomolgus macaque was then extensively used, primarily by Flynn and colleagues, to study aspects of *Mtb* infection (Capuano *et al.* 2003; Lin *et al.* 2009), especially early infection (Lin *et al.* 2006), reactivation due to SIV co-infection (Diedrich *et al.* 2010) and for vaccine evaluation (Lin *et al.* 2012; Mehra *et al.* 2013). The work of several other groups

simultaneously led to a renewed interest in the rhesus macaque as a model for TB pathogenesis (Gormus *et al.* 2004; Lewinsohn *et al.* 2006; Qiu *et al.* 2008; Chen *et al.* 2009; Sharpe *et al.* 2009; Verreck *et al.* 2009, Sharpe *et al.* 2010). *Mtb*-infected rhesus macaques have since been utilized extensively to study the role of bacterial determinants of pathogenesis (Dutta *et al.* 2010), e.g. stress-response transcription factors (Mehra *et al.* 2012, 2015). These studies generated results different to those obtained in the typical mouse model and extended our knowledge of how *Mtb* has evolved to manage stress in its attempt to persist. This model has been invaluable to replicate the immune responses that lead to human granuloma formation and their failure because NHPs are able to produce granulomas that are comparable with those found in humans (Figure 1) (Mehra *et al.* 2010, 2013; Slight *et al.* 2013). The NHP model has been vital for testing vaccines and vaccine-induced responses (Kaushal *et al.* 2015; Foreman *et al.* 2017), in elucidating *Mtb*/HIV co-infections using SIV as a surrogate (Diedrich *et al.* 2010; Sestak *et al.* 2011; Foreman *et al.* 2016) and in ascertaining the role of individual cell types (Kuroda *et al.* 2018) and immune/metabolic pathways/molecules (Diedrich, Mattila and Flynn 2013; Cheng *et al.* 2017).

Old World NHPs originating from Africa and Asia, including macaques, are prevalent as an NHP model in TB research. New World NHPs, originating from Central and South America as well as Mexico, e.g. marmosets, have also been used for TB research. A challenge of the marmoset model remains that even low-dose infection has been shown to yield intensely pathological outcomes, making it difficult to faithfully model TB latency (Via *et al.* 2013; Cadena *et al.* 2016). Marmosets remain a captivating model organism, however, due to the ability to standardize experiments using twins, particularly for comparisons of strain virulence and disease progression (Via *et al.* 2013). Marmosets have also been a useful model for investigating the effect of different drug regimens because they produce fully formed granulomas that can be collected and evaluated to determine if a drug has fully sterilized a granuloma (Via *et al.* 2015). This is a method that has been evaluated additionally in cynomolgus macaques (Gideon *et al.* 2015). There is limited work on TB in baboons, although that species of monkeys is also susceptible to *Mtb* under experimental conditions (Martino, Hubbard and Schlabritz-Loutsevitch 2007). It may be useful to look at the effectiveness of the baboon as a model of TB co-infection because extensive work has been done in baboons to study T2D (Rincon-Choles *et al.* 2012). T2D is known to promote *Mtb* infection and TB disease (Gauld and Lyall 1947; Kapur and Harries 2013). Treatment strategies for T2D have exhibited promise as anti-TB host-directed therapeutics (Singhal *et al.* 2014).

In terms of determining which species to use as a model for TB, an understanding of what one wishes to accomplish must be reached, considering both the strain of *Mtb* being used as well as what effect is being investigated. In side-by-side comparisons of rhesus macaques and cynomolgus macaques, the former have been found to be more susceptible to developing active TB infections when infected with a virulent strain, *Mtb* Erdman (Langermans *et al.* 2001; Sharpe *et al.* 2009; Maiello *et al.* 2017). The lowest dose used of *Mtb* Erdman by Sharpe *et al.*, however, was ∼30 CFU, with an upper limit of 500 CFU (Sharpe *et al.* 2009). In a later study, where a retained aerosol exposure was calculated to be as low as 3 CFU of *Mtb* Erdman, Sharpe *et al.* still found that rhesus macaques were overall more likely to progress to active disease and that cynomolgus macaques possessed a reduced disease burden (Sharpe *et al.* 2016). When a less virulent strain is used, such as CDC1551, it is possible to investigate latency even in rhesus macaques (Sestak *et al.* 2011; Foreman *et al.* 2016).

VALIDATION OF MOLECULAR DETERMINANTS OF INFECTION, DISEASE AND PROTECTION IN THE NHP MODEL

Over the last decade, important discoveries have been made using innovative approaches, including novel genomic techniques, to validate molecular determinants of infection and disease in NHPs. In 2007, Huang *et al.* established and validated a real-time quantitation system that identified 78 immune genes that were induced in rhesus macaques that were vaccinated/challenged with BCG (Huang *et al.* 2007). The first application of a system-wide approach in the NHP model of TB infection showed that gene expression in recently formed lung granulomas is characterized by a massive cytokine storm (Mehra *et al.* 2010). However, with the establishment of the chronic state of infection, this intense expression of pro-inflammatory cytokines was rapidly inhibited in later granulomas (Mehra *et al.* 2010). This study provided significant insights into the functioning of the primate granuloma. The initial study, conducted in rhesus, was then validated in cynomolgus macaques, where the effect of BCG vaccination on the progression of granuloma gene expression was also studied (Mehra *et al.* 2013). These studies indicated that primate lung TB granulomas were characterized by a high degree of expression of markers of adaptive immune dysfunction, such as indoleamine 2,3-dioxygenase (IDO), Arg1, LAG-3 and PD-1 (Mehra *et al.* 2013). Many of these observations have since been validated (Mattila *et al.* 2013; Phillips *et al.* 2015; Gautam *et al.* 2017). These results suggest that either immune exhaustion or suppression events may be common in granulomas. In support of this, T cells derived from NHP TB granulomas appear to be dysfunctional (Gideon *et al.* 2015), although it has recently been suggested that exhaustion may not be the main mechanism for this (Wong *et al.* 2018). Inhibition of IDO activity relieves the inhibition on T cells (Gautam *et al.* 2017) and it may soon be possible to test the impact of inhibiting other immune checkpoints, e.g. LAG-3 and PD-1 in this model, using either an antibody/depletion or inhibitor/antagonism approach. Numerous other cellular pathways that have been identified to be involved in TB pathogenesis using murine models of TB can be validated using NHPs to assess the feasibility of designing host-directed therapies directed against these pathways. These include the C-Abl pathway (Napier *et al.* 2011, 2015), the SIRT pathway (Cheng *et al.* 2017) and the AMPK pathways (Singhal *et al.* 2014). The C-Abl pathway can be modulated by the inhibitor Imatinib, a tyrosine kinase inhibitor (TKI). Inhibition of the C-Abl pathway by Imatinib correlates with better control of TB via myelopoesis (Napier *et al.* 2011, 2015). The SIRT pathway can be induced using agonists like pterostilbene (PTS) and its induction correlates with better control of TB due to enhanced macrophage function (Cheng *et al.* 2017). The AMPK pathway can be induced by the antidiabetic drug metformin (Singhal *et al.* 2014). Several of these pathways suggest the interface between immunity and cellular metabolism during *Mtb* infection. *Mtb* is already well described to preferentially alter the host's cellular metabolism, in addition to its own metabolism. Furthermore, these pathways also suggest mechanisms by which T2D and other metabolic syndromes could affect the immunity to TB in individual patients.

System-wide transcriptomic approaches in NHPs infected with *Mtb* have also been used to identify correlates of risk of TB development in the blood (Gideon *et al.* 2016) as well as lung tissue (Kaushal *et al.* 2015). These results show that transcriptional changes in this model translate well to the human situation and can be used for novel discoveries. In studies of macaques

infected with specific *Mtb* mutants, the global host response has identified correlates of lack of disease despite infection (these responses may or may not translate to correlates of protection) (Mehra *et al.* 2012). By investigating global responses in the lungs of rhesus macaques infected with *Mtb*:Δ*dosR* mutants which are deficient in responding to hypoxia, we have found that *Mtb* actively subverts the recruitment of Th1 cells into the early granuloma (Mehra *et al.* 2015). System-wide measurements have also been applied to the granuloma level in both rhesus (Mehra *et al.* 2010) and cynomolgus (Mehra *et al.* 2013) macaques. In both models, we conclude that early lesions are characterized by a proinflammatory cytokine storm but later lesions are reprogrammed to suppress such responses (Mehra *et al.* 2010). It has also been found that a balance of pro- and anti-inflammatory cytokines, including IL-10 and IL-17, are associated with sterilization (Gideon *et al.* 2015). We have since successfully used lung and bronchoalveolar lavage (BAL) responses to study vaccine-induced protection (Kaushal *et al.* 2015), as well as the effect of host-directed therapeutics (Gautam *et al.* 2017).

Some of the recent advances in the NHP model have resulted from the use of high-definition live imaging using [^{18}F]2-fluoro-deoxy-D-glucose ([^{18}F]FDG) positron emission tomography–computed tomography (PET/CT) scanning (Lin *et al.* 2013; Coleman *et al.* 2014; Lin *et al.* 2014; Martin *et al.* 2017; White *et al.* 2017). PET has proven particularly useful in determining the inflammatory status of individual lesions. Generally, these studies suggest that PET-hot granulomas are prevalent in early time points and later time points are characterized by increased PET-cold lesions (Coleman *et al.* 2014). These studies confirm previous transcriptomics results that showed a cooling of proinflammatory signatures in granulomas over the course of time (Mehra *et al.* 2010, 2013). The most important message from these studies is the extensive heterogeneity in granuloma phenotype. Each granuloma appears to be an individual entity and factors governing their inflammatory status are not well understood. Recent cutting-edge molecular studies in this model include the use of digitally barcoded strains (Martin *et al.* 2017) to identify the founder bacilli for individual lesions and blockade of individual immune pathways and cells (Diedrich, Mattila and Flynn 2013; Gautam *et al.* 2017). Some of the recent advances in anti-TB vaccine discovery have involved the use of isogenic *Mtb* mutants (Kaushal *et al.* 2015; Foreman *et al.* 2017) or specific cytomegalovirus-based viral vectors in this model.

MICE

Rodent, and primarily murine, research is ubiquitous in science and is utilized in the study of nearly every human disease as ∼99% of all mouse genes possess a human homolog (Consortium 2002). Mice also have the benefit of posing a low cost to laboratories and having a wide variety of available genetic knockouts for immunological studies. Cost is of particular importance to TB research since the requirement for specialized containment facilities leads to increased expenses to begin with. In terms of an animal model for TB, mice are susceptible to disseminating infection and are typically incapable of controlling the infection (Kramnik, Demant and Bloom 1998). Most strains do not form classical granulomas like those found in humans (see Figure 1) and instead form diffused, non-caseating structures, such as the C57BL/6 strain that forms non-necrotic lesions primarily comprised of neutrophils (Flynn 2006; Kramnik and Beamer 2016). Thus, while the mouse model of TB has been extremely useful in identifying key immune mechanisms responsible for the control of TB (Orme 2003), as well

as for the assessment of drugs, therapeutics and regimens, its utility in effectively modeling the progression of *Mtb* infection, its pathology and the host-pathogen interactions that lead to either finite control or progressive TB disease, has been questioned. It has been suggested that the microenvironment within human TB lesions so profoundly impacts the physiology and the metabolism of the pathogen that the study of TB in systems that do not recapitulate these specific stress conditions (e.g. hypoxia) present within human TB lesions can be fundamentally misleading (Kaufmann *et al.* 2005). Recently, however, murine systems have been developed that recapitulate specific conditions. For example, granulomas formed in C3HeB/FeJ (Kramnik) mice infected with *Mtb* develop caseating necrosis and become centrally hypoxic (Driver *et al.* 2012). B6.C3H^{sst1} mice provide stable hypoxic lesions to study (Kramnik 2008) and CBA/J IL-10 KO are able to model mature fibrotic granulomas (Cyktor *et al.* 2013). Of particular interest also are mouse models of *Mtb*/HIV co-infection that utilize humanized mice. HuMouse develop a progressive, disseminating *Mtb* infection producing caseous granulomatous inflammation (Calderon *et al.* 2013). Endsley and colleagues showed that increased *Mtb* replication (≥1-log) and larger and diffused lesions are observed in these mice following HIV/*Mtb* co-infection (Nusbaum *et al.* 2016).

Studies built on the comparison of inbred strains of mice, with varying degrees of susceptibility to *Mtb* infection (Fortin *et al.* 2007), are vital for deciphering the role of singular changes to the immune response that may have diverse effects on the overall host. These models have been used for multimodal *in vivo* and *ex vivo* imaging to assess necrosis, fibrosis, RNAseq of whole granulomas, macrophage recruitment and death, as well as IFN and ISR pathway activation for comprehensive quantitative evaluation of the dynamics of TB granulomas.

Recently, two additional mouse models have captured the imagination of the field, largely due to their ability to offer maximal allelic variation within a mouse model that more closely resembles human population dynamics. One, known as the Collaborative Cross (CC) model, is a large panel of new inbred mouse strains derived from an eight-way cross using a set of Jackson Labs mice that included three wild-derived strains. CC mice display a broad range of susceptibility to *Mtb* infection, which are expectedly heritable (Smith *et al.* 2016). The other, known as the Diversity Outbred (DO) model, represents a far more diverse underlying genotype and was produced by a novel outbreeding strategy that maintained a balanced mixture of the founder genomes and avoided allelic loss and inbreeding (Churchill *et al.* 2012). Each DO mouse is genetically unique and, unlike inbred strains, DO mice have normal levels of heterozygosity, recapitulating that of humans (Svenson *et al.* 2012). The Jackson Labs DO model was generated using 160 breeding lines as founders, some which were also used to construct the CC mice (Churchill *et al.* 2012). These DO mice have since been established as the models of choice to study qualitatively different outcomes in behavior (Logan *et al.* 2013), cancer (Winter *et al.* 2017) and infectious diseases (McHugh *et al.* 2013). DO mice display a wide range of pathologic phenotypes following influenza virus or bacterial co-infection (McHugh *et al.* 2013). These results offer a glimpse of the powerful potential that these newer mouse models, especially the DO outbred model, provide in the study of complex human diseases with varied phenotypes. TB is one such disease, with *Mtb* infection leading to varied outcomes over the course of time. Genetics has been strongly implicated in this diversity of phenotypes. When DO mice were infected with standard doses of *Mtb* via aerosol, super-susceptible, susceptible and resistant phenotypes were readily observed (Niazi *et al.* 2015).

In addition to experiencing highly variable outcomes, DO mice exhibited a spectrum of necrotic (human-like) lesions including visible necrosis of individual macrophages, necrotizing granulomas with a fibrotic capsule, tuberculous pneumonia with intraalveolar neutrophilic exudates and thrombosis of the lung capillaries (Niazi *et al.* 2015; Kramnik and Beamer 2016).

More recently, the DO mouse collection has been used to measure the impact of host-directed therapeutics (specifically vitamin D) on the progression of *Mtb* infection (Bhatt *et al.* 2016). In these studies, changes in host-specific immune responses, e.g. cellular recruitment and phenotype, its impact on the granuloma structure and changes in bacterial burden could be readily measured (Bhatt *et al.* 2016). DO mice exhibit a range of bacterial burdens following aerosol infection with *Mtb*, enabling DO mice to act as an improved mouse co-infection model (Monin *et al.* 2015). The DO mouse model has been able to recapitulate complex phenotypes related to *Mtb* infection, such as helminth co-infections (Monin *et al.* 2015), as well as vaccine efficacy (Griffiths *et al.* 2016). Furthermore, several genes in a TB susceptibility signature identified in an adolescent cohort study of human household contacts (Zak *et al.* 2016) that predicted progression to TB far earlier than when clinical signs were apparent can be detected in the lungs of *Mtb*-infected DO mice, but not conventional B6 mice, by RNAseq (our unpublished data).

In the process of testing other toll-like receptor (TLR) agonists to similarly activate innate DC pathways in mice, we identified monophosphoryl lipid A (MPL) as a safe adjuvant for pulmonary delivery (Ahmed *et al.* 2017). We next tested whether the delivery of MPL and CD40 agonist FGK 4.5 in DO mice improves *Mtb* control, as seen in inbred B6 mice (Ahmed *et al.* 2017). Accordingly, individual DO mice were vaccinated with BCG, and at the time of *Mtb* infection received MPL, FGK4.5 along with IL-10R neutralizing antibody. IL-10 was identified as a key anti-inflammatory cytokine that limits T cell responses (Griffiths *et al.* 2016). These new data show that DO mice that received the DC activation therapy resulted in improved *Mtb* control and formed enhanced B cell follicles, which serve as a correlate of improved *Mtb* control in both murine and NHP animal models (Slight *et al.* 2013 ; Kaushal *et al.* 2015; Foreman *et al.* 2016). DO mice mimic the spectrum of disease seen in human TB and, similar to humans, exhibit variability in control upon *Mtb* challenge (Gopal *et al.* 2013). These model systems are, therefore, likely to advance our understanding of TB pathogenesis and immunity in the years to come. Furthermore, these will serve as initial screening tools for therapeutic agents in the setting of human-like genetic variability.

Mice are immensely valuable for their ability to provide a clear analysis of protection in vaccine studies and in mechanistic studies using genetic knockouts. This latter attribute is particularly important since the interaction of the *Mtb* bacillus with the host is complex and multi-parametric. Numerous cell types of the immune system and several immune pathways are involved, giving rise to variability and heterogeneity in phenotypes, which mechanistic approaches can better control.

VALIDATION OF MOLECULAR DETERMINANTS OF INFECTION, DISEASE AND PROTECTION IN THE MURINE MODEL

Seminal murine studies have established principles of *Mtb* infection progression and TB disease in the murine model and identified bacterial determinants of disease and host contributions into both protection from and pathology of TB. The current iteration of the murine model of TB was optimized in the early 1990s with the advent of the ability to infect mice with aerosols of *Mtb*. These studies showed that *Mtb* was more virulent when the exposure route was aerosol instead of the previously used intravenous route (North 1995). This led to the adoption of the aerosol route of murine infection as the model of choice by the field. Further experimentation with this route of infection showed that, unlike systemic infection with *Mtb*, adaptive immune responses are not initially elicited following *Mtb* infection via the aerosol route (Cooper 2014), a result which has since been confirmed in other experimental models (Mehra *et al.* 2010).

Using a mouse model where the route of infection is comparable to humans was a defining achievement. Simultaneously, landmark studies by Jacobs, Bloom and others allowed the development of genetic systems for the manipulation and the investigation of *Mtb* (Jacobs *et al.* 1989, 1990; Cirillo *et al.* 1991; Jacobs *et al.* 1991; Balasubramanian *et al.* 1996). These systems allowed the generation of isogenic mutants of *Mtb* for testing in murine virulence experiments. Eventually, genome-wide libraries of *Mtb* transposon-interrupted mutants were generated which allowed a system-wide investigation of the bacterial genes required for the survival of the pathogen in murine lungs (Sassetti and Rubin 2003; Lamichhane, Tyagi and Bishai 2005). Successful execution of these projects has since allowed a limited number of similar investigations to be performed in guinea pigs and macaques (Jain *et al.* 2007; Dutta *et al.* 2010). One approach was to generate Mariner-transposon mutants of *Mtb* with lesions in individual genes and to pool them for use in *in vitro* or *in vivo* studies, known as Designer Arrays for Define Mutant Analysis (DeADMAn). This allowed >1/3rd of all *Mtb* genes to be mutated and individual mutants included in the library (Lamichhane *et al.* 2003). Bayesian statistical analyses of this library revealed that at least a third of the *Mtb* genes may be essential and it may not be possible to interrupt these. The DeADMAn approach has been used to study molecules required for bacterial survival and persistence in mice (Lamichhane, Tyagi and Bishai 2005). It was found that 6% of the *Mtb* mutants were essential for growth in the mouse model, including those encoding for mycobacterial membrane large and small protein family (mmpL/S), as well as genes involved in the synthesis of (p)ppGpp, the alarmone that signals stringent response due to stress and amino acid starvation (Haseltine and Block 1973). Most of these observations have since been confirmed using specific isogenic mutants and the molecules implicated in these murine screens have been validated to be critical for TB disease progression (Varela *et al.* 2012; Weiss and Stallings 2013; Degiacomi *et al.* 2017). Another approach that was used to test the consequences of genome-wide mutagenesis of *Mtb* genes in the mouse model was Transposon Site Hybridization (TraSH). This approach combined high-density insertional mutagenesis with either microarray- or TnSeq-based mapping of mutant pools enriched or diminished post-infection (Sassetti, Boyd and Rubin 2001; Sassetti and Rubin 2003; Griffin *et al.* 2011, 2012; DeJesus *et al.* 2017). These studies identified a core group of ∼200 genes (5% of the genome, a number that compares favorably to the one arrived at by the DeADMAn approach), which were required for the survival of *Mtb* in mice. Many of these genes were unique to mycobacteria and the majority were of hitherto unknown functions (Sassetti and Rubin 2003). Many of the core genes required for virulence in the mouse model have since been individually validated to encode molecules important for *in vivo* survival and/or persistence of the bacillus.

Since the first draft of the *Mtb* genome sequence became available, it has become abundantly clear that this remarkable pathogen has evolved to respond to a variety of stress conditions during its *in vivo* life cycle (Cole *et al.* 1998). Numerous stress conditions that could be biologically relevant *in vivo* have been identified, e.g. damage to *Mtb* cell wall, or DNA, acidic pH in the phagosomal environment, nitrosative and oxidative stresses, nutritional starvation including phosphate, and hypoxia (Stallings and Glickman 2010). These stressors do not work in isolation but may in fact be interlinked. Furthermore, *Mtb* genes and pathways have been implicated to be involved in defense against such stress conditions and specific knock-out of molecules or pathways analyzed for phenotype relative to wild-type *Mtb* in the mouse model of aerosol infection. Thus, a mutant in the *kasB* gene, which encodes an enzyme in the mycolic acid synthesis pathway, was susceptible to treatment with antimicrobial peptides that damage cell wall and found to be highly susceptible to killing in the murine model. These results showed a connection between the specific molecular function of an *Mtb* protein and its ability to encounter and respond to *in vivo* relevant stress. Numerous other mutants in genes involved in cell wall component synthesis, maintenance and modification (e.g. phthiocerol dimycocerosate, mycolic acids and cyclopropanes) have been observed to be deficient for survival in murine lungs (Glickman, Cox and Jacobs 2000; Rao *et al.* 2005). Similarly, mutants in *Mtb* genes that protect against or repair DNA damage also have significant defects for growth in the murine model. For example, Rv1633, which encodes for UvrB, forms a critical part of the nucleotide excision repair machinery (Darwin *et al.* 2003). Interestingly, there was a significant defect in the killing of mice by a strain unable to synthesize UvrB, but the difference in tissue bacterial burdens was minimal (Darwin *et al.* 2003). These results suggest that while the murine model may be good for replicating several stress conditions that *Mtb* faces in human lungs, it is possible that specific mechanisms that restrict the replication of the bacillus in human lungs may be somewhat differently invoked in murine lungs. *Mtb* mutants in serine protease genes Rv3671c and Rv2224c were unable to survive in murine lungs, validating their role in protecting the bacillus from acidic pH by preserving intrabacillary pH (Vandal *et al.* 2008; Vandal, Nathan and Ehrt 2009). *Mtb* uses a multitude of pathways to counter oxidative burst of the phagosome, indicating the importance of being able to survive in the wake of this stress. These include a catalase gene *katG* (Pym, Saint-Joanis and Cole 2002) and two superoxide dismutase genes, *sodA* (Edwards *et al.* 2001) and *sodC* (Piddington *et al.* 2001). Mutants in *katG* have strong defects in murine growth (Pym, Saint-Joanis and Cole 2002) and *sodA* is an essential gene. When the expression of *sodA* was perturbed by antisense RNA approach, the resulting strain survived poorly in mice (Edwards *et al.* 2001). *Mtb* also experiences oxidoreductive stress and has extensive means to respond to it. *Mtb* encodes a stress response network orchestrated by an alternate, extracytoplasmic factor response sigma factor *sigH*, which is induced in response to reductive, acidic pH, heat shock and a variety of other stresses (Fernandes *et al.* 1999; Manganelli *et al.* 1999). SigH orchestrates an antioxidative response by inducing the expression of thioredoxin/thioredoxin reductase proteins (Bell *et al.* 2016). Surprisingly, a mutant in the *sigH* gene, while being susceptible to oxidoreductive stress, could grow in mice to levels comparable with wild-type *Mtb* despite evidence of lesser pathology (Kaushal *et al.* 2002).

The murine model has perhaps been most beneficial in establishing the key role of various components of the immune system in protection from TB. Thus, seminal studies by Flynn and Bloom, and Cooper and Orme, established the importance of CD4$^+$ T cells in protecting from TB, especially those expressing IFN-γ and IL-12 and those involved in TNF-α induction (Cooper *et al.* 1993; Flynn *et al.* 1993a, 1993b, 1995a, 1995b; Cooper *et al.* 1997). Since then, mouse studies have revealed a relatively smaller role of CD8$^+$ T cells in protection from *Mtb* infection (Lin and Flynn 2015) and an even more miniscule contribution of B cells and humoral immunity (Chan *et al.* 2014). Work by Khader and Cooper has since revealed an interesting role for the Th17 branch of immunity in TB (Khader *et al.* 2005, 2007). More recent work suggests that the role of some immune pathways, particularly Th17, may be governed by the strain of *Mtb* used and its relative pathogenicity (Gopal *et al.* 2014). Similarly, Turner and colleagues have established the negative role of IL-10 signaling in the control of *Mtb* infection (Beamer *et al.* 2008). These studies have since allowed us to focus on the function of specific lymphocytes. Thus, it has been postulated that murine granuloma T cells may have compromised cytokine section and functional activity (Egen *et al.* 2008; Bold *et al.* 2011; Egen *et al.* 2011).

RABBITS

Early on, rabbits of different origins were shown to have differential susceptibility to TB (Lurie, Zappasodi and Tickner 1955). Rabbits have commonly been infected via the aerosol route (Manabe *et al.* 2008), however human strains of *Mtb* are not fully virulent in rabbits (Dannenberg and Collins 2001). During infection with *M. bovis*, rabbits are able to form pulmonary cavitation, which has been considered to resemble *Mtb*-induced granulomas in humans more faithfully than those formed in mice and guinea pigs (Dannenberg 2006). Most of the species available today are resistant to *Mtb* as Lurie's susceptible breed had died out (Dharmadhikari and Nardell 2008). Seminal studies by Dannenberg *et al.* showed that alveolar macrophages are the target cell for *Mtb* and are required for resistance to TB (Henderson, Dannenberg and Lurie 1963; Rojas-Espinosa *et al.* 1974). Resistance to TB required proteolytic enzymes functioning in the endocytic pathway (Henderson, Dannenberg and Lurie 1963; Rojas-Espinosa *et al.* 1974). Dannenberg also illuminated the phenomenon of delayed type hypersensitivity in the rabbit model (Dannenberg 1991). More recently, the model has been employed to study mechanisms of *Mtb* persistence and reactivation (Geiman *et al.* 2004; Kesavan *et al.* 2009), cavitary dissemination (Nedeltchev *et al.* 2009), response of *Mtb* to host-mediated hypoxia in the granuloma (Converse *et al.* 2009), correlates of immune-mediated latency (Subbian *et al.* 2013a, 2013b) and both the environment of failed immunity and its correction by host-directed therapeutics (Subbian *et al.* 2011a, 2011b, 2016). Manabe *et al.* have developed the only animal model of TB-related immune reconstitution inflammatory syndrome (IRIS) in rabbits to study the progression and severity of the immune reconstitution inflammatory syndrome (Manabe *et al.* 2008). The model has now been employed for testing drug effectiveness, particularly pharmacokinetics and pharmacodynamics, to estimate drug penetration into different types of lesions in experimentally infected rabbits (Rifat *et al.* 2018). Rabbits also form caseating, necrotic granulomas (Figure 1) which, under the correct conditions, may liquefy and cavitate (Nedeltchev *et al.* 2009). Bishai and colleagues have developed a reliable model of cavitary disease in rabbits and confirmed pathological and molecular correlates of human disease. This model permits *in vivo* imaging to observe and quantify the events leading to cavitation (Kubler *et al.* 2015) and thus has tremendous potential in the study of granuloma breakdown.

VALIDATION OF MOLECULAR DETERMINANTS OF INFECTION, DISEASE AND PROTECTION IN THE RABBIT MODEL

As discussed above, Subbian *et al*. have characterized various outcomes of *Mtb* infection in the rabbit model (Subbian *et al*. 2011a, 2012, 2013b; Tsenova *et al*. 2014). They also applied whole genome transcriptomic evaluation to better understand universal host responses as they relate to the progression of infection (Subbian *et al*. 2012). Rabbits infected with low doses of low-virulence strain CDC1551, which elicits strong immune responses, initially exhibited bacterial growth and pathology, but eventually controlled infection in a latent form (Subbian *et al*. 2012). Genome-wide transcriptional analysis supported this notion, with the number of genes involved in both inflammatory responses and in lung remodeling (required for granuloma formation) peaking 2 weeks after infection in rabbit lungs and then progressively reducing in extent (Subbian *et al*. 2012). Similar results were earlier obtained in both the rhesus (Mehra *et al*. 2010) and the cynomolgus (Mehra *et al*. 2013) macaque models, which is discussed subsequently. The use of PET/CT imaging was first employed in the rabbit model of TB to study the dynamics of infection. These studies also confirmed earlier transcriptomic outcomes that low-dose experimental infection in this model involved an initial inflammatory phase followed by a chronic phase with reduced granuloma-associated inflammatory signal (Via *et al*. 2012). Thus, the use of state-of-the-art molecular approaches in animal models have extended our understanding of the granuloma formation/maturation kinetics in a manner not possible in humans. Circulatory human cytokine/chemokine profile-based molecular analytes are now being used to successfully study *Mtb* infection in rabbits (Dehnad *et al*. 2016).

Transcriptomics has also been employed in the rabbit model to successfully evaluate responses to therapy, including host-directed therapy (Subbian *et al*. 2011a). However, the most striking use of global 'omics' approach in this model has been to compare infection with a high-immunogenic vs. a high-virulence strain of *Mtb* (Subbian *et al*. 2013b). According to these reports, *Mtb* CDC1551, and perhaps other hyper-immunogenic strains, express early lung transcriptional profiles of infection that result in reduced STAT1 induction and polymorphonuclear leukocyte (PMN) recruitment and inflammation. Hence, the usual outcome of infection in rabbits with this strain is LTBI (Bishai *et al*. 1999; Subbian *et al*. 2012, 2013b). On the contrary, STAT1 and inflammatory signals were highly expressed following comparable infection with the hypervirulent HN878 strain and led to high levels of PMN recruitment and cavitary disease (Subbian *et al*. 2013b). While further research across experimental models is necessary, these results provide a potential mechanism for how hyper-virulent strains may have acquired the ability to elicit lung pathology as a means of overcoming the ability of the large majority of human beings to restrict *Mtb* infection as LTBI.

GUINEA PIGS

Guinea pigs are susceptible to *Mtb* and form granulomas, so they have been useful in immunohistopathological stages of pulmonary granuloma formation (Dannenberg and Collins 2001; Turner, Basaraba and Orme 2003). Guinea pigs have also been useful in investigations of responses to antigenic lipopeptides due to their possession of type I CD1 presentation molecules (Kaufmann *et al*. 2016). Initially considered the gold standard for vaccine testing against TB, the guinea pig model is considered highly susceptible (Clark, Hall and Williams 2014) and

has suffered from a lack of immunological tools such as guinea pig-specific antibodies for immune analysis in the past. It is expected, however, that more tools will be available in the future. Thus, a genome-wide interrogation of transcriptional responses to *Mtb* infection in this model is now possible (Jain, Dey and Tyagi 2012). An immune-gene specific array platform has been available for over a decade now and has been used to study *Mtb* infection in this model (Tree *et al*. 2006). This reagent has validated that *Mtb* infection of guinea pigs elicits strong and immediate Th1 responses (Tree *et al*. 2006), indicating its ability to recapitulate aspects of the human immune response to *Mtb*.

VALIDATION OF MOLECULAR DETERMINANTS OF INFECTION, DISEASE AND PROTECTION IN THE GUINEA PIG MODEL

Guinea pigs have been successfully leveraged to study *Mtb*-specific metabolic fate. With the increasing understanding that the immune system interacts with metabolism with influential consequences on the fate of various infections, interest in the guinea pig model of TB has also increased. Recently, important studies have investigated persisters in lung granulomas of this animal model (Lenaerts *et al*. 2007). Basaraba *et al*. have recently been successful in developing a model of TB/T2D in these guinea pigs (Podell *et al*. 2014, 2017). This is an exceptionally significant advance. T2D is a major cause of morbidity and mortality worldwide amongst non-infectious causes and accounts for ~90% of the entire diabetic burden (Deshpande, Harris-Hayes and Schootman 2008). Furthermore, the rates of T2D have been significantly increasing both globally as well as in areas of the world where TB is endemic. Thus, globally, it is predicted that the number of T2D patients will double between 2010 and 2030 (Rowley *et al*. 2017). It has been proposed that T2D is a comorbidity that is fueling the TB pandemic in certain parts of the world at levels comparable with HIV co-infection, especially in regions where TB is already endemic (Dooley and Chaisson 2009). When T2D was induced in guinea pigs, *Mtb* infection caused a significantly higher degree of TB disease with reduced survival and severe pathological presentation than controls (Podell *et al*. 2014). This coincided with uncontrolled inflammatory responses. Thus, the guinea pig model allows an in-depth study of T2D/TB comorbidity, including the testing of novel approaches for therapeutic control.

The guinea pig model has also been used in mutant infection screening experiments to better understand the bacillary determinants of pathogenesis (Jain *et al*. 2007). When compared with mice infected with the identical mutant library, guinea pigs permitted an accelerated detection of genes required for the *in vivo* survival of *Mtb* (Jain *et al*. 2007). These results suggest that TB-related pathology in guinea pig lungs results in relatively greater immune pressure on the bacillus than the corresponding murine pathology.

RATS

Rats remain a more recent and less developed model of TB infection out of all mammalian models. Recently, Lewis rats (Sugawara, Yamada and Mizuno 2004a), American cotton rats (Elwood *et al*. 2007), Wistar rats (Gaonkar *et al*. 2010; Cardona *et al*. 2011; Singhal *et al*. 2011) and diabetic rat strains (Sugawara, Yamada and Mizuno 2004b; Sugawara and Mizuno 2008) have been used to develop the rat model of TB infection. They have also been used to demonstrate Th1-shifts in immunity

following BCG vaccination (Cardona *et al.* 2011). Unlike mice, rats have been found to form well-organized granulomas and accumulate foamy macrophages (Figure 1), which have been associated with pulmonary lesion resolution (Singhal *et al.* 2011). Rats have been used successfully as an animal model for testing drug efficacy (Gaonkar *et al.* 2010; Foo *et al.* 2011) and antigen-specific T cell responses (Cardona *et al.* 2011; Foo *et al.* 2011; Singhal *et al.* 2011) and have been a valuable animal model for pharamacokinetic and toxicology studies by pharmaceutical companies (Gumbo *et al.* 2015). Similar to NHP models of TB infection, Wistar rats exposed to a low dose infection were found to control or clear bacilli by 4 months post-infection, depending on the strain used (Singhal *et al.* 2011), indicating that they have the potential to model latency. As a co-infection model, rats would be of benefit in the exploration of diabetes as Goto Kakizaki T2D rats (McGillivray *et al.* 2014) and Komeda diabetes-prone rats (Sugawara and Mizuno 2008) have been shown to have a higher susceptibility to TB compared with wild-type rats. Like mice, rats are simple to house, however they can withstand repeated blood collections and could be used during long-term infection studies.

ZEBRAFISH

Despite the fact that zebrafish do not possess lungs, researchers have been successful in utilizing their infection with *M. marinum* to uncover the mechanisms of microgranuloma formation prior to antigen-specific responses (Volkman *et al.* 2010; Ramakrishnan 2013). This model is useful in the determination of elementary mycobacterial pathogenesis and treatment. Many seminal recent discoveries about granuloma induction and the role of various mycobacterial virulence factors in this process have been initiated in this model (Volkman *et al.* 2010; Cambier *et al.* 2014). Zebrafish were vital in showing that macrophage death spreads bacilli to adjacent macrophages encapsulated within the same granuloma (Volkman *et al.* 2004). Many of these observations were extended from the fish model and validated in clinical human samples (Tobin *et al.* 2010). Recently, investigations in this system have identified specific mechanisms by which granulomas mature, with specialized phagocyte morphology contributing to the pathology of the lesion and promoting bacillary replication and persistence (Cronan *et al.* 2016). The zebrafish embryo model was used to show innate immune mechanisms that lead to granuloma formation, including induction of innate immune aggregates (Figure 1) and activation of *M. marinum* granuloma-specific genes (Davis *et al.* 2002). Zebrafish are small, easy to image (especially in the larval stage where the lesions can be directly visualized) and offer the advantage of significantly powered group sizes while posing minimal increases in overall cost (Cronan and Tobin 2014). The lack of host homology, however, limits the ability of zebrafish to completely model human infection, particularly latency. Special facilities are required for studies utilizing the fish model, however these studies have an additional benefit of being conducted at BSL2 since most studies utilize the strain *M. marinum,* which rarely infects humans, rather than *Mtb*.

IMPORTANCE OF ANIMAL MODELS FOR DRUG AND VACCINE EFFICACY

Animal models are important for evaluating new drug regimens prior to introduction in humans. With humans, the threat of developing drug or multidrug resistant strains prevents rapid introduction and animals, particularly mice, provide a niche where drug regimens can be used quickly to determine if new regimens are efficacious in treating and eliminating TB. Mice were useful in showing that rifapentine and moxifloxacin could be used to eliminate TB within 2 months, rather than the standard 6 months with rifampin (R), isoniazid (H) and pyrazinamide (Z) (Rosenthal *et al.* 2007). The use of different strains is vital to the evaluation of drug efficacy for later human use. Dual and triple drug combinations of H, R and Z have shown that different challenge strains yielded different *in vivo* efficacy in BALB/c mice (De Groote *et al.* 2012), leading to the need to develop improved drug regimens for eventual human use. Animal models allow an avenue to explore these new drugs prior to human use. Different quinolones (Lenaerts *et al.* 2008) and nitroimidazo-oxazines (Nuermberger *et al.* 2008) have recently been investigated to replace the current three-drug regimen. Recently, a subclass of quinolones called 2-pyridones has been investigated, including KRQ-10 018, and was found to be similar in *in vivo* efficacy to isoniazid and have less activity than moxifloxacin (Lenaerts *et al.* 2008). When the nitroimidazo-oxazine PA-824 was used with moxifloxacin and pyrazinamide regimen as a substitute for rifapentine, it cured mice more quickly than the standard regimen, but not within 3 months of treatment (Nuermberger *et al.* 2008).

As early as 1997, it was suggested that vaccine efficacy is altered based on the number of passages that the BCG strain has undergone (Behr and Small 1997). Behr *et al.* found that significant genetic changes had accumulated in many of the BCG strains, including the loss of region of difference 2 (RD2) in BCG-Denmark, BCG-Tice and BCG-Glaxo (Behr and Small 1999). In mice, it was found that the Prague and Japanese strains of BCG had lower recovery rates compared with three other BCG strains and were unable to protect against a second challenge with *Mtb* (Lagranderie *et al.* 1996). These studies highlight that, despite the presence of a vaccine that has been accepted for a long period, proper maintenance of the vaccine strain is vital in ensuring its efficacy. Experiments in animal models are thus necessary to ensure that efficacy is maintained over time to protect vulnerable human populations.

It has been observed for some time that BCG is primarily efficacious in the prevention of disseminated TB in children, so studies in other animal models are vital in investigating improvements that must be made to improve protection. Animal models have supported the lack of widespread protection produced by the BCG vaccine. Using *Mtb* Erdman and HN878 challenge to evaluate parenteral BCG vaccination efficacy, Verreck *et al.* showed that protection widely varies in rhesus macaques, as is the case in humans (Verreck *et al.* 2017). Until we possess a vaccine that prevents adult pulmonary TB, it will be impossible to eliminate the disease. Due to the existence of multidrug and extremely drug-resistant TB, treatment improvements alone will not be sufficient to contain the infection. Improved developments in vaccines, treatments and patient support for improved compliance will be necessary to ultimately eliminate TB.

IMPORTANCE OF STRAIN-STRAIN COMPARISON IN ANIMAL MODELS

It has been estimated that humans can be infected with *Mtb* with an infectious dose as low as 10 CFU. Humans have a 10% lifetime

risk of progressing to active disease once they have acquired TB (Society AT 2000). As a result, using an animal model with a higher susceptibility rate would make sense in replicating the natural, human response to infection. As humans have different susceptibilities to different strains of *Mtb*, it is ideal to compare the susceptibilities of each animal model with different strains of *Mtb*, as well as using multiple strains of *Mtb* to model treatment efficacy (De Groote *et al.* 2012). Several animal models have been used for side-by-side susceptibility comparisons of different strains such as *Mtb* Erdman, H37Rv and CDC1551 including mice (Kelley and Collins 1999; Manca *et al.* 1999; Ordway *et al.* 2007; De Groote *et al.* 2012), rabbits (Manabe *et al.* 2003; Tsenova *et al.* 2005), rats (Singhal *et al.* 2011) and NHPs (Cadena *et al.* 2016). In early comparisons over the course of 3 months, mouse studies failed to show differential susceptibilities among different strains of *Mtb* in intravenous or aerosol challenge (Kelley and Collins 1999). A study involving comparisons of rabbits aerosol-challenged with *Mtb* Erdman, CDC1551 and H37Rv found that Erdman was the most virulent strain and caused a broader spectrum of disease within 16–20 weeks post-challenge (Manabe *et al.* 2003). Several studies of strain-strain comparisons have been conducted within the NHP model as well. Despite marmosets having high susceptibility, they have been used to reveal that strain and infectious dose can lead to heterogenous responses by comparing low-dose Erdman and CDC1551 infection ranging from 1–12 CFU (Cadena *et al.* 2016).

The use of animal models in strain-strain comparison are beneficial in that they enable one to investigate both differences in the host's responses to the individual strains as well as differential virulence mechanisms of the strains themselves. Using New Zealand white rabbits, Tsenova *et al.* found that phenolic glycolipid (PGL) biosynthesis is critical in subverting the host's ability to produce protective TNF-α by comparing rabbits infected with CDC1551, HN878 and HN878*pks1*–15::*hyg* (Tsenova *et al.* 2005). Using CDC1551 and HN878 infection, Subbian *et al.* showed that differential leukocyte activation and recruitment within the first 3 h of infection led to vastly different outcomes despite identical infectious doses being used (Subbian *et al.* 2013b). HN878-infected rabbits that developed cavitary disease had increased expression of pro-inflammatory genes that are associated with extravasation and activation of PMNs in the lungs, including those producing TNF-α, IL-8, IL-15, MCP-1 and CXCL10. Other studies have found that CDC1551 induces a more rapid and robust response in mice, with granulomas containing high levels of TNF-α, IL-6, IL-10, IL-12 and IFN-γ mRNA (Manca *et al.* 1999). Mice were also vital in showing that some of HN878 hypervirulence is due to the induction of stronger Th1 responses and rapid Treg emergence compared with other strains that result in their decreased survival (Ordway *et al.* 2007). Studies utilizing multiple challenge strains are also vital in evaluating the protection offered by different vaccines. BCG has shown efficacy in protecting mice from various strains of *Mtb*, however these differ incredibly from clinical observations in humans (Jeon *et al.* 2008).

ADDITIONAL GENOMIC APPROACHES IN THE ANIMAL MODELS OF TB

The availability of genome sequences in the past two decades has spawned the sciences of systems biology, where the understanding of the entire genome is leveraged to understand the responses of both the pathogen and the host on a 'system-wide' level. Initial studies focused on a simplistic design where the response of *Mtb* to various *in vitro* conditions directly or indirectly corresponding to stresses that the pathogen might be subjected to *in vivo* were measured. These experiments clearly illuminated that *Mtb* has developed an intricate network of transcriptional networks to fine-tune responses to different stresses. Key molecules in this network included sigma factors, which serve to guide the RNA polymerase to specifically initiate gene expression. The *Mtb* genome encodes about a dozen stress- or condition-specific sigma factors whose expression are often intricately controlled at multiple levels (Gomez, Chen and Bishai 1997). Thus, it was shown that *sigE* responds to stress that weakens the *Mtb* cell wall and, unlike wild-type *Mtb*, $\Delta sigE$ mutants are effectively killed by phagocytes (Manganelli *et al.* 2001). SigE is one of the few molecules known to be expressed in *Mtb*-infected macrophages by selective capture of transcribed sequences (SCOTS), an early genome-wide RNA measurement technique. Since those early days, the animal models of TB have progressed tremendously, and state-of-the art techniques are now routinely used in these to study both the pathogenesis of *Mtb* as well as the evaluation of host responses involved in immunity as well as pathology. These include comparative genomics, post-genomic mutations, transcriptomics (beginning with DNA microarrays, but now including RNAseq and single cell RNAseq), proteomics (including to detect proteins with modifications such as phosphorylation, etc.), lipidomics, metabolomics and whole animal live imaging by magnetic resonance imaging (MRI) and PET/CT.

CONCLUSIONS

No one animal model recapitulates the breadth of heterogeneity in human TB. However, several new mouse models can portray specific events in the granuloma such as hypoxia or fibrosis. Similarly, guinea pigs, zebrafish and rabbit infection models have significantly contributed to our understanding of *Mtb* infection and the resulting immune response. Perhaps the best recapitulation of *Mtb*-specific pathogenesis events and immunity occurs in NHPs. Mtb-infected macaques have been used for transcriptome-wide immune response evaluation in peripheral blood to back-translate key findings for humans. These studies have been extended to evaluate BAL and lung samples. State-of-the-art molecular imaging tools such as PET/CT have been successfully employed in this model. The use of bar-coded strains and isogenic mutants to better understand TB pathogenesis has revealed interesting information from this model. Finally, the use of the NHP model is informing vaccine and therapeutic discovery critical to the future control of the TB pandemic. Overall, the use of animal models improves our understanding of *Mtb* disease pathogenesis and provides us with models that recapitulate aspects of human disease for the testing of new vaccine and treatment regimens that are vital to ultimately eliminate TB.

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