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Modeling pathogen and host: *in vitro*, *in vivo* and *in silico* models of latent *Mycobacterium tuberculosis* infection

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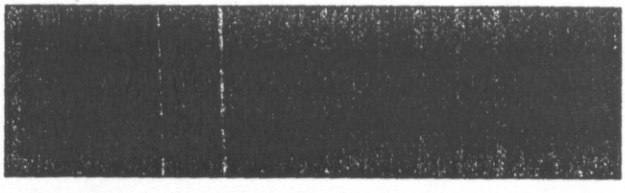
Tuberculosis remains a significant global health burden. Little is understood regarding the critical pathogen-driven factors and host immune responses that result in latent infection and reactivation. The Wayne model is an *in vitro* model of latent infection in which *Mycobacterium tuberculosis* undergoes a hypoxia induced nonreplicating persistent state of metabolism. Using this model, important findings in genomic and proteomic factors involved in newly defined metabolic pathways and drug susceptibility have been identified. The mouse remains the most popular *in vivo* model latent infection. The Cornell model, reflecting chronic infection altered by antibiotic treatment, and the low to moderate dose chronic infection model have been used. Studies using this model have revealed important insights regarding TNF, IFN, reactive nitrogen intermediates, IL-10, CD4 and CD8 function during latent infection. However, the immune responses in the murine model most likely reflect chronic infection rather than true latency. The non-human primate model is the only animal model in which a true latent state occurs. However, its limited availability, high cost and support required are impractical for frequent use. Disparate data from multiple studies can be used to predict complex biologic interactions through mathematical simulations (*in silico* model). Mathematical modeling can be use to foresee important insights into host–pathogen interactions that can then be confirmed by *in vivo* experiments. Since no single perfect model of latent infection exists, the use of multiple models has and will continue to provide significant contributions in our understanding of latency and reactivation of tuberculosis.



Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a significant global health burden, worsened by the AIDS epidemic. One-third of the world's population is currently infected with *M. tuberculosis* while almost two million deaths are attributed to TB each year. What differentiates *M. tuberculosis* infection from some other pathogens

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Glossary

Latent infection: a state in which a person has proven *M. tuberculosis* infection (either by skin test or other immunologic test) without clinical signs or symptoms of tuberculosis. This represents a state in which the host immune response is able to contain the infection.
Reactivation: a clinical state in which the host who was previously in a latent state of infection has developed clinically apparent disease. This represents a change in the immune response in which the host is no longer able to contain the infection as compared to previous.
Tuberculosis: the clinical disease caused by *M. tuberculosis* infection representing a state of disease in which the immune system is unable to contain the infection.

is its ability to establish latent infection, defined as proven evidence of infection (i.e. positive tuberculin skin test) without clinical evidence of disease. The mechanisms that contribute to the establishment of latent infection and reactivation to active TB remain poorly understood.

Human infection results from inhalation of aerosolized bacilli from a person with active TB. Once infected, a cascade of immunologic events that involve both the innate and adaptive immune response is activated. Primary TB occurs in a minority of patients (5–10%) within the first two years after infection. Although some people might clear the infection, the majority of persons (90%) develop LATENT INFECTION as a result of an adequate immune response in which the bacilli are contained but not eradicated. Latently infected healthy individuals can develop REACTIVATION at a rate of 10% per lifetime, and treatment with isoniazid, an antibiotic used specifically to treat mycobacterial infections, can reduce this risk. HIV infection increases the risk of reactivation to 10% per year. The large numbers of latently infected people worldwide represent a significant reservoir of infection and make eradication of TB difficult. Effective treatment of active TB requires multiple drugs for a long duration (e.g. 6–12 months) of time. The cost of medications, lack of compliance and burden on limited health systems make eradication of TB extremely difficult. Thus, drugs that significantly shortened therapy for both active and latent infection would have substantial impact on this disease worldwide. Little is currently understood regarding the host and pathogen factors important in latent infection. To date, several models have been utilized to examine aspects of the host immune response as well as pathogen-driven factors important during latency and reactivation.

In this review, the advantages, disadvantages and contributions made by the current models of latent *M. tuberculosis* infection are discussed.

In vitro

Mycobacterium tuberculosis might lay dormant in an infected host and reactivation could result in active disease year to decades later. The Wayne model focuses on the nonreplicating persistent (NRP) state of the bacilli that is believed to exist during latent infection. The basic principal of the Wayne model assumes that *M. tuberculosis* should undergo metabolic alterations as a result of reduced oxygen concentrations within the host's microenvironment, i.e. the granuloma [1]. In this model, a NRP state of *M. tuberculosis* can be generated in a chamber with artificially controlled states of hypoxia.

Use of the Wayne model has resulted in several important findings. It was identified that *M. tuberculosis* could shift into the glyoxylate pathway, an important alternate metabolic pathway for NAD production. Mice infected with a strain of *M. tuberculosis* in which the gene for isocitrate lyase (the first enzyme involved in the glyoxylate pathway) was interrupted had reduced persistence compared to wild type *M. tuberculosis*. [2]. Genomic- and proteomic-expression comparisons have identified several important proteins that are up-regulated under oxygen deprivation, including glycine dehydrogenase and nitrate reductase [3] and those genes in the DosR regulon [4]. Reduced anti-mycobactericidal activity with rifampin and isoniazid but increased activity with metronidazole during this metabolic NRP phase has also been observed [5]. This bolstered speculation that the requirement for multiple drug regimens inherent to TB therapy might be, in fact, an attempt to kill the various metabolic forms of *M. tuberculosis* within the host. Studies in this area could potentially identify new drug targets in hopes of making a significant treatment impact. Additionally, this model might allow us to understand mechanisms of bacterial persistence and how the host response might vary based on the metabolic conditions of the pathogen.

In vivo

Murine model

Current animal models of *M. tuberculosis* infection include mouse, guinea pig, rabbit (primarily using *M. bovis*), non-human primate (NHP), and zebra fish (using *M. marinum*). Although the histopathology of *M. tuberculosis* infection in guinea pigs mimics that seen in progressive human disease, they are highly susceptible to low-dose infection and therefore do not establish latent infection. Rabbits infected with *M. bovis* (rabbits are more resistant to *M. tuberculosis*) bear close similarity in histopathology to human granulomas with formation of caseum and cavities. Though able to sustain *M. bovis* infection for several months, rabbits eventually succumb to fatal infection. It has been speculated that the inherently resistant nature of rabbits to *M. tuberculosis* infection might indicate latent infection although this has yet to be established. Embryonic zebra fish infected with *M. marinum* have been used to visualize the formation of granulomas during acute infection. All of these models mentioned, either have been or have the potential for studies in mutant *M. tuberculosis* to test virulence but are limited by lack of latent infection and the limited availability of reagents (reviewed in [6]).

The mouse is the most popular animal model of *M. tuberculosis* infection. The inherent advantages of this model include the cost, size and availability, the enormous potential for manipulation, and the technical reagents available, including genetically modified strains. Two models of latent infection exist in the mouse model. In the low-dose infection mouse model, resistant strain C57BL/6 mice are infected intravenously or via aerosol with a moderate and low-dose inoculum, respectively. By three to four weeks, a bacterial burden plateau in the lung is reached (~10^5 to 10^7 CFU) followed by a steady state of infection maintained for up to one-year postinfection, when they begin to succumb to TB. In this chronic model, mice appear clinically well and are able to control the infection provided an adequate cell-mediated immune response is present, mimicking human latent infection. Bacteria might enter a NRP state of dormancy during infection in this model [7,8], although it appears to be nitric oxide-mediated [9,10]. However, given the relatively high-bacterial numbers and progressive pathology, this model is more akin to a chronic infection, rather than human latent infection.

The second model is the Cornell model and its variations. The original description of the Cornell model involved intravenous infection at 1–3 × 10^6 of virulent *M. tuberculosis*. Infection was established while concurrently treating mice with isoniazid and pyrazinamide for 20 weeks [11,12]. Despite undetectable levels of *M. tuberculosis* after treatment, mice were capable of developing spontaneous or induced reactivation [13]. The advantages of this model include low-level infection among mice that appear clinically well with episodes of reactivation mimicking human latent infection. Lack of standardization and stability of the model is a significant disadvantage in that numerous factors that can alter outcome such as genetic strain of mouse, *M. tuberculosis* strain, method of stock preparation, route of infection, dose of inoculum, and time interval between treatment and infection [14]. Moreover, the effect of anti-mycobacterial treatment as an artificial means of controlling infection introduces another variable that cannot be easily related to human latent infection. There are also differences between humans and mice with respect to pathology. The human granuloma is much more organized, consisting of epithelioid macrophages surrounded by lymphocytes, with the center frequently necrotic or caseous. In contrast, the mouse granuloma is less structured, although macrophages and lymphocytes do tend to cluster in areas of infection. Necrosis in the mouse occurs primarily in response to very high bacterial numbers in the lung.

Despite their limitations, investigations using the murine model have made significant contributions to our understanding of latency and reactivation. The murine model has been used in numerous studies to understand host response to different strains and mutants of *M. tuberculosis*. Functional tumor necrosis factor (TNF)-alpha is important in preventing reactivation. Neutralization of TNF results in reactivation of latent infection associated with poor survival, high bacterial burden and poor granuloma structure [15–17]. This phenomenon appears to be clinically relevant as an increased incidence of TB was reported among humans treated with Infliximab, a TNF neutralizing antibody. It is presumed that these were cases of reactivation from latent infection [18]. Although the mechanism of TNF influence on granuloma formation has not been completely elucidated, neutralization of TNF reduces chemokine expression among CD11b positive lung cells [17]. Studies in the Cornell model show that interferon (IFN)-gamma probably also plays an important role in latent infection. Treatment with anti-IFN antibody resulted in higher bacterial burden and increased rates of reactivation compared to controls depending on the type of Cornell model used. However, it was not recapitulated in the low-dose model [14]. Both IFN and TNF play important roles in macrophage activation and induction of reactive nitrogen intermediates (RNI) [9,19]. Reactivation TUBERCULOSIS occurred in both murine models of latent infection after treatment with aminoguanidine, an inhibitor of nitric oxide synthase [2], suggesting the important role of macrophage activation via the RNI pathway during latent infection [20]. IL-10 also appears to be important in latent infection as demonstrated by increased rates of reactivation among transgenic mice with high expression of IL-10. This was associated with decreased expression of TNF and IL-12p40, reduction in IFN production, decreased expression of lymphocyte expression of CD11a and formation of macrophage dominant granulomas [21].

CD4 and CD8 T cells play an important role in *M. tuberculosis* infection in part due to production of IFN. Reactivation was observed among low dose chronically infected mice depleted of CD4 T cells. Although there was an increased bacterial burden present, no decrease in IFN (or nitric oxide sythase 2) was noted [22]. These results could not be confirmed in the Cornell model. CD8 T cell depletion in the Cornell model resulted in reactivation of the infection [23].

Non-human primate model

While NHPs have been used in TB research for decades, there has been a recent resurgence of interest with regard to vaccine trials, diagnostic assays and drug development. It was recently described that latent infection could be observed in a macaque model. Cynomolgus macaques (*Macacca fascicularis*) infected with a low dose (~25 CFU) of *M. tuberculosis* via bronchoscopic instillation into the lung results in a spectrum of disease that mimics the human infection outcomes. Data from 46 macaques infected in our lab demonstrates that 60% of infected NHPs will develop latent infection. *M. tuberculosis* infection is confirmed by conversion from a negative to positive tuberculin skin test and as well as

by lymphocyte proliferation assay using several mycobacterial antigens. By six months postinfection, NHP can be classified as active disease or latent infection. Latent infection is defined as having no radiographic or clinical evidence of TB and negative bacterial cultures during the first six months postinfection. We have observed spontaneous reactivation as early as nine months and as late as almost three years postinfection. Reactivation can also be induced by immune suppression. Findings at the time of necropsy in a latent NHP are generally limited to enlargement of the hilar lymph nodes with or without granulomas and a small number of granulomas typically within only one lower lung lobe. These granulomas are often fibrotic and can be calcified. Bacterial burden is generally minimal (10^3–10^5 CFU/g granuloma tissue at most) in latent NHPs. By contrast, NHPs with clinically significant disease (e.g. cough, weight loss and abnormal chest radiograph) have numerous granulomas throughout the lungs with or without pneumonia and can have evidence of extrapulmonary dissemination. The histopathologic characteristics of NHP granulomas are extremely similar to human granulomas. Various types of granulomas exist (caseous, solid or fibrotic) even within the same NHP and even mineralization can occur in non-active granulomas [24]. Cavitary lesions with liquefaction of caseum can occur in NHP as they do in humans.

Clearly, cynomolgus macaques have the capability to control the infection and clinically mimic latent infection. It is an ideal model for studying granuloma formation and function as its pathology is almost identical to human. Unlike the other animal models that have similar histopathology (e.g. guinea pig and rabbit), immunologic reagents are readily available. NHPs are also unique in that they can be used to study co-infections, such as with SIV and *M. tuberculosis* (as a model for HIV and *M. tuberculosis*). Given their genetic similarity to humans, results from this model are probably directly translatable to human disease. However, the overall cost of maintaining a biosafety level 3 facility for NHPs with dedicated staff can be extremely difficult if not prohibitive. In addition, NHPs are expensive and a limited resource. Sample sizes for studies are necessarily small,which impairs the ability to study subtle but significant differences. Like human studies, genetic variability between each monkey can result in experimental variability. Despite these issues, the NHP model of *M. tuberculosis* infection most accurately reflects human latent infection to date.

In silico

Mathematical models have been used to describe interactions between *M. tuberculosis* and its host during infection [25–27]. Mathematical modeling of biological systems is a method whereby experimental theory and data can be evaluated and predictions made. In brief, the known characteristics of a system (e.g. immune cell interactions, specific effects of bacteria on host cells, etc.) are described mathematically, the rates of these interactions quantified via parameter estimations, and the model is then simulated on the computer by which an outcome value is calculated. Specific host and bacterial factors can then be perturbed to determine their contribution to the predicted outcome. In addition, models can be derived to capture any system: human or animal models. So parameters and known differences between animal systems can be included to enhance the ability to address specific questions. The strength of a mathematical modeling approach lies in its ability to capture the complex interactions of biological systems and in its ability to utilize large amounts of disparate data about these systems to make predictions that would otherwise not be perceptible or even feasible in animal or human models (either because of ethical or practical limitations). Simulations of experiments can be performed very fast, making predictions that can then be tested in an experimental setting [28]. Finally, negative and positive control testing of the model, as in experiments is a necessary task. Models should predict key aspects of known biology (such as the relevance of IFN-gamma and IL-12 in infection, etc.), as an important step towards validating the system. Drug development and testing is also very accessible using mathematical models. This can take place at the level of the drug action [29] or even at a 'virtual clinical trial' level [30]. Regardless, mathematical models can be used as a way to identify irrelevant experiments and trial protocols while predicting useful ones, allowing for a more focused determination of important approaches.

For *M. tuberculosis*, a key advantage is an ability to establish a model system whereby latency, active disease and even clearance can be observed. Then, predictions can be made regarding what system components are decisive in directing the dynamics towards one outcome versus another. For example, some models are developed to capture changes in the behavior of the system over time [25,26] while others can capture both time and space dynamics [27]. For TB, both approaches can yield insights into the important dynamics governing the behavior of the host–pathogen interactions. In particular for *M. tuberculosis*, the developing granuloma can be captured as an evolving time-series movie that allows for tracking the formation of this structure. Having access to a complete picture of the formation of granulomas can aid in the development of interventions to enhance or in some instances, halt this process.

Mathematical models in TB have predicted several features that now require experimental testing. For example, IL-10 knockout mice showed no phenotypic differences upon challenge with *M. tuberculosis* as compared with wild type. However, in mathematical models of a virtual human infection, simulating IL-10 knockout predicted an insight that the experiments missed: namely, that IL-10 stabilizes the system making reactivation more probable in its absence [25]. This is

Table 1.

In vivo		*In vitro*	*In silico*
Mouse (low dose and Cornell model)	**Non-human primate**	**Wayne model**	**Mathematical model**
Pros			
Large numbers available for use	True latent infection established	Hypoxia likely to occur in granuloma microenvironment (unpublished data)	Long-term experiments can be quickly performed
Inexpensive	Granuloma histology identical to human	Inexpensive	Experiments not limited by technical considerations
Genetic variant strains available	Findings are likely to be directly translatable to human disease		Inexpensive
Reagents readily available	Reagents readily available		Can mimic human infection
			Easy to perform multiple experiments and test drug efficacy
Cons			
Immunologic state of chronic infection rather than true latency	Expensive	Granuloma likely to have more complicated microenvironment that might not be reflected by hypoxia alone	Might miss unknown factors
Granuloma histology unlike human	Limited resource requiring studies with small sample size	Lack of host response	Requires data on various aspects
Biosafety level 3 containment	Biosafety level 3 containment		
Veterinary staff required (less intense than NHP)	Dedicated veterinary staff required		
Not all immunologic factors of the human are represented in the murine model (e.g. granulysin)			
Discordance results between murine models can occur			
Best use of model			
Immunology	Immunology	Drug screening	Provides predictions that can be experimentally tested in both immunology and pathogenesis
	Pathogenesis	Pathogenesis	
	Drug testing		

being studied further in the animal models. Regarding granuloma formation, models predicted that an overabundance of resting macrophages at the site of infection, particularly early in infection, can exacerbate infection, tipping the scales in favor of uncontrolled bacteria growth [27].

Conclusion

Currently, little is understood about the immunologic and pathogen driven features of latent *M. tuberculosis* infection. We have briefly reviewed the highlights of the current *in vitro*, *in vivo* and *in silico* models currently used in the field (Table 1). Given the advantages and disadvantages of each, it seems most likely that our perceptions of latent infection will be derived from a combination of models. Thus far, the Wayne model is and will continue to be the primary model for nonreplicating persistent form of *M. tuberculosis*. There is growing evidence that hypoxia likely represents the microenvironment of a subset of granulomas. For practical reasons, the murine model of latency will likely continue to predominate the field with regard to studies on immunologic responses of both wild type and genetically altered pathogen strains. Mathematical models can lend important insights into the dynamics of infection and they probably will become more common as an additional tool. Given the degree of expense and resources required for NHP research, NHPs will probably be used only for specific areas that cannot be addressed by using other animal models or human epidemiologic data. The NHP model is still a model in development such that potential advantages and disadvantages might not have been identified at this time. Although there does not appear to be a perfect model of latent infection, it is clear that elucidating important factors during latency and reactivation will be a significant contribution to our understanding of tuberculosis.

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