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The non-human primate model of tuberculosis

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Abstract

Non-human primates (NHPs) are used to model human disease owing to their remarkably similar genomes, physiology, and immune systems. Recently, there has been an increased interest in modeling tuberculosis (TB) in NHPs. Macaques are susceptible to infection with different strains of *Mycobacterium tuberculosis* (*Mtb*), producing the full spectrum of disease conditions, including latent infection, chronic progressive infection, and acute TB, depending on the route and dose of infection. Clearly, NHPs are an excellent model of human TB. While the initial aim of the NHP model was to allow preclinical testing of candidate vaccines and drugs, it is now also being used to study pathogenesis and immune correlates of protection. Recent advances in this field are discussed in this review. Key questions such as the effect of hypoxia on the biology of *Mtb* and the basis of reactivation of latent TB can now be investigated through the use of this model.

Keywords

latent; *Mycobacterium tuberculosis*; non-human primate; reactivation; TB/AIDS coinfection

Global impact of TB

Tuberculosis (TB) is a major infectious disease of mankind, annually causing about 1.5 million deaths [1]. The situation is worsening with an increase in drug-resistant *Mtb* and the spectacular failure of the global TB vaccination strategy [2, 3]. Rampant coinfection with HIV, the causative agent of AIDS, is also believed to have contributed to a global resurgence in TB cases [4]. *Mtb* is a highly successful human pathogen and infects over 1/3 of the human population. However, a great majority of humans exposed to *Mtb* are able to immunologically contain infection in a latent state. With waning immune response, however, latent TB can be reactivated. It is believed that coinfection with HIV contributes to a significant increase in the number of reactivation TB cases [5].

Experimental models of TB

There are no natural hosts of *Mtb* other than humans. As humans cannot be knowingly exposed to *Mtb*, numerous experimental models of TB have been employed. Historically,

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TB was studied in guinea pigs since the times of Koch in the 19th century [6]. Infected guinea pigs produce symptoms and pathology similar to humans and can be used to model chemotherapy and vaccination. However, these animals exhibit exquisite susceptibility to the disease [7]. Rabbits have also been employed as models of TB [8]. Once again, this model faithfully represents the pathophysiological aspects of TB infections, in particular the study of cavitary lesions. However, the limited availability of molecular and immunological research resources for both these models represents a challenge.

The mouse is the most utilized model of TB [9]. It has been extensively utilized to not only study the bacterial factors of virulence, but has also contributed tremendously to our understanding of the immune protection mechanisms that control *Mtb* infections. Additionally, tools for molecular and immunological research and the availability of defined transgenic and knockout mice render the murine model invaluable. However, the mouse model has some shortcomings. A classical latent infection, represented by immunological response to the infection in the absence of clinical and microbiological evidence of disease, is not attained in the mouse model [10]. Moreover, the gross and microscopic pathology of murine TB is significantly different from TB in humans. This is a major drawback because different host responses reflected in the histopathology of the lesions are predicted to present different challenges and varying degrees of *in vivo* stress on *Mtb*, thus resulting in varying response from the pathogen and different outcomes to infection.

Fish infected with *Mycobacterium marinum* have recently emerged as an excellent surrogate model for studying *Mtb* infections. Infected zebrafish generate granulomatous lesions [11]. Infected zebrafish embryos are transparent, allowing the emergence of granulomatous pathology to be visually assayed in real time. Coupled with the relative ease of performing host genetics in zebrafish, this renders this fish/*M. marinum* model attractive for studying factors responsible for both bacterial pathogenesis and host immunity [12].

Non-human primates as surrogate models of human diseases

Non-human primates, because of their genomic, physiological, and immunological similarities to humans, are attractive models of a wide range of infectious diseases. NHP models of AIDS have significantly contributed to our understanding of AIDS pathogenesis including viral latency and development of vaccines, therapeutics, and microbicides to prevent HIV transmission [13–17]. Additionally, NHP models are being utilized to study malaria [18] and a variety of infectious agents such as smallpox [19, 20], ebola [21], Venezuelan equine encephalitis [22], nipah [23], Marburg [24], SARS [25], parainfluenza viruses [26], pneumocystis/AIDS coinfection [27], *B. anthracis* [28], *S. mansoni* [29], *C. tracomatis* [30], *L. monocytogenes* [31], group-A *Streptococcus* [32], *Leismania* [33], *Coxiella burnetti* [34], and *Burkholderia pseudomallei* (D. Kaushal, S. Mehra and C. J. Roy, unpublished data). The common element in all these studies has been the similarity between the biology of the species being modeled (humans) and the model itself (NHPs), resulting in the recapitulation of key aspects of the diseases, as they occur in humans.

The non-human primate model of TB

All of the experimental models of TB infection described above have contributed immensely to our understanding of the various phases of *Mtb* infection and the resulting host response. However, it is widely believed that none of these models reasonably reproduce classical latency and the various types of pathological lesions observed during human infections. Moreover, the lack of a sufficient repertoire of molecular and immunological reagents for some of these models limits their use. It is in this light that the NHP model of TB has gained importance in the last decade. A crucial advantage of the NHP models of TB is the ability to generate clinical correlates of infection, for example, blood CBC counts and chemistries,

serum C-reactive protein and differential centrifugation values, thoracic X-rays, tuberculin skin tests, and interferon gamma release assays.

By the 1970s, it was generally agreed that primates can be robustly and reproducibly infected with *Mtb* and generate several aspects of human-like disease, including protection by vaccination with Bacille Calmette–Guerin (BCG) [35, 36]. Much of this work was performed in the Indian rhesus macaque (*Macaca mulatta*). The reduced availability of this species in the 1970s because of political reasons and in the 1980s because of the recruitment of this model for AIDS research, coupled with the assumption that infectious disease in general, and TB in particular, was waning, contributed to declining interest in the macaque model of TB. In the modern era, pioneering work by Walsh et al. [37] reinvigorated interest in the NHP model of TB. Most of the recent work on the NHP model of TB has primarily utilized either the cynomolgus macaque (*Macaca fascicularis*) of Philippine or Mauritian origin [37–50] or captive bread rhesus macaque (*Macaca mulatta*) of Indian or Chinese origin [51–72]. Significant advances in this field are summarized in Table 1. Important variables such as the inoculum dose, route of infection, and the strain of *Mtb* used are also listed in Table 1. As part of these studies, macaques have been routinely infected with *Mtb* strains Erdman, H37Rv, and CDC1551 (Table 1). These inoculations typically used either the intratracheal instillation using a bronchoscope or a true, head-only aerosol delivery method. There are advantages to both methods. While employing the former method, it is relatively easy to consistently quantitate the delivered dose of the inocula, while this is difficult to achieve via the aerosol method. This is due to the fact that NHPs are expensive and unlike mice cannot be euthanized at day 1 with the aim of identifying the initial inocula. Therefore, techniques such as whole-body plethysmography are used in conjugation with aerosol delivery to accurately identify the animals breathing rate and voidal volume. On the other hand, aerosol delivery of *Mtb* accurately mimics the natural infection of human beings. Intratracheal deposition is localized to one lobe, while with aerosol route, infection can be initiated in all lobes of both lungs.

The cynomolgus macaque as a model of human TB

Cynomolgus macaques infected with a very low dose [10^1–10^2 colony-forming units (cfu)] of virulent *Mtb* Erdman strain via the intratracheal route generated a spectrum of TB disease conditions based on the choice of dose and the relative infectiousness of the strain. A high dose of the *Mtb* Erdman strain (10^4–10^5 cfu) instilled into the trachea resulted in acute and fatal tuberculous pneumonia in all cynomolgus macaques, while a moderate dose of *Mtb* Erdman (~10^3 cfu) delivered via the identical route led to the development of localized, slowly progressing TB in a majority of animals [37] (Table 1). The extremely low dose (10^1–10^2 cfu *Mtb* Erdman) caused the animals to maintain infection in a subclinical, latent state for long periods of time [37]. Similar results with this species were obtained in a later study [38]. More than one-third of the animals infected with a low dose (~25 cfu) of *Mtb* Erdman strain failed to develop any clinical signs of disease in this study, in spite of signs of infection, thus mirroring the human population latently infected with *Mtb*. In this model, gross granulomatous lesions can be observed as early as 3 weeks post-infection and were characterized by extensive necrosis. Adaptive immune response, characterized by IFNγ production, could only be discerned after 4 weeks of infection [40]. A subsequent, more detailed description of the lung pathology using identical strain and dose of *Mtb* points to a spectrum of granulomatous lesions, primary as well as post-primary in the lungs of these animals, akin to those observed in chronically infected humans [42]. This model has since been utilized to study the effectiveness of candidate anti-tubercular vaccines with varying degrees of success [39, 43, 44, 50]. Recently, this model has also been used to explore the central role of tumor necrosis factor (TNF) in controlling latent TB infection [47] and to study whether regulatory T cells play a role in this process [46]. These macaques are also

being used to model the reactivation of latent *Mtb* infection through the use of anti-TNF antibodies to neutralize TNF activity [47] and through coinfection with simian immunodeficiency virus (SIV) to model TB/AIDS coinfections in the human population [45, 48] (Table 1).

The rhesus macaque as a model of human TB

It was initially believed that rhesus macaques do not exhibit latent phase of Mtb infection. This notion stemmed from the fact that a comparable BCG vaccination even appeared to better protect cynomolgus rather than rhesus macaques from challenge with highly virulent *Mtb* Erdman [72]. However, recent data from the same research institute point out that rhesus macaques can be protected against *Mtb* Erdman by BCG vaccination [59]. It is now accepted that rhesus macaques, much like the cynomolgus, can develop asymptomatic [56], chronic latent [68], or acute, rapidly fatal pulmonary TB [62, 64, 66] following infection with appropriate doses of *Mtb* (Table 1).

Much of the earlier work with rhesus macaques utilized the vaccine strain BCG as the mycobacterial infecting agent [52–54, 57, 58]. In these studies, high doses of BCG (10^3 as a low dose, 10^6 as a moderate dose, and 10^9 as a high dose) were employed, and simultaneous coinfection with SIV was performed. As a result of the high dose of BCG and simultaneous coinfection with an immunodeficiency-inducing viral agent, BCG was able to generate a TB-like disease, and it was possible to study the reactivation and progression of the disease [52, 53, 57]. This model laid the foundation for significant studies on the role of $CD8^+$ [60] and Th17 [65, 70] cells in the immune control of *Mtb* infections (Table 1).

Infection via the aerosol route has been established in the rhesus macaque model, allowing a more natural deposition of infecting bacilli onto the pulmonary surfaces [62–64, 66, 70]. However, the aerosol route is challenging for administration of extremely low cfus to infect macaques. The use of *Mtb* strains with reduced pathogenicity, for example, CDC1551, has allowed the development of a model of human TB where macaques can be exposed to moderate numbers of viable bacilli via the inhalation route and result in a latent to chronic, rather than an acute, disease outcome [70].

The rhesus macaque model has also been used to test the efficacy or safety of numerous anti-tubercular vaccine candidates [59, 61, 63, 69]. It has been shown beyond doubt that BCG-vaccinated rhesus macaques exhibit protection from disease [59]. These new results clarify the confusion that has existed in the past about the high susceptibility to *Mtb* infection and low levels of protection following BCG vaccination in rhesus, relative to cynomolgus (Table 1).

Key advantages of the NHP model of TB

By definition, no animal model can completely mimic human disease. This is particularly true for TB, which presents as remarkably different diseases based on strain virulence, host genetics, and a variety of confounding environmental factors. However, *Mtb*-infected macaques offer a significant advantage while modeling key aspects of human TB.

Macaques develop true, human-like latent infection characterized by the absence of any clinical signs of infection in the presence of antigen-specific immunological response, which can be measured by a tuberculin skin test (TST) or an IFNγ release assay (IGRA) developed specifically for primates (PRIMAGAM) [62]. Clinical and radiological diagnostic means to assess *Mtb* infection are well developed for NHPs and comparable to humans [38–40, 62, 66, 68].

As discussed earlier, one of the key advantages of the macaque model is its ability to recapitulate the complete spectrum of granulomatous lesions that occur in human disease. As shown in Fig. 1, in rhesus macaques infected with *Mtb*, classical lesions with central caseous and necrotic cores are commonly found (Fig. 1A). However, other types of lesions such as fibrotic (Fig. 1B) or cavitary (Fig. 1E) may also be present, albeit more rarely. In some cases, mineralization or calcification of caseous lesions is also observed (Fig. 1C). Often, caseous lesions also exhibit the formation of numerous multinucleated giant cells in the peripheral region (Fig. 1D). In rhesus macaques coinfected with SIV, post-primary lesions were readily observed in the vicinity of primary lesions (Fig. 1F). These results strongly reinforce the point that macaque models of TB recapitulate the wide variety of pathologic TB lesions observed in infected humans.

In all of the macaque models described in this review, it has been possible to reactivate latent or chronic TB into an acute form characterized by rapid multiplication of bacilli and pneumonia using either SIV coinfection or blockade of the TNFα pathway [46, 47, 68].

Coinfection with AIDS is a major reason for the global resurgence of TB in the last several decades. It is expected that these robust TB/AIDS coinfection models using *Mtb* (or BCG) and SIV will not only allow preclinical testing of therapeutics and vaccines but also lend insights into the molecular and cellular mechanisms of reactivation. Typically, coinfection of rhesus or cynomolgus macaques already latently or chronically infected with *Mtb* via either aerosol or intratracheal route is performed by inoculating with SIV_{mac239} or SIV_{mac251}, intravenously. For rhesus macaques, a dose of 3×10^2 $TCID_{50}$ appears to be sufficient for reactivation of chronic TB. The required dosage may be higher for cynomolgus macaques because SIV_{mac} viruses are host adapted for rhesus macaques. It has recently been possible to identify lung as well as lymph node cells from coinfected rhesus macaques as harboring both *Mtb* and SIV [68]. We have recently identified these coinfected cells as lung macrophages by multilabel confocal microscopy to colocalize cell type–specific and TB-specific antigens in the same cell (S. Mehra et al., unpublished data). Such novel observations on the biology of TB/AIDS coinfection are impossible to generate in any other model besides the natural host itself. The macaque model also provides advantages over studying *Mtb*/HIV coinfection in humans including but not limited to defined timing of infection, control of the dose, and delivery method of *Mtb* and SIV including the availability of defined gene deletion mutants of both SIV and *Mtb*, the ability to collect longitudinal samples, and the control of environmental, dietary, and social factors. In future, it will be important to study whether coinfected macrophages in the lung are deficient in the control of bacterial replication.

Application of novel technologies to the NHP model

In the last few years, several novel applications have been incorporated into the NHP model for TB. The ability to infect NHPs via the inhalation aerosol route is one such example. It is conceivable that the ability to use this natural route with the NHP model would have a similar positive impact on TB research, as did the ability to infect mice via the aerosol route two decades ago.

Use of imaging techniques in the NHP model of TB

Several NHP studies have used thoracic radiography to image and determine the progression and extent of TB in NHPs. However, state-of-the-art imaging tools are now being applied to the NHP model. Once such major advance occurred, Lewinsohn et al. [73] employed X-ray computed tomography (CT) scanning to capture high-resolution real-time images of lung lesions in rhesus macaques infected with high doses of *Mtb*. These results significantly correlated with pulmonary histopathology, thus providing an accurate, yet noninvasive

assessment of lesion development and disease progression. Flynn et al. have used positron emission tomography (PET) coupled with CT (PET/CT) to enable an assessment of both functional (PET) and structural (CT) aspects of the development and progression of pulmonary granulomatous lesions [J.L. Flynn, personal communication]. Using this technology, these researchers can differentiate lesions that have the potential to reactivate *Mtb* replication from those that are truly latent. Clearly, such advanced imaging technology can be useful while assessing the protective efficacy of candidate vaccines or the therapeutic potential of candidate drugs.

Use of *Mtb* mutant libraries to study mechanisms of pathogenesis in the NHP model of TB

Mtb encodes over 4000 different open reading frames. A significant number of these genes do not have any defined homologs in other species. Therefore, it is difficult to assign function to many such genes. Researchers have resorted to the use of *Mtb* mutant libraries to define genes that are absolutely required for pathogenesis *in vitro* and *in vivo*, using the murine model [74–76]. Our laboratory recently applied this approach to the rhesus model. Mixed pools of over 300 defined, distinct *Mtb* mutants were used to infect rhesus macaques via the aerosol route, and their infection phenotype was studied using microarray-based survival readouts [62]. We found that during acute TB, over 33% of all mutants tested were attenuated for survival and multiplication in macaques. This was in stark contrast to when these mutants were tested in mice, where a significantly lower number of mutants (6–10%) were attenuated. It is conceivable that better structural organization of the NHP TB lesions contributes to a more effective and robust immune response, which is able to clear mutants lacking certain proteins required by the pathogen to survive *in vivo*. This study identified potentially novel bacterial pathways that could be targeted for anti-*Mtb* drug development, such as DNA repair [77] and molybdenum biosynthesis [78].

Study of hypoxia in NHPs infected with *Mtb*

It is believed that human TB lesions evolve highly necrotic centers filled with debris and cellular contents from destroyed infected macrophages. As lesions mature over time, this environment becomes hypoxic [79]. It has been speculated that hypoxia must have a significant effect on the biology of the pathogen [80], primarily because *Mtb* is known to mount a significant transcriptional response to changes in oxygen concentration, governed by the transcription factor DosR [81] via sensor kinases DosT and DosS [82]. This hypothesis was tested by analyzing lesions obtained from macaques infected with *Mtb* [41]. Pimonidazole hydrochloride, an imaging agent bioreductively activated specifically in hypoxic environment, was injected into macaques prior to necropsy. Discrete regions exhibiting activated pimonidazole adduct in the central caseous regions of tubercular granulomas were evident, clearly confirming that NHP lesions are hypoxic. These experiments begin to provide the tools to examine the role of the local microenvironment on *Mtb* biology and virulence *in vivo*.

Use of genome-wide techniques to study TB infections in NHPs

Genome-wide systems biology approaches have been extensively used to study biological problems in the last decade. These include transcriptome- and proteome-wide investigations. Recently, some of these approaches have been applied to the NHP model of TB. In the first such study, we employed rhesus macaque–specific whole-genome microarrays to study host gene expression in tuberculous lesions obtained from the lungs of infected animals, at an acute and a chronic stage of infection. Our results indicate that change in *Mtb* biology rapidly modulates the host granuloma environment, with the initial Th1 type gene expression signatures giving way to anti-inflammatory gene expression markers [64]. Recently, macaque transcriptomics has also been used to identify pathogen-specific gene

expression signatures in peripheral blood [83]. It is expected that several such studies will be reported in the near future.

Another advantage of the NHP models is the rapidly growing availability of the rhesus/cynomolgus macaque genome sequence and their similarity to the human genome. We expect that in the recent future, MHC typing data from the infected macaques will allow us to validate resistance and susceptibility hypotheses derived from the analyses of human populations.

Future directions

The interest in the NHP model of TB stems from its accurate portrayal of key aspects of human syndrome. These models were initially developed with a focus on preclinical testing of diagnostic, therapeutic, and vaccine candidates. While this aim remains important, the NHP model appears to be extremely valuable for the study of pathogenesis including both host and bacterial determinants of disease and immune protection. In particular, we think this model will be crucial to examining TB/AIDS coinfections and reactivation TB in general. The model will also be useful for the design of computational models of TB latency, reactivation, and reinfection based on macaque response to different types of *Mtb* infections. Key questions related to *Mtb* virulence can also be addressed in the model including the effect of hypoxia on the pathogen and its long-term survival; the ability of *Mtb* to reprogram its metabolism to adapt to the lung environment and the nutrients available therein; how a productive adaptive response is unable to provide sterilizing immunity and how *Mtb* modulates the host immune response to its benefit.

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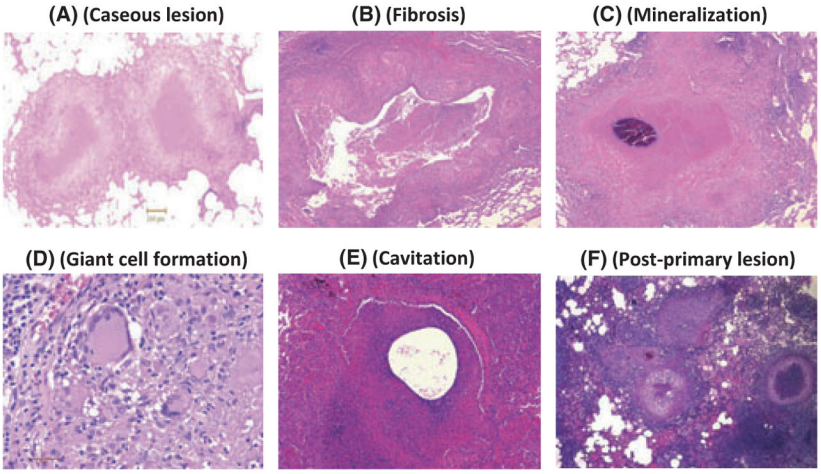

Fig. 1.

Different types of histopathological lesions observed during various *Mtb* infections of rhesus macaques. A. Centrally caseous lesion with peripheral rim of immune cells is the most typical type of pathology observed in NHPs infected with *Mtb*. B. A rare fibrotic lesion in a rhesus macaque infected with *Mtb*. C. Mineralization of a caseous lesion over time in a rhesus macaque infected with *Mtb*. D. Formation of highly inflamed multinucleated giant macrophages in a lesion from a rhesus macaque infected with *Mtb*. E. A rare lesion in a rhesus macaque infected with *Mtb* with a pathology that could be a precursor for cavitation. F. Post-primary lesions in the vicinity of primary, centrally caseous lesions in a rhesus macaque coinfected with *Mtb* and simian immunodeficiency virus.

Table 1

Key advances in the research on the NHP model of TB are tabulated, along with the description of *Mtb* strains, route of infection, and the dose inoculated

Year	Mycobacterial agent	Dose/route of infection	Significant advance	Citation
1996	*Mtb* Erdman	10^1–10^2 (low), 10^3 (moderate), 10^4–10^5 (high); intratracheal	Establishment of the cynomolgus macaque as a model of human TB	[37]
2001	*M. bovis* BCG (Pasteur)	10^3 (low), 10^6 (moderate), 10^8 (high); intravenous	Reactivation of BCG infection in rhesus macaques by SIV coinfection	[52]
2004	*Mtb* Erdman *Mtb* H37Rv	10^1 (low), 3×10^1 (moderate), 1.5×10^2 (high); intratracheal 3×10^1 (low), 2×10^2 (moderate), 10^6 (high); intratracheal	Establishment of a rhesus macaque model of asymptomatic infection with *Mtb*	[55]
2005	*Mtb* Erdman	3×10^3 cfu; 10^3 cfu; intratracheal	Use of the NHP model to test a candidate vaccine against TB	[39]
2006	*Mtb* Erdman	~2.5×10^1 cfu; intratracheal	Description of the early events upon infection of cynomolgus macaques with *Mtb*	[40]
2008	*Mtb* Erdman	~2.5×10^1 cfu; intratracheal	Study of hypoxia in caseous lesions of NHPs infected with *Mtb*	[41]
2010	*Mtb* Erdman	~2.5×10^1 cfu; intratracheal	Reactivation of latent TB in cynomolgus macaques by SIV coinfection	[45]
2010	*Mtb* Erdman	~2.5×10^1 cfu; intratracheal	Reactivation of latent TB in cynomolgus macaques by TNF depletion	[46]
2010	*Mtb* CDC1551	~5×10^3 cfu; aerosol	Infection phenotype of *Mtb* mutants in rhesus macaques via the aerosol route	[63]
2011	*Mtb* CDC1551	~2.5×10^2–5×10^2 cfu; aerosol	Reactivation of latent TB in rhesus macaques by SIV coinfection	[67]

BCG, Bacille Calmette–Guerin; NPHs, non-human primates; SIV, simian immunodeficiency virus; TB, tuberculosis; TNF, tumor necrosis factor.