

*Alle grote dingen komen voort uit het onbelangrijke.
Daarom streeft de wijze nooit naar het grote.
Op die manier kan hij grote dingen bereiken.*

(Lao-Tse)



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Animal models to study tuberculous meningitis

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ABSTRACT

Tuberculosis (TB) has the highest mortality of any bacterial pathogen in history. Tuberculous meningitis (TBM) is the most severe complication of this disease and frequently occurs in childhood. The interaction between mycobacteria and the host consists of pathogen recognition and subsequent specific inflammatory responses.

The majority of our knowledge on pathogenesis of TB in general and specific local inflammatory responses in case of TBM derives from experimental studies in animals.

In this review animal models to study TBM are summarized, focusing on the pathogenesis of this disease.

INTRODUCTION

Worldwide 2 billion people are infected with *Mycobacterium tuberculosis* (*M. tub*). In 2009, more than nine million million people developed active TB and 1.7 million people died of the consequences of this disease [1]. TB is the world's second largest killer among infectious agents behind HIV/AIDS. Besides, HIV infection increases the risk to develop active TB and results in more complications and a higher case fatality rate [2]. An emerging problem, especially in Eastern Europe, China and sub-Saharan Africa, is the development of (extensive) multidrug-resistant TB, which is very difficult to treat and indicates the failure of local TB control programs [3].

Tuberculous meningitis (TBM) is the most severe complication of TB and frequently occurs in childhood. TBM occurs in approximately 1% of all patients with active TB and has a high mortality rate. Survivors often develop severe neurological sequelae. TBM usually starts insidiously with non-specific symptoms, such as fever and vomiting and the diagnosis is often only considered once irreversible neurological damage has already occurred [4].

The pathogenesis of TB and the development of TBM is a complicated interplay between mycobacteria and the specific host immune response. Granuloma formation is a very characteristic hallmark of mycobacterial infection [5]. In case of TBM, the local inflammatory response inside the central nervous system (CNS) is aimed at mycobacterial clearance but also creates a harmful environment for neuronal tissue.

The majority of our knowledge about TB pathogenesis in general and specific immune responses inside the CNS in case of TBM originates from studying disease phenomena in animal models. These disease models have also been used to study antimicrobial and vaccine responses, as well as immunomodulating strategies.

We will focus on the pathogenesis of TBM and summarize animal models to study TBM in this perspective.

PATHOGENESIS OF TUBERCULOUS MENINGITIS

M. tub is an acid-fast, slow growing bacillus. The target cell for *M. tub* in mammalian species is the mononuclear phagocyte. After inhalation, *M. tub* enters the alveolar macrophage and survives inside. Phagocytosis also triggers innate immune recognition of *M. tub* inside these cells. The innate immune system is able to recognize highly conserved molecular motifs on pathogens that are not found on the cells of higher eukaryotes. These motifs are called pathogen-associated molecular patterns (PAMPs) and bind to pattern recognition receptors (PRRs). Activation of these receptors on antigen-presenting cells induces a signaling cascade by the expression of

co-stimulatory molecules and cytokines and consecutive activation of the adaptive immune system [6]. Toll-like receptors (TLRs) are a group of cellular adaptor proteins that play a major role as PRRs in the initiation of innate immune responses. TLR activation results in various functional outcomes, including the secretion of inflammatory cytokines and chemokines, the regulation of phagocytosis and the induction of host defense mechanisms leading to direct antimicrobial activity but also responsible for local tissue damage [7].

Mycobacteria consist of several structures that act as PAMPs, which will be recognized by TLRs. The TLRs known to be involved in recognition of *M. tub* are TLR2, TLR4, *TLR9*, and possibly TLR8 [8]. TLR2 binds lipoarabinomannan of the mycobacterial cell wall. In experimental studies, triggering of TLR2 by mycobacteria leads to killing of intracellular *M. tub* in both murine and human macrophages [9]. Heat shock proteins, secreted by *M. tub*, activate TLR4. Mice lacking functional TLR4 were more susceptible to develop pulmonary TB and showed a reduced capacity to produce the protective type 1 cytokine interferon-gamma (IFN- γ) upon macrophage activation, indicating a protective role for TLR4 in host defense against pulmonary infection by *M. tub* [10]. TLR8 and *TLR9* are intracellular PRRs, located in the endosome and very closely related to each other. *M. tub* is an intracellular pathogen that resides in characteristic phagosomes, which interact with early endosomes [11]. Single-stranded RNA derived from pathogens such as *M. tub* has been proposed as a likely ligand of TLR8 [12]. *TLR9* recognizes unmethylated Cytosine-phosphate-Guanine (CpG) oligodeoxynucleotides, highly frequent motifs in (myco-) bacterial DNA and rare in the mammalian genome [13]. TLR8 was recently reported to play a possible role in susceptibility to pulmonary TB in adults as shown by a genetic association of four TLR8 polymorphisms in two independent cohorts of TB patients compared to healthy controls. Expression levels of TLR8 transcripts and protein showed a marked increase during bacterial infection [14]. Experiments with *TLR9* *-/-* mice infected with high doses of *M. tub* showed markedly enhanced susceptibility to infection in association with combined defects in proinflammatory cytokine production in vitro compared to wild type mice [15].

Figure 1 illustrates mycobacterial recognition receptors and their intracellular signaling pathways leading to an inflammatory response.

Cells inside the CNS also express TLRs, especially on astrocytes and microglia, antigen presenting and phagocytosing cells inside the CNS and key players in the immune response in this compartment [16]. Microglia are resident macrophages in the brain and are able to recruit and activate peripheral immune cells, such as monocytes and T-lymphocytes, leading to leukocyte migration and cellular influx in the cerebrospinal fluid (CSF). Upon pathogen recognition TLRs activate a cell-signaling cascade, ultimately leading to the local production of pro-inflammatory cytokines

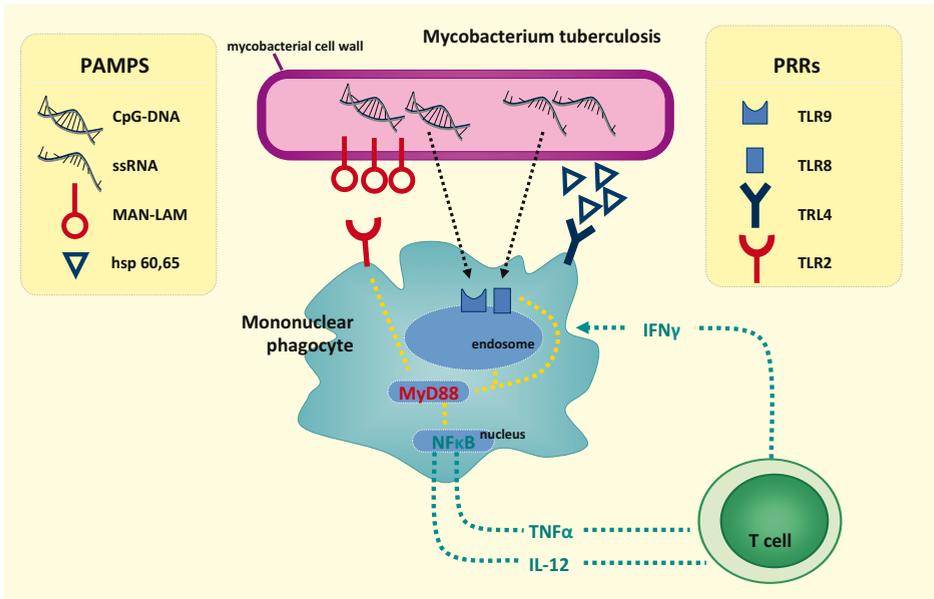


Figure 1. Pathogen-associated molecular patterns of *Mycobacterium tuberculosis* and its cell wall and the pathogen-recognition receptors of the mononuclear phagocyte, interacting via cytokines

and chemokines such as $\text{TNF-}\alpha$, IL-1, IL-6, IL-8, IL-10 and IL-18 by microglia [17]. These cytokines lead to activation and migration of more inflammatory cells and to lysis of invading pathogens in case of CNS inflammation but are also toxic and harmful for neuronal cells [18].

Our understanding of the pathogenesis of TBM originates from experiments in guinea pigs and rabbits and post-mortem data of children described by Arnold Rich and Howard McCordock in 1933 [19]. They found that TBM results from hematogenous dissemination of *M. tub* to the CNS after primary pulmonary infection. However, TBM develops not directly by hematogenous spread of bacilli to the meninges but by release of bacilli into the CSF from focal granulomatous lesions, called *Rich foci* that are typically located subpial or subependymal. Recent studies show the dynamic interactions between mycobacteria and granuloma: infected macrophages attract uninfected macrophages and aggregate in an organized way to form granuloma, the hallmark of mycobacterial infection. These early granuloma contribute to mycobacterial growth, contrary to the classical paradigm of being host-protective structures [20].

Once macrophages are infected, they are able to leave the granuloma and hematogenously form distant secondary foci of infection. The predominant location of *Rich foci* in the brain parenchyma, rather than the meninges, illustrates secondary granuloma formation during a preceding bacteremia. Bacteria remain dormant inside

the granuloma for months or years and meningitis occurs only when bacteria are released into the subarachnoid space [21]. Entrance of bacteria into the CNS and the subsequent inflammatory response causes the three features responsible for the clinical manifestations of TBM: 1) development of tuberculoma, 2) basal inflammatory exudates that cause cranial nerve palsies and obstruction of CSF pathways, resulting in hydrocephalus, and 3) obliterative vasculitis which leads to infarctions [19, 22].

Figure 2 summarizes the chain of events in TBM pathogenesis with the essential *Rich focus*.

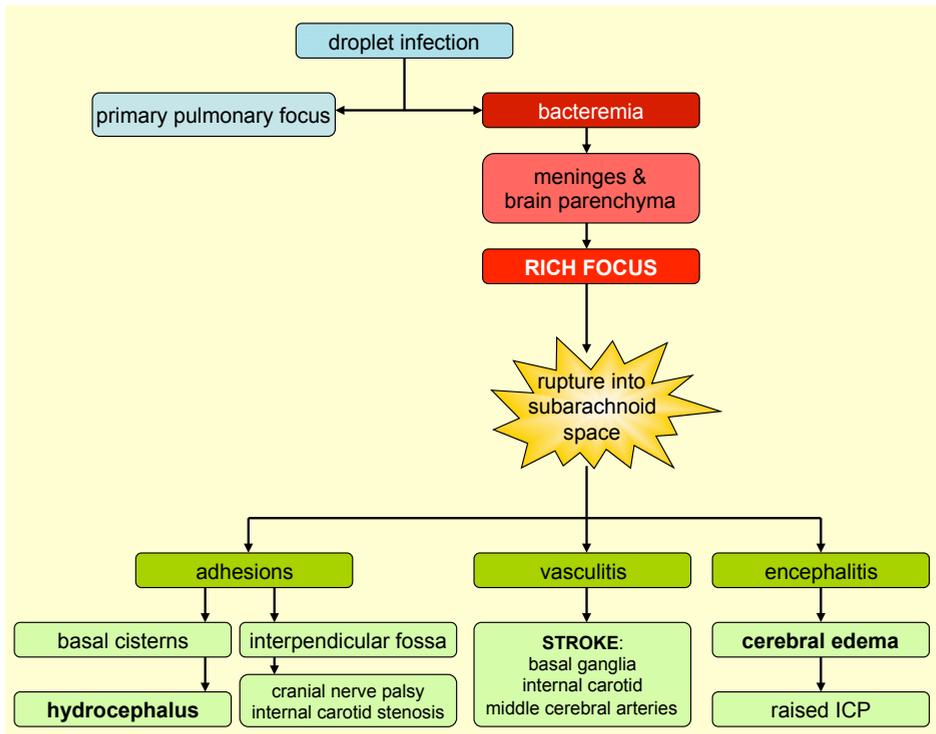


Figure 2. Schematic presentation of chain of events in TBM pathogenesis

ANIMAL MODELS OF TUBERCULOUS MENINGITIS

Animal models have contributed substantially to our understanding of the pathogenesis, pathology and immunology of TB and TBM throughout the years. In their thorough description of TBM in 1933, Rich and McCordock also used animal models. Next, we will describe which models have been designed to study TBM since the first descriptions in 1933. Available animal models will be summarized focusing on the understanding of host and bacterial factors in TBM. Primary purpose, advantages, and limitations of each model will be discussed.

Guinea pig

The guinea pig (*Cavia porcellus*) has been used extensively in TB research in general. Already in 1867 the first use of these rodents was reported in the literature by Sanderson and Fox, describing inoculation of guinea pigs with human lesion exudate and subsequent development of progression, mimicking natural human disease [23, 24]. Robert Koch used guinea pigs in his famous experiments to isolate and identify the causative agent of human TB and also to test tuberculin as vaccine [25]. Guinea pigs are believed to be the most sensitive animals to TB, according to the classic experiments by Riley, showing that TB is an airborne infection [26]. He used guinea pigs as living air samplers for airborne tubercle bacilli generated by patients on an experimental TB ward. Because of the progressive nature of the pathology of TB in guinea pigs it replicates many aspects of TB, especially childhood TB and disseminated TB in immunocompromised hosts. Granuloma formation is well organized and the granulomas become necrotic, similar to human granuloma. It has also been a useful model to study dissemination and secondary granuloma formation [27].

Rich and McCordock used the guinea pig in their classical model to study the pathophysiology of TBM [19]. In a series of experiments they showed that both normal and hypersensitive guinea pigs showed clear meninges after direct injection of tubercle bacilli in the carotid artery, implying that TBM is not merely the simple result of direct meningeal infiltration from the bloodstream in miliary TB. The guinea pigs in their experiments indeed developed generalized miliary TB after mycobacterial injection in the carotid arteries but this did not result in diffuse inflammatory meningitis. TBM lesions could however be readily reproduced by introducing tubercle bacilli directly into the subarachnoid space. They also observed that the animals that had been injected with tubercle bacilli in the bloodstream developed a focal tubercle adjacent to the meninges. These lesions developed to the point of caseating and discharging bacilli into the subarachnoid space with diffuse meningitis as a result [28].

The main advantage of the guinea pig model to study TB in general is that it displays well-structured granulomas and caseous necrosis. It has also proven to be a very effective model to test vaccines, especially BCG. Major limitation of the guinea pig model is the lack of available immunological reagents what makes it difficult to study the exact nature of the immune response in TBM in terms of receptor and protein expressions [29].

Rabbit

Classical experiments by Lurie *et al.* in 1952 described pulmonary TB pathogenesis in rabbits genetically inbred to be either susceptible or resistant to airborne human *M. tub* [30]. Most studies in rabbits however, use *M. bovis* because rabbits are extremely susceptible to *M. tub* and very rapidly succumb when infected. The pathology in rabbits infected with *M. bovis* is remarkably similar to human infection with *M. tub*,

showing pulmonary granulomas of similar organization as in human TB, including caseous necrosis.

The group of Kaplan *et al.* used the rabbit to model acute mycobacterial infection in the CNS. They injected 2×10^7 colony forming units (CFU) of *M. bovis* Ravenel directly in the cisterna magna of New Zealand White rabbits and collected CSF samples 0, 2, 4, 6, and 24 h and every next 24h up to 8 days after inoculation [31]. These experiments induced leukocytosis (predominantly mononuclear cells), high protein levels, and release of tumor necrosis factor-alpha (TNF- α) into the cerebrospinal fluid within 1 day. Histologically, severe meningitis with thickening of the leptomeninges, prominent vasculitis, and encephalitis was apparent, and mortality was 75% by day 8. This model was also used to describe the potential therapeutic effect of thalidomide in TBM. Adding thalidomide to antituberculous drugs in TB infected rabbits substantially reduced TNF- α concentrations in CNS and plasma and 100% of animals survived compared to 50% mortality in rabbits not additionally treated with thalidomide [32]. Using the same stereotactic rabbit model they also described the therapeutic effect of IMiD3, a thalidomide analog showing limited pathological neurologic changes and improved survival of experimental animals [33].

Although very useful to specifically study TBM and closely mimicking well-structured granulomas and caseous necrosis and moderate range of immunological reagents, rabbits are costlier to maintain and have larger laboratory space requirements [29, 34].

Mouse

Mice are generally resistant to pulmonary TB compared to guinea pigs, rabbits and humans. They develop non-caseating granulomas in the lung and manifest a chronic inflammatory disease. On the other hand, parallels exist between murine and human innate immune responses in terms of pathogen recognition and cytokine responses. Another advantage of this experimental to study the pathogenesis of TB is the availability of inbred and targeted genetic knockout strains of mice that help to elucidate the role of specific cytokines, cells and surface markers in response to mycobacterial infection. Mice are also the most common available experimental animals with the obvious advantage of low cost in housing and feeding and a wide spectrum of available immunological reagents.

Mazzolla *et al.* inoculated susceptible Balb/c mice and resistant DBA2 mice with *M. bovis* by intracranial injection. Natural differences in systemic inflammation susceptibility in these mice strains were determined by the presence or absence of natural resistance-associated protein 1 (Nramp1). After direct intracisternal inoculation with 1×10^5 *M. bovis* BCG mycobacterial growth in the CNS was very different for both mouse strains on day 14: DBA/2 mice showed low colonization that reached

the maximal peak of 22×10^3 CFU/brain on day 14. In contrast, at the same time point, BALB/c mice showed as much as 134×10^3 CFU/brain. Both strains cleared the infection completely after 28-35 days. They also observed different patterns of local immune responses between the two mouse strains (time-dependent differences were observed between Balb/c and DBA/2 mice for macrophage inflammatory protein (MIP)-2, interleukin (IL)-12, IL-1 β , interferon (IFN)- γ , IL-6 and TNF- α and concluded that Nramp1 is important in anti-mycobacterial defenses. In both strains of mice, infection did not result in granuloma formation, giant-cell formation or necrotic areas [35].

More recently, we described a C57Bl/6 murine model to study the pathogenesis of TBM. We found perivascular leukocyte infiltration in the meninges after intracerebral inoculation with 1×10^5 CFU *M. tub* H37Rv. We cultured up to 1×10^5 CFU of mycobacteria from brain homogenates after 3, 7, 12, 18 and 24 weeks after inoculation, reflecting intracerebral growth of mycobacteria. Besides, in the CSF we could measure concentrations of cytokine-induced neutrophil chemoattractant (KC) (mean concentrations of 123 ± 16 pg/mL 3 weeks after infection) and MIP-2 (mean concentrations of 695 ± 97 pg/mL 24 weeks after infection), chemokines that were absent in uninfected mice, reflecting chronic infection by attraction and activation of phagocytic cells inside the CNS. However, we did not observe brain exudate or infarction, characteristic for human TBM, we did not find the characteristic pro-inflammatory cytokine responses (TNF- α IL-6 and IFN- γ) upon our first measurements 3 weeks after infection and we found no infection related mortality in the experimental animals [36]. Because of the availability of a wide variety of genetically inbred mice, this model seems promising for future research to unravel the details of the inflammatory response to TBM in terms of immune cells, cell surface receptors and cytokine production.

In order to study a crucial step in the pathogenesis of TBM, which is entering the CNS after primary pulmonary infection upon bacteremia, Be *et al.* developed a murine model based on their *in vitro* experience with cell cultures mimicking the blood-brain barrier. They used BALB/c mice and infected them intravenously with 1×10^6 CFU *M. tub* H37Rv to mimic hematological dissemination. Gross pathological examination of the brain did not show any granulomas or infectious lesions in the brain at any time point. Significantly higher numbers of *M. tub* were found in the brain than in the whole-blood volume in the mouse, indicating that CFU counts from the brain were not due to contamination from the blood. CFU count of mycobacteria in brain tissue peaked at day 14. They also observed cytokine responses inside the CNS. TNF- α (300-400 pg/mL) and IL-10 (1000 pg/mL) were found in brain tissue of infected mice compared to mice injected with saline. The model was developed to identify genetic determinants for CNS invasion. By means of a pooled infection

with genotypically defined *M. tub* mutants, bacterial genes required for invasion or survival were determined in the CNS. They identified mutants for 5 *M. tub* genes (Rv0311, Rv0805, Rv0931c, Rv0986, and MT3280) with CNS-specific phenotypes [37].

Lee *et al.* described the specific immune response in the CNS after direct intracerebral infection of C57Bl/6 mice with 1×10^5 CFU *M. bovis* BCG. At 3 and 5 weeks post infection, BCG bacilli were detected not only in the CNS but also at a low level in the periphery, which suggests systemic dissemination of BCG. The bacterial load declined from 3 to 5 weeks post infection in all tissues tested, but the reduction was most significant in the brain and the lymph nodes draining the CNS. The significant decline in the number of CFU in the brain implies that immunity within the CNS is as efficient at controlling bacterial growth as it is in the periphery. Histopathology was evaluated 3 and 5 weeks post infection by assessing infiltrating mononuclear cells in H&E stained brain sections. In the infected mice, infiltrating mononuclear cells were found in the perivascular areas, ventricles, and parenchyma. They found IFN- γ producing CD4+ T-cells in the brain, similar as in peripheral blood but also a subset of activated T-cells (IFN- γ IL-17 producing double-positive CD4+ T-cells) unique to the CNS. These cells originate from microglial and dendritic sources indicating a specific local inflammatory response inside the CNS [38].

Limitations of the murine model to study TB in general are the loosely organized granulomas. Compared to the rabbit model to study TBM, the murine model is limited in pinpointing the optimal location to infect the animals intracerebrally.

Swine

In order to develop a TB model that better mimics human disease Bolin *et al.* developed a swine model in 1997. In a sub-experiment the infected swines were divided in two groups and infected with 1×10^4 and 1×10^8 *M. bovis* intrathecally. These pigs showed less signs of respiratory distress compared to swine that were infected intravenously or inside the lymph nodes. Mycobacteria were cultured from brains, lungs and lymph nodes in the three different groups of inoculation indicating dissemination. The granuloma found in lungs and lymph nodes were very similar in architecture details when compared to human granulomas [39]. They concluded that swine are an excellent model to study pulmonary and disseminated TB. To study TBM they later switched to murine models.

Non-human primate

The obvious advantage of non-human primates, such as the cynomologous macaque, is the similarity of disease compared to humans, both clinically and pathologically. Another advantage is that most human reagents can be used in primates. Because

of cost and space requirements and ethical issues, research on primates should be reserved for those questions that cannot be answered with in vitro techniques or with unvertebrated species or rodents. They mainly concern vaccine and drug testing and the issue of reactivation as well as the interaction with HIV. We are not aware of primate models that were primarily designed to study TBM. However, we did find a communication on a primate model that also described granuloma formation in the CNS [40]. In 1996 a cynomologous macaque model to study TB was described, closely resembling human disease. Three groups of four monkeys were described and infected with 10^5 , 10^4 , and 10^2 CFU *M. tub* Erdman strain respectively. In a second experiment three groups of four animals were infected with 10^3 , 10^2 , and 10^1 CFU. All infected animals were compared to healthy animals. In this study, the authors describe that two animals developed TBM. They displayed weakness of the extremities, loss of coordination, and irritable behavior. At necropsy, military tubercles were observed on the meninges.

Zebra fish

The zebra fish is relative new animal used to study TB, especially pathogenesis, host-pathogen interactions and granuloma formation. Ramakrishnan *et al.* described in 2002 the real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos using *M. marinum* [41]. No zebra fish model has been published that studies central nervous system TB or neuronal granuloma formation. Currently our group is developing such a model. The zebra fish model is excellent for imaging early pathology and ideal to study innate immunity and also suitable for making mutants [29].

CONCLUSIONS

Evaluating the available animal models to study TBM, the rabbit model seems to most closely mimic human TBM. The murine model however, is superior in terms of studying the immunological response. The non-human primates are irreplaceable for drug and vaccine testing. Animal models in general have been and continue to be crucial to our understanding of both host and bacterial factors in TB and TBM. Still unresolved questions range from the genetics of host defense and microbial virulence to drug therapy and vaccination. Animal models promise to be useful immunologic, genetic, molecular and pharmacological tools in future research of TBM.

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