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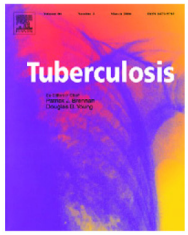

Experimental tuberculosis: the role of comparative pathology in the discovery of improved tuberculosis treatment strategies

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Summary
The use of laboratory animals is critical to the discovery and in vivo pre-clinical testing of new drugs and drug combinations for use in humans. *M. tuberculosis* infection of mice, rats, guinea pigs, rabbits and non-human primates are the most commonly used animal models of human tuberculosis. While granulomatous inflammation characterizes the most fundamental host response to *M. tuberculosis* aerosol infection in humans and animals, there are important species differences in pulmonary and extra-pulmonary lesion morphology which may influence responses to drug therapy. Lesions that progress to necrosis or cavitation are common, unfavorable host responses in naturally occurring tuberculosis of humans, but are not seen consistently in experimental infections in most animal model species. The importance of these unique lesion morphologies is that they represent irreversible tissue damage that can harbor persistent bacilli which are difficult to treat with standard therapies. Understanding the differences in host response to experimental tuberculosis infections may aid in selecting the most appropriate animal models to test drugs that have been rationally designed to have specific mechanisms of action in vivo. A better understanding of lesion pathogenesis across species may also aid in the identification of novel therapeutic targets or strategies that can be used alone or in combination with more conventional tuberculosis treatments in humans.
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Introduction

The relatively recent resurgence of human tuberculosis now with multi- and extensively-drug resistant strains has prompted the need to develop new, effective and safe drugs as quickly and efficiently as possible.[1,2] Animal models have and will continue to aid in early discovery as well as the pre-clinical testing phase of new drugs for efficacy and toxicity. This is particularly true in the search and testing of badly needed tuberculosis drugs. This review is intended to briefly summarize our current knowledge of the pathogenesis of experimental tuberculosis in the commonly used animal models of the human disease. Until recent years, there have been relatively few new drugs developed that have undergone testing in the classical tuberculosis animal models. As a result, we have a poor understanding of the response of the various animals to drugs or drug combinations currently used or being tested for use in humans. This review focuses on animals historically used in tuberculosis research and more specifically, on the morphologic features or pathologic changes that characterize responses to aerosol or

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airway infections with virulent *M. tuberculosis*. Where data are available, responses to drug therapy in the various models are described. The goal in modeling tuberculosis in animals is to mimic as closely as possible the pathology and clinical progression of the naturally occurring disease. An attempt has been made here to highlight the major morphologic features of experimental tuberculosis, particularly lesion types that are known to occur in humans as well. The specific goal is to provide a better understanding of the pathogenesis of experimental tuberculosis as it pertains to drug therapy. These data may aid in the selection of animal models that best meet the needs of rationally designed, hypothesis driven research related to the development of new tuberculosis drugs. Additionally, by critically comparing disease features shared by people and animals, new therapeutic targets can be identified and tested as alternatives or adjuncts to current therapy. Lastly, there is increasing interest in how the existing models can be modified to more closely reflect specific lesion types, particularly those that are known to respond poorly to drug treatment.[3,4] These efforts will benefit from a better understanding of the pathogenesis of the major lesion types in both humans and animals.

Use of animals in tuberculosis drug research

Essentially all drugs approved for use in humans by the Food and Drug Administration (FDA) undergo extensive testing in two or more species of laboratory animals. For practical or economic reasons, some species are more widely used for efficacy studies while others are preferred for pharmacokinetic and toxicity studies. Similar considerations influence the selection of animal models in tuberculosis research. One example is that despite the documented differences in the immune response between mice and humans, mice are still the most widely used animal model for studying the immunological responses to *M. tuberculosis* infection and tuberculosis vaccines.[5,6] For the same reasons, mice are widely used in early tuberculosis drug discovery and efficacy research. However, due to species specific differences in disease progression and lesion morphology, responses to drug therapy in mice may or may not reflect the desired effects in people. The response of animal models to experimental therapy for other human diseases has recently been called into question due to lack of agreement between animal and human studies.[7,8] The main reasons given for the differences in outcomes were a lack of stringent experimental design in animal studies compared to human clinical trials and the failure of animal models to adequately reflect the naturally occurring disease in people.[8] Similarly, what has prompted recent interest in considering more appropriate models to test tuberculosis drugs is not only the urgent need for new drugs, but also relevant differences between animals in their response to experimental *M. tuberculosis* infections.

The susceptibility of various species of animals to the human tubercle bacillus was explored long before Robert Koch re-isolated the organism from experimentally infected guinea pigs.[9–12] Even these early studies demonstrated marked differences between species in their susceptibility to experimental infection. Currently, the animals most widely used in tuberculosis research are the various strains of resistant and susceptible mice, rats, guinea pigs, rabbits, and non-human primates.[13–20] The major advantages and disadvantages of each of these species for tuberculosis research have been the subject of recent reviews.[21–31] Because laboratory rodents share many basic physiologic, metabolic and anatomic similarities with people, they have and continue to play a critical role in evaluation of fundamental drug effects and toxicity.[32–36] Mice have and will continue to be a valuable model in the early pre-clinical stages of tuberculosis drug discovery. In the mouse model, relatively small amounts of experimental compounds can be used to obtain toxicity, pharmacokinetic, tissue distribution as well as initial efficacy data. Because of their larger size, rats are routinely used in late pre-clinical pharmacokinetic studies but their use requires larger amounts of experimental compounds to achieve dose responses similar to that of mice. However, rats are less commonly used for tuberculosis research mainly because of the added purchase and colony expenses compared to mice and the fact that the lesion morphology in outbred strains like mice fail to mimic some features that are commonly seen in human tuberculosis.[13,37–40] Since there are important practical advantages to using rats for late pre-clinical drug studies, there has been recent interest in using the cotton rat, S*igmadon hispidus* and S*igmadon fulviventer* as a model of tuberculosis, which do develop a wider variety of lesion types.[23]

Pathogenesis of experimental tuberculosis

Since the human tubercle bacillus rarely if ever infects animals naturally, experimental aerosol exposure of laboratory species to *M. tuberculosis* is inherently artificial, which is reflected by the varied clinical and pathological responses, some of which are species specific. At the most fundamental level, the host inflammatory responses in animals and people are similar, with the important differences being in the rate of disease progression and in the array of lesion types.[14,23,39,41–45] While the more rapid rate of clinical tuberculosis in animals is advantageous in the experimental setting, the differences in pathology have prompted the need to critically evaluate common tuberculosis models more from a morphologic perspective. This approach is necessary to identify those that best mimic the natural disease or that are appropriately suited to test specific hypotheses related to drug discovery.

Tuberculosis lesions in all species are typically a mixture of macrophages, lymphocytes, plasma cells and granulocytes that encroach upon the cellular elements that make up the pulmonary parenchyma.[5,42,45–48] The predominance of macrophages that differ in morphology (mononuclear or multinucleated giant cells) as well as differentiation and activation state are the hallmark of

mycobacterial infections. Main species differences are seen in the structural organization of the granulomata, specifically the propensity to progress toward lesion necrosis and cavitation. Arguably, lesion and host tissue necrosis is the most important consequence to *M. tuberculosis* infection in people and animals. Lesion necrosis causes irreversible tissue damage with loss of cell and tissue function and is the prelude to cavity formation. More importantly in the context of drug therapy, lesions with necrosis or cavitation, more so than other lesion morphologies, often harbor viable bacilli that are difficult to treat with conventional antibiotic therapy.[41,49]

The difficulty in modeling human tuberculosis lies in the variety of clinical presentations which can be influenced by a wide array of host and environmental factors. Known tuberculosis risk factors in humans like age, nutritional status, exposure to cigarette smoke or pollution, and concurrent infections or chronic diseases like diabetes can also be modeled in animals.[38,50–60] The presence of risk factors in both humans and animals may influence the rate of disease progression, pathology, and bacterial load as well as treatment responses. Lesions from different patients or even within a single infected individual display a range of morphologies usually related to stage of progression and anatomic locations (pulmonary vs. extra-pulmonary) which may vary in response to therapy.[41,61]

The basic lesion morphologies in people and in animals infected with *M. tuberculosis* are broadly classified as non-necrotic or solid, necrotic, cavitary, calcified and fibrotic. Pathology of experimental tuberculosis in animals can further be influenced by the route of infection, strain of animal or dose and strain of *M. tuberculosis*.[5,62–64] In a given species and with all variables held constant, the differences in lesion morphology in animals are mainly due to genetic resistance, the stage of disease and the presence or absence of an adaptive immune response that is acquired during infection or conferred by vaccination.[5,65] This is particularly true in species that develop a wide spectrum of lesion morphologies similar to those seen in people.

It is widely accepted that proper and rapid structural organization of the tuberculous granuloma is a favorable host response as it contains bacilli locally, thus preventing the progression of disease and spread between individuals by infected aerosols.[47,66–68] However, in the context of drug therapy and sterilizing immunity, the well formed granuloma, especially with necrosis or cavitation can also represent a barrier to effective treatment, and therefore represents an unfavorable rather than favorable host response.[41,48,61,69] Surprisingly, little is known about the ability of commonly used tuberculosis drugs to reach bactericidal or bacteristatic concentrations in lesions in humans or animals and moreover, what morphologic features influence local pharmacokinetics and tissue distribution of the various drug formulations.

What has re-emerged recently from testing new tuberculosis drug candidates in animals is the persistence of drug tolerant bacilli and more importantly, their distribution within specific lesion types.[70] One of the most comprehensive descriptions of the association between persistent tubercle bacilli and microscopic lesion morphology in people was made by the physician Georges Canetti. Canetti conducted a thorough and systematic description of human tuberculosis between 1940 and 1944, in which he described the "histobacteriology" of over 1500 cases.[41] While Canetti's approach was simplistic by today's standards, he established that certain lesion types were associated with the persistence of viable tubercle bacilli that resisted drug therapy. Even during this period of early tuberculosis drug development, Canetti suggested that "practically all bacilli will develop resistance to drugs, individually and less so with drugs in combination". Canetti concluded that lesion morphology contributed to the development of tuberculosis drug resistance, particularly those lesions that were more likely to harbor difficult to treat bacilli.[41]

In the course of carefully examining thousands of human tuberculosis lesions, Canetti broadly classified responses as benign or unfavorable. The benign lesions were those with minimal or no necrosis, with complete or near complete healing by calcification, fibrosis or even bone formation (ossification). These changes were viewed as non-progressive with limited irreversible tissue damage containing few or no visible or cultureable bacilli. Unfavorable lesions on the other hand were classified as such because the progressive inflammation and necrosis resulted in extensive irreversible tissue damage that often harbored relatively large numbers of visible and cultureable bacilli. These features have served as the basis of lesion classification schemes that can also be used to evaluate responses in animals, particularly those that develop a range of lesion morphologies similar to humans.[61,71]

Non-necrotic or solid lesions

Solid or non-necrotic lesions occur as an early manifestation of *M. tuberculosis* infection in all species. Solid lesions represent the initial non-suppurative or granulomatous inflammatory response that precedes organization into the classical granulomas typical of human tuberculosis and experimental infections.[14,23,41,61,72,73] With few exceptions, solid lesions are the predominant lesion morphology, irrespective of stage of disease, in most strains of resistant and susceptible mice.[5,16,42,45] Solid lesions also characterize post-primary or secondary lesions that have been best characterized in the guinea pig model.[31,71,73–75] Similar post-primary lesions occur in other species as the result of hematogenous or chronic intra-pulmonary dissemination but are often indistinguishable from primary lesions by routine histology. In the remaining models the pathogenesis of post-primary lesions has not been systematically characterized.[14,23,42,76,77]

A better understanding of tuberculosis lesion pathogenesis is emerging from evaluating animal models that demonstrate a varied in vivo response to experimental infections. Differences in lesion morphology can be species specific or influenced by presence or absence of acquired immunity. In the guinea pig model, post-

primary or secondary lesions are generally thought to originate from hematogenous lung reinfection during the bacillemic phase of disease.[30,44,65] The lack of lesion necrosis and thus calcification of post-primary lesions in guinea pigs is likely influenced more by the development of a systemic adaptive immune response which is coincident with bacillemia and hematogenous lung reinfection.[5,31,42,44,65,77] Morphologic differences between primary and post-primary lesions in immunologically naïve animals have provided the best evidence in experimental tuberculosis that lesion morphology significantly influences the effectiveness of drug therapy.[70,74,78] In drug treated guinea pigs, early post-primary lesions resolve and are prevented from developing further, whereas primary lesions remain unresolved but continue to heal by calcification and fibrosis. In a study by Dhillon, responses to isoniazid or rifampicin treatment were determined in mice and guinea pigs that were first vaccinated with *M. bovis* BCG (BCG).[78] While the beneficial responses to drug therapy in BCG vaccinated guinea pigs was interpreted from the perspective of adaptive immunity, we know from past and more recent studies that BCG vaccination significantly improves lesion morphology primarily by preventing lesion necrosis.[31,71] In guinea pigs but not in mice, BCG vaccination prevents necrosis and calcification of primary lesions, thus changing the lesion from necrotic to a solid phenotype. Despite the immunity conferred by BCG vaccination, treatment of mice had no impact on the bactericidal activity of either rifampicin or isonizid in lungs compared to non-vaccinated animals. However, in BCG vaccinated guinea pigs, both drugs were more effective, suggesting that lesion morphology differences had a greater influence on drug efficacy than did immunity conferred by BCG vaccination.[78]

Lung lesions that develop during the chronic stages of infection (immune phase) are also more responsive to drug therapy than necrotic lesions that develop following initial exposure (pre-immune phase).[70,74,78] These data further suggest that the relationship between immune status and response to drug therapy is mostly due to differences in lesion morphology. Even one of the most promising new tuberculosis drugs is effective at reducing the size and bacterial burden of the solid, post-primary lesions compared to necrotic primary lesions.[70]

Necrotic lesions

Caseous necrosis, as much as the granuloma itself, defines the response to *M. tuberculosis* infection in humans and experimental infections in some animals. Caseation describes the macroscopic and microscopic appearance of inspissated (cheese-like) exudate associated with lesion necrosis. In his historic presentation in 1882, Robert Koch observed that "in all tissues in which the tuberculosis process has recently developed and is progressing most rapidly, these bacilli can be found in large numbers, especially at the edge of large, cheesy masses. The bacilli occur almost exclusively in large numbers free of the tissue cell".[11] What Koch described in these early microscopic studies was that the majority of acid-fast bacilli were concentrated extra-cellularly in rapidly progressing lesions with caseous necrosis.

Lesion necrosis represents irreversible tissue damage that undergoes healing in some species by calcification, fibrosis and sometimes ossification. However, incomplete healing creates a microenvironment that harbors bacilli that are visible by acid-fast staining, some of which are confirmed viable by culture.[49,70,79] More importantly, bacilli sequestered by these lesions are often more tolerant to drug therapy.[41,61,70,74] In general, animals that develop necrosis following aerosol or intra-tracheal infection include non-human primates, rabbits, guinea pigs, cotton rats and a small group of highly susceptible mouse strains (Figure 1).[14,23,43,64,72,75,80–82] The pathogenesis of lesion necrosis is poorly understood but likely involves both host and pathogen factors.[4,83–86] Host factors include the combined effects of early delayed type hypersensitivity (DTH) as well as necrosis associated with neutrophil infiltration and vascular thrombosis as suggested by some recent animal studies.[28,43,61,72,81] In species that develop primary lesion necrosis, solid lesions begin to progress to central necrosis between 3–4 weeks post-infection, often associated with the late phase of a biphasic granulocytic inflammatory response.[14,44,64] Interestingly, strains of mice that fail to develop lesion necrosis also show a biphasic granulocytic inflammatory response, suggesting that this is a common feature of infection but is not the sole mediator of lesion necrosis.[46,80,87–90] A better understanding of the pathogenesis of necrosis through the study of appropriate animal models is needed. Therapeutic strategies aimed at preventing or minimizing necrosis may be beneficial in eliminating bacilli that persist in necrotic lesions in the face of conventional antibiotic therapy.

Lesion hypoxia

One of the important consequences of inflammation with necrosis is lesion hypoxia resulting in the loss of structural organization of the pulmonary parenchyma including the local blood supply.[70,91–93] Hypoxia is thought to be an important determinant in the pathogenesis of tuberculosis since *M. tuberculosis* has long been classified as an obligate aerobe. Bacilli grown under low oxygen conditions have altered physiology with a reduced rate of replication, making them less susceptible to some drugs.[94,95] Bacilli grown in low iron containing media combined with gradual depletion of oxygen are more virulent in animals.[96] Most of what is known about the influence of low oxygen on the response of *M. tuberculosis* to drug treatment comes from in vitro studies.[95,97,98] Besides confirming the in vivo hypoxic state, the difficulty in studying the effect of low oxygen on *M. tuberculosis* response to drug therapy is in isolating and characterizing the relatively few organisms within appropriate lesions. Lesions in most strains of mice, unlike those in people, show minimal necrosis and no hypoxia.[91,93] In highly susceptible mouse strains that do develop lesion necrosis, lesion hypoxia is likely to

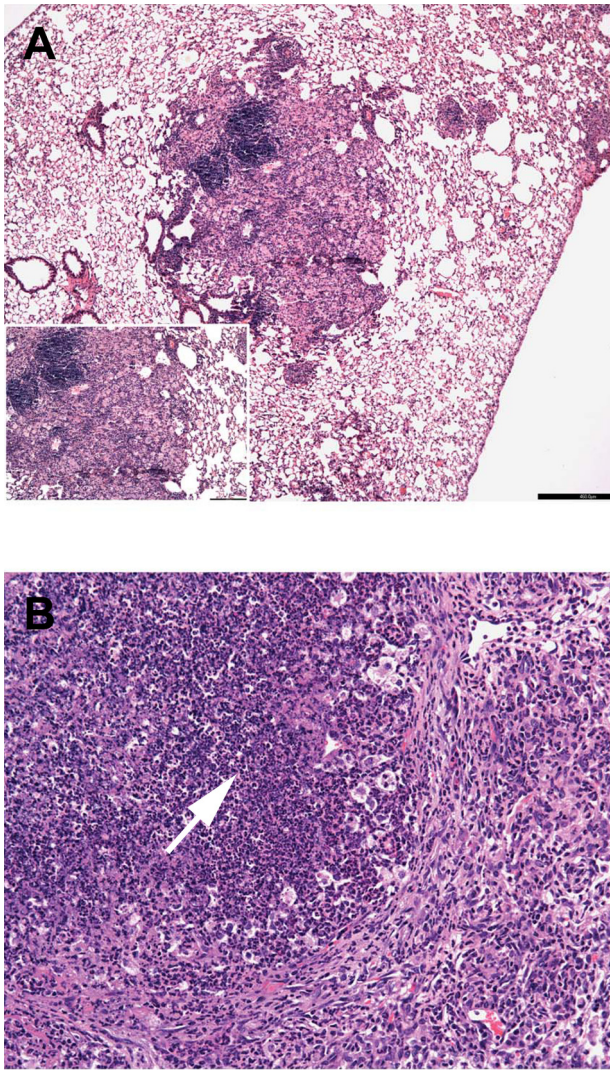

Figure 1 Lesion morphology differs between different inbred strains of mice infected with virulent *M. tuberculosis*. A. At 58 days following low-dose aerosol infection in the resistant C57Bl/6 strain of mice, lesions are composed of macrophages and lymphocytes (inset) and show no evidence of necrosis. B. In contrast the highly susceptible IFN-γ knock out mouse at 29 days after infection has more extensive lesions with necrosis and neutrophilic infiltrates (arrow). Hematoxylin and eosin stain. A, 40X magnification (Bar = 460 μm), inset 200X magnification, B, 200X magnification.

exist but has not been confirmed. In guinea pigs and people, the use of oxygen sensitive dyes in tissue sections demonstrates that bacilli co-localize to lesions with progressive inflammation and central zones of necrosis.[70,93,99]

Because of extensive lung involvement with chronic inflammation, progressive tuberculosis in people and animals can also be associated with whole body hypoxia (hypoxemia). Lowered blood and tissue oxygenation can be a direct effect of decreased lung perfusion and gas exchange as a consequence of progressive lung disease or indirect from decreased circulating erythrocytes and hemoglobin concentrations reflecting anemia of chronic infection.[92,100–102] These effects can be measured in people and animal models by decreased peripheral blood oxygen saturation and decreased packed erythrocyte volumes and hemoglobin concentrations, respectively[100–102] (Basaraba unpublished data). Hypoxia therefore may not be restricted locally to pulmonary or extra-pulmonary lesions but may also be a systemic effect during chronic infections.

Mineralized lesions

Dystrophic mineralization or calcification is a pathologic process associated with intra- and extra-cellular deposition of mixed calcium salts at sites of tissue necrosis.[103–108] The hydroxyapatite mineral complex found within foci of tissue necrosis with dystrophic calcification is similar to that found in normal bones and teeth. As cells degenerate, calcification is initiated first within mitochondria and progresses intra-cellularly by a process referred to as propagation. Extra-cellular calcification can be initiated from free iron or phospholipids originating from organelles and membranes of dead and dying cells.[104,105,107,109] Calcification is a progressive process and if complete, is considered along with fibrosis as a favorable healing response. While there are differences in the propensity of different species to form calcified lesions, it is generally seen in all models that develop lesion necrosis with the exception of the highly susceptible mouse strains. The lack of calcification in these animals is likely due to the shortened life-span from the rapidly progressive infection rather than the inability to form calcified lesions.

Calcification of lesions can be complete or incomplete. Complete calcification is a favorable response whereas incomplete calcification is unfavorable since residual lesion necrosis may harbor viable bacilli that are drug tolerant.[41,61,70] Patients with healed lesions including those with calcification have a 2 fold higher chance of reactivation compared to patients with no radiographically visible lesions.[54] The degree of lesion calcification may be influenced by the relative presence or absence of a variety of endogenous inhibitors of calcification.[104,110,111] The persistence of residual primary lesion necrosis is best characterized in the guinea pig but is present in other species as well (Figure 2).[70,71,109] It has been suggested that the benefits of exposure of tuberculosis patients to sunlight was in part due to the role of increased endogenous vitamin D in promoting dystrophic calcification and thus more complete lesion healing.[112]

Residual lesion necrosis in the persistence of *M. tuberculosis*

We have recently shown that drug treatment failed to clear *M. tuberculosis* from regions of residual primary lesion necrosis in immunologically naïve guinea pigs.[70] The persistent lesion necrosis in partially calcified primary lesions can be likened to a biofilm-like structure which has important implications with regard to

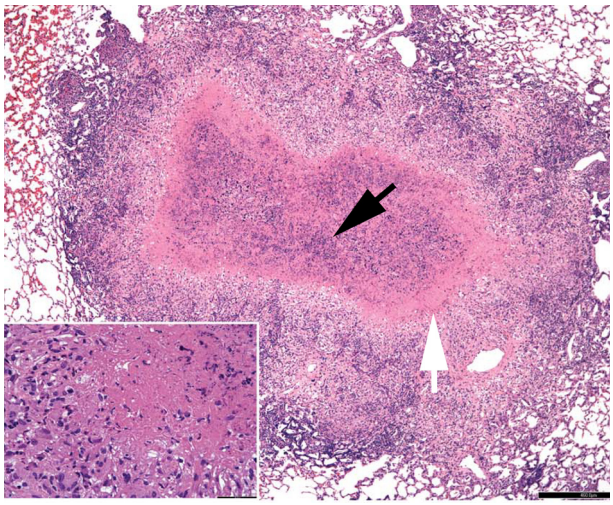

Figure 2 Immunologically naïve cotton rats S*igmadon spp*. develop primary lesion necrosis following aerosol infection with virulent *M. tuberculosis*. Thirty days following infection, there are foci of early dystrophic calcification (black arrow) with a rim of residual necrosis (white arrow and inset). The rim of residual necrosis is devoid of calcified foci and resembles a biofilm-like structure similar to that described in other animals that develop primary lesion necrosis. Hematoxylin and eosin stain. 40X magnification (Bar = 460 μm), inset 400X magnification.

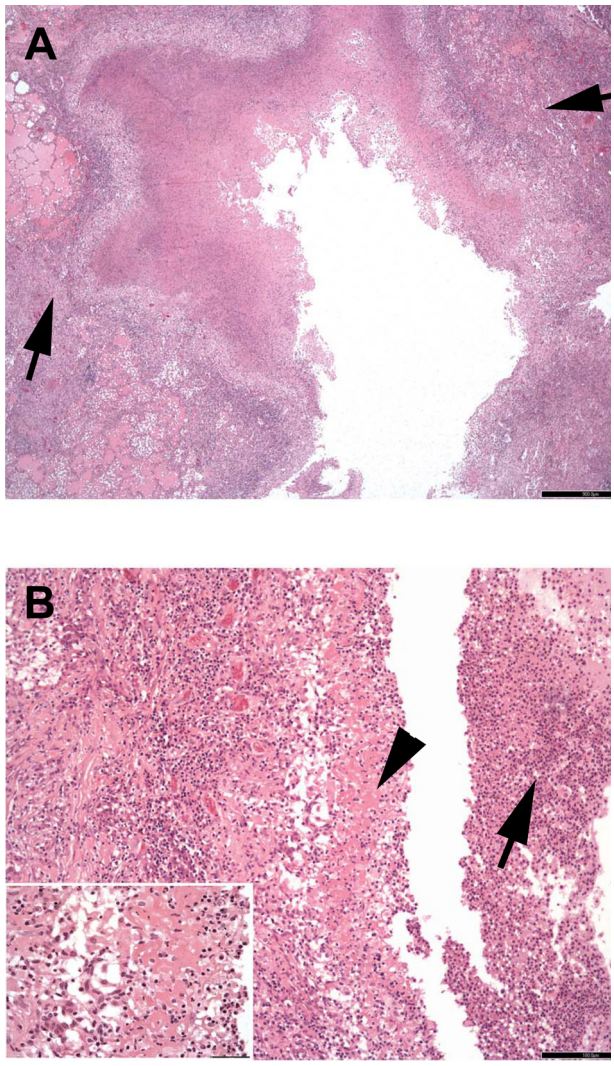

Figure 3 In non-human primates (Rhesus macaque, *Macaca mulatta)* infected with *M. tuberculosis*, the primary lesions associated with airways often progress to form large cavities as the result of extensive lung tissue necrosis. A, liquifactive necrosis leaves an extensive cavitation that is delineated from more normal lung parenchyma by extensive mixed inflammatory cell infiltrates (arrows). B, the cavity lining contains sheets of inflammatory cells, mostly neutrophils (arrow) and a layer of homogenous, acellular material that resembles a biofilm-like structure (arrowhead and inset). Hematoxylin and eosin stain, A, 20X magnification (Bar= 900 μm), B, 200X magnification (Bar= 180 μm), inset = 400X magnification.

tuberculosis drug therapy (Figure 2).[70,113,114] Biofilm-associated bacteria are in general resistant to antibiotics and are inaccessible to cellular and humoral host defenses.[115–118] The formation of biofilms is initiated by bacterial colonization and formation of pathogen derived, extra-cellular matrices, however, host components derived from necrotic cells can also contribute to the formation of biofilms.[119,120] Besides residual primary lesion necrosis, a similar layer of organized, acellular debris may also represent a biofilm that lines the interior surface of cavitary lesions as well (Figure 3B). [69]

The formation of biofilms by *M. tuberculosis* is controversial but non-tuberculous mycobacterium form well characterized biofilms associated with opportunistic infections or persistence in the environment.[113,116,121–124] In vivo biofilms are best characterized in chronic lung infections caused by *Pseudomonas aeruginosa* (*P. aeruginosa*) and are composed of both pathogen and host factors. In preliminary studies we have shown that residual necrosis in guinea pig primary lesions also contains host cell nuclear and cytoplasmic contents similar to *P. aeruginosa* biofilms (Basaraba unpublished data).[115,120] It stands to reason that in all models that develop lesion necrosis with incomplete calcification or cavitation, similar biofilm-like structures occur but further study and characterization is needed.

Another lesion in tuberculosis that has features of an in vivo biofilm is the filling and obstruction of small airways with senescent neutrophils and macrophages. This feature is commonly associated with post-primary lesions in the chronic stages of infection in both guinea pigs and mice and is likely to occur in other species as well. The importance of this particular lesion is that it represents the early stages of the animal equivalent of sputum formation and often contains large numbers of bacilli (Basaraba unpublished data).[44,125] Bacilli are often intracellular within degenerate macrophages but also can be found embedded in an extra-cellular matrix composed of host cell debris similar to that seen in necrotic primary lesions within incomplete calcification.[44,126] In addition, similar to the mucoid airway plugs seen in chronic *Pseudomonas* infections, these lesions in tuber-

culosis may harbor a population of bacilli that are concentrated in areas that are almost completely devoid of oxygen.[127] The importance of biofilms in tuberculosis is that these structures may contain difficult to treat bacilli and represent a potential target for alternative therapy that can be tested in appropriate animal models.[117,118]

Iron accumulation is another feature of primary lesions with residual necrosis and incomplete calcification. Iron is an essential micronutrient and like other pathogenic bacteria, *M. tuberculosis* has evolved virulence mechanisms to scavenge host iron.[128–132] Iron may be important in the pathogenesis of tuberculosis biofilm formation and may be involved in the pathogenesis of primary lesion necrosis through the generation of tissue damaging reactive oxygen intermediates.[133] Recently, we have shown that iron accumulates both intra-cellularly and extra-cellularly in the primary lesions of guinea pigs infected with *M. tuberculosis*, a process that is abrogated by BCG vaccination.[109] Extracellular iron accumulation is stratified within lesions and is concentrated in a transition zone between the calcified center and residual necrosis that harbors the majority of the bacilli.[125,109] It is unclear based on these preliminary studies whether iron is biologically available or if bacilli remain in an iron-poor microenvironment. Iron chelation therapy has been suggested as an alternative therapy in tuberculosis patients as well as those co-infected with HIV.[128,134,135] Therefore, the targeting of lesion-associated iron with iron chelator represents an alternative strategy to treat tuberculosis either alone or in combination with conventional therapy. Iron chelation may deprive *M. tuberculosis* of host iron, aid in the disruption of biofilms or influence the activity of anti-tuberculosis drugs, all of which can be tested in appropriate animal models.[128,130,131,133,136–139]

Cavitary lesions

Cavitary lesions are considered the most destructive and thus the least favorable of host responses in humans with tuberculosis, yet are seen consistently only in a few animal models. Necrotic lesions associated with airways are the prelude to cavity formation. It is generally thought that cavities progress from caseous necrosis to liquefaction to leave large voids that replace normal lung parenchyma (Figure 3).[69,140] The loss and fragmentation of mineralized debris from calcified lesions is a common tissue processing artifact and should not be confused with true cavitary lesions.[28] Non-human primates and rabbits are the models that most often develop cavitary lesions; however, the pathogenesis likely differs from that of humans.[15,43,83,85] In people, cavitary lesions typify post-primary or reactivation tuberculosis that can develop decades after initial infection; however, it can also be an extension of progressive primary disease in patients with lowered resistance.[41,48,61,140–142] The large numbers of bacilli combined with communication of cavities with airways (open cavities) are considered important risk factors for *M. tuberculosis* transmission.[41,69] The pathogenesis of post-primary tuberculosis with cavitation in people is unclear but is likely to involve mediators of inflammation and necrosis similar to those responsible for inciting primary lesion necrosis. The initiation of reactivation tuberculosis with subsequent cavity formation is thought to originate from bacilli that persist from the bacillemic phase of the primary infection.[141]

Post-primary cavitary tuberculosis in people has a characteristic apical lobe distribution that corresponds to post-inflammation scarring. Radiographic surveys suggest that these fibrotic or inactive lesions are associated with a 30 fold higher risk of reactivation compared to patients without apical lobe scars.[54] A similar pattern of regional tissue scarring at the site of subsequent reactivation has not been described in animals. Interestingly, the propensity for cavitary lesions to form in apical lung lobes in human reactivation tuberculosis corresponds to preferential accumulation of iron in apical lung lobes associated with known tuberculosis risk factors like smoking.[143] Similar to the suggestion that lesion iron accumulation may be involved in the pathogenesis of primary lesion necrosis, tissue iron accumulation may also be involved in the pathogenesis of reactivation tuberculosis in humans.[109,125,143]

To develop new strategies to effectively treat cavitary tuberculosis, there is a need for animal models that reliably develop similar lesions. Therefore, there is increasing interest in promoting necrosis and cavity formation in animal models that don't typically develop these distinct lesion morphologies.[3,64] However, this is not possible without a better understanding of the pathogenesis of cavity formation. It is generally accepted that cavity formation results when there is a transition from caseous necrosis to liquifactive necrosis by an unknown mechanism. Caseous necrosis is called such because of the dry crumbly appearance resembling cheese whereas liquifactive necrosis is softening of the caseum that is thought to result from fluid accumulation and the lytic enzymes released from infiltrating inflammatory cells, particularly neutrophils.[41] Similar to the role neutrophils may have in the initiation of primary lesion necrosis, they are the predominant cell type in cavitary lesions in people (Figure 3B).[43,144]

Cavitary lesions in animal models usually develop as a continuation of the primary infection. However, even in documented cases of cavitary lesions developing in latently infected non-human primates, the distribution of reactivation disease appears to be random rather than apical as is typically seen in people.[76] Rabbits develop cavitary lesions as part of the primary disease process following intra-tracheal infection with *M. bovis*.[43,72,145,146] Recent evidence, however, suggests that highly virulent clinical isolates of *M. tuberculosis* in rabbits also promote cavitary lesions as part of the primary infection.[146] Challenge with more virulent clinical isolates in the rabbit model represents one strategy that has been used to modify existing models to promote specific lesion morphologies that more closely mimic a specific lesion morphology seen in the naturally occurring human disease.[43,146]

The staging of cavity formation in non-tuberculosis mycobacterium infections may provide important clues

to the pathogenesis of *M. tuberculosis* cavitary lesions. The initial step involves airway associated necrosis which is followed by progressive thickening of airway walls with subsequent obstruction and eventually airway dilation.[147] Airway involvement may be direct (endobronchial) or secondary to the expansion of lesions originating from the peribronchial and peribronchiolar lesions that involve pulmonary lymphatics.[10,126,147] The failure of peribronchial lesions to progress to cavitation in some species may be in part, associated with differences in pulmonary anatomy or the amount of peribronchial and perivascular connective tissue.[126] If animal models can be made to consistently develop cavities, they would be extremely useful in developing therapies targeting this difficult to treat lesion morphology in people.

Extra-pulmonary tuberculosis

An element of experimental tuberculosis in animals that is often overlooked and has important implications during drug therapy is the distribution and severity of extra-pulmonary lesions. Because experimental tuberculosis is a progressive disease in most animals, disseminated extra-pulmonary lesions occur relatively early following experimental aerosol infections.[14,39,64,65,74,80] As in people with progressive disease, the characteristic weight loss associated with tuberculosis is the result of extra-pulmonary disease as well as lesions restricted to the lungs.[50] Extra-pulmonary dissemination is frequently documented by culture in experimental *M. tuberculosis* infections but morphologic features are rarely reported in the literature.[39,44,64–66,71,148]

The first site of extra-pulmonary spread is usually the pulmonary and mediastinal lymph nodes that are infected via draining, afferent lymphatics.[5,14,21,69] In people, the primary infection resulting in the combination of lung and lymph node lesions is common and is referred to as the Ghon complex. In contrast, spread to other extra-pulmonary organs is hematogenous through the blood vasculature or through the gastro-intestinal tract from swallowed bacilli.[14,66,149] Gastrointestinal infection can occur from swallowing bacilli during aerosol infection or soon after as the result of grooming, especially if whole body rather than nose-only aerosol exposure methods are used. Additionally, gastrointestinal infection can occur in the chronic stage of infection from swallowing bacilli rich, respiratory secretions produced by mucocilliary clearance.[14] In a recent study, besides the typical lung and pulmonary lymph node involvement, extra-pulmonary *M. tuberculosis* lesions in guinea pigs were found in brain, small intestine, hepatic and mesenteric lymph nodes, pancreas, adrenal gland and heart.[150]

The importance of extra-pulmonary lesions in the evaluation of tuberculosis drug therapy is that tissues respond differently and pose a unique challenge to drug therapy.[35,97,151,152,153] The differences may be tissue specific, relating to type and distribution of vascularity (lymphatic vs peripheral blood), differences in tissue susceptibility, propensity to develop a unique lesion morphology or differences in tissue distribution of drugs.[66,71,126,148] Lymph node involvement either with or without lung involvement is a unique therapeutic challenge in people that can also be modeled in animals.[153]

In summary, the differences in lesion morphology among the different animal species infected with *M. tuberculosis* provide different levels of stringency for testing new drugs. Mouse strains that develop only solid lesions are best suited for discovery and early testing of drugs for in vivo effects and toxicity.[35,36] Certain highly susceptible mouse strains have the added benefit of not only developing necrotic lesions but also having a more rapid disease progression, thus shortening the in vivo testing intervals.[34,64] Species such as guinea pigs and cotton rats provide a wider variety of lesion types that include necrotic and mineralized lesions for a higher level of in vivo testing stringency and to test adjunct therapies against novel therapeutic targets. The non-human primate and rabbit models develop an even wider variety of lesion types and are the most appropriate models to test drugs specially designed to treat cavitary lesions. Since experimental *M. tuberculosis* infections are progressive in the majority of model species, all are suitable for testing the effects of drugs on extra-pulmonary lesions.

Understanding the pathogenesis of the various lesion types and their response to conventional drug therapy and vaccination is important as it may aid in identifying new drugs and novel therapeutic targets that can be used alone or as adjunct therapy in people.[154,155] These strategies will be aided by a better understanding of how unique morphologic features like necrosis, cavitation and calcification influence drug penetration, distribution and metabolism in vivo. Animal models have shed light on the importance of the granulomatous inflammatory response in containing bacilli, but also how lesions may represent a barrier to conventional or newly developed drugs.

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