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# The Pathogenesis of Post-primary Tuberculosis. A Game Changer for Vaccine Development.

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## Abstract

A vaccine that prevents transmission of infection is urgently needed in the fight against tuberculosis (TB). Results of clinical trials have been disappointing. Major problems include lack of biomarkers and understanding of the mechanisms of disease and protection. A more fundamental problem is that the scientific community seldom recognizes that primary and post-primary TB are distinct disease entities. Nearly all vaccine candidates have been designed and tested in models of primary TB, while transmission of infection is mediated by post-primary TB. Post-primary TB is seldom studied because no animal develops the disease as it exists in humans. Nevertheless, mice, guinea pigs and rabbits all develop infections that at certain points appear to be models of human post-primary TB. Slowly progressive pulmonary TB in immunocompetent mice is an example. It is characterized by an alveolitis with infected foamy macrophages that have multiple characteristics of the human disease. We demonstrated that inclusion of an immune modulating agent, lactoferrin, with a BCG vaccine in this model induced a sustained reduction in lung pathology, but not numbers of organisms in tissue. Since the animals die of expanding pathology, this demonstrates the feasibility of using selected animal models for studies of vaccines against post-primary TB.

### Keywords

Tuberculosis; vaccine; post-primary; lactoferrin; pathology

In 2009, the National Academy published a monograph “A New Biology for the 21st Century” stating that molecular biology of the 20$^{th}$ century was too narrowly focused to successfully address problems in areas such as evolution, morphogenesis and infectious

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disease (1). “Advancing from identifying parts to defining complex systems is well beyond its present capabilities.” The New Biology requires integration of the knowledge from many disciplines to permit deeper understanding of biological systems.

Carl Woese, a thought leader in this area put it differently: “Science is impelled by two main factors, technological advance and a guiding vision (overview). A properly balanced relationship between the two is key to the successful development of a science. Without the proper technological advances the road ahead is blocked. Without a guiding vision there is no road ahead” (2). Biology hits the ‘wall of biocomplexity’ meaning that simply defining molecular components is insufficient to understand higher level biologic functions. The discipline of molecular biology has produced an astounding harvest in areas such as the gene and the nature of the cell. However, it chose to ignore other problems such as evolution and the nature of biological form that are fundamentally not understandable as collections of parts typically dismissing them as inconsequential (2). This becomes a problem as we try to address issues such as the pathogenesis of tuberculosis that exceed capabilities of molecular biology.

## Tuberculosis is an excellent example.

Prior to the rise of molecular biology, investigators had a ‘guiding vision’ of TB, but lacked ‘technological advances’. They understood the pathology, radiology and clinical course of each stage of TB, but could not understand the biologic processes. Today, we have many ‘technological advances’, but have forgotten the ‘guiding vision’ and replaced it with a convenient but erroneous notion that granulomas are the only key lesion of TB (3). The result is that research on the pathogenesis of TB has hit the ‘wall of biocomplexity’. Modern publications on the pathogenesis of TB describe increasing numbers of mechanisms and contributing parts, but with an inaccurate guiding vision these pieces cannot be assembled to form a coherent explanation (4).

Most contemporary research on TB follows the guiding vision (overview or paradigm) that granulomas are the important lesion of all TB (3). This is a late 20$^{th}$ century concept that has no support among those who actually studied tissues of people with untreated pulmonary TB (5–7). As documented in a recent review, *M. tuberculosis* (MTB) produces, not just one, but two distinct disease entities known as primary and post-primary TB (8). Both are necessary for the continued survival of MTB. Primary TB mediates protective immunity to disseminated infection while post-primary TB causes tissue damage that results in formation of cavities. Primary TB has been extensively studied in humans and animals. Post-primary TB is seldom recognized or studied. It begins as an asymptomatic early infiltrate that may resolve or progress by bronchogenic spread and necrosis to become caseous pneumonia that fragments to produce cavities or is retained to produce post-primary granulomas and fibrocaseous disease (9). Primary and post-primary TB differ in histopathology, x-ray appearance, genetic predisposition and immune status of the host, age of onset, organ distribution, clinical course and susceptibility to protection induced by BCG (8). MTB is a highly successful human parasite because it produces both primary and post-primary TB as distinct disease entities in humans.

It has long been known that the most important risk factor for the type of TB disease is the existence of prior TB infection (5, 10). Post-primary TB is defined as the type of disease that develops after primary TB. It remains confined to the lungs, spreads via the airways as bronchogenic TB and causes far more necrosis than primary TB (6, 11). The fundamental pathologic and radiologic features of post-primary TB that were reported by many investigators in the preantibiotic era were ignored in the early days of molecular biology and have now been largely forgotten.

Failure to recognize the differences between primary and post-primary TB impedes vaccine research. For example, global leaders in TB vaccine development from six countries recently wrote that an improved vaccine is ‘an inevitability’ because the Bacille Calmette Guerin (BCG) vaccine can “provide decades of protection against tuberculosis disease” (12). The paper did not mention the fact that while BCG is effective against primary TB, it has little or probably no effect on post-primary TB. They reported that we now have greater ability than ever to manipulate vaccines to induce desired immune responses including choice of vaccine technology, choice of antigen, choice of adjuvant and route of administration. The big question is what type of immune response should be induced? There are many options for this question, but no way other than clinical trials to decide among them since we have no biomarkers of protective immunity for adult pulmonary TB. With little knowledge of postprimary TB, research on vaccines has ‘hit the wall of biocomplexity’. While new knowledge is being generated at an unprecedented rate, little progress is being made on the fundamental questions of the nature of host immunity to post-primary TB or to the goal of producing a vaccine that inhibits transmission of infection (4, 13–16).

Since transmission of infection occurs during late stage TB, investigators have logically sought to develop vaccines with late stage antigens (17). Transcriptomics has been used to identify genes expressed during each of the disease stages (18–20). Great care has been exercised to insure that the antigens are recognized by human T cells as well as those generated by experimental animals (21). This led to identification of vaccine antigen candidates that represent ‘late-stage’ molecular targets. Many of these antigens, alone or in combination with early stage antigens, have proven successful as vaccines demonstrating improved survival, bacterial load and/or reduced extra pulmonary dissemination in animal models (21–24). While some results are impressive, their relevance for human disease is doubtful. Prevention of extra pulmonary TB and bacterial burden a few weeks after infection are functions of primary TB, not post-primary TB. Furthermore, tissue damage in TB is caused largely by immune responses (25). It is important to develop vaccines that do not augment tissue damage (26, 27). Finally, animal models for TB vaccine development have not been designed to mimic postprimary TB (26–28).

## Animal models of post-primary TB:

The most prevalent conception today is that TB is a ‘war of attrition’ between MTB and the host. Can MTB divide faster than they are killed by activated macrophages or do they evade, overwhelm and eventually kill the macrophages so that they are free to divide extracellularly (14). This conception misapprehends the human post-primary disease. There is no consistent correlation between the numbers of MTB in tissue and the severity of disease in people (5).

Many people with post-primary TB die with paucibacillary infections in which very few viable mycobacteria are demonstrable by either culture or AFB staining. Subclinical pulmonary lesions, the early infiltrates, frequently develop asymptomatically for months before onset of clinical TB (8, 29). Most early infiltrates regress and resolve completely while others progress to caseous pneumonia and clinical post-primary TB.

Several animal models of TB show patterns of organisms in tissue and pathology that resemble developing post-primary TB. Progressive pulmonary TB in rabbits, mice and guinea pigs is not due to increasing numbers of viable bacilli but is due to a continuous host response to mycobacterial products (30). After containing the initial infection, animals develop a low load of MTB in their lungs. This number of organisms remains constant for months until the animals die of progressive pathology. In recognizing this, North wrote “A central problem in tuberculosis research is to explain why immunity to infection does not enable mice, guinea pigs, rabbits or susceptible humans to resolve lung infection and thereby stop development of the disease” (31). This is characteristic of post-primary, not primary TB.

Lung infections in the mouse, guinea pig, and rabbit do not resolve but persist at stationary levels from approximately days 40-60 of infection, and onward. A stationary level of MTB is also characteristic of developing human post-primary TB. The infection is maintained by the continuous expression of $T_H1$ immunity as evidenced by the demonstration that depleting mice with stationary lung infection of $CD4^+$ T cells results in a resumption of MTB growth, as does treatment with a NOS2 inhibitor (32). Stationary lung infection has been shown to be associated with the presence in the lungs of replicating $CD4^+$ T cells capable of making IFN-$\gamma$ in response to MTB antigens. (33, 34). The lesions in these animals are models of the asymptomatic early infiltrate of developing human post-primary TB (8). $T_H1$ immunity restricts systemic pathology, but cannot prevent development of pulmonary disease.

It is generally assumed that primary MTB infection fails to resolve because of the generation of an inadequate level of $T_H1$ immunity. Accordingly, the purpose of vaccination is to induce a sufficient level of MTB-specific $T_H1$ cells. Most attempts to design a vaccine that is more protective than BCG are based on the assumption that BCG is of insufficient immunogenicity (35). Such vaccines typically enable vaccinated mice to maintain an MTB challenge infection at about one log lower level than in unvaccinated mice. However, the lower level of lung infection eventually causes progressive pathology (34). In addition, using chemotherapy to reduce the MTB load in the lungs by 2 logs does not enable immunity to cause the much lower level of infection to resolve (34).

The data from humans also suggests that simply increasing the magnitude of $T_H1$ immunity is the wrong approach, that MTB actually needs and uses our strongest immune responses for its benefit. Recovery from TB does not cause protection, but leaves a person more susceptible to new infection (36, 37) . Humans cured of tuberculosis by chemotherapy can become reinfected with a different strain of MTB within weeks (37). Finally, young immune-competent adults with the strongest tuberculin skin tests are far more susceptible to clinical TB than those with small skin test reactions demonstrating that a strong immune

response can be detrimental (38–40). MTB has evolved to avoid destruction by innate and adaptive immune mechanisms of immune-competent humans and to use our immune responses to induce lesions that facilitate transmission (31)

The foregoing implies that development of a vaccine that prevents post-primary TB, and thereby blocks development of disease and transmission of infection, will be difficult. However, there is hope. At least 95% of early infiltrations of post-primary TB resolve spontaneously in immune-competent people (5, 41). If we knew why, it might be possible to design a vaccine or other therapy to make them all regress and thereby have a tool to eradicate MTB completely. North identified a persistent low-level infection (a signature of developing post-primary TB) in most of the commonly used animal models of TB even though they have different pathologies (31). The common features of these models could be valuable in identifying the key components.

Studies with experimental TB vaccines typically measure CFUs, weight loss and survival (21). However, in human TB, there is no consistent relationship between disease pathology and numbers of organisms (5). Similarly, death from TB in many immune-competent animal models is due to increasing immunopathology, not increasing numbers of organisms (30). We reported that slowly progressive TB in C57/BL6 mice is a model of the preclinical stage of human post-primary TB (9). In addition, we reported that a vaccine can be modified to inhibit development of the characteristic pathology of post-primary TB in that model (42).

This was accomplished by inclusion of lactoferrin with BCG in vaccination of mice. Lactoferrin is an iron binding protein present in mucosal secretions and secondary granules of neutrophils (43, 44). It possess a wide variety of immune regulatory activities (45), including increasing lymphocyte and natural killer cell activity (46), and increasing surface expression of antigen presentation and co-stimulatory molecules in both MTB-infected macrophages (47, 48) and dendritic cells (47, 49, 50).

As expected, vaccination with BCG produced about one log reduction of CFU counts in the lungs of mice subsequently challenged with virulent MTB and a sustained IFN-$\gamma$ recall responses to mycobacterial antigens (51). Surprisingly, the inclusion of lactoferrin with the BCG vaccine had no significant effect on the numbers of organisms in tissue, but dramatically reduced the alveolitis in tissue surrounding the resolved granulomas, (Figure 1). This is significant because, like the disease in many humans, it is the spreading alveolitis, and not the numbers of organisms that eventually kills the animals. The alveolitis is composed primarily of interstitial lymphocytes and foamy alveolar macrophages that are sparsely infected with MTB demonstrated by AFB staining. The lactoferrin adjuvanted BCG vaccine was able to induce long-lasting pathological protection (greater than 6 months) and limited proinflammatory mediators in lung tissue without reducing CFU’s more than the BCG alone (49). Since the onset of clinical disease in human post-primary TB are due to expansion of this type of pathology, not to increasing numbers of MTB (30), then these animals may represent a model where mice continue indefinitely with subclinical or latent disease. If this could be transferred to humans, it would be the basis for an anti-transmission vaccine.

MTB does not follow the usual rules of infection and immunity. It has evolved with humans to produce primary and post-primary disease as separate disease entities both of which are required for continued survival of the organism (8). Primary TB protects the host from disseminated infection. Post-primary TB, in contrast, manipulates our strongest immune response in parts of the lung to produce cavities that support massive numbers of organisms in proximity position to be exhaled to infect new people. Vaccines that simply increase the natural response have not shown efficacy against post-primary TB. Fortunately, most nascent post-primary lesions regress spontaneously so that progression to clinical disease is a rare event. The challenge is to learn why these lesions regress and to find ways to make all lesions regress in a similar manner.

In conclusion, progress on understanding the pathogenesis of post-primary TB has been inhibited by an excessively narrow focus on molecular biology. We must abandon the concept that granulomas are the important lesion of all TB, and reactivate knowledge of the actual human disease learned over nearly 200 years of study of the pathology, radiology and clinical presentation of untreated TB. We must use this knowledge with our best modern tools to advance understanding of long standing questions of immunity, susceptibility and pathogenesis of TB. As an example, we have shown that use of an adjuvant molecule (lactoferrin) with BCG can inhibit the characteristic pathology of developing postprimary TB in a mouse model. Other models of parts of post-primary TB have been described (27, 28). We suggest that further studies in such models will lead to a better understanding of immunity that protects most adults from clinical TB, and help establish the basis for a vaccine that prevents transmission of infection.

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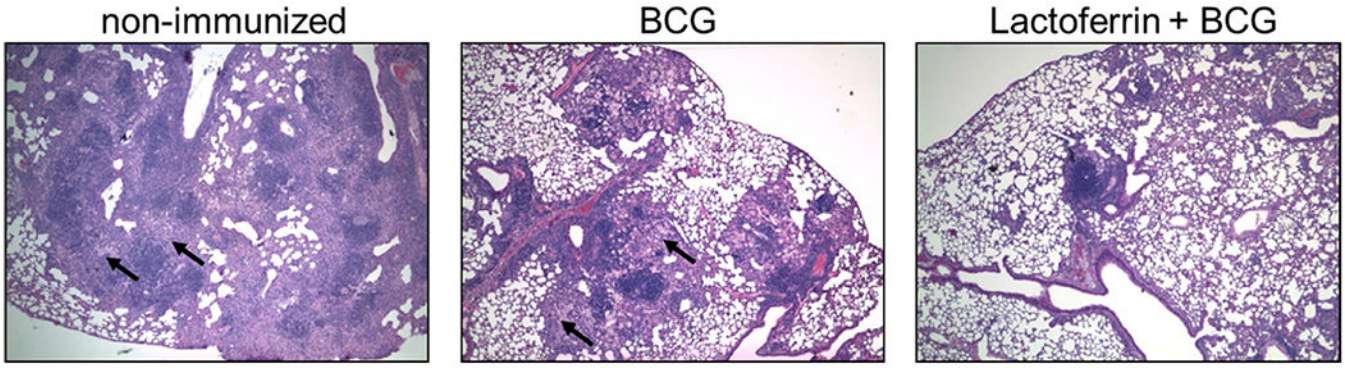


**Figure 1. C57BL/6 mice immunized with BCG and lactoferrin demonstrate long lasting diminished inflammation and destructive pulmonary histopathology upon aerosol challenge with virulent MTB.**

Mice demonstrated reduced histological manifestation of disease in the BCG and recombinant human lactoferrin immunized group at day 150 post challenge with <100 CFU Erdman strain (methods detailed in (49, 51)). Activated monocytes are apparent in the BCG and non-immunized groups (arrows). Lactoferrin adjuvant immunized mice revealed reduction in alveolitis with evidence of lymphocytic clusters and limited focal pockets of inflamed monocytes. H&E staining (20×; N≥5 mice per group).