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Guinea Pig Model of *Mycobacterium tuberculosis* Dormant Infection

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Abstract

Most experimental studies of latent or dormant tuberculosis infection have been conducted in murine models. Although guinea pigs are considered one of the animals that best reproduce human tuberculosis, surprisingly little data exist describing latent or dormant infection in these animals. The present work addresses this issue and shows that guinea pigs infected with a streptomycin auxotrophic mutant of *Mycobacterium tuberculosis* (strain 18b) replicate most of the known clinical, immunological, and pathological manifestations of this phase of the infectious process in humans. To establish infection, animals were inoculated with the mutant followed by administration of streptomycin for three weeks. Withdrawal of streptomycin caused microbial dormancy and few microorganisms remained viable and could be recovered from the animals' lungs and spleen several months later. Guinea pigs with dormant infection steadily gained weight and presented no clinical signs (scuff fur and lethargy) of active disease. The histopathology of the tuberculous lesions mimicked human lesions well. In addition, PBMC from infected animals strongly responded to stimulation with PPD or soluble culture filtrate proteins of *M. tuberculosis* throughout the duration of the experiment (six months). Finally, the tuberculin skin test (a clinical hallmark of latent infection) performed in guinea pigs infected with the auxotrophic mutant remarkably reproduced the response of humans to this test. Together, these findings confirm that infection of guinea pigs with *M. tuberculosis* strain 18b results in an infectious process that can be used as an interesting alternative model of latent or dormant tuberculosis infection in this animal specie.

Keywords

Latent infection; dormant infection; *Mycobacterium tuberculosis*; guinea pigs

INTRODUCTION

Tuberculosis remains a major infectious cause of morbidity and mortality worldwide (13). The incidence of the disease remains high and is increasing in many parts of the world due in part to its association with human immunodeficiency virus (HIV) infection (41). Despite the existence of specific anti-microbial agents and the widespread application of the Bacille Calmette-Guérin (BCG) vaccine, it is estimated that a third of the world's population is infected with *Mycobacterium tuberculosis* (34), with 8 million newly diagnosed cases of tuberculosis and up to 2.5 million deaths per year (31). Treatment of tuberculosis is complex due to the necessity of several medications over extended periods of time and because over 50 million

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people in the world are already infected with multi-drug resistant *M. tuberculosis* strains. BCG is the only available vaccine, which has been in use since the early 1920s. However, BCG only protects children from disseminated tuberculosis, but does not prevent pulmonary disease (9, 12), the most common and contagious form of tuberculosis. Moreover, the limited efficacy of BCG has varied considerably in clinical trials in geographically distinct populations (9,16).

Over the past years much progress has been achieved in antigen discovery and selection of promising tuberculosis vaccine antigen candidates as well as in the development of safe and potent adjuvant for human use (36). However, most of these studies have used animal models of either primary tuberculosis or disseminated tuberculosis. In humans, these forms of the disease account for less than 5% of the tuberculosis burden (20).

Most cases of adult or pulmonary tuberculosis occur in individuals who have been previously exposed to *M. tuberculosis* and developed a localized and self healing inflammatory reaction. This limited infection, known as Ghon's complex, is comprised of a focal multiplication of the mycobacterium in the lung tissue associated with lymphangitis, infection and massive enlargement of the corresponding hilar draining lymph node. In approximately 95% of these individuals the inflammatory reaction is spontaneously contained and in many cases calcifies and persists for the remainder of the person's life (28). However, several *M. tuberculosis* organisms remain viable for life, a condition known as latent or dormant tuberculosis (23,28).

Latent tuberculosis usually stimulates strong and long lasting cellular immune response to *M. tuberculosis* antigens. However, immunosuppressive conditions such as malnutrition and AIDS can facilitate the development of pulmonary tuberculosis, which can be consequent to either reactivation of the latent infection or to secondary exogenous re-infection with *M. tuberculosis*. Because over 90% of all cases of human tuberculosis occur in individuals who carry latent or dormant infection a successful anti-tuberculosis vaccine needs to be efficacious in both immunologically naïve and particularly in individuals with latent infection. Unfortunately, thus far most anti-tuberculosis vaccine under development have been tested or validated only in immunologically naïve animals due to the lack of a simple and reliable animal model of latent or dormant infection.

We have recently described and began the characterization of a unique mouse model of dormant tuberculosis (22), which we believe will significantly facilitate and improve studies designed to evaluate anti-tuberculosis vaccine efficacy. Because this model uses a streptomycin auxotrophic *M. tuberculosis* mutant that can be easily manipulated *in vitro* and *in vivo* to interchangeably enter latent and growth phases of the mycobacterium life cycle, we began experiments to verify if this mutant could be used to establish dormant infection in guinea pigs as well. Guinea pigs infected with *M. tuberculosis* constitute an animal model that best recapitulates the pathology of human tuberculosis (18,27,33,39,42).

In this work we present evidence that indicate that infection of guinea pigs with the streptomycin auxotrophic mutant *M. tuberculosis* strain 18b results in an infectious process that clearly resembles the clinical, microbiological, immunological and pathological characteristics of humans with latent tuberculosis infection.

MATERIALS & METHODS

Mycobacterium and animal infection

Streptomycin-auxotrophic *M. tuberculosis* strain 18b was originally provided by Stewart Cole, Institute Pasteur, Paris, France. Microorganisms were isolated from mice after two cycles of infection and were cultivated in Difco Middlebrook 7H9 supplemented with OAC (BD Microbiology Systems, Sparks, MD), glycerol, Tween 80 and 100 µg/ml of streptomycin

sulfate (Sigma) for 10 days. Glycerol stocks of *M. tuberculosis* 18b were preserved at -80°C . Colony Forming Unit (CFU) determination of cultured *M. tuberculosis* 18b was performed by plating different dilutions of bacteria suspension on Difco Middlebrook 7H10 agar enriched with glycerol, OADC and streptomycin. Agar plates were incubated at 37°C and CFU were enumerated 3–4 weeks later. Outbred female Hartley guinea pigs with average weight of 300 g obtained from Elm Hill Breeding Labs (Chelmsford, MA) were i.v. (humeral vein) infected with 10^7 CFU of *M. tuberculosis* 18b (groups of 4–5). All animals were injected with 20 mg (s.c.) of streptomycin two hours before infection followed by daily inoculations of the antibiotic for three weeks thereafter. Guinea pigs' weights were recorded at several time-points during the course of infection. All experiments were conducted in compliance with The Forsyth Institute Animal Care and Use Committee guidelines.

Histopathology

For histology, animal's lungs, spleen, and liver were removed and embedded in paraffin. Sections, 4 μm thick, were cut, formaldehyde fixed and stained with hematoxylin and eosin, or by the Ziehl-Neelsen method for acid-fast bacilli (AFB). Scanning electron microscopy (SEM) was performed on deparaffinized histological sections. Glass slides with sections of all three organs were air dried from 100% ethanol, then coated with palladium/gold and analyzed in a JEOL 6400 SEM operating at 15 kv.

CFU

At different time points after infection, animals were euthanized, and lungs and spleen were removed. Organ homogenates were prepared in PBS and plated at a 10-fold serial dilution on Middlebrook 7H10 Bacto agar with or without 100 $\mu\text{g}/\text{ml}$ of streptomycin sulfate. Agar plates were incubated at 37°C and the CFU were counted 3–4 weeks later.

Assay for antigen specific peripheral blood mononuclear cells (PBMC) response

Approximately 15 ml of blood were harvested from animals at five and 14 and 28 weeks after infection. Before blood collection, animals were anesthetized with a cocktail of ketamine (44mg/kg) and xylazine (5mg/kg). Blood from each guinea pig was centrifuged over Ficoll/Hypaque (Sigma) to prepare PBMC. Cells were suspended in complete RPMI containing Hepes, L-glutamine, penicillin-streptomycin (Sigma) and 10% fetal bovine serum (Hyclone, Logan, Utah). PBMC were cultured in 96-well microtiter plates and stimulated with either PPD (2.5 $\mu\text{g}/\text{ml}$) or early culture filtrate proteins (CFP, 5 $\mu\text{g}/\text{ml}$) of *M. tuberculosis* (Colorado State University, NIAID/NIH Tuberculosis Research Materials contract no. 1-A125174). In addition, PBMC were cultured with medium alone or with the mitogen Concanavalin A (5 $\mu\text{g}/\text{ml}$, Sigma) as negative and positive controls, respectively. Proliferative response was assayed in 5-day cultures after pulsing with 0.5 μCi of [^3H]-thymidine (GE Healthcare Bio-Sciences, Piscataway, NJ) for 8h; radionucleotide incorporation was measured using a β -scintillation counter (Beckman, Palo Alto, CA). Data are presented as stimulation index (S.I.) which is the ratio of the counts per minute (cpm) of [^3H]-thymidine incorporation by cells cultured in the presence of antigens to the cpm incorporation by cells cultured in the presence of medium alone (non-stimulated cells).

Delayed type hypersensitivity (DTH) reaction

Skin tests (Mantoux technique) were performed with intradermal injection of PPD (5 $\mu\text{g}/0.1\text{ml}$) into a small shaved area of the animal flank. Twenty-four hours later transverse diameters of induration reaction were measured in millimeters.

RESULTS

Infection of guinea pigs with *M. tuberculosis* strain 18b results in no clinical signs of disease

To begin the characterization of the infection caused by the *M. tuberculosis* strain 18b in guinea pigs the animals were inoculated intra-venously with a high dose of microorganisms (10^7 CFU) followed by daily sub-cutaneous administration of streptomycin for three weeks. External clinical changes related to tuberculosis in guinea pigs (lethargy, scuffed fur, and weight loss) were monitored for the duration of the experiment (six months). Over this time period the animals did not show any sign of scuffed fur, lethargy or any changes in the overall behavior. Moreover, as illustrated in Fig. 1 all infected guinea pigs steadily gained substantial weight throughout the experiment and no death occurred. These observations clearly indicate that infection of guinea pigs with *M. tuberculosis* strain 18b does not cause any of the typical clinical signs of the progressive tuberculosis caused by virulent strains of this organism. Rather, these findings suggest that infection of guinea pigs with the mutant recapitulates the infection of mice with this organism (22). In other words, results in an infectious process compatible with latent or dormant infection and not active tuberculosis because in guinea pigs, as for humans, tuberculosis causes lethargy, cachexia, weight loss, and death.

Low mycobacteria burden recovered from guinea pig tissues after infection with *M. tuberculosis* strain 18b

To confirm that infection of guinea pigs with the mutant strain results in latent or dormant infection, the animals were inoculated with strain 18b and treated for three weeks with daily administration (s.c.) of streptomycin to favor bacterial growth and to establish the initial infectious process. Guinea pigs were sacrificed at several time points (1, 15, 30, 90 and 180 days) after infection and CFU were enumerated in lungs and spleens. As can be seen in Fig. 2, viable *M. tuberculosis* organisms were recovered from both organs at all time points. During the first 15 days after infection, the microorganisms proliferate in the guinea pigs tissues and the CFU recovered from both lungs and spleen declined steadily afterwards. However, low numbers of the mutant remained viable *in vivo* in guinea pig tissues for approximately six months in the absence of substrate. These experiments suggest that infection of guinea pigs with *M. tuberculosis* strain 18b can be manipulated to generate an infection process that reproduces human primary tuberculosis followed by latent infection. In other words, a primary infectious process characterized by a high bacteriological burden that spontaneously subsides followed by a condition in which few or undetectable microorganisms remain viable in the host tissues after the resolution of the initial infection. Therefore, a condition consistent with latent tuberculosis in humans (23,28).

Histology of the inflammatory reaction in tissues of guinea pigs infected with *M. tuberculosis* strain 18b

Organized granuloma is a hallmark of the inflammatory reaction induced by infection with *Mycobacterium* in humans (1). Therefore, to assess if guinea pigs infected with *M. tuberculosis* strain 18b develop similar type of inflammation, histopathological analysis was performed in lungs, spleen and liver harvested from infected animals. Tissue sections were embedded in paraffin and stained with hematoxylin-eosin. As can be seen in Fig. 3, well organized granulomas were present in the guinea pigs organs at 30 and 180 days after infection (9 and 159 days after substrate starvation respectively). The granulomas were characterized by accumulation of individual macrophages and giant cells circumscribed by epithelioid and lymphoid cells, which is a pattern that resembles the cellular and spatial organization of the granulomas present in humans with either active or latent tuberculosis. However, the overall inflammatory reaction observed in the three organs at the early time point post-infection (30 days) was clearly different from the inflammation seen at six months post-infection. At the early stage of infection inflammation in lungs was characterized primarily by diffuse

mononuclear cell infiltration with no obvious granuloma organization. In contrast, organized granulomas were seen in the spleen red pulp as well as throughout the liver parenchyma, both in the lobules and in the portal triad areas. At the six-month time point the inflammation present in the liver was restricted to sparse patches of mononuclear cell infiltration primarily seen in the portal triad. In spleen, no obvious inflammatory reaction could be observed. Interestingly, in lungs, in contrast to the early time point of infection, the inflammatory reaction was characterized by well circumscribed and organized granulomas distributed throughout the organ's parenchyma. The interstitial mononuclear cell infiltration was no longer present and the overall lung structure was clearly normal. These two markedly different histological patterns seen in the lungs of guinea pigs infected with *M. tuberculosis* strain 18b for 30 or 180 days clearly resemble the histopathology seen in lungs of humans with primary tuberculosis and latent infection respectively.

To evaluate the sub-cellular localization of the infectious process with *M. tuberculosis* 18b in guinea pigs, we performed conventional scanning electron (SEM) microscopy of lungs, spleen, and liver tissue sections obtained from guinea pigs infected for 30 days. SEM, in contrast to transmission electron microscopy is very convenient for this type of analysis because large areas of tissue sections that can be scanned with high resolution and sufficient magnification to easily discern the bacteria. This approach clearly defined that the mycobacterium was localized inside macrophage vesicles. Figure 4 illustrates this intracellular localization in lung macrophages. The same pattern of infection was also seen in the spleen and liver macrophages (not shown).

T cell mediated recognition of *M. tuberculosis* antigens by guinea pigs infected with *M. tuberculosis* strain 18b

A distinctive immunological characteristic of latent tuberculosis in humans is the presence of strong T cell mediated responses to *Mycobacterium* antigens. These responses can be manifested or detected either *in vivo* or *in vitro*. *In vivo*, most individuals with latent infection develop marked delayed type hypersensitivity (DTH) response when skin tested with purified protein derivative of tuberculin (PPD). In fact, tuberculin skin test (TST) has been used worldwide for approximately 100 years as an adjunct test for the diagnosis of both latent and active tuberculosis (2,3,21). *In vitro*, PBMC of positive TST individuals respond vigorously to stimulation with numerous antigen preparations of *M. tuberculosis* including PPD and *M. tuberculosis* culture filtrate proteins (10,11). To evaluate if dormant infection of guinea pigs with *M. tuberculosis* strain 18b exhibits strong recognition of *M. tuberculosis* antigens the next experiments were designed to reproduce these two typical immunological characteristics of latent disease in humans. Infected and control guinea pigs were initially used to evaluate the development of DTH to PPD over a six month period. DTH in guinea pigs is strikingly similar to human DTH (27), therefore, a reliable TST in an animal model can be an invaluable asset for studies related to latent tuberculosis. Infected and control guinea pigs were PPD skin tested using the Mantoux technique and DTH (induration reaction) was read 24h later. Figure 5 shows that all infected guinea pigs developed strong TST that was detected at 5 (18 ± 3 mm), 14 weeks (19 ± 0.7 mm) and 28 weeks (15.5 ± 1.9 mm) after infection. No induration reaction was observed in non-infected guinea pigs skin tested with PPD (not shown). These results indicate that guinea pigs with dormant infection with *M. tuberculosis* strain 18b typically reproduce the TST response of humans, a unique feature of tuberculosis that is not achievable in any other animal models of the disease including mice, rabbits and non-human primates.

Finally, to evaluate the *in vitro* T cell mediated recognition of *M. tuberculosis* antigens, PBMC from guinea pigs with latent infection were stimulated with either PPD or *M. tuberculosis* culture filtrate proteins (CFP) and assayed for proliferative response. Figure 6 indicates that all infected animals (n=5) developed strong responses to both PPD and CFP with stimulation

indexes (S.I.) slightly over 100, approximately 1,000, and 500 at 3, 14, and 28 weeks after infection, respectively. S.I. of PBMC from control animals were <5 to both antigens (not shown). This pattern of response clearly reproduces the typical strong response of PBMC from humans with latent tuberculosis stimulated with the same antigens (10,11).

DISCUSSION

Ever since Robert Koch first described *M. tuberculosis* as the etiological agent of human tuberculosis, guinea pigs have been extensively used as one of the most reliable animal models of this disease. Guinea pigs are highly susceptible to infection with *M. tuberculosis* either by the intravenous route or via the respiratory tract by aerosols containing low levels of virulent bacteria. Guinea pigs were the animal of choice used by Calmette and Guérin to test the BCG vaccine. These animals are still used today in quality control procedures designed to evaluate the efficacy of BCG preparations. In addition, guinea pigs constitute the animal model used to test the efficacy of new antibiotics or drug combinations for the treatment of tuberculosis (19,27).

Moreover, since the discovery of the tuberculin reaction by Robert Koch over a century ago, guinea pigs have been the animal model for studies concerning delayed type hypersensitivity (DTH). The DTH response observed in infected guinea pigs is remarkably similar to that seen in humans skin tested with PPD (27). Finally, the pulmonary granuloma followed by necrosis observed in these animals resembles the pathology observed in human tuberculosis (42). For these reasons guinea pigs are generally accepted to be the best animal model of tuberculosis for studies designed to investigate DTH and tuberculosis lung pathology.

Conversely, guinea pigs are extremely susceptible to infection with *M. tuberculosis*. Low dose infection of these animals either via intra-venous or the respiratory routes invariably results in progressive tuberculosis characterized by marked weight loss, cachexia, and death occurring usually within 15–20 weeks after infection (4,27). Unfortunately, latent infection does not occur in these animals following challenge with virulent *M. tuberculosis*. Remarkably, attempts to develop a guinea pig model of this phase of tuberculosis infection have not been successful.

In contrast, murine models of latent infection have been described over the past years. In one of these models (33), mice are infected (aerosol route) with a low dose of *M. tuberculosis* and approximately three months later the bacillary burden reaches $\sim 10^4$ CFU/lung and remains controlled at these levels for several months. However, at unpredictable time point the infection takes off again and the mice succumb to tuberculosis. This model has the advantage of keeping the bacterial burden under control for some time but it has the disadvantage of a high bacillary burden that is unlikely to reproduce the actual low bacterial multiplication that is found in human latent tuberculosis. Furthermore, the uncontrollable and unpredictable time point of the bacterial resurgence in the mice tissue followed by the animals' death constitutes a serious limitation of this model for most experiments. In another model, also called the Cornell model (24–26,37), mice are inoculated intravenously (i.v.) with $1-3 \times 10^6$ viable bacilli of virulent *M. tuberculosis* and the resultant infection is treated for 12 weeks with the anti-mycobacterial drugs. From this point on, no tubercle bacilli can be cultured from the organs of those animals for several months. However, administration of cortisone (at immunosuppressive doses) at 2–3 months after the interruption of the antibiotic therapy reverts this condition, and *M. tuberculosis* can be cultured from lungs and spleens of $\sim 50\%$ of the animals, thus indicating that in these animals the mycobacteria were present in their tissues in a dormant state. This model has the advantage of achieving and maintaining very low numbers of the tubercle bacilli for many weeks in the tissues of infected mice. However, this model has two major limitations: 1. Dormancy is difficult to standardize because the optimal antibiotic concentration and duration of treatment to achieve low numbers of bacilli varies from experiment to experiment;

2. Only 50% of the animals successfully treated with antibiotic develop dormant infection, which imposes a major complication in the planning and interpretation of the experiments (16).

In an attempt to present an alternative to these limitations, we have recently described a murine model of dormant infection (22). In this model, mice are infected with a unique *M. tuberculosis* streptomycin auxotrophic mutant strain (strain 18b), followed by daily supplementation of the substrate for 2–3 weeks. During this period the bacteria grows in the animal's tissues and stimulates a strong T cell immune response and typical mycobacterium-induced granulomas. After withdrawn of the substrate the bacteria stop their growth but remain viable for at least 6 months. *In vitro* studies indicated that the mutant grows unimpaired in the presence of streptomycin and no longer grows but remains viable for long periods of time after substrate removal shifting from log growth phase to latent stage as indicated by augmented production of α -crystallin (22).

Unfortunately, none of these murine models of latent infection have been convincingly proven to be useful in other animal species. Therefore the present studies were undertaken to expand to guinea pigs the murine model of latency that uses the streptomycin auxotrophic mutant *M. tuberculosis* strain 18b.

Our results indicate that guinea pigs infected with this mutant develop an infectious process that reproduces in great detail latent tuberculosis infection in humans. In humans, infection with *M. tuberculosis* in general leads to the so called primary tuberculosis or Ghon complex, which is characterized by intense initial focal multiplication of the bacteria in the lung tissue and draining mediastinum lymph node followed by spontaneous resolution of the infection in immunocompetent individuals. The resolution of inflammation is not sterilizing and it is generally accepted that only a few microorganisms remain viable for life in individuals with latent tuberculosis (14,17,32,40). Moreover, reactivation of tuberculosis due to conditions that lower the host immunity constitutes circumstantial evidence that during latency *M. tuberculosis* organisms, although rarely detectable by conventional culture procedures, remain present in the host tissues. Infection of guinea pigs with *M. tuberculosis* strain 18b, resulted in initial multiplication of the organisms in the animals' tissues followed by resolution of the infection to almost undetectable levels of the microbial burden but without complete sterilization. The animals stayed healthy through the infectious process and developed granulomas and immune reactivity to PPD (lasting at least 28 weeks post-infection) remarkably similar to that seen in humans with latent tuberculosis.

Unfortunately we have not yet been able to clearly demonstrate reactivation of the dormant infection in guinea pigs infected with *M. tuberculosis* strain 18b. One of the possible reasons for this failure is the lack of established protocols to induce partial immunosuppression in this animal species. Drugs such as cortisone (26) and nitric oxide synthase inhibitors (15,37) which have been used in mice models of latent infection to trigger reactivation do not work well as immunosuppressive agents in guinea pigs (6,8,29,44). Notwithstanding this limitation, the proposed model constitutes an important alternative for studies of tuberculosis disease caused by exogenous re-infection with *M. tuberculosis* of individuals with latent infection, which in contrast to reactivation of latent infection, is considered the most common cause of disease development in tuberculosis endemic countries (5,7,30,38,43).

Nevertheless, one concern with experiments focused in measuring efficacy of anti-tuberculosis vaccine candidates is that the animal models (mouse, guinea pigs and cynomolgus monkeys) are, in contrast to humans, highly susceptible to developing progressive primary tuberculosis after infection with virulent *M. tuberculosis*. Following infection of guinea pigs and cynomolgus monkeys, death occurs in the majority of animals. In mice, most strains keep in

check high bacterial burden for approximate 12 months but the animals succumb of tuberculosis thereafter (27). Because these animals do not develop latent or dormant infection, they are useful to measure vaccine efficacy against progressive tuberculosis, which happens in ~5% of humans (primarily children). Therefore, the results of vaccine efficacy obtained from these models cannot necessarily be translated to adult tuberculosis, which happens in ~95% of humans and results from either reactivation of latent infection or exogenous reinfection of individuals with previous latent infection. Consequently, the guinea pig model of infection with *M. tuberculosis* strain 18b is an attractive alternative for anti-tuberculosis vaccine development studies in the context of pre-sensitized animals.

In addition, this proposed model can be a valuable tool to study the pathology of adult tuberculosis. As mentioned before, guinea pigs, although constituting an excellent animal model of progressive tuberculosis they do not develop pulmonary cavity, a pathological hallmark of the human disease. Nevertheless, humans only develop cavity, after reactivation of latent infection or exogenous re-infection with *M. tuberculosis*. Therefore, establishing a latent infection with *M. tuberculosis* strain 18b in guinea pigs should favor the development of caseous lesions and cavities after re-infection with virulent *M. tuberculosis*. Finally this model should be helpful to understand at the immunological basis the tuberculin shock a phenomenon described over 100 years by Robert Koch. This serious complication of the TST, thought to be of historic interest only, may become again a major concern with the introduction of highly immunogenic new recombinant vaccines and skin test reagents, which are currently being evaluated in humans. For example, we have recently described that skin testing guinea pigs with active tuberculosis with a highly purified *M. tuberculosis* recombinant protein (CFP-10) caused serious tuberculin shock in most animals resulting in 50% mortality (35). Because guinea pigs with latent infection, in contrast to animals with active disease caused by infection with virulent *M. tuberculosis*, should not develop tuberculin shock upon skin test with the antigen like CFP-10 or other molecules this model can be an important asset for evaluation of vaccine or skin test candidates that elicit either protective or pathogenic immune responses.

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Abbreviations

PPD

purified protein derivative of tuberculin

CFP

culture filtrate proteins of *M. tuberculosis* culture

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Figure 1. Guinea pigs weight gain subsequent to infection with *M. tuberculosis* strain 18b
Five guinea pigs were infected i.v. with *M. tuberculosis* strain 18b. Animals were injected (s.c.) daily for a period of three weeks (starting 24 hours before infection) with streptomycin sulfate (20mg/animal). Guinea pigs were weighted before infection and every 2 weeks afterwards. Animals were observed for a period of 28 weeks. Results are expressed as means of the weights in grams $\pm$ SD.

Figure 2. Persistence of viable *M. tuberculosis* strain 18b in tissues of infected guinea pigs

Guinea pigs were infected and treated as described in legend to Fig. 1. Groups of five animals were euthanized at 1, 15, 30, 90, and 180 day time-points after infection. Spleens and lungs homogenates were plated on Middlebrook 7H10 Bacto agar containing 100 µg/ml of streptomycin sulfate. CFU was counted after incubation for 4 weeks at 37°C. Results are represented by means of CFU ± SE. Organ homogenates were also inoculated on Middlebrook 7H10 Bacto agar without streptomycin. No bacterial growth was observed on these plates (not shown).

Figure 3. Histopathology of lung, spleen, and liver tissues from guinea pigs infected with *M. tuberculosis* strain 18b

Guinea pigs were infected and treated as described in the legend to Fig. 1. Animals were euthanized 30 (upper panel) and 180 days (lower panel) after infection. Lungs, spleen, and liver were removed and tissue sections were stained with hematoxylin-eosin (H&E) or by the Ziehl-Neelsen method for acid-fast bacilli. At 30 days post-infection inflammation in lungs is characterized by diffuse mononuclear cell infiltration. Organized granulomas can be seen in the spleen red pulp as well in the liver parenchyma, both in the lobules and in the portal triad area. At 180 post-infection well organized granulomas are seen only in the lung's parenchyma. The interstitial mononuclear cell infiltration seen at 30 days post-infection is no longer present in the organ's structure. No obvious inflammatory reaction can be seen in spleen. In liver, sparse cell infiltration is seen only in the portal triad. No infiltration is seen in the lobules. Insets illustrate the presence of acid-fast *M. tuberculosis* organisms in the lung tissue at both 30 and 180 days post-infection. Original magnification, H&E, 200X; Insets, 1,000X.

Figure 4. Scanning electron micrograph of a lung section from guinea pig infected with *M. tuberculosis* strain 18b

Guinea pigs were infected and treated as described in the legend to Fig. 1. Animals were euthanized 30 days after infection. Lungs were removed and tissue sections were submitted to scanning electron microscopy. Arrows (A) point to several mycobacteria inside a macrophage (3,000 x). Area delineated by rectangle is shown at higher magnification in (B). Arrow head (B) highlights the intra-vesicular location of a mycobacterium (18,500 x).

Figure 5. PPD skin test in guinea pigs infected with *M. tuberculosis* strain 18b

Guinea pigs were infected and treated as described in the legend for Fig. 1. Animals were then skin tested with PPD (5 µg) using the Mantoux technique (0.1ml of PPD solution injected intradermally) at 5, 14, and 28 weeks post-infection. DTH (skin induration) was measured 24 h later and is expressed as the means of mm of transverse induration reaction observed for five animals per time-point. Bars represent the SD of the means.

Figure 6. Proliferative response of PBMC from guinea pigs infected with *M. tuberculosis* strain 18b Guinea pigs were infected as described in the legend to Fig.1. Animals were euthanized by exsanguination at 5, 14, and 28 weeks post-infection. PMBC were obtained and stimulated for 5 days with PPD (2 $\mu\text{g}/\text{ml}$) or CFP (5 $\mu\text{g}/\text{ml}$). Proliferation was assessed by determining the incorporation of [^3H]-thymidine. Data are expressed as the means of stimulation index (S.I.) obtained for five animals per time-point. Bars represent the SD of the means.