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Drug Treatment Combined with BCG Vaccination Reduces Disease Reactivation in Guinea Pigs Infected with *Mycobacterium tuberculosis*

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Abstract

Bacillus-Calmette-Guerin (BCG), the only human tuberculosis vaccine, primes a partially protective immune response against *M. tuberculosis* infection in humans and animals. In guinea pigs, BCG vaccination slows the progression of disease and reduces the severity of necrotic granulomas, which harbor a population of drug-tolerant bacilli. The objective of this study was to determine if reducing disease severity by BCG vaccination of guinea pigs prior to *M. tuberculosis* challenge enhanced the efficacy of combination drug therapy. At 20 days of infection, treatment of vaccinated and non-vaccinated animals with rifampin, isoniazid, and pyrazinamide (RHZ) was initiated for 4 or 8 weeks. On days 50, 80 and 190 of infection (10 weeks after drug were withdrawn), treatment efficacy was evaluated by quantifying clinical condition, bacterial loads, lesion severity, and dynamic changes in peripheral blood and lung leukocyte numbers by flow cytometry. In a separate, long-term survival study, treatment efficacy was evaluated by determining disease reactivation frequency post-mortem. BCG vaccination alone delayed pulmonary and extra-pulmonary disease progression, but failed to prevent dissemination of bacilli and the formation of necrotic granulomas. Drug therapy either alone or in combination with BCG, was more effective at lessening clinical disease and lesion severity compared to control animals or those receiving BCG alone. Fewer residual lesions in BCG vaccinated and drug treated animals, equated to a reduced frequency of reactivation disease and improvement in survival even out to 500 days of infection. The combining of BCG vaccination and drug therapy was more effective at resolving granulomas such that fewer animals had evidence of residual infection and thus less reactivation disease.

Keywords

Tuberculosis; BCG vaccination; Drug therapy; Guinea pig

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Introduction

Drug treatment and vaccination are the main strategies used to control clinical tuberculosis and the spread of human *M. tuberculosis* infections worldwide. The live, attenuated strain of *M. bovis*, Bacillus-Calmette-Guerin (BCG) is the only vaccine currently approved for use in humans. The greatest benefit of BCG vaccination is to lessen the severity of disease and reduce the risk of developing the life threatening manifestations of extra-pulmonary tuberculosis especially in children [1, 2]. However, BCG in general, fails to provide lifelong immunity and to prevent *M. tuberculosis* infections or reinfections in adolescents and adults [3]. The variable efficacy of BCG among the general population is the driving force behind the ongoing and urgent search for improved tuberculosis vaccines or new strategies to improve the overall effectiveness of BCG [4].

Similar to what is observed in children, BCG vaccination of laboratory animals is only partially protective when administered prior to experimental *M. tuberculosis* challenge. In mice and guinea pigs, BCG vaccination prolongs survival by reducing the bacterial burden and delaying the manifestations of progressive pulmonary and extra-pulmonary disease [5–7]. In guinea pigs, a model species that consistently develops caseous granulomas, BCG vaccination reduces the severity of disease including the incidence and severity of pulmonary and extra-pulmonary granuloma necrosis [7]. However, the beneficial effects of BCG in animals have been studied primarily using the less virulent H37Rv strain of *M. tuberculosis* [5, 7, 8]. What is unknown is whether BCG provides a similar level of protection in the face of a more virulent challenge and whether lessening the disease severity by vaccination in combination with drug therapy, improves overall treatment outcome.

Recently, it has been shown that the reduction of *M. tuberculosis* lung CFUs in guinea pigs treated with isoniazid monotherapy is biphasic and that residual bacilli persist through phenotypic drug tolerance rather than the spontaneous emergence of drug-resistant mutants [9]. Studies from our laboratory and others using the guinea pig model, have shown that bacilli concentrated within necrotic granulomas, survive combination drug therapy yet remain drug susceptible *in vitro* [10–12]. The mechanisms of *in vivo* drug tolerance are unknown but the most widely accepted hypotheses is that drugs fail to adequately penetrate certain lesion types [13, 14] or that during the course of infection, bacilli are forced into a non- or slow replicating state of persistence and therefore are less drug susceptible [15–17]. The *in vivo* persistence of drug-tolerant *M. tuberculosis* may reflect a survival strategy that enables bacilli to adapt to the complex host response to infection, including the low oxygen microenvironment represented by necrotic granulomas [9, 18, 19]. Collectively, studies using the guinea pig model suggest that at least one population of drug tolerant bacilli are entrapped in a complex, extra-cellular matrix composed mostly of necrotic host cells [10, 20, 21]. These bacilli not only survive standard combination drug therapy but persist even after 6 weeks of treatment with the most promising new anti-tuberculosis drug TMC 207 [10].

In a study by Dhillon and Mitchison, BCG vaccination prior to infection of guinea pigs and mice with *M. tuberculosis* H37Rv, enhanced drug treatment responses in guinea pigs but had no benefit in mice [22]. However, Guirado et al. showed that vaccination of mice with fragments of *M. tuberculosis* incorporated into liposomes were effective at potentiating chemotherapy [23]. In the Dhillon study, the authors attributed the species-specific differences in drug treatment responses to host immunity, which they characterized as “mainly bacteriostatic in mice and bactericidal in guinea pigs”. An alternative hypothesis however is that, especially in guinea pigs, BCG vaccination prior to challenge and drug therapy, lessens the incidence and severity of necrotic granulomas and thus the persistence of extra-cellular, drug-tolerant bacilli. The purpose of our study therefore was to use guinea

pigs challenged with the highly virulent Erdman KO1 strain of *M. tuberculosis* to determine first, whether BCG was as protective as what has been shown using less virulent challenge strains and secondly, whether BCG combined with drug therapy was more effective at eliminating persistent bacilli compared to combination drug therapy alone.

Material and Methods

All experimental protocols were in accordance with the National Research Councils Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Usage Committee of Colorado State University under protocol number 08-212A-02.

Guinea pigs

Specific pathogen free, female Hartley guinea pigs (approximately 500 gm) were purchased from the Charles River Laboratories (North Wilmington, MA, USA). In experiment one, 80 animals (n=5 for each group) were randomly assigned to one of 4 treatment groups to be terminated at each of 4 predetermined end-points. Treatment groups included animals that were vaccinated with BCG or saline that received either drugs or sucrose alone as a carrier by oral gavage. Guinea pigs were euthanized and necropsied at 20, 50, 80 and 190 days of infection. In a second experiment 40 animals (n=10 for each treatment group as described above) were allowed to survive or were euthanized for humane reasons. Prior to *M. tuberculosis* challenge, animals were appropriately acclimatized, microchipped for individual animal identification and under barrier conditions in a Biosafety Level III containment laboratory.

BCG Vaccination

Half of the guinea pigs in each experiment were vaccinated intradermally with 10^4 *M. bovis* BCG Pasteur in 200 μ l of saline six weeks prior to aerosol challenge as previously described [7]. The remaining animals were sham-vaccinated with intradermal injections of 200 μ l of saline alone. Successful BCG vaccination was evident by the expected, greater than 1 log reduction in bacterial numbers in the lung, spleen and lymph node and the differences in lung lesion burden and morphology by day 50 of infection compared to animals sham-vaccinated [7].

Low-dose aerosol infection

Guinea pigs were infected with approximately 20 CFU/ animal with *M. tuberculosis* Erdman KO1 by aerosol exposure using a Madison chamber aerosol generation device as previously described [24]. Determination of bacterial loads from tissue homogenates was used to compare the rate and course of *M. tuberculosis* infection among all treatment groups. The lung, spleen and lymph node bacterial loads were determined from tissue homogenates and the data expressed as mean colony forming units (CFUs) per gram of tissue prior to the start of drug treatment (Day 20) in a representative group of BCG and saline vaccinated animals and then in all groups on days 50 (4 weeks of drug therapy), 80 (8 weeks of drug therapy) and 190 (10 weeks after drug therapy was discontinued) (Figure 1). Tissue homogenates were plated on nutrient 7H11 agar containing 10 μ g/ml cycloheximide and 50 μ g/ml of carbenicillin to prevent contamination and colony-forming units (CFU) determined after 6 weeks of incubation at 37°C. The baseline bacterial burden prior to the initiation of drug therapy was determined on a separate group of 4 guinea pigs on day 20 of infection.

Drug treatments

Combination drug treatment was administered once daily 5 days per week for 8 weeks. To minimize the adverse side-effects of drug treatment, individual animals were hand fed a

carrier containing a mixture of 40% sucrose (wt/vol), 20% pumpkin puree (wt/vol) (Libby's Brand 100% pure pumpkin), additional vitamin C (50 mg/kg) and commercial *Lactobacillus* (BD Lactinex) alone (sham treated), or the carrier containing rifampin (R) (50 mg/kg of mean body weight), pyrazinamide (Z) (100 mg/kg of mean body weight), and isoniazid (H) (30 mg/kg of mean body weight), guinea pig doses calculated based on previous PK/PD studies [25]. To minimize adverse drug interactions a split-feeding protocol was used in which H and Z were combined in a single dose followed by R given 6–8 hours later. Animals were monitored daily and clinical condition was scored using a modified Karnovsky scale [26]. At pre-determined or humane end-points, animals were heavily sedated and euthanized by intra-cardiac injection with pentobarbital and tissues and blood collected for culture, pathology and flow cytometric analysis as previously described [7].

Non-invasive Clinical Parameters

To determine the value of non-invasive parameters for monitoring long-term responses *M. tuberculosis* infection and drug therapy, changes in body temperature and weight, were determined weekly and the peripheral oxygen saturation measured on sedated animals just prior to euthanasia and necropsy. Because progressive lung inflammation interferes with the oxygenation of circulating erythrocytes and thus reduces oxygen delivery to tissues [27], the percent oxygen saturation of blood from individual guinea pigs was measured prior to necropsy using a portable pulse oximeter (Nonin pulse oximeter Model number 8600, NONIN medical, Inc. MN). Guinea pigs were sedated with an intramuscular injection of a cocktail of ketamine (30 mg/kg of mean body weight) and xylazine (1.1 mg/kg of mean body weight). In fully sedated guinea pigs, a LED probe was placed on a hind footpad, which was thoroughly cleaned with ethanol. Pulse oximetry measures oxyhemoglobin and its deoxygenated form in the peripheral blood as detected by two diodes that emit light at different wavelengths. The oxygenated/deoxygenated hemoglobin ratio is displayed and the mean percent oxygen saturation was calculated for each treatment group.

Since weight loss is a common clinical manifestation of progressive tuberculosis in humans and animals, the mean body weight (in grams) was calculated weekly and at the time of necropsy. Previous studies have suggested that the change in body temperature may serve as an indicator of active inflammation and disease progression in the guinea pig model of tuberculosis [28]. During the acclimatization period, prior to aerosol challenge, each guinea pig was implanted subcutaneously with an electric transponder (IPTT-300, Bio Medic Data Systems, Seaford, DE). Temperature readings (degrees C) were recoded twice weekly and at the time of necropsy, using a probe (IPTT-6007, Bio Medic Data Systems, Seaford, DE). The data was expressed as mean body temperature calculated for each treatment group.

Pathology

Random samples from the left caudal lung lobe, spleen, and lymph nodes from each animal (n= 5) was collected at necropsy and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Tissue sections selected at random were embedded in paraffin and cut to 5 μ m, mounted on glass slides, deparaffinized and stained with hematoxylin and eosin and carbol fuchsin using the Ziehl-Neelsen acid-fast method as previously described [29]. The lesion burden was determined by quantifying the area of normal tissue affected by lesion using the area fraction fractionator method as previously described with the investigator blind to all treatment groups [25]. Briefly, the area from regions of interest were determined from a total of 8 to 12 random, computer selected fields from each tissue section using a counting frame (2,000 μ m²) containing probe points with a grid spacing of 200 μ m. The data were expressed as the mean percent area of tissue affected by lesions from all animals within a treatment group at each time point. Photomicrographs were based on tissue sections from

individual animals that had lesion areas that were the closest to the mean value for each treatment group.

Flow cytometry

Flow cytometry was used to characterize and follow the dynamic changes in leukocyte phenotypes in the blood and lung of *M. tuberculosis* infected guinea pigs as previously described [26]. Briefly, the lungs were perfused through the pulmonary artery with 25 ml of ice-cold PBS containing 50U/ml of heparin (Sigma, St Louis, MO). Tissue sections were transferred and minced in cold, incomplete D-MEM then incubated for 30 min at 37°C in D-MEM containing type IV bovine pancreatic DNase (SigmaChemical, 30mg/ml) and collagenase XI (Sigma Chemical, 0.7mg/ml). Tissue digests were added to an additional 10 ml of media and cells pushed gently through screens. Contaminating erythrocytes were lysed with 4.0ml Gey's solution (0.15MNH₄Cl, 10mM KHCO₃) and the cells washed with Dulbecco's modified Eagle's minimal essential medium. Cells were plated at 1×10^6 per well in 96-well plates. Total cell numbers were determined by flow cytometry using BD™ Liquid Counting Beads, as described by the manufacturer (BD PharMingen, San Jose, CA). In addition, leukocytes were separated from 10 ml of guinea pig blood as previously described [26].

Single cell suspensions from individual guinea pigs were incubated with primary antibodies to guinea pig CD4 [30], CD8 [30], pan T cells [30, 31], CD45 [32], MIL4 [33], B cells [33], macrophage [34] and class II [30, 35] antibodies at 4°C for 30 minutes and washed in PBS containing 0.1% sodium azide (Sigma-Aldrich). Prior to incubating cells with anti-macrophage [30, 35] and anti-MHC Class II [35] antibodies, cells were permeablized using Leucoperm (Serotec Inc, Raleigh, NC) according to the manufacturers recommendations. Data acquisition and analysis were done using a FACscalibur (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences, San Jose, CA). Compensation of the spectral overlap for each fluorochrome was done using CD4 or MIL4 or CD3 antigens from cells gated in the FSC^{low} versus SSC^{low}; FSC^{mid/high} versus SSC^{mid/high}; SSC^{low} versus MIL4⁺; SSC^{high} versus MIL4^{neg} and SSC^{high} versus MIL4⁺ region respectively. Analyses were performed with an acquisition of at least 100,000 total T cells events.

Survival study

To determine whether combining BCG vaccination and drug therapy reduced the rate of disease relapse, guinea pigs (n=10 for each treatment group) RHZ treatment was discontinued after 8 weeks, and survival and disease progression was monitored out to 500 days of infection. Guinea pigs with clinical evidence of disease relapse were allowed to survive to humane end-points using a modified Karnovsky scale or were euthanized 500 days after infection [26]. At necropsy, tissues were collected for CFU determination and histologic analysis as described above.

Statistical analysis

Data is representative of the mean values from individual guinea pigs within each group (n=5) $\pm$ standard error of the mean. Student T test was used to compare differences in tissue bacterial burden and cell phenotypes by flow cytometry. The data comparing differences in lesion area and necrosis areas between treatment groups was compared by 2 way ANOVA and post-hoc multiple comparisons were made using Tukey's post-test with the level of significance set at $P \leq 0.05$. Kaplan Meier analysis was used to illustrate differences in survival and analyze by log rank statistics.

Results

The effect of BCG vaccination and drug therapy on disease progression

The rapid progression of *M. tuberculosis* infection in unvaccinated and untreated control animals was evident in the first 20 days of infection. The bacterial loads in the lung, spleen and lymph nodes of sham-vaccinated guinea pigs peaked by day 50 and all animals succumbed to infection by day 80. BCG vaccination was effective at prolonging survival out to 190 days and resulted in a 1.5 log reduction in CFUs in the lung (Figure 1A) and spleen (Figure 1B), however the CFU reduction in the lymph node (Figure 1C), were not statistically different from control animals by day 80. In addition to prolonging survival, animals treated with RHZ with or without BCG vaccination responded with a rapid decrease in bacterial numbers in the lung (Figure 1A), spleen (Figure 1B) and lymph node (Figure 1C), values that were statistically different from untreated control animals and those that received BCG alone. Despite the fact that the animals in the RHZ alone group had significantly higher CFUs in all tissues prior to the initiation of treatment, there was no significant difference in the rate of reduction of bacterial numbers by drug treatment whether BCG vaccinated or not.

To determine whether residual bacilli persisted in lesions from guinea pigs receiving BCG and drug therapy, lung, spleen and lymph node homogenates were cultured from animals 190 days after infection, which was 10 weeks after drugs were discontinued. The bacterial numbers in the lungs of RHZ treated animals with and without BCG vaccination remained below detectable limits out to 190 days (Figure 1A) whereas 1 of 5 animals had relapse of bacterial growth in the spleen (Figure 1B) treated with RHZ alone and 1 of 5 animals had relapse growth in the lymph nodes (Figure 1C) that received both BCG and RHZ.

In a separate experiment, guinea pigs were treated and infected similarly to determine if BCG vaccination combined with RHZ had any effect on long-term survival. The survival differences between the treatment groups out to 500 days of infection are shown in Figure 2. Infection of guinea pigs with the Erdman KO1 strain of *M. tuberculosis* resulted in 100% mortality of the vehicle control group by 100 days of infection similar to our previous studies [36]. BCG vaccination alone failed to significantly prolong survival compared to the sham vaccinated controls, which had 100% mortality by 190 days of infection. In animals that were sham-vaccinated with saline and treated with RHZ, 4 of 10 animals died by day 500 compared to only 1 of 10 guinea pig in the BCG vaccinated and RHZ treated group.

Use of clinical parameters to monitor disease progression and reactivation

Currently there are no sensitive, ante-mortem biomarkers of disease progression or reactivation in *M. tuberculosis* infected guinea pigs, especially for long-term drug efficacy studies. Therefore we investigated whether monitoring the changes in body weight, temperature or percent oxygen saturation of peripheral blood could be used as reliable indicators of disease progression and reactivation. Figure 3, shows reduced body temperature (Figure 3A), percent oxygen saturation of peripheral blood (Figure 3B) and body weight (Figure 3C) in control animals with rapidly progressive disease. Animals receiving RHZ whether BCG vaccinated or not, maintained a constant weight gain (Figure 3A) and normal percent oxygen saturation (Figure 3B) compared to control animals or those that received BCG alone. Interestingly, the weight gain in guinea pigs that received drug therapy and BCG was significantly less than animals, which received BCG or RHZ alone (Figure 3A). There were no significant differences in the change in body temperature between any of the treatment groups (Figure 3C).

The effect of BCG vaccination combined with drug therapy on granuloma size

At 20 days of infection, prior to the initiation of drug therapy, BCG vaccination failed to significantly reduce the lesion burden in the lung (Figure 4A), and the development of extra-pulmonary lesions in the spleen (Figure 4B) and lymph node (Figure 4C). Treatment of guinea pigs with RHZ alone or combined with BCG vaccination significantly reduced lesion severity at all time points in lung (Figure 4A), spleen (Figure 4C) and lymph node (Figure 4B) compared to controls and animals that received BCG alone. In the lung, (Figure 4A), spleen (Figure 4B) and lymph node (Figure 4C), BCG vaccination combined with drug therapy significantly reduce lesion burden compared to control and BCG alone groups. At day 190 of infection (10 weeks after drugs were discontinued) there was histological evidence of disease reactivation in the spleen in the drug treated only group (Figure 4B) that was not evident in the group that was BCG vaccinated prior to drug therapy. At all time points, there were residual lesions in the lymph nodes, which were significantly more severe in the group that received RHZ alone (Figure 4C).

The effect of BCG vaccination combined with drug therapy on lung granuloma morphology

Photomicrographs in Figure 5 show the progressive increase in granulomatous inflammation by day 50 in control animals sham-vaccinated with saline and fed drug carrier alone (Figure 5A). In contrast, sham-vaccinated animals that received 4 weeks of drug therapy (Figure 5B) had significantly less inflammation but residual primary lesions with central necrosis (inset). In animals that were BCG vaccinated and drug treated for 4 weeks, there was a significant reduction in lung involvement (Figure 5D) compared to non-vaccinated carrier controls (Figure 5C). Combining BCG vaccination and drug therapy, reduced the lesion burden and prevented the development of primary lesion necrosis by day 50 (Figure 5D inset).

By day 80 of infection, the protection conferred by vaccination in non-drug treated animals had been lost. In animals that received saline (Figure 5E) or BCG alone (Figure 5G), the foci of granulomatous inflammation were extensive with prominent lesion necrosis. In animals that received saline and RHZ, lesions were calcified (Figure 5E, 5F inset) indicative of healing of necrotic granulomas by dystrophic mineralization. Drug therapy significantly reduced the lesion burden in sham-vaccinated saline controls (Figures 5B and F) but remnants of necrotic lesions corresponding to granulomatous lesions within the pulmonary lymphatics [7, 19] were calcified by day 80 (Figure 5F). The failure of BCG vaccination to protect against progressive disease was evident in animals that were BCG vaccinated and sham treated with drug carrier alone (Figures 5C, G). There was extensive lung involvement and progressive necrosis by day 50 of infection (Figure 5C inset). However, even by 80 days, guinea pigs that received BCG and RHZ (Figure 5H) had lesion remnants visible microscopically (Figure 5H inset). By day 190, 4 of 5 animals that received RHZ drug therapy alone, had residual granulomas with evidence of active inflammation (Figure 6B) or calcified lesions (Figure 6A) within bronchus associated lymphoid tissue of the lung. In contrast only 1 of 5 animals that received BCG and RHZ had residual lesions (Figure 6C).

The changes in blood and lung leukocyte populations in response to BCG vaccination and drug therapy

Flow cytometric analysis was used to quantify and characterize the dynamic changes in circulating and lung leukocytes subpopulations in guinea pigs that were BCG vaccinated and treated with combination drug therapy. The changes in the total numbers of CD4⁺ and CD8⁺ T cells, MHC class II⁺ macrophages, B cells and neutrophils in the lung are illustrated in Figure 7. In all animals except those that received drug therapy alone, CD4⁺ T cells expressing a marker of T cell activation CD45^{hi} (common leukocyte antigen), peaked and then decreased by day 190 of infection. In contrast to control guinea pigs or those that

received BCG or RHZ alone, the increases in CD4⁺ CD45^{hi} T cells in the BCG and RHZ group was minimal (Figure 7A). In addition to CD45^{hi}, CD4⁺ T cells expressing the homing receptor CT4⁺ were also quantified (Figure 7B). Similar to CD4⁺ CD45^{hi}, there was a rapid spike in cell numbers that returned to basal levels by day 190 of infection (Figure 7B). Animals that received BCG and RHZ had the smallest increase in CD4⁺ CT4⁺ T cells compared to those that received BCG or RHZ alone. A similar spike and return to basal levels was seen in CD8⁺ CT4⁺ T cells (Figure 7C), MHC class II⁺ macrophages (Figure 7D), B cells (Figure 7E) that was less in the BCG/RHZ group but differences in neutrophil numbers were not significantly different (Figure 7F). Among all treatment groups, the animals that received BCG and RHZ had the least dynamic change in numbers of all cell types within the lung.

The changes in the total numbers of CD4⁺ and CD8⁺ T cells, MHC class II⁺ macrophages, B cells and neutrophils in the peripheral circulation are illustrated in Figure 8. As in the lung, BCG combined with RHZ prevented a spike in CD4⁺ CD45^{hi} T cells until late in infection (Figure 8A). A similar pattern was seen in numbers of CD4⁺ CT4⁺ (Figure 8B) and CD8⁺ CT4⁺ T cells (Figure 8C). A significant spike in CD8⁺ CT4⁺ T cells was also seen in animals in the late stages of infection that received BCG alone and less so in animals that received RHZ alone (Figure 8C). In contrast, it was the BCG alone group that had histologic evidence of active disease, which showed the most significant increase in numbers of circulating MHC class II⁺ macrophages (Figure 8D), B cells (Figure 8E) and neutrophils (Figure 8F).

Discussion

The guinea pig model of *M. tuberculosis* infection is especially valuable for studying the effect vaccination and drug therapy has on the *in vivo* progression of experimental tuberculosis [5, 37, 38]. However, there is little known about how drug therapy or vaccination combined with drug therapy, influences host responses in this or other tuberculosis animal models [23, 39–41]. Our original hypothesis was that despite using a highly virulent challenge strain of *M. tuberculosis*, BCG vaccination alone would lessen the disease severity and therefore, enhance the efficacy of combination drug therapy. We designed this study to first, determine whether BCG vaccination was as effective in the face of a more virulent *M. tuberculosis* challenge and secondly whether BCG vaccination combined with drug treatment would prevent the persistence of drug-tolerant bacilli and thus reduce the rate of disease reactivation.

The main finding of this study is that BCG vaccination combined with drug therapy was more effective at reducing the lung lesion burden and the risk of reactivation disease in guinea pigs even when challenged with a more virulent strain of *M. tuberculosis*. Challenge strain virulence has a significant impact on the rate of disease progression and on the ability of BCG to provide immune protection in guinea pigs [7, 28]. When guinea pigs are challenged with the relatively low virulence H37Rv strain of *M. tuberculosis*, BCG vaccination extends survival from less than 30 weeks for saline controls to greater than 80 weeks [42]. In this study, BCG vaccination of guinea pigs infected with the Erdman KO1 strain of *M. tuberculosis*, extended the mean survival interval from 13 weeks in sham vaccinated animals, to only 17 weeks. Moreover, BCG vaccination failed to prevent rapid extra-pulmonary dissemination of bacilli and necrosis of lung and extra-pulmonary granulomas as is seen in studies using H37Rv as the challenge strain [7, 8].

BCG vaccination of guinea pigs in these studies reduced the bacterial burden and delayed the progression of pulmonary and extra-pulmonary disease in the early stages of infection. However, the effect was short lived with no significant differences in disease burden by day

80 in animals that received BCG alone compared to those that were sham-vaccinated with saline. These data are in agreement with recent studies that show that immunity primed by BCG vaccination fails to prevent the rapid progression of pulmonary and extra-pulmonary disease when guinea pigs are challenged with highly virulent, clinical strains of *M. tuberculosis* [36, 43]. In contrast, treatment of guinea pigs with RHZ was effective at rapidly reducing the pulmonary and extra-pulmonary bacterial burden and reversing the progression of disease in both BCG-vaccinated and non-vaccinated guinea pigs. Despite having higher CFUs in all tissues in the RHZ alone group prior to the initiation of treatment, drug therapy was equally effective whether animals were BCG vaccinated or not.

We show here that at 190 days of infection (10 weeks after the withdraw of drug therapy), there was no bacilli growth in the lung but 1 of 5 animals had growth in either the spleen or lymph node. More importantly, when the study was extended out to 500 days of infection, 1 of 10 animals that received BCG and RHZ and 4 of 10 that received RHZ alone succumbed to disseminated disease as a result of reactivation tuberculosis. This is in contrast to a previous study by Ahmad et al. that showed no relapse in growth at 4 months in guinea pigs treated with RHZ twice weekly for 2 months [39]. The difference in outcome between the former study and ours may be explained at least in part by the difference in treatment schedule and the length of time guinea pigs were allowed to survive, 4 months compared to 500 days (16.5 months) in our study. In addition, the use of different challenge strains could also account for differences in the two studies however, we've shown that the Erdman KO1 and CDC 1551 behave similarly in the guinea pig model [36]. Collectively, these data support the hypothesis that even following 8 weeks of combination drug therapy, guinea pigs harbor a viable population of bacilli that are capable of causing lethal reactivation disease [10].

While there was early culture and histologic evidence of extra-pulmonary lesion reactivation by day 190 in two animals, more conclusive evidence of bacterial persistence was seen when animals were allowed to survive for up to 500 days. There was histologic evidence of residual lesions in the lymph node at all time points in the drug treated animals whether they received BCG or not. The presence of visible lesions increases the likelihood that affected lymph nodes harbor persistent bacilli. Being able to confirm *in vivo* persistence of viable bacilli following drug therapy in animals even by culture remains a challenge yet is essential to adequately evaluate the efficacy of new drugs and new drug combinations intended for use in humans. This study reaffirms that extra-pulmonary lesions are an important site of bacterial persistence and a site of disease reactivation, which is often overlooked in experimental infections in animals. The disadvantage of using histopathology or culture to confirm disease reactivation in long-term survival studies is that animals succumb to disseminated disease making it difficult to confirm the original site of reactivation. There is clinical evidence in humans suggesting that persistent bacilli within extra-pulmonary lesions are more difficult to eradicate with drug therapy [44, 45].

One of the most striking findings of this study was the near complete healing of residual lesions in the lung and spleen but not in the lymph nodes in BCG vaccinated guinea pigs receiving drug therapy. In the lung, the most consistent site of residual lesions even in the drug treated animals, were within the perivascular and peribronchial lymphatics, which were often calcified [29]. Our previous studies like that of Ahmed et al., demonstrated that acid fast bacilli can be found entrapped within the mineralized matrix of calcified granulomas in *M. tuberculosis* infected guinea pigs [21, 29, 39]. In human studies, calcified lesions can also harbor viable bacilli but in general there are fewer culturable organisms compared to non-calcified lesions [44]. Therefore, persistent calcified lesions have the potential to harbor viable bacilli and serve as a source of disease reactivation [5, 21, 29, 45].

In an attempt to identify clinical parameters that could serve as biomarkers of disease progression and reactivation, body weight and temperature as well as peripheral blood oxygen saturation was quantified. None of the clinical parameters proved to be sensitive indicators of disease reactivation but correlated with active disease in animals that received BCG alone. The most significant differences between treatment groups were in change in weight gain and percent oxygen saturation. The failure of the untreated, BCG vaccinated animals to gain weight is a reflection of the delayed but progressive disease associated with the inability of BCG vaccination to provide effective immune protection. The gain in body weight in the two groups that received RHZ reflects the effectiveness of drug therapy at controlling infection whether animals were BCG vaccinated or not. The reason why animals vaccinated with BCG combined with drug therapy gained significantly less weight compared to the RHZ alone group is unclear. One possible explanation is the release of proinflammatory cytokines like TNF- α which have been shown to contribute to the weight loss seen in human patients with active *M. tuberculosis* infections [46]. Among the few differences that was seen in the change in body temperature was an increase in the animals that received BCG and RHZ. Moreover, the flow cytometric data showed that combining BCG vaccination and drug therapy stimulated an increase in circulating leukocytes compared to animals that received drug treatment alone. While BCG combined with drug therapy may stimulate peripheral blood leukocytes, anti-inflammatory rather than pro-inflammatory cytokines appear to dominate in lesions from BCG vaccinated animals compared to non-vaccinated animals [47]. Pro-inflammatory cytokines have also been shown to play a role in toxic side-effects of anti-tuberculosis drug therapy, which can manifest clinically as weight loss [48].

Combining flow cytometry with histologic analysis in the guinea pig tuberculosis model allows us to more precisely characterize the cell types that make up the pulmonary and extra-pulmonary lesions seen microscopically [7]. We have previously compared flow cytometry and histopathology to show that *M. tuberculosis* infection in guinea pigs stimulates a progressive increase in lymphocyte, macrophage and neutrophil numbers in the blood and tissues which is abrogated by drug therapy [7, 26, 49]. In these studies, drug treatment either alone or combined with BCG vaccination, prevented the spike in CD4⁺ T cells that express CD45^{hi} and CT4⁺ as well as CD8⁺ T cells expressing CT4⁺, MHC class II expressing macrophages, B lymphocytes and neutrophils. We have also shown previously that the partial protection conferred by BCG vaccination, delays the appearance of a spike in macrophages, B cell and neutrophils in the blood, which was also seen here. However, the increase in numbers of circulating MHC class II expressing macrophage, B cells and neutrophils in the BCG alone group proved to be a sensitive, ante-mortem indicator of active disease, which was confirmed histologically.

Combining BCG and drug therapy stimulated an increase in activated CD4⁺ T cells as measured by co-expression of CD45^{hi} and CT4⁺ in the blood, which was not seen in animals receiving BCG alone. A similar spike in CD8⁺ T cells expressing the homing receptor CT4⁺ in the blood was also seen in animals receiving BCG alone. This increase in activated lymphocytes in circulation may indicate that combining BCG and drug therapy is more effective at stimulating a protective cellular immune response than either drug therapy or BCG alone. Fewer residual lesions seen histologically and the decreased incidence of disease reactivation in animals that received BCG and RHZ support this interpretation.

The results of this study suggest that while prior vaccination of guinea pigs with BCG has a considerable benefit by lessening the lesion severity in the early stages of infection, there is not an added benefit related to the rate of clearance of bacilli in the early stages of disease. Despite the inability of BCG to prevent the rapid dissemination of bacilli, combining vaccination and drug therapy reduced the incidence of reactivation disease compared to

animals receiving RHZ alone. As shown by others, even small numbers of persistent bacteria have the potential to reactivate from 12 to 41 months in this model [10, 25, 50]. In this study, the long-term survival study was effective at confirming the persistence of viable bacilli following combination drug therapy. What remains unknown is whether bacilli that resume growth are associated with residual pulmonary or extra-pulmonary lesions. These findings suggest that the long-term guinea pig studies even though costly, may be necessary to demonstrate how effective new drug combinations are at eliminating persistent bacilli *in vivo*. Moreover, monitoring the dynamic changes in circulating leukocytes as an ante-mortem indicator of disease progression or reactivation in animal models may prove useful for the *in vivo* evaluation of new drug or drug combinations intended for use in people.

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Highlights

Guinea pigs develops lesions that harbors drug tolerant bacilli when infected with *M. tuberculosis*

BCG vaccination prior to challenge decreases the severity of caseous granulomas

BCG vaccination improved antimicrobial treatment outcome in the guinea pig tuberculosis model

BCG vaccination combined with drug therapy had better clinical and survival outcomes

BCG vaccination combined with drug therapy reduces the incidence of disease reactivation

Figure 1.

Combination drug therapy significantly reduces the bacterial burden in the lungs, spleen and lymph node in BCG vaccinated and non-vaccinated guinea pigs infected with the Erdman KO1 strain of *M. tuberculosis*. Bacterial counts in the lungs (A), spleen (B) and lymph node (C), on days 20, 50, and 80 from guinea pigs infected with *M. tuberculosis* Erdman-KO1 by low dose aerosol. Treatment groups include guinea pigs (n=5) that were sham vaccinated with saline and received 40% sucrose and nutrient vehicle orally as a drug carrier (solid squares), those receiving BCG and drug carrier (open squares), those receiving saline and chemotherapy with RHZ in drug carrier (open diamonds) and those receiving the combination of BCG and RHZ chemotherapy in drug carrier (solid diamonds). Results are expressed as the mean (n=5) log₁₀ CFU ($\pm$ SEM) for each treatment group. * = p < 0.050.

Figure 2. Combination drug therapy and BCG vaccination prolongs survival of guinea pigs infected with the Erdman KO1 strain of *M. tuberculosis*. The survival of individual guinea pigs (n=10 per group) were either sham vaccinated with saline and received 40% sucrose and nutrient vehicle orally as a drug carrier (solid squares), BCG and drug carrier (open squares), or saline and chemotherapy with RHZ in drug carrier (open diamonds) or the combination of BCG and RHZ chemotherapy in drug carrier (solid diamonds). Kaplan Meier analysis, n=10. * = $p < 0.05$.

Figure 3.

Combination of BCG vaccination and RHZ chemotherapy prevents progressive clinical disease in *M. tuberculosis* infected guinea pigs as measured by changes in body weight, percent peripheral blood oxygen saturation, and body temperature. Treatment groups included *M. tuberculosis* infected guinea pigs (n=5) that were either sham vaccinated with saline and received 40% sucrose and nutrient vehicle orally as a drug carrier (solid squares), BCG and drug carrier (open squares), or saline and chemotherapy with RHZ in drug carrier (open diamonds) or the combination of BCG and RHZ chemotherapy in drug carrier (solid diamonds). Guinea pigs that received RHZ chemotherapy either alone (open diamonds) or in combination with BCG vaccination (solid diamonds) gained weight (A), retained normal percent oxygen saturation (B) and normal body temperature out to 190 days of infection. Untreated control animals (solid squares) or animals that received BCG alone (open squares) showed evidence of progressive disease with a progressive decrease in body weight (A), percent oxygen saturation (B) and body temperature. Results are expressed as the mean (n=5) of body weight in grams, percent oxygen saturation and body temperature [degree C] ($\pm$ SEM). * = $p < 0.05$.

Figure 4.

Treatment of BCG vaccinated guinea pigs with RHZ drug therapy significantly reduces the lesion burden in *M. tuberculosis* infected guinea pigs. On day 20, 50, 80 and 190 of infection, the lung, spleen and lymph node lesion severity was quantified in *M. tuberculosis* infected guinea pigs that were either sham vaccinated with saline and received 40% sucrose and nutrient vehicle orally as a drug carrier (control), BCG and drug carrier (BCG), saline and chemotherapy with RHZ in drug carrier (RHZ) or the combination of BCG and RHZ chemotherapy in drug carrier (BCG/RHZ). Data is expressed as the mean value for the percent area of lung, lymph node and spleen affected by granulomatous inflammation and the area of lesion affected by necrosis. The data is the mean value for 5 animals per treatment group and at each time point. * = $p < 0.05$.

Figure 5.

Treatment of BCG vaccinated guinea pigs with RHZ drug therapy significantly reduces the lung lesion severity in *M. tuberculosis* infected guinea pigs on days 50 and 80 of infection. Photomicrographs are representative sections of lung from individual animals that best represents the mean values shown in panel 4A. Panels C,G represent sections were taken from *M. tuberculosis* infected guinea pigs that were either sham vaccinated with saline and received 40% sucrose and nutrient vehicle orally as a drug carrier (saline/No RHZ). Panels C and G represent infected animals that received BCG and drug carrier (BCG/No RHZ). Panels B and F were from infected animals that received, saline and chemotherapy with RHZ in drug carrier (saline/RHZ). Panels D and H are from infected animals that received a combination of BCG and RHZ chemotherapy in drug carrier (BCG/RHZ). Images illustrate

that BCG vaccination failed to prevent foci of granulomatous inflammation from developing central necrosis on days 50 (C, inset) and 80 (G, inset) compared to those that received BCG and RHZ chemotherapy D and H out to 50 and 80 days respectively. RHZ chemotherapy alone failed to resolve primary lesions within the perivascular and peribronchial lymphatics that became calcified and persisted to day 50 (B) and 80 (F) of infection. Combination BCG and RHZ almost completely resolved lung lesions by day 80 (H) leaving only residual foci of interstitial fibrosis (H, inset). Hematoxylin and eosin staining. Magnification = 40X, inset magnification 200X.

Figure 6.

Combination BCG and RHZ chemotherapy prevented the relapse of inflammation in the perivascular and peribronchial lymphatics of *M. tuberculosis* infected guinea pigs. At 500 days after infection with the Erdman KO strain of *M. tuberculosis*, 4 of 5 animals mock-vaccinated with saline prior to treatment with RHZ had residual lesions within perivascular and peribronchial lymphoid aggregates (A, white arrows) that were either calcified (C) or showed evidence of reactivation of granulomatous inflammation (G) in panel B. In contrast, 4 of 5 guinea pigs vaccinated with BCG prior to infection followed by 8 weeks of treatment with RHZ showed no evidence of residual lesions (panel C) but had normal lung associated lymphoid aggregates (white arrow) associated with peribronchial and perivascular lymphatics (black arrows). Hematoxylin and eosin. Magnification = 200X.

Figure 7.

Flow cytometric analysis of lung cells collected from *M. tuberculosis* infected guinea pigs that were CD4⁺ CD45⁺ (panel A), CD4⁺ CT4⁺ (panel B), CD8⁺ CT4⁺ (panel C), MR-1⁺ and MHC class II⁺ macrophages (panel D), B cells (panel E) and MIL4⁺ neutrophils on days 20, 50, 80 and 190 of infection. Guinea pigs infected with Erdman KO1 strain of *M. tuberculosis* were sham vaccinated with saline and received 40% sucrose and nutrient vehicle orally as a drug carrier (solid squares), received BCG and drug carrier (open squares), received saline and chemotherapy with RHZ in drug carrier (open diamonds) or received combination of BCG and RHZ chemotherapy in drug carrier (solid diamonds). Each data point represents mean ($\pm$ SEM) total cell numbers per gram of tissue from 5 guinea pigs for each treatment and each time point. * = $p < 0.05$.

Figure 8.

Flow cytometric analysis of peripheral blood cells collected from *M. tuberculosis* infected guinea pigs that were CD4⁺ CD45⁺ (panel A), CD4⁺ CT4⁺ (panel B), CD8⁺ CT4⁺ (panel C), MR-1⁺ and MHC class II⁺ macrophages (panel D), B cells (panel E) and MIL4⁺ neutrophils on days 20, 50, 80 and 190 of infection. Guinea pigs infected with Erdman KO1 strain of *M. tuberculosis* were sham vaccinated with saline and received 40% sucrose and nutrient vehicle orally as a drug carrier (solid squares), received BCG and drug carrier (open squares), received saline and chemotherapy with RHZ in drug carrier (open diamonds) or received combination of BCG and RHZ chemotherapy in drug carrier (solid diamonds). Each data point represents mean ($\pm$ SEM) total cell numbers per gram of tissue from 5 guinea pigs for each treatment and each time point. * = p<0.05.