Comparative Medicine
Copyright 2020
by the American Association for Laboratory Animal Science

Vol 70, No 1
February 2020
Pages 45–55

Original Research

Evaluation of Peripheral Blood Markers as Early Endpoint Criteria in Guinea Pigs (*Cavia porcellus*) when Testing Tuberculosis Vaccine Candidates

Wendy R Williams,[1,*] JoLynn Troudt,[2] Elizabeth Creissen,[2] Helle Bielefeldt-Ohmann,[4] Matthew S Johnston,[3] Lon V Kendall,[2] and Angelo A Izzo[2]

The guinea pig model of tuberculosis is used extensively to assess the efficacy of novel tuberculosis vaccines. There are established parameters to determine vaccine efficacy in this model, but the science community currently lacks established biomarkers for early detection and monitoring of experimental disease in guinea pigs. To define a set of biomarkers that could be used as benchmarks for disease progression and early endpoint criteria, we assessed serum biochemical and hematology parameters in 2 groups of guinea pigs—one vaccinated with the attenuated *Mycobacterium bovis* vaccine strain (BCG) and one sham-vaccinated with saline—and then experimentally infected with a virulent strain of *Mycobacterium tuberculosis*. After infection, WBC showed the strongest differences between saline-inoculated and vaccinated animals, with more subtle changes in other serum biochemical parameters, including ALT and ALP. Therefore, this study provides a starting point for evaluating the utility of blood values as possible early endpoint criteria in the guinea pig model of tuberculosis.

Abbreviation: BCG, Bacillus Calmette–Guerin

DOI: 10.30802/AALAS-CM-19-000047

Infection with *Mycobacterium tuberculosis* remains a global public health concern for which the success of developing a new vaccine for protection against tuberculosis relies heavily on animal models to prove concept, safety, and efficacy.[10] Although no animal model is a perfect replica of the immune response elicited in humans infected with *M. tuberculosis*, several models are used for different phases of study and are imperative for scientific and biomedical advancement. The mouse model is the most widely used animal model, applied in all types of research globally, including for tuberculosis research.[18,19] However, the experimental tuberculosis infection mouse model comes with some significant shortcomings. Anatomically, the mouse bronchial tree is not only very small but is less complex compared with humans. The mouse bronchial tree lacks accessory vasculature and has limited alveolar lymphatic drainage.[7] In addition, a true latent form of tuberculosis is difficult to replicate in mice. Although they do show a chronic stage of infection where the mycobacterial organism replicates at a much slower rate, mice are not in a truly latent stage, where replication has ceased completely. This situation is in contrast to humans in the latent stage, in which bacterial numbers are undetectable by conventional methods. Because of a constantly elevated bacterial load in multiple organs, the disease is always progressing in mice, albeit slowly.[7,21]

Received: 11 Apr 2019. Revision requested: 29 May 2019. Accepted: 26 Jul 2019.
[1]Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma;[2]Departments of Microbiology, Immunology, and Pathology, [3]Department of Clinical Sciences, Colorado State University, Fort Collins, Colorado; [4]University of Queensland, Australian Infectious Diseases Research Centre, St Lucia, and School of Veterinary Science, Gatton Campus, Queensland, Australia
[*]Corresponding author. Email: wendytuttle@gmail.com

Another significant drawback to using mice in tuberculosis studies is the difference in pathology between mice and humans. Humans develop distinct pulmonary granulomas in response to *M. tuberculosis* infection. The mouse response to *M. tuberculosis* infection is similar in that it includes granulomatous inflammation, but does not result in a true tubercle or granuloma with primary lesion necrosis.[8,7,19] Human granulomas are well circumscribed, with a central core of neutrophils and lymphocytes, surrounded by macrophages.[21] In contrast, mice develop a more diffuse granulomatous inflammation that lacks the structure of a true granuloma; although it still contains lymphocytes and macrophages, it is not organized into a well-circumscribed lesion.[21]

In addition, granulomas in humans can become necrotic (caseous), fibrotic, mineralized, or even cavitary due to lytic necrosis. And multiple granulomas of different types can coexist in human lung infected with *M. tuberculosis* at any given time. In contrast, mice do not have different stages of granulomatous inflammation. Instead, there is little heterogeneity in the granulomatous response, and lesions do not typically become necrotic or cavitated.[21] Because the various stages of granulomas in the human create different microenvironments at the same time, it is important to be able to model this type of bacterial pathogenesis and effects due to the microenvironment. One useful rodent model is the C3HeB/FeJ mouse (Kramnik model of tuberculosis), which develops highly organized encapsulated necrotic granulomas after *M. tuberculosis* infection.[6] C3HeB/FeJ mice have a recessive allele that is responsible for a decreased ability to control *M. tuberculosis* multiplication in the lungs and has been shown to control the formation of caseous necrosis of

pulmonary lesions.[6] Therefore, mice are used to obtain information regarding immunogenicity and protective immunity during early vaccine development.[1]

In contrast to mice, guinea pigs are an excellent preclinical animal model for the evaluation of tuberculosis vaccine candidates to prevent disease.[1] Once exposed to a low-dose aerosol of virulent *M. tuberculosis*, guinea pigs develop progressive and fatal diseases usually within a matter of weeks.[5,10] The 3 phases of disease following exposure are divided into acute, subacute, and chronic stages which are based on patterns of bacterial growth, dissemination, and pathology.[3,17,26] The acute phase occurs in the first 2 wk and is characterized by rapid bacterial proliferation in the lung and draining lymph nodes, as well as the progression of granulomatous inflammation and necrosis in the lungs. The subacute phase, which lasts 2 to 4 wk, is characterized by a stationary phase of bacterial replication in the lung and lymph nodes. It is during this stage that the most severe inflammation begins to subside. It is also in this phase that multiple extrapulmonary lesions develop. Finally, the chronic stage is characterized by continued bacterial replication in extrapulmonary tissues. The morbidity and mortality at this stage are due to the combined effect of progressive pulmonary and extrapulmonary lesions.

The lung pathology in guinea pigs, compared with mice, more closely reflects that of human infection.[12,18] The granulomas developing in guinea pigs are well circumscribed with a central core of heterophils (the guinea pig equivalent of human neutrophils) and a surrounding population of lymphocytes and macrophages. Granulomas can become centrally necrotic and cavitated, with varyious degrees of fibrosis and mineralization. However, the granulomas are not as protective to guinea pigs as to humans, because the lesions do not adequately contain the bacteria.[3,12,15,19] Because of the pattern of susceptibility to low-dose infection and subsequent development of fulminant tuberculosis, guinea pigs are often thought to be the animal model most sensitive to tuberculosis infection.[10,12] Due to their high susceptibility and inability to control infection, guinea pigs are also thought to be an ideal animal model to test vaccine candidates, with the rationale that the best vaccine needs to protect against the most susceptible host.[3,12] Furthermore, guinea pigs have demonstrated their use as a model of dissemination, given that late-stage tuberculosis is often found not only in lungs but also commonly in liver, spleen, and lymph nodes, and less commonly in the adrenal glands, mammary glands, eye, and brain.[20] The primary drawback of the guinea pig model has been the relative lack of specific immunologic reagents; however, this disadvantage is disappearing as PCR-based techniques and antibodies are developed to better characterize the guinea pig immune response.[17]

Clinical presentation of primary tuberculosis infection in humans can be insidious. Typical clinical signs include a fever of unknown origin, night sweats, and weight loss in conjunction with persistent and productive cough.[14] These signs are vague in nature and can be associated with many other infectious agents. Although the clinical presentation of tuberculosis in humans can be indistinguishable from other infectious causes, there are detectable signs to indicate fulminant pulmonary infection. This situation is in contrast to guinea pigs, which are great maskers of disease. Vaccines are expected to prevent or delay weight loss and limit pulmonary pathology, thus prolonging the survival of an infected guinea pig.[10] However, due to their high level of susceptibility, this is rarely achieved. The guinea pig model nevertheless represents a critical link between preclinical testing and human trials in the pipeline of tuberculosis vaccine candidates. Therefore, a better understanding of the model and how it fits into the progression of vaccines is ongoing[10,12,20] Weight loss and clinical signs remain the most common correlates of disease progression, but often these signs are noticed only when the disease is quite severe.[2,20] Therefore, there is still a need to characterize the clinical progression of disease in guinea pigs to better evaluate vaccine candidates.

To achieve this goal, we assessed peripheral blood biomarkers of guinea pigs in 2 groups infected with the virulent strain of *M. tuberculosis*, H37Rv. One group was vaccinated with Bacillus Calmette–Guerin (BCG), and the other group was sham-vaccinated with saline. In addition, these data were examined against data collected from naive control animals. To our knowledge, the assessment of peripheral blood biomarkers as possible criteria for early endpoints in guinea pigs undergoing vaccine studies for tuberculosis has never been done. Analysis of blood data indicated that WBC had the most change in the face of infection, whereas other serum enzymes did not demonstrate clinically relevant differences despite disseminated disease as evidenced by histopathology.

Materials and Methods

Animals. Female, outbred Hartley guinea pigs weighing 450 to 500 g were purchased from Charles River Laboratories (Wilmington, MA). Guinea pigs were maintained under ABSL3 barrier conditions in isolator cages (Thoren, Hazleton, PA) for the entire period of the experiment. They had access to standard guinea pig chow (Harlan, Madison, WI), water was provided without restriction, and hay cubes (Rocky Mountain Lab Supply, Centennial, CO) were provided daily. Guinea pigs were housed on 1/8-in. corncob bedding (Harlan, WI), with red huts (Bio Serve, Frenchtown, NJ) for enrichment. All animals were pair-housed until inoculated and then singly housed until the end of the experiment. All experimental procedures were conducted in accordance with the PHS Policy on the Humane Care and Use of Laboratory Animals and were approved by the Colorado State University IACUC.

Mycobacteria. *M. bovis* BCG, Pasteur (TMCC 1011) strain, was grown in Proskaur and Beck (P and B) medium with 0.05% Tween 80 (Sigma, St Louis, MO) to midlog phase, as described previously.[10] Aliquots were stored at -80 °C and thawed before use. *M. tuberculosis* H37Rv (TMCC 102) was initially grown for 3 passages as a pellicle on Proskaur and Beck medium to produce seed stocks. Working stocks with a maximum of 6 passages were expanded from the seed stocks in Proskaur and Beck medium with 0.1% Tween 80. Working stocks were prepared at the midlog phase, and aliquots were stored at –80 °C.

Inoculation and infection. Ten guinea pigs were vaccinated with 10^3 cfu of BCG Pasteur, and another 10 guinea pigs were inoculated with saline via the intradermal route on the ventrum, using a tuberculin syringe attached to a 26-gauge, 1/2-in. needle. Two weeks after vaccination, all guinea pigs were bled (details later) for baseline values. Guinea pigs were rested for 10 wk before exposure to 10 to 20 cfu of *M. tuberculosis* through the respiratory route by using a Madison Aerosol Exposure Chamber (University of Wisconsin, Madison, WI).

Weight and body temperatures. Guinea pigs were monitored for the duration of study. Body temperature was measured daily by using an RFID microchip (IPT-300, Bio Medic Data Systems, Seaford, DE), which was implanted subcutaneously over the dorsum, and a scanner transponder (DAS-6006/7, Bio Medic Data Systems). This subcutaneous microchip implant allowed measurement of temperature and carried information regarding experiment number and animal ID. The body temperatures of individual guinea pigs were assessed each day at approximately the same time in the morning. Body weight was measured weekly by placing individual animals in a weigh boat on a gram scale.

Blood collection. To facilitate blood collection prior to inoculation and every 30 d afterward, guinea pigs were briefly anesthetized with isoflurane gas at 3% to 5%, with an oxygen flow rate of 0.75 to 1.0L/min, and placed in dorsal recumbency. Approximately 3 mL of blood was collected from the cranial vena cava by using a 26 5/8-gauge, 1/2-in. needle attached to 3-mL Luer lock syringe (BD Biosciences, Mountain View, CA). Blood was immediately transferred into appropriate tubes containing lithium heparin or potassium EDTA (BD Biosciences). Blood for hematology was submitted to the Colorado State University Diagnostic Lab and run on an ADVIA 120 Hematology System (Siemens, Loveland, CO). Blood for serum chemistry was run on an Abaxis VS2 (Abaxis, Union City, CA). Guinea pigs were sedated by using ketamine (45 mg/kg; MWI, Boise, ID) and xylazine (5 mg/kg; Akorn, Decatur, IL) administered intramuscularly into the proximal hindlimb. Once at a surgical plane of anesthesia (assessed by lack of response to toe pinch), guinea pigs were placed in dorsal recumbency. The ventral thorax was saturated with 70% isopropyl alcohol (Sigma–Aldrich, St Louis, MO), and blood was collected from the cranial vena cava by using a 3-mL Luer lock syringe with a 20-gauge, 1-in. needle attached. Blood was then transferred to appropriate tubes for CBC and serum chemistry analyses, as previously described. Guinea pigs were allowed to recover fully from anesthesia prior to return to the home cage. In addition to the infected groups, we used a naïve, uninfected control group of 10 guinea pigs for blood collection only to determine average baseline levels.

Time to necropsy. Infected guinea pigs were monitored for disease and when they met the established set of endpoint criteria approved by the IACUC, they were euthanized. Criteria included a moribund state or greater than 15% weight loss or evidence of respiratory compromise (increased respiratory effort and/or rate). Of note, one animal exceeded 15% weight loss precipitously and was euthanized as soon as the research team became aware. At the time of necropsy, guinea pigs were sedated as described earlier and then euthanized through intracardiac administration of sodium pentobarbital (MWI). Organs were harvested for both culture and histology. The right cranial lung lobe, half of the spleen, one liver lobe, one kidney, half of the brain, and one eyeball were evaluated for bacterial load, which was determined by plating tissue homogenates onto nutrient 7H11 agar supplemented with oleic acid–albumin–dextrose–catalase (Sigma). Colonies were counted after 21 d of incubation at 37 °C. The remaining lung lobes, spleen, liver, kidney, brain, and eyeball, as well as the stomach, small intestine, colon, cecum, and mediastinal and mesenteric lymph nodes, were processed for pathology assessment.

Pathology. Organs were placed in 10% neutral-buffered formaldehyde for 2 wk then trimmed and routinely processed, and paraffin sections cut and stained with hematoxylin and eosin (H and E; IHC Tech, Aurora, CO). Pathology was scored according to published criteria by a board-certified pathologist without prior knowledge of the groupings.[20] Briefly, slides were evaluated on a scale of 0 to 15, with 0 indicating no appreciable lesions and 15 indicating very severe and complicated lesions. Lungs were scored according to total organ involvement, primary lesions, secondary lesions, necrosis, mineralization, and fibrosis. Spleens and livers were scored on the basis of lesion severity and extent. Accessory organs were scored according to the presence or absence of lesions and their severity. Photomicrographs were taken by using a microscope (Eclipse 51E, Nikon, Tokyo, Japan), camera (DS-Fi1 with a DS-U2 unit, Nikon), and NIS Elements F software (Nikon).

Statistical analyses. Sample size was established based on our prior determination of the efficacy of BCG in the guinea pig model as measured by survival and indicated that a sample size of 10 would be sufficient to provide us with the power to perform statistical analyses with 90% confidence. Guinea pigs were randomized at the time of vaccination as well as at the time of infection, and data acquisition were not blinded. Data points were excluded when the sample was left at unfavorable conditions known to affect the results. Statistical analysis was performed by using SAS 9.3 (SAS Software, Cary, NC). A separate repeated-measures analysis was performed for each response variable. Response variables included albumin, globulin, total protein, ALP, ALT, total WBC, heterophils, lymphocytes, and monocytes. The within-subjects factor was time (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, and 420 d), and the between-subjects factor was treatment (BCG or saline). In addition, a treatment over time (time×treatment) interaction term was included in the model, and a random effect for animal (nested within treatment) was used to account for repeated measures. Contrasts were used to estimate and test comparisons of interest. Tukey adjustment was used to control for multiple testing (separately for each response variable).

In addition, a repeated-measures analysis based on differences (compared with prechallenge) was conducted. Using only the first and last (scheduled) measurement for each animal, a paired *t* test was performed for each response variable. A 2-sample *t* test was run for each response variable to identify differences between the 2 treatment groups. The difference was calculated as last – first.

Ten naïve control guinea pigs were sampled 5 times over a 150-d period, and blood chemistry, hematology, and urine were analyzed to determine baseline data. For analysis, the data for each readout from each guinea pig at each time point were combined and used to establish a naïve control standard and are represented as a horizontal line in the figures. Individual guinea pig values used as naïve controls are also presented for total protein, albumin, glucose, and ALT. Values from naïve guinea pigs were similar to the published previously reference ranges for serum biochemistry analyses in guinea pigs.[11]

Results

Body temperature. The average temperature for the BCG-vaccinated group prior to infection was 38.9 °C (1 SD, 0.15) °C, with a minimum of 37.0 °C and a maximum of 39.9 °C (Figure 1) The average temperature for the saline-treated group prior to infection was 38.8 °C (1 SD, 0.19 °C), with a minimum of 37.2 °C and a maximum of 39.8 °C. Both groups of guinea pigs had similar body temperatures throughout the experiment, within their thermoneutral zone[25] and consistent with homeostasis. After infection, the average temperature for the BCG-vaccinated group was 38.6 °C (1 SD, 0.16 °C), with a minimum of 36.9 °C and a maximum of 39.7 °C (Figure 1). The average temperature for the saline-treated group after infection was 38.8 °C (1 SD, 0.17), with a minimum of 37.2° C and a maximum of 40.6 °C. In addition, the saline-treated guinea pigs had significant increases in the temperature at days 25 and 26 after infection as compared with BCG-vaccinated animals. Both groups showed a decreasing trend in temperature over time, and neither group showed evidence of pyrexia or hypothermia associated with disease. One animal in the saline-treated group had an increase above 40 °C, to 40.6 °C, but then returned back to 40 °C prior to euthanasia within 4 d of that increase. No other animals in either group exhibited temperatures above 40° C at any time.

At the last temperature recorded for each guinea pig (on the day of euthanasia), the BCG-vaccinated group had an average of 38.3 °C (1 SD, 0.36 °C), with a minimum of 37.8 °C and a maximum

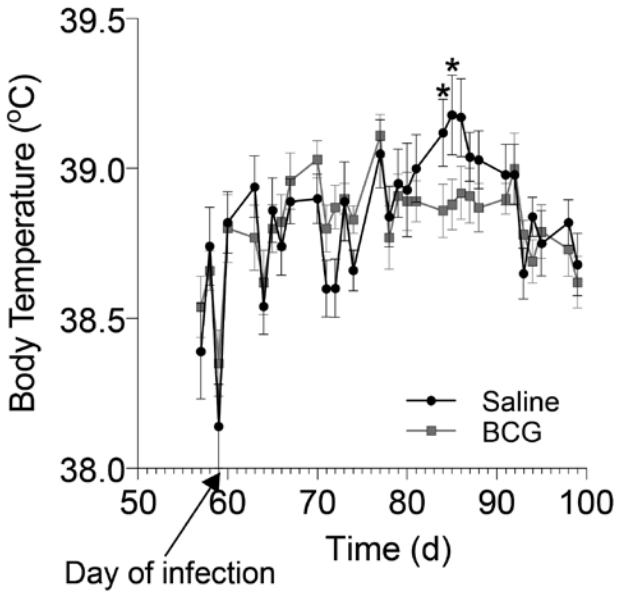

Figure 1. Guinea pig body temperatures (in °C; mean ± SEM) were measured from the day of infection until euthanasia. Guinea pigs were given a subcutaneous microchip prior to vaccination and challenge, and temperatures were monitored daily. The data presented focus on temperatures immediately after infection, to highlight the period when there was a difference between the groups. There was no statistical difference between groups at all the other time points measured. *, $P < 0.05$ for comparison between groups at each weight evaluation time point.

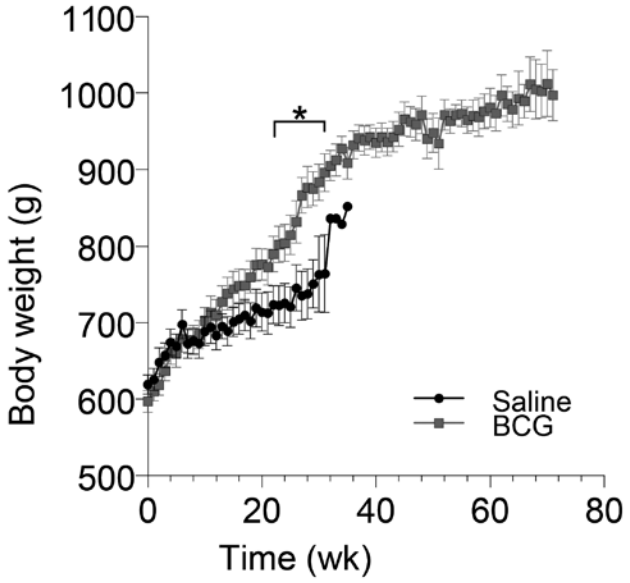

Figure 2. Guinea pig body weight (in grams; mean ± SEM) was measured weekly from the day of infection until euthanasia. *, $P < 0.05$ for comparison between groups at each weight evaluation time point.

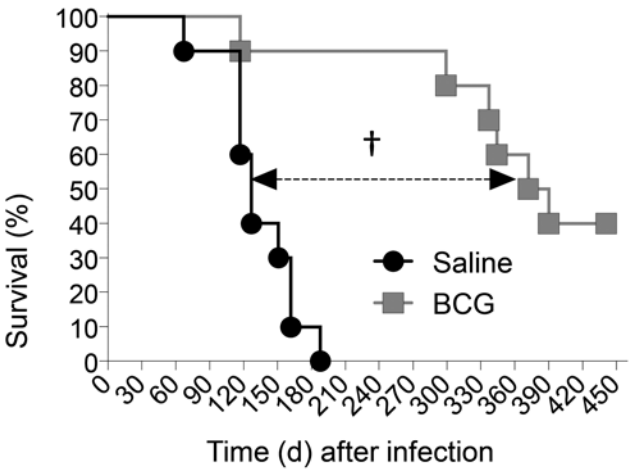

Figure 3. Kaplan–Meier plot of guinea pig survival after low-dose aerosol infection with virulent *M. tuberculosis* H37Rv. Guinea pigs were either vaccinated intradermally with 10^3 cfu BCG Pasteur or inoculated with sterile pyrogen-free saline at 10 wk prior to infection. Guinea pigs were monitored as described in the Materials and Methods and euthanized when the disease criteria had been attained. Survival time differed significantly between BCG-vaccinated and saline-treated guinea pigs after infection. †, $P < 0.01$ (determined by log-rank analysis of the time-to-necropsy for each guinea pig).

of 38.9 °C. The saline-treated group had an average of 38.8 °C (1 SD, 0.51 °C), with a minimum of 38.0 °C and a maximum of 39.7 °C.

Body weight. Preinfection body weights were lower than the average adult weight because guinea pigs were young when the study started and continued to gain weight for most of the experiment (Figure 2) BCG-vaccinated guinea pigs had an average prechallenge bodyweight of 664 g (1 SD, 63 g), and saline-treated guinea pigs had an average prechallenge bodyweight of 668 g (1 SD, 58 g). We evaluated guinea pig weights from the maximum prevaccination weight to the terminal weight, which was an overall decrease in both groups in comparison to healthy uninfected guinea pigs. In the BCG-vaccinated group, the average postinfection weight was 896 g (1 SD, 106 g) and an average terminal bodyweight of 918 g. The saline-treated group had an average postinfection bodyweight of 739 g and an average terminal bodyweight of 739 g. Few guinea pigs reached 10% total bodyweight loss from their greatest weight, and only one guinea pig in the BCG-vaccinated group lost more than 10%, with 21% total body weight loss over 10 wk' time. However, several guinea pigs in both groups were at their maximal weight at the time of the terminal measurement, specifically one animal in the BCG-vaccinated group and 4 animals in the saline-treated group. The average body weight at the time of euthanasia was typical of average for an adult female guinea pig in both groups. The BCG-vaccinated group had an average terminal bodyweight of 916 g, and the saline-treated guinea pigs had an average terminal bodyweight of 743 g.

Postinfection survival and bacteriological assessment. Intradermal BCG vaccination significantly prolonged the time to necropsy of guinea pigs after low dose aerosol *M. tuberculosis* infection (Figure 3) Four of the 10 guinea pigs in this group had not succumbed to infection at the time the experiment was terminated (day 441 postinfection). The prolonged survival was associated with lower numbers of bacterial colonies in the lungs and spleens of guinea pigs, compared with the saline-treated animals (Figure 4) For the BCG vaccinated group, there was a correlation between bacterial colony count and survival in lung ($r^2 = 0.7$, $P = 0.003$) and spleen ($r^2 = 0.41$, $P = 0.046$) but not in the saline-treated group. Other organs such as liver, kidney, brain, lung associated lymph nodes and eyes were also examined. Dissemination, as determined by colony count, to these organs varied but was generally lower in the BCG-vaccinated group (Table 1) Serum was collected from guinea pigs throughout the infection period to assess hematology and blood chemistry to determine whether there was a correlation with time taken to disease.

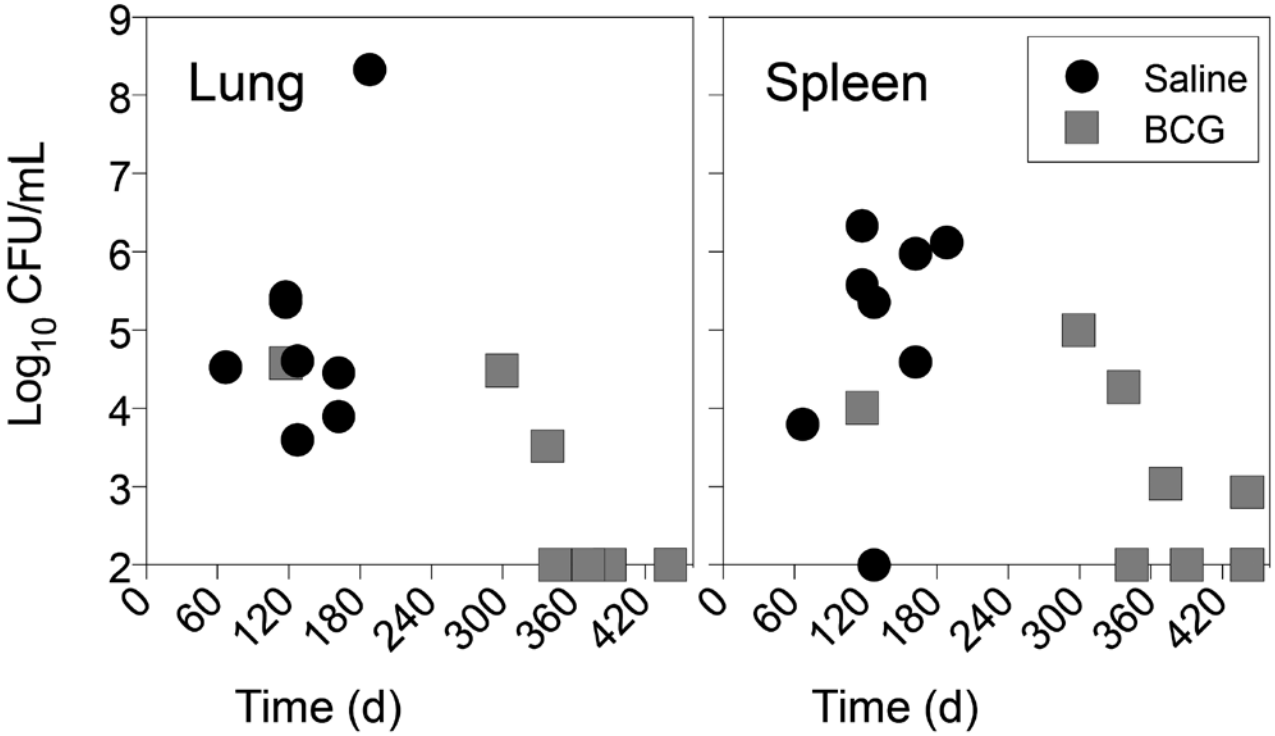

Figure 4. At the time of necropsy, portions of lung and spleen were taken to determine the colony counts in each organ, which were plotted against the time at which the guinea pig was euthanized. There was no significant correlation between CFU and time-to-necropsy, regardless of preinfection immune status.

Table 1. Bacterial colony counts (in cfu) from infected guinea pigs

	BCG group									
Animal no.	1	2	3	4	5	6	7	8	9	10
Lung	0	31	0	0	0	0	0	3	0	0
Spleen	0	TNTC	0	0	0	0	0	20	0	0
Liver	0	TNTC	0	0	0	0	0	17	0	0
Kidney	0	0	0	0	0	0	0	1	0	0
Brain	0	0	0	0	0	0	0	5	0	0
Eye	0	0	0	0	0	0	0	0	0	2
MLN	0	1	0	0	0	0	0	4	0	0

	Saline group									
Animal no.	11	12	13	14	15	16	17	18	19	20
Lung	29	34	TNTC		4		0	41	TNTC	TNTC
Spleen	TNTC	13	TNTC		0		0	TNTC	TNTC	TNTC
Liver	52	18	52		2		0	28	TNTC	TNTC
Kidney	0	0	0		0		0	0	2	0
Brain	0	0	0		2		0	0	3	2
Eye	0	0	0		0		0	0	0	0
MLN	52	16	8		0		0	2	0	20

MLN, mediastinal lymph node; TNTC, too numerous to count.
Colonies were counted after a 1:10 dilution of tissue homogenates.
Cells for animals 14 and 16 are blank due to missing data.

Histopathology. All but one of the animals in the saline group had typical primary (granulomas) and secondary (diffuse granulomatous inflammation) lesions in the lungs, and all 10 animals had extrapulmonary lesions consistent with tuberculosis in one or more organs, including spleen, liver, heart, and lymph nodes (Table 1). Necrosis and mineralization of the granulomas and interstitial fibrosis were prominent features in the lungs of this group. In contrast, only 5 of the 10 animals in the BCG group had pulmonary lesions; of these only 2 had primary granulomas, neither of which was mineralized (Table 2)

Table 2. Lung, spleen, and liver lesions in infected guinea pigs

		BCG										Saline								
	Animal no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	19	20
Lung	Total involvement	0	2	0	1	0	0	0	3	2	1	3	4	3	3	0	1	3	3	3
	Primary lesions	0	0	0	0	0	0	0	2	1	0	2	2	2	3	0	2	2	3	0
	Secondary lesions	0	2	0	1	0	0	0	3	2	1	3	4	3	2	0	1	3	3	3
	Necrosis	0	2	0	0	0	0	0	2	2	0	2	2	2	2	0	1	2	0	0
	Mineralization	0	0	0	0	0	0	0	0	0	0	2	1	2	0	0	2	0	2	0
	Fibrosis	0	2	0	0	0	0	0	2	2	0	2	2	2	2	0	2	2	2	2
	Total score	0	8	0	2	0	0	0	12	9	2	14	15	14	12	0	9	12	13	8
Spleen	Lesion severity	1	3	0	0	0	0	0	3	2	0	3	3	3	4	0	3	3	1	4
	Extent	1	4	0	0	0	0	0	3	2	0	4	1	4	4	0	3	4	3	4
	Total score	2	7	0	0	0	0	0	6	4	0	7	4	7	8	0	6	7	4	8
Liver	Lesion severity	0	3	0	3	0	0	0	3	3	0	3	3	3	3	3	4	4	3	4
	Extent	0	2	0	1	0	0	0	2	2	0	3	2	4	4	2	2	2	2	4
	Total score	0	5	0	4	0	0	0	5	5	0	6	5	7	7	5	6	6	5	8

Lesions were scored by a veterinary pathologist who had no prior knowledge of the groups. Scale is from 0 to 15, with 0 being no lesions and 15 having the most severe lesions.

Moreover, the secondary granulomatous inflammation, as a result of hematogenous spread of the bacteria, was far less extensive in this group compared with the saline treatment group (Tables 1 and 2).

Lung tissues had the highest overall scores, with the saline-treated group having the highest scores among animals (Table 1 and Figure 5) The average lung score was 3.3 for the BCG animals and 9.7 for those given saline. The same trend continued with the other organs—BCG-vaccinated animals had lower scores than saline-treated animals. Some animals in both groups had lesions in the liver, lungs, and spleen (Figure 6) All 4 survivors in the BCG-vaccinated group had 0 as a score for the lungs, whereas one had lesions in the spleen (total score = 2), and one had lesions in the liver (total score, 4). The animal with the most severe lung lesions (total score, 15) was saline-treated and lived the shortest time (67 d).

Total protein, albumin, and globulin. Total protein is a composite value reflecting both albumin and globulin levels in the serum. In the current study, total protein was significantly different between groups only at the prechallenge blood draw (Figure 7 A) The BCG-vaccinated group had significantly higher levels of total protein at day 0 when compared with the naïve group values, likely reflecting the effects of intradermal vaccination. Subsequently, total protein concentrations in the BCG-vaccinated group were similar to naïve guinea pigs until day 180, after which they again became elevated, albeit not higher than the day 0 value. The BCG-vaccinated group showed differences at several time points after infection, relative to the saline-treated group, which showed a steady increase from prechallenge values. Over the life of the infected animals, there was a statistically significant effect of treatment over time compared with the one prechallenge levels.

Differences in the measured total protein at day 0 were due to significant differences between the groups in both albumin and globulin concentrations, which were higher in the BCG-vaccinated group. The serum albumin trend was similar in both infected groups: a decrease within 30 d of infection and then an increase over time (Figure 7 B). However, the degree of increase was greater in the saline-treated group compared with the BCG vaccinated group. In addition, there was a significant effect of treatment over time. Neither infected group had serum albumin concentrations significantly greater than the naïve controls. Serum globulins increased within 30 d of infection with a continual rise thereafter in the saline-treated group, which maintained levels greater than in naïve guinea pigs (Figure 7 C). In the BCG-vaccinated group, serum globulin levels decreased initially and then increased, ultimately resulting in no appreciable net change over time. All 3 proteins showed significant differences between preinfection and last blood draws in the BCG-vaccinated group (Figure 7 E through G).

Liver enzymes. ALT, a measure of liver function, can provide early evidence for the ability of the immune system to limit organism dissemination[10] and disease (Figure 8 A) By day 30, the saline-treated group had minimal change in serum ALT compared with day 0, whereas the BCG-vaccinated group showed a significant decrease. Beyond day 30, serum ALT concentrations increased in the saline-treated group, predominantly above the naïve guinea pig values and in contrast to BCG vaccinated animals, which showed no appreciable change. Comparison of the preinfection and final postinfection blood draws after BCG vaccination revealed a net reduction of ALT at the end of the study (Figure 8 C), whereas the saline-treated group showed a nonsignificant trend ($P = 0.03$) toward an increased ALT. These data suggest that serum ALT might provide an indicator of tissue damage associated with infection after day 60.

WBC counts. WBC comprise several cell types, the majority of which includes lymphocytes, heterophils, and monocytes. Total WBC counts in the saline-treated group increased significantly over time from prechallenge levels in response to infection, whereas the BCG-vaccinated group did not show a significant change (Figure 9 A) The saline-treated guinea pig response to infection was due to steady increases in both heterophils and lymphocytes (Figure 9 B and C) but not monocytes. In the BCG-vaccinated group, cell numbers rarely varied from the naïve guinea pig value. However, determined as the number of heterophils or lymphocytes, WBC showed an effect of treatment over time when evaluated as a single parameter (Figure 9 D through F).

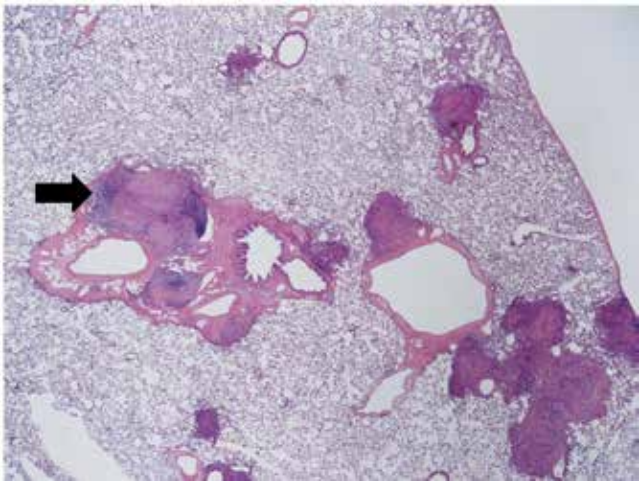

Figure 5. Severe lung lesion from a saline-treated guinea pig. Presence of discrete granuloma with central necrosis, fibrosis, and mineralization (arrows). Hematoxylin and eosin stain; magnification, 40×.

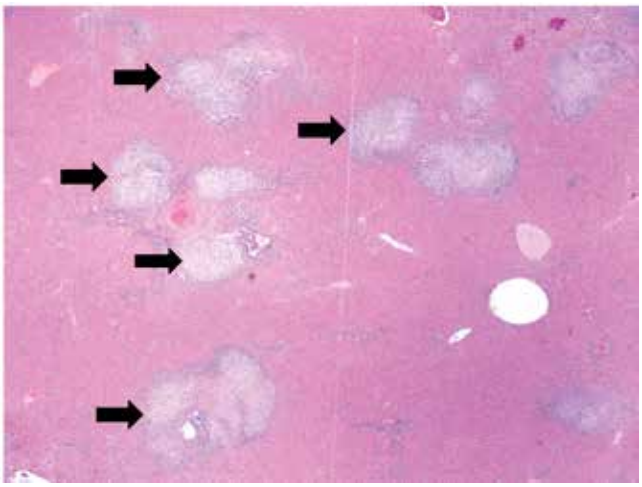

Figure 6. Liver granuloma from saline-treated guinea pig. Evidence of necrosis and fibrosis (arrows). Hematoxylin and eosin stain; magnification, 100×.

Discussion

The premise of this study was that a set of parameters could be identified and used as criteria for disease monitoring, to aid in establishing early indicators in guinea pigs experimentally infected with *M. tuberculosis.* We aimed to identify biomarkers that changed specifically due to shifting metabolic demands secondary to infection with *M. tuberculosis*, with or without systemic dysfunction. We conducted a thorough search and found that many commonly evaluated blood parameters did change, but many of these changes were not clinically relevant and, therefore may not have the potential as criteria for earlier intervention. WBC components showed the most appreciable differences between groups, and may be useful in tracking disease progression in the guinea pig model of tuberculosis.

In regard to body temperature, normal body temperature in guinea pigs ranges from 37.2 to 39.5 °C,[25] however our lab typically uses 40 °C as the upper limit, due to historical data from normal guinea pigs housed under ABSL3 conditions at our institution.[13] One animal had a transient increase in body temperature as high as 40.6 °C; however that value returned to a normal level within 4 d and was not correlated with impending mortality. We consistently see body temperatures rise within 25 to 30 d of infection, and BCG prevents this rise. However, the value of this aberrant readout needs further investigation to determine whether it could be used as an endpoint criterion.

Our data corroborated earlier studies that indicated tuberculosis pathogenesis in guinea pigs is very similar to that in humans.[4,10,17] In a previous study, human tuberculosis patients showed a slowly progressive, mild, nonregenerative anemia without prominent leukocytosis, but often with neutrophilia.[14] Although our guinea pigs were not anemic according to established reference ranges, there was a decrease in Hct in animals classified as survivors. It is possible that in guinea pigs that sufficiently control infection to survive, a form of anemia of chronic disease may be predictable and reproducible; further studies are needed to fully evaluate this. In addition, previous human tuberculosis patients had relatively normal liver function tests but increased serum immunoglobulins.[14] Our animal data parallel these findings, with minimally changed serum liver enzyme values and an elevation in serum globulins. Interestingly, despite seemingly normal bloodwork, bone marrow and liver biopsies in humans often show abnormalities including granulomatous inflammation without the presence of detectable organisms,[14] which we also found to be true with regard to liver histopathology in guinea pigs.

M. tuberculosis disseminates to the liver of guinea pigs, but the degree is variable and somewhat dependent on immune status.[10,17,20] BCG vaccination does not protect against the development of disease in guinea pigs, as in humans, but it does provide some protection against acquiring very severe and disseminated forms of disease.[17,26] Measures to evaluate the liver include the evaluation of enzymes detectable in the blood. ALP, ALT, glucose, albumin, and globulin were measured parameters in the serum biochemistry assay used for this study. Briefly, ALP is an inducible enzyme where elevations can be due to changes in isoenzymes of the liver, bone or gastrointestinal tract.[9,10] ALT is more indicative of hepatocellular damage but is not organ-specific in guinea pigs.[11] Glucose is highly variable and can be increased or decreased due to many factors including cirrhosis, stress, postprandial sampling, sepsis, chronic renal insufficiency, artifact of sample storage, and many others.[11] Because changes in glucose can be affected by many variables other than the liver specifically, we did not evaluate glucose as a liver-function enzyme. Albumin and globulin are both serum proteins that are made by the liver. Albumin decreases as both a negative acute-phase protein[16] and is directly related to decreased production by the liver. Globulin, however, is not as specific, given that it is a measure of both liver function and immune function, involving the production of antibodies.

Guinea pigs differed in their preinfection ALT values, with the BCG-vaccinated group higher than the saline-treated group, perhaps reflecting vaccination status. Over time, the saline group had an increasing trend in serum ALT, but values stayed within the normal reference ranges, and differences were not significant. Histopathologically, guinea pigs in the saline-treated group had much more severe and disseminated lesions affecting the hepatic parenchyma; these changes correlated with increased serum ALT over time as a direct result of hepatocellular damage. These data also corresponded to what is observed in humans: relatively normal liver function tests but abnormal liver biopsies in 92% of patients.[14]

Although the saline-treated group had more severe liver lesions than the BCG-vaccinated group on gross necropsy and histopathology (Tables 1 and 2), neither group showed extreme changes in serum levels of liver enzymes.

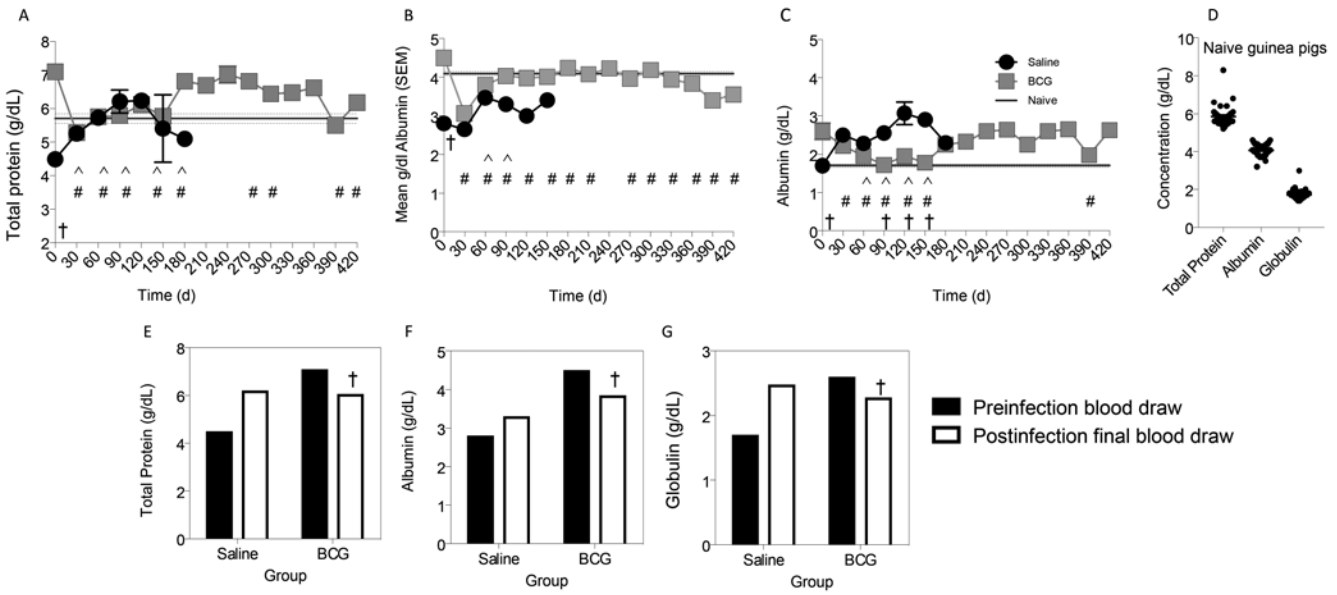

Figure 7. Comparison of serum total protein (A and E), albumin (B and F), and globulin (C and G) levels (mean ± SEM) in BCG-vaccinated, saline-treated, and negative control animals from prechallenge through the last scheduled blood draw. (D) In addition, individual naïve guinea pig blood draws taken at 5 different time point over 150 d are shown for each protein analyzed. For each serum component, significant (†, $P < 0.01$) differences occurred between groups at the prechallenge time point. (A) For total protein, significant differences occurred within the saline-treated group when comparing prechallenge values to all the following time points (^, $P < 0.01$) and within the BCG-vaccinated group when comparing prechallenge values with days 30, 60, 90, 120, 150, 300, 330, 390, and 420 (#, $P < 0.01$; day 330, $P = 0.01$). (B) For albumin, significant differences occurred within the saline-treated group when comparing prechallenge values with days 60 and 90 (^, $P < 0.01$) and within the BCG-vaccinated group when comparing prechallenge values with all the following time points, except day 240 (#, $P < 0.01$; day 180, $P = 0.03$). (C) For globulin, significant differences occurred between groups at prechallenge and days 90, 120, and 150 (†, $P < 0.01$); within the saline-treated group when comparing prechallenge values with all following time points (^, $P < 0.01$; day 150, $P = 0.02$); and within the BCG-vaccinated group when comparing prechallenge values with days 60, 90, 120, 150, 180, and 390 (#, $P < 0.01$; day 120, $P = 0.02$; day 180, $P = 0.03$). (E) Comparison of serum total protein between BCG-vaccinated and saline-treated animals from prechallenge to the last scheduled blood draw. There was a significant effect of treatment over time between groups (†, $P < 0.01$). (F) Comparison of serum albumin between BCG-vaccinated and saline-treated animals from prechallenge to the last scheduled blood draw. There was a significant effect of treatment over time between groups (†, $P < 0.01$). (G) Comparison of serum globulin between BCG-vaccinated and saline-treated animals from prechallenge to the last scheduled blood draw. There was a significant effect of treatment over time (†, $P < 0.01$).

ALP at the preinfection time point was significantly higher in the saline-treated group compared with the BCG-vaccinated group, but postinfection, there was no significant difference between groups. This result could indicate either an elevation in serum ALP from physiologic levels in the saline-treated group, an effect of BCG vaccination, or a combination of both. Although ALP, ALT, albumin, and globulin are not used as evaluation of liver function specifically, perturbations in these parameters can be due to hepatocellular leakage and damage to the liver.[11,23,25] Unfortunately, none of these parameters measured in the current study were significant enough to use as early endpoint criteria.

Infection caused a similar effect on serum albumin regardless of vaccination status, which was reflected as a predicted decrease due to the fact that albumin is a negative acute phase protein.[23] Given that the serum albumin level over time in naïve guinea pigs was stable, we are confident that the changes in the infected groups are sequelae of infection. The saline-treated group started with a lower serum albumin concentration prior to infection, but these values were not significantly lower than for the BCG-vaccinated group and likely represent normal average albumin levels for this subset of animals.

Serum globulin measurements showed a significant effect due to infection over time in the saline-treated group but not the BCG-vaccinated group. Although statistically significant, this change was small and not a reliable means to evaluate disease status in guinea pigs. Further studies are required to validate the use of serum globulin as a marker of disease status.

Other parameters for evaluating liver-specific pathology in humans and other animals include GGT, AST, sorbitol dehydrogenase, LDH, and total bilirubin; these parameters may be worth evaluating in future studies. Because hepatic forms of extrapulmonary tuberculosis occur in both BCG-vaccinated and saline-treated guinea pigs, identifying a specific hepatic biomarker for early endpoint criteria would be beneficial but is beyond the scope of the current study.

Leukocytosis was observed in the saline-treated group but was not present in the BCG-vaccinated group or naïve guinea pigs, thus reflecting a systemic inflammatory response over time in the saline-treated group. The leukocytosis observed in the saline-treated animals was due to increases in all subsets of leukocytes; however it most closely mirrored changes in heterophils.

BCG-vaccinated and saline-treated animals showed similar peripheral blood heterophil counts at the preinfection time point; after infection, the saline-treated group had a significantly higher heterophil count only at some points during disease progression. Interestingly, the saline-treated group experienced greater tissue inflammation and more severe tuberculosis-associated lesions, but these differences did not correlate with similar severity in peripheral blood counts. Together, these data suggest that heterophils in the saline-treated group may extravasate to the tissue in higher numbers and may become more aggressive and degranulated at a higher

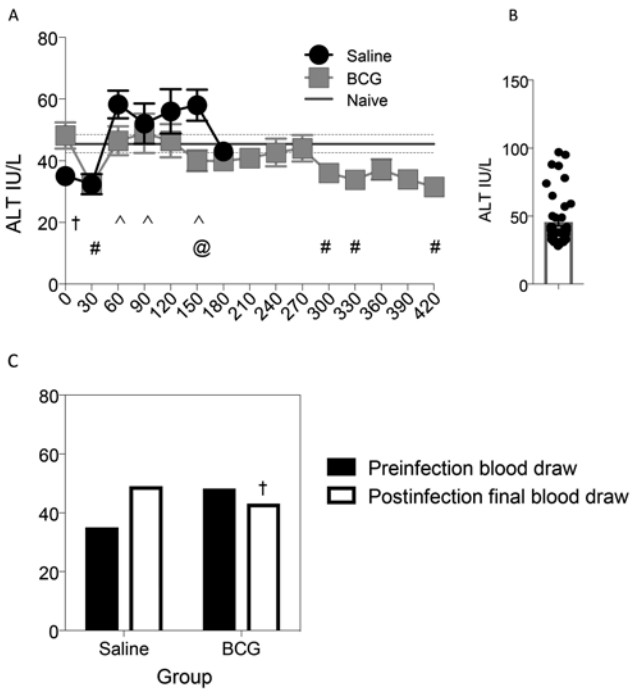

Figure 8. (A) Comparison of serum ALT levels (mean ± SEM) in BCG-vaccinated, saline-treated, and negative control animals from prechallenge through the last scheduled blood draw. Significant differences occurred between groups at preinfection blood draw and day 150 (@, $P = 0.03$); within the saline-treated group when comparing preinfection blood draw with days 60, 90, and 150 (^, $P < 0.01$); and within the BCG-vaccinated group when comparing preinfection blood draw with days 30, 300, 330, and 420 (#, $P < 0.01$; days 300 and 420, $P = 0.02$). (B) Serum ALT values from individual naïve guinea pig blood draws taken at 5 time points over 150 d are shown also. (C) Comparison of serum ALT between BCG-vaccinated and saline-treated animals from preinfection blood draw to the postinfection final blood draw. There was a significant effect of treatment over time (†, $P < 0.01$).

rate than those in the BCG-vaccinated group, consequently causing greater tissue destruction and pathology.[22,24] We know that heterophils are recruited to the pulmonary parenchyma and that a common route from the bone marrow to the tissue is through the vasculature; it may be that heterophils were in the vasculature only briefly before extravasating into tissue, thus further confirming that tuberculosis is a disease of tissues rather than blood.[14,21] Another possibility is that heterophils travel more in the lymph fluid than in the vasculature (similar to lymphocytes), and once in tissues, they remain there until they function or die. More extensive evaluation of heterophil trafficking needs to be conducted to fully evaluate these possibilities, given that the structure and function of efferent lymphatics are less well understood compared with emigration into the vasculature.[27]

When comparing guinea pig lymphocytes to human lymphocytes and the response to infection, guinea pigs are unique in that daily lymphocyte production is much higher than in humans, and the thymus is a major contributor.[28] In contrast to the situation in nearly every other mammalian species, including humans, lymphatic vessels are the main route of transfer of thymocytes to the general circulation in guinea pigs, largely due to an extensive efferent lymphatic network around the thymic artery.[15,16] This effect could be occurring in our guinea pigs in the lymphocyte response over time between the 2 groups, because the BCG-vaccinated group did not show an exaggerated response. Even in the saline-treated group, lymphocytosis was not of the magnitude described in humans.[14] With regard to peripheral blood lymphocytes, there were no significant differences between the groups of saline-treated and BCG-vaccinated guinea pigs at any time point. There was a significant elevation in both groups at 30 d, which indicated an initial increase in lymphocytes in the peripheral blood that remained constant throughout the disease process, independent of immune status. Again, this finding may not be due to an undetectable peripheral inflammation in guinea pigs but rather to a more severe tissue inflammatory component and lymphocyte travel through lymph fluid; additional studies might answer these questions.

Our data showed that although BCG-vaccinated guinea pigs developed classic tuberculosis lesions, the severity and dissemination was reduced when compared with the saline-treated animals, but slight differences were detectable in the hematologic readouts. These differences may be due to a synergistic effect caused by a decreased mycobacterial burden and the opposite in nonvaccinated animals. These trends correlated with a longer lifespan (Figure 2 A), decreased severity of pathology score (Table 1), and lower bacterial burden (Figure 2 B) in the BCG-vaccinated group. However, the question remains as to why the BCG-vaccinated guinea pigs succumb to infection, given that they do it does not appear to develop severe and disseminated tuberculosis or show marked changes observed in serum biochemical and hematologic analyses.

The overall inflammatory response in the peripheral blood of guinea pigs infected with *M. tuberculosis* showed few statistically significant differences between saline-treated and BCG-vaccinated guinea pigs. However, the histopathology score was more severe in the saline-treated group. This apparent dichotomy may be due to the methods for detecting inflammation. Alternatively we may be seeing what has been described previously,[15] briefly: that 1) guinea pigs do not have high levels of circulating inflammatory cells, 2) inflammatory cells may only circulate for a short period, and 3) these cells may also travel through lymph fluid as well as blood. There may be other parameters of greater interest for evaluating inflammation, which will require further investigation and may be used in conjunction with those used in the current study.

Our hypothesis was that one or more parameters would be associated with the progression of disseminated tuberculosis disease and have the potential to identify criteria for early predictive endpoints in guinea pigs experimentally infected with *M. tuberculosis.* After extensive data collection from 2 groups of guinea pigs exposed to *M. tuberculosis*—one group vaccinated with BCG and the other treated with saline—and comparison with naïve guinea pigs, we found that of the biomarkers we evaluated, only lymphocytes have potential to be used to monitor disease progression. However the majority of data fell within normal reference ranges for guinea pigs,[9,17] and therefore its utility may be limited. In general, our studies provide a starting point that can be used for further analysis and a better understanding of *M. tuberculosis* infection in the guinea pig model. More research needs to be conducted in this area to better characterize biomarkers to detect liver function, tissue, and lymphatic inflammatory responses in the peripheral blood, which would aid considerably in tracking disease progression among guinea pigs with differing vaccine status.

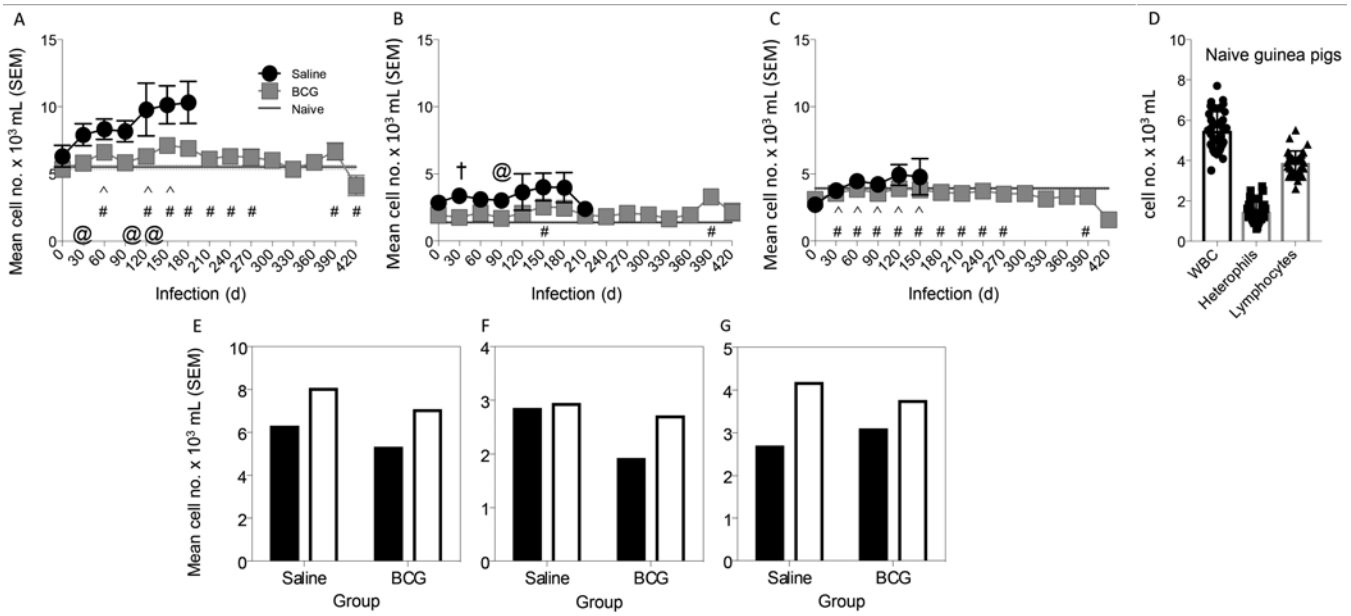

Figure 9. Comparison of (A and E) WBC, (B and F) heterophils, and (C and G) lymphocytes in BCG-vaccinated, saline-treated, and negative control animals from prechallenge through the last scheduled blood draw. (D) Data from individual naïve guinea pig blood draws taken at 5 time points over 150 d are shown also. (A) For WBC, significant differences occurred between groups at days 30, 90, and 120(@, $P = 0.02$); within the saline-treated group when comparing prechallenge with days 60, 90, 120, and 150 (^, $P < 0.01$); and within the BCG-vaccinated group comparing prechallenge with days 60, 120, 150, 180, 210, 240, 270, 390, and 420 (#, $P < 0.01$; days 120, 240, and 420, $P = 0.02$; day 210, $P = 0.04$; and day 270, $P = 0.03$). (B) For heterophils, significant differences occurred between infected groups at day 30 (†, $P < 0.01$) and day 90 (@, $P = 0.02$). There were no significant differences within the saline-treated group when comparing prechallenge with all following time points. There were significant (#) differences within the BCG-vaccinated group comparing prechallenge with day 150 ($P = 0.03$) and day 390 ($P < 0.01$). (C) For lymphocytes, there were no significant differences when comparing time points between infected groups. Significant differences occurred within the saline-treated group when comparing prechallenge with all following time points (^, $P < 0.01$) and within the BCG-vaccinated group comparing prechallenge with all following time points except days 270, 330, 360, and 390 (#, $P < 0.01$; day 300, $P = 0.01$). (E) Comparison of peripheral blood leukocytes between BCG-vaccinated and saline-treated animals from prechallenge to the last scheduled blood draw. There was no significant effect of treatment over time. (F) Comparison of peripheral blood heterophils between BCG-vaccinated and saline-treated animals from prechallenge to the last scheduled blood draw. There was no significant effect of treatment over time. (G) Comparison of peripheral blood lymphocytes between BCG-vaccinated and saline-treated animals from prechallenge to the last scheduled blood draw. There was no significant effect of treatment over time.

Acknowledgments

We appreciate the support of the NIH/NIAID program Advanced Small Animal Models for the Testing of Candidate Therapeutic and Preventative Interventions against Mycobacteria (HHSN-272201000009I-003, task order 12) at Colorado State University (AAI).

References

1. **Aceves-Sánchez MF-VM, Shanley C, Orme I, Bielefeldt-Ohmann H.** 2018. Vaccination of guinea pigs with BCGΔBCG1419c transiently reduces hematogenous spread of M. tuberculosis to the spleen. Pathog Dis **76:**1–4. https://doi.org/10.1093/femspd/fty088.
2. **Baldwin SL, D'Souza C, Roberts AD, Kelly BP, Frank AA, Lui MA, Ulmer JB, Huygen K, McMurray DM, Orme I.** 1998. Evaluation of new vaccines in teh mouse and guiena pig model of tuberculosis. Infect Immun **66:**2951–2959.
3. **Basaraba RJ, Izzo AA, Brandt L, Orme I.** 2006. Decreased survival of guinea pigs infected with *Mycobacterium tuberculosis* after multiple BCG vaccinations. Vaccine **24:**280–286. https://doi.org/10.1016/j.vaccine.2005.07.103.
4. **Dannenberg AM Jr.** 1982. Pathogenesis of pulmonary tuberculosis. Am Rev Respir Dis 125**:**25–29.
5. **Dharmadhikari AS, Basaraba RJ, Van Der Walt ML, Weyer K, Mphahlele M, Venter K, Jensen PA, First MW, Parsons S, McMurray DN, Orme IM, Nardell EA.** 2011. Natural infection of guinea pigs exposed to patients with highly drug-resistant tuberculosis. Tuberculosis (Edinb) **91:**329–338. https://doi.org/10.1016/j.tube.2011.03.002.
6. **Driver ER, Ryan GJ, Hoff DR, Irwin SM, Basaraba RJ, Kramnik I, Lenaerts AJ.** 2012. Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against Mycobacterium tuberculosis. Antimicrob Agents Chemother **56:**3181–3195. https://doi.org/10.1128/AAC.00217-12.
7. **Flynn JL,** 2006. Lessons from experimental *Mycobacterium tuberculosis* infections. Microbes Infect **8:**1179–1188. https://doi.org/10.1016/j.micinf.2005.10.033.
8. **Flynn JL, Capuano SV, Croix D, Pawar S, Myers A, Zinovik A, Klein E.** 2003. Non-human primates: a model for tuberculosis research. Tuberculosis (Edinb) **83:**116–118. https://doi.org/10.1016/S1472-9792(02)00059-8.
9. **Grover A, Troudt J, Arnett K, Izzo L, Lucas M, Strain K, McFarland C, Hall Y, McMurray D, Williams A, Dobos K, Izzo A.** 2012. Assessment of vaccine testing at three laboratories using hte guinea pig model of tuberculosis. Tuberculosis (Edinb) **92:**105–111. https://doi.org/10.1016/j.tube.2011.09.003.
10. **Grover A, Taylor J, Troudt J, Keyser A, Arnett K, Izzo L, Rholl D, Izzo A.** 2009. Kinetics of the immune response profile in guinea pigs after vaccination with *Mycobacterium bovis* BCG and infection with *Mycobacterium tuberculosis*. Infect Immun **77:**4837–4846. https://doi.org/10.1016/j.tube.2004.09.00810.1128/IAI.00704-09.
11. **Harkness J WP, VandeWoude S, Wheeler C.** 2010 Clinical procedures, p 122–130. Biology and medicine of rabbits and rodents. Ames (IA): Wiley–Blackwell.
12. **Izzo A, Brandt L, Lasco T, Kipnis AP, Orme I.** 2005. NIH pre-clinical screening program: overview and current status. Tuberculosis (Edinb) **85:**25–28. https://doi.org/10.1016/j.tube.2004.09.008.
13. **Keyser AT, Troudt JM, Taylor JL, Izzo AA.** 2011. BCG substrains induce variable protection against virulent pulmonary *Mycobacterium tuberculosis* infection, wtih the capacity to drive Th2 immunity. Vaccine **29:**9308–9315. https://doi.org/10.1016/j.vaccine.2011.10.019.

14. **Kim JH, Langston AA, Gallis HA.** 1990. Miliary tuberculosis: epidemiology, clinical manifestations, diagnosis, and outcome. Rev Infect Dis **12:**583–590. https://doi.org/10.1093/clinids/12.4.583.
15. **Kotani M, Seiki K, Yamashita A, Horii I.** 1966. Lymphatic drainage of thymocytes to the circulation in the guinea pig. Blood **27:**511–520. https://doi.org/10.1182/blood.V27.4.511.511.
16. **Kotani M, Manabe A, Yamashita A, Horii I.** 1967. Experimental formation of lymphatic nodules in the thymus of guinea pigs after injection of serotonin. J Anat **101:**805–812.
17. **Ordway D, Palanisamy G, Henao-Tamayo M, Smith EE, Shanley C, Orme IM, Basaraba RJ.** 2007. The cellular immune response to *Mycobacterium tuberculosis* infection in the guinea pig. J Immunol **179:**2532–2541. https://doi.org/10.4049/jimmunol.179.4.2532.
18. **Orme IM.** 2003. The mouse as a useful model of tuberculosis. Tuberculosis (Edinb) **83:**112–115. https://doi.org/10.1016/S1472-9792(02)00069-0.
19. **Orme IM, Ordway DJ**. 2016. Mouse and guinea pig models of tuberculosis. Microbiol Spectr **4:**1–20. https://doi.org/10.1128/microbiolspec.TBTB2-0002-2015.
20. **Palanisamy GS, Smith EE**, **Shanley C, Ordway D, Orme I, Basaraba R.** 2008. Disseminated disease severity as a measure of virulence of mycobacterium tuberculosis in the guinea pig model. Tuberculosis (Edinb) **88:**295–306. https://doi.org/10.1016/j.tube.2007.12.003.
21. **Pedrosa J, Saunders BM, Appelberg R, Orme IM, Silva MT, Cooper AM.** 2000. Neutrophils play a protective nonphagocytic role in systemic *Mycobacterium tuberculosis* infection of mice. Infect Immun **68:**577–583. https://doi.org/10.1128/IAI.68.2.577-583.2000.
22. **Radi ZA, Kehrli ME** Jr**, Ackerman MR.** 2001. Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration. J Vet Intern Med **15:**516–529. https://doi.org/10.1111/j.1939-1676.2001.tb01586.x.
23. **Ritchie RF, Palomaki GE, Neveux LM, Navolotskaia O, Ledue TB, Craig WY.** 1999. Reference distributions for the negative acute-phase serum proteins, albumin, transferrin, and transthyretin: a practical, simple and clinically relevant approach in a large cohort. J Clin Lab Anal **13:**273–279. https://doi.org/10.1002/(SICI)1098-2825(1999)13:6<273::AID-JCLA4>3.0.CO;2-X.
24. **Sawant KV, McMurray DN.** 2007. Guinea pig neutrophils infected with *Mycobacterium tuberculosis* produce cytokines which activate alveolar macrophages in noncontact cultures. Infect Immun **75:**1870–1877. https://doi.org/10.1128/IAI.00858-06.
25. **Shomer NH, Holcombe H, Harkness J.** 2015. Biology and diseases of guinea pigs, p 247–283. In: Fox JG, Anderson LC, Otto GM, Pritchett KR, Whary MT, editors. Laboratory animal medicine. San Diego CA): Elsevier.
26. **Turner OC, Basaraba RJ, Orme IM**. 2003. Immunopathogenesis of pulmonary granulomas in the guinea pig after infection with Mycobacterium tuberculosis. Infection Immun **71:**864–871. https://doi.org/10.1128/iai.71.2.864-871.2003.
27. **Weinreich MA, Hogguist KA.** 2008. Thymic emigration: when and how T cells leave home. J Immunol **181:**2265–2270. https://doi.org/10.4049/jimmunol.181.4.2265.
28. **Yoffey JM.** 1956. The mobilization and turnover times of cell populations in blood and blood-forming tissue. J Histochem Cytochem **4:**516–530. https://doi.org/10.1177/4.6.516.