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Establishment of a Neonatal Rhesus Macaque Model to Study *Mycobacterium tuberculosis* Infection

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Summary

Mycobacterium tuberculosis (Mtb) is the causative agent of human tuberculosis (TB) with an estimated 8.8 million new TB cases and 1.4 million deaths annually. Tuberculosis is the leading cause of death in AIDS patients worldwide but very little is known about early TB infection or TB/HIV co-infection in infants. A clinically relevant newborn animal model to study TB infection is urgently needed. We have successfully established an aerosol newborn/infant model in neonatal nonhuman primates (NHPs) that mimics clinical and bacteriological characteristics of Mtb infection as seen in human newborns/infants. Further, this model will allow the establishment of a TB coinfection model of pediatric AIDS. Aerosol versus intra broncho-alveolar Mtb infection was studied. Interestingly, 42 days post infection specific lesions were detected suggestive of the classic Ghon focus in human children. Concurrently, specific cellular immune responses developed 4–6 weeks after Mtb infection. Using the enzyme-linked immunospot (ELISPOT) assays, we found that IL-12 production correlated with early Mtb infection lesions seen by routine thoracic radiographs. Overall, this work represents the first example of early Mtb infection of newborn macaques. This study gives us a unique opportunity to further characterize immunopathogenesis and establish a TB/SIV co-infection model for pediatric AIDS.

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Ethical approval

Not required.

Competing interests

None declared.

Keywords

TB; rhesus macaque; newborn; Ghon focus; ELISPOT; IL-12

1. Introduction

Tuberculosis (TB) is a reemerging infectious disease in humans with over 14 million new infections and 8.8 million deaths annually¹. Tuberculosis is caused by *Mycobacterium tuberculosis* (Mtb)², which affects the lungs (pulmonary TB)³ but can affect other sites (extrapulmonary TB)^{4, 5}. Disease-causing organisms are spread into the air when people with pulmonary TB expel bacteria, for example, by coughing. The tubercle bacilli are transmitted from person to person as an infectious aerosol; the most contagious people are those whose respiratory secretions contain more than 10⁴ organisms/ml. Among those initially infected, a small percentage (5–10%) fail to control initial infection and develop primary TB disease. The majority of humans infected with this organism mount an effective immune response resulting in latent infection. However, reactivation can occur years to decades later in a small subset of infected persons, leading to active TB⁶. Symptoms considered suggestive of TB include cough for more than two weeks, persistent fever, night sweats and weight loss⁷.

Diagnosis of TB is complicated especially in children since Mtb isolation from clinical specimens is extremely difficult⁸. Tuberculosis in children is often imperceptible and can rapidly progress to active disease⁹. Under 2 years old children often progresses to disease within 12 months. During childhood (5–10 years old) the risk of disease progression is at its lowest but increases again during adolescence. Immune-competent children of at least 3 years old with latent TB carry a risk of future reactivation and of becoming a reservoir for future disease in adulthood^{10, 11}. Initial Mtb infection is characterized by a Ghon focus with regional lymphadenitis, called Ghon complex or primary complex¹². In most children, this complex resolves spontaneously leaving scarring or calcification. However, some children can develop progressive primary tuberculosis leading to hematogenous dissemination to lung and other organs¹³.

Childhood tuberculosis diagnostic represents a major challenge and usually relies on poorly validated clinical case definitions¹⁴. The most common radiological finding and hallmark of children primary TB is hilar lymphadenopathy with/without parenchymal lesions. Bacteriological confirmation is limited because childhood TB tends to be paucibacillary with poor bacteriologic yield. Sputum smear microscopy, which is how most TB cases are diagnosed worldwide, is positive in less than 10–15% of all pediatric samples. In addition, traditional tuberculin skin test has poor sensitivity in HIV-infected children and poor specificity in BCG-vaccinated children, but still remains a fairly accurate measure in immune-competent children. Gamma-Interferon (INF- γ) release/T-cell based assays compared to Tuberculin skin test demonstrate moderately improved performances in children over 2 years old^{15, 11}.

There are no natural Mtb hosts other than humans. Several animal models such as mice, guinea pigs, rabbits and nonhuman primates (NHP), are currently used for vaccines

development and drug candidates¹⁶, with routes of inoculation ranging from aerosol, intratracheal, intranasal, intravenous to intraperitoneal^{16, 17}. Despite their cost, NHP models are often used to study infectious diseases because of their genomic, physiologic and immunologic similarities with humans¹⁸. Most commonly used include Rhesus macaques (*Macaca mulatta*), which are shown to be susceptible to TB, and cynomolgus macaques (*Macaca fascicularis*), which are more resistant¹⁶. However, both species have very similar clinical and pathological Mtb manifestations, including pulmonary granulomata with calcification, caseation, cavitation and infection of tracheobronchial lymph nodes¹⁹, and mimic all pathological features observed in human TB^{20, 21}.

There is a critical need for a neonatal primate model that mimics TB disease in human children to better understand the early immune responses, pathogenicity and pediatric Mtb disease progression. In this study, we established a neonatal macaque model to investigate clinical, bacteriological and immunological characteristics of pediatric tuberculosis. We produced evidence, within limitations of the small animals number studied, that newborn macaques can be infected with aerosolized Mtb, which make them an ideal animal model to study Pediatric TB.

2. Materials and methods

2.1. Animals

Newborn rhesus macaques (*Macaca mulatta*) of Indian origin were used and obtained from the SIV-negative and type D retrovirus-free colony at the Southwest National Primate Research Center (SNPRC) in San Antonio (TX). Animals were bred, hand reared, and housed on site in accordance to the Guide for the Care and Use of Laboratory Animals and standards outlined by the American Association for Accreditation of Laboratory Animal Care. Animals were transferred to the ABSL-3 prior to experimental Mtb infection.

2.2. *Mycobacterium tuberculosis* stock and inoculums

Mtb Erdman strain was obtained from the ATTC (#35801) as frozen suspension. This stock was thawed and cultured in Middlebrook 7H9 broth with ADC supplement for 6 weeks at 37°C (5% CO₂). Serial dilutions were performed using the McFarland 1.0 as turbidity standard to a final concentration of 1×10³ colony-forming units (CFU)/ml. This stock was stored in aliquots at -80°C and used to prepare consecutive Mtb doses (serial dilutions in sterile saline). Prior Mtb-infection, each inoculum was confirmed by plating an aliquot onto 20 Middlebrook 7H10/ADC plates, and by calculating bacterial numbers (Figure 1A). The average CFU for each dose was: i. 4.8CFU (±1.105) for the estimated 5CFU dose; ii. 9.95CFU (±3.561) for the estimated 10CFU dose; and, iii. 49.2CFU (±2.088) for the estimated 50CFU dose.

2.3. Experimental *Mycobacterium tuberculosis* infection

Uninfected newborn macaques were housed at the SNPRC for 5 weeks prior transfer to the ABSL-3 (Figure 1C). At 6–7 weeks old, animals were inoculated with Mtb Erdman via the bronchoalveolar or aerosol inoculation routes. Both routes were used in parallel to determine the minimum dose necessary to mimic mother-to-child Mtb transmission.

2.4. Bronchial-alveolar exposure

Animals were fasted for 12 hours to lessen the chance of anesthesia-induced emesis and were sedated with an intramuscular sedative (Telazol 5mg/kg) to promote muscle relaxation and loss of consciousness. Infants were placed in ventral recumbence with heads slightly arched to help the veterinarian positioning a 2-mm diameter flexible pediatric endotracheal tube at the laryngeal folds to deliver the appropriate Mtb inoculum. Lidocaine was applied to the tracheal and bronchial mucosa to alleviate laryngospasm and coughing. After inoculation, 0.25–0.30 ml of saline was flushed through the endotracheal tube to ensure that the entire dose was given. The infant was monitored until fully recovered.

2.5. Aerosol exposure

An aliquot of Mtb stock (1×10^3 CFU/ml) was thawed and diluted appropriately with sterile distilled water, as described above. The dose was adjusted for a final volume of 1 ml thereby increasing the total volume of inoculum to improve proper aerosol delivery. A beetle neb compact ultrasonic nebulizer, specifically adapted to newborn/infant macaque nose, was used to convert the inoculum into a mist (Figure 1B). The nebulizer rate flow was 0.5ml/minute and the dose administered was in CFU/ml. The inoculum was administered until the chamber was empty of its content. The nebulizer was then rinsed with an additional 2 ml of sterile water and run again to ensure that all the inoculum was inhaled. Animals were only lightly sedated to promote muscle relaxation and were monitored throughout the procedure. Each inoculum was confirmed through samples collected before and after inoculation by plating an aliquot onto 20 Middlebrook 7H10/ADC plates to determine the bacterial numbers in each sample after 3 weeks incubation (37°C +5% CO₂).

2.6. Monitoring post-Mtb infection

At least twice per day, trained veterinary technical staff closely monitored all animals for changes in behavior, activity level, hydration status, and stooling. Body weights and temperature were recorded weekly. Physical examination and laboratory testing such as CBC were performed every two weeks or whenever deemed necessary. Animals were monitored daily for any clinical symptoms associated with TB (e.g.: breathing difficulty; coughing; increased mucus secretion; lethargic behavior; weight loss; opportunistic infections).

2.7. Thoracic Radiographs

Thoracic radiographs (SP VET 3.2; Xograph Imaging Systems Ltd.) were acquired (ventral-dorsal, right-left lateral thoracic radiographs) before and every two weeks thereafter Mtb-inoculation. A pediatric TB specialist and a board-certified veterinary radiologist (Veterinary Imaging Center of South Texas) evaluated all thoracic radiographs.

2.8. Blood collections

Blood samples were collected at week 0 (baseline) and every two weeks, as shown in the overall animal's procedures (Figure 1C). EDTA-treated bloods were used for Complete Blood Counts (CBC) determination using a VetScan HM2 Hematology System (Abaxis, WA). Peripheral blood mononuclear cells (PBMC) were separated by centrifugation using a

standard Lymphocyte Separation Medium (MP Biomedical, Solon, OH) and counted using Trypan blue to identify dead cells. PBMC were kept on ice for flow cytometry and ELISPOT, as described below.

2.9. Broncho-alveolar lavages

Animals were fasted for 12 hours, sedated with Telazol (5mg/kg) and placed in ventral recumbence with heads slightly arched. A 2.5-mm-outer-diameter flexible bronchoscope (Wolf Medical Instruments Incorporated, Vernon Hills, IL) was used to instill 5 ml of sterile saline. The broncho-alveolar lavage (BAL) recovery was generally 70%. BAL fluids were decontaminated and plated for bacterial burden determination. Isolated PBMC were kept on ice for flow cytometry and ELISPOT, as described below.

2.10. Gastric Lavages

Gastric lavages (GL) were collected under sterile conditions pre- and at weeks 4, 6, 8, 10, and 12 post-Mtb. GL were decontaminated and plated for bacterial burden determination, as described below.

2.11. Necropsy procedures

Blood, spleen, thymus, lymph nodes (inguinal, axillary, mesenteric, hilar, cranial-bronchial), lungs (right, left), liver, BAL and GL were collected during necropsy. All tissues were placed in RPMI medium 1640 supplemented with 1% L-glutamine, 1% 1M HEPES buffer, 1% penicillin-streptomycin, 10% FBS) and processed immediately.

2.12. Pathology

Gross pathology evaluation and tissues/organs collection were performed at necropsy. Samples were divided and either snap-frozen for Mtb culture or fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5µm, and stained with hematoxylin and eosin (H&E) according to standard protocols. A board certified veterinary pathologist and a pediatric TB specialist evaluated all histologic sections.

2.13. Tissues homogenization

Portions of liver, spleen, and lung (right, left) were sampled for viable Mtb bacteria postmortem. Each sample weight was determined prior tissue homogenization. Briefly, tissues were macerated, washed several times with PBS, cut into small pieces, placed in MACS M-Tubes system and processed to homogenization using a specified program (RNA-01). Once all cycles were completed, M-Tubes contents were transferred into 50ml conical tubes ready for tissue decontamination and bacterial burden determination.

2.14. Tissues and fluids decontamination

Equal volumes of homogenized-tissue solution and NALC-NaOH digestant solution were added to each sample, vortexed for 15 seconds (or until liquefied) and incubated 15 minutes at room temperature. Recovered suspensions from GL and BAL were decontaminated with addition of NALC-NaOH digestant solution, as described above. Each mixture was vortexed

with addition of PBS (40 ml, final volume) and centrifuged for 30 minutes at 3200 RPM. Each pellet was resuspended in 1ml cold PBS and kept on ice until used.

2.15. Bacterial recovery

Following decontamination, 10 μ L of each final solution (tissues, BAL, GL suspensions) were plated in 7H11 Middlebrook media (tissues) or onto 20 7H10 agar plates (BAL, GL) and incubated for 4 weeks (37°C +5% CO₂).

2.16. ELISPOT

ELISPOT assays were performed on freshly isolated PBMC from bloods collected at week 0, 2, 4, 6, 8, 10, and 12 post-infection, and various tissues/organs collected at necropsy. Interferon- γ and IL-12 ELISPOT kits were used (MABTECH, Stockholm, Sweden), as previously described¹². Briefly, 96-well multiscreen immunoprecipitation filtration plates (Millipore Corp., Bedford, MA) were coated with monoclonal antibody GZ-4 (100 μ L) diluted in PBS from 1mg/ml to 15 μ g/ml (IFN- γ) or 0.5mg/ml to 10 μ g/ml (IL-12). Plates were incubated overnight (4°C) or for 2 hours (37°C), washed with PBS (200 μ L) and blocked for 30 minutes with 200 μ L of complete RPMI (room temperature). The blocking media was removed by inverting the plate over a waste container. Both ESAT-6 and PPD were used as Mtb-specific antigens. Concanavalin A (ConA, Sigma) and RPMI were used as positive and negative controls. PBMC from 10 uninfected newborn macaques (SNPRC breeding colony) were used as negative controls. The cut-off was set at 20 dots per million cells, determined by the non-specific responses detected with both ELISPOT kits.

According to the experiment, 100 μ L of designated cell suspension was added to each well (3.5 $\times$ 10⁵/well) with ESAT-6 (20 μ g/ml, antigen), PPD (1 μ L/100 μ L, antigen), Concanavalin A (5 μ g/ml, positive control), or RPMI (negative control). Plates were incubated for 10–16 hours (37°C), washed with PBS, and 100 μ L of detection antibody (7-B6-1-biotin) diluted from 1mg/ml to 1 μ g/ml (PBS +0.5% FBS) was added per well. Plates were incubated for 2 hours (room temperature), washed with PBS and incubated with Streptavidin-alkaline phosphatase (100 μ L, at 1:1000)¹². Plates were washed again, incubated with 100 μ L of substrate and kept until dark spots emerged (15–20 minutes). Plates were washed with 200 μ L distilled water to stop the reaction, left overnight to dry and analyzed (Zellnet Consulting, Inc., NY).

2.17. Flow Cytometry Surface Staining

PBMC were incubated for 30 minutes (dark) with monoclonal antibodies for surface staining: CD3 (phycoerythrin [PE], Alexa 700, or V500; clone SP34; BD), CD8 (Pacific Blue; clone 3B5; Invitrogen), CD4 (peridinin-chlorophyll-protein complex [PerCP]-Cy5.5; clone L200; BD), CD16 (FITC; clone 3G8; Caltag). After incubation, samples were washed and fixed with 300 μ L of 2% formaldehyde (Polysciences, Inc., Warrington, PA). Flow samples were processed using a Dako Cyan ADP flow cytometer and analyzed with FlowJo software (Treestar, Inc.).

3. Results

3.1. Pediatric Mtb infection

The primary goal was to develop a neonatal primate model of *Mtb* infection that reflected clinical and bacteriological characteristics of the disease as seen in human newborns/infants. Three newborn macaques were infected with Mtb Erdman using: i. a low Mtb dose (5CFU) via the broncho-alveolar route of infection; ii. an intermediate Mtb dose (10CFU) via the aerosol route of infection; or, iii. a higher Mtb dose (50CFU) using the aerosol route of infection (Table 1). The objectives were to: (i) Favor the aerosol route of Mtb inoculation to mimic natural neonatal Mtb exposure, and (ii) Determine which dose would be high enough to cause infection, but not so high that animals would succumb too quickly and preclude experimental interventions (Table 1). The broncho-alveolar route was used as a positive control to assess the *in vivo* effectiveness of low inoculum (5 CFU). Each Mtb Erdman dose was confirmed *in vitro* by plating 10 μ l of the inoculum onto 20 7H10 agar plates to determine the bacterial numbers for each sample, as described above (Figure 1A).

Aerosol Mtb inoculations were performed using a pediatric nebulizer, which was modified to fit the nose of each newborn macaque (Figure 1B). For the broncho-alveolar infection route, a flexible small feeding tube was used (not shown). To avoid any type of distress and/or discomfort during Mtb infection, the newborns were lightly sedated. Both routes of infection were well tolerated; however the broncho-alveolar route was only performed as a comparison to optimize the low-dose aerosol necessary to infect the animal. An overall summary of the experimental design is shown in Figure 1C. Evidence of infection was detected in all animals using at least one assay, as described below.

3.2. Weight and body temperature

Newborn/infant macaques were observed daily for signs of illness or distress, as described above. Weight and body temperature measurements were performed weekly (Figure 2). Each animal weight gain was gradual (Figure 2, left) and body temperature remained at a normal range (Figure 2, right). Overall, the growth curve of each *Mtb*-infected newborn/infant was comparable to that of uninfected age-matched animal.

3.3. Phenotypic characterization of PBMC from Mtb-infected newborn macaques

Flow cytometry was performed to define the phenotypic characteristics of cells from blood, BAL fluid, and tissues at necropsy. The percentage of CD4⁺CD3⁺ T cells (68% $\pm$ 7%) and CD8⁺CD3⁺ T cells (21% $\pm$ 6%) in PBMCs did not change over the course of infection and did not differ significantly from pooled data from uninfected age-matched infants macaques (CD4⁺CD3⁺, 65% $\pm$ 9%; CD8⁺CD3⁺, 25% $\pm$ 14%). The Figure 3 represents the longitudinal results plotted as absolute count/mm of total T lymphocytes (CD3⁺ T cells) and true CD4⁺CD3⁺CD8⁻ Helper T cells and true CD3⁺CD8⁺CD4⁻ cytotoxic T cells.

Natural killer (NK) cells were also evaluated using the phenotype of CD3⁻CD8⁺. This phenotype is generally considered to be appropriate for the identification of NK cells in macaques as CD56, which is used in humans, occurs at a high frequency on monocytes in macaques, and has been shown to be inadequate to identify rhesus NK cells. Furthermore,

CD16 alone may underestimate the number of NK cells defined by the phenotype CD3⁻CD8⁺. Overall, a significant increase of CD3⁻CD8⁺ and CD3⁻CD8⁺CD16⁺ Natural Killer (NK) T cells was seen post-Mtb infection in all infected animals (Figure 3). In cells recovered in the BAL, the T-cell percentages were variable (CD4⁺CD3⁺, 44% $\pm$ 8%; range, 36 to 52%; CD8⁺CD3⁺, 46% $\pm$ 15%; range, 31 to 61%). This profile was not significantly different from BAL fluid of uninfected animals (data not shown). There was no obvious trend towards decreasing or increasing T-cell absolute numbers or percentages over the 12 weeks of Mtb-infection. At necropsy, no obvious change in T cell percentages in various LN were detected compared to age-matched infants macaques.

To determine the distribution of leukocyte subsets in newborn macaques, blood was collected at multiple time points, ranging from birth and up to week 84. Complete blood counts were performed using a VetScan HM2 Hematology System (Abaxis) in which the cell size discriminators were adjusted for rhesus macaques.

3.4. Monitoring by chest X-radiograph

Chest X-rays were performed on each newborn prior to infection and every two weeks thereafter until the animal was euthanized. The low-dose (5CFU) Mtb Erdman delivered directly into the bronchus (bronchoscope) resulted in a discrete nodule at day 42 post-infection (Figure 4, animal 31242). The nodule was absent at day 28 but was clearly visible on the CXR anteroposterior (AP) view taken on day 42 (Figure 4). By day 56, the lesion had radiographically resolved.

An intermediate dose of Mtb Erdman (10CFU) was introduced via nebulized aerosol into the lungs of animal 31338. At day 28 post-infection, a right upper lobe lung lesion was noted on the CXR, which became more prominent at day 42 but started to resolve at day 56 (Figure 4, animal 31338). Animal 31669 was infected with higher dose (50CFU) of Mtb Erdman via the aerosol route and the CXR showed a small infiltrate in the left lower lobe that was not present at baseline but resolved at day 42 (Figure 4, animal 31669).

The timing of these lesions were significant, as it is known that the initial incubation-period post-Mtb infection can last 2–6 weeks before any evidence of acquired immunity can be seen, as indicated by a positive tuberculin skin test in human infants. These transient lesions in the lung are due to focal replication of Mtb at the site of uptake by macrophages post-infection. In human infants, such lesion would be referred to as a Ghon focus. Lesions appeared visible in the AP CXR of all animals between day 28 and 56 post-infection but were most prominent at day 42 (Figure 4). Overall, more diffuse lesions were noted on CXR from neonates infected by the aerosol route compared to the lesion seen in the animal infected by the broncho-alveolar route.

3.5. Histopathology and clinical outcome

To thoroughly assess the level of Mtb infection in newborn/infant macaques, several tissues collected at necropsy were evaluated for TB pathology, i.e., (i) the lung as the primary site of TB-specific pathology, (ii) the lung-draining bronchial LNs, and (iii) the spleen as a more distal lymphoid indicator of Mtb dissemination (Table 2). Microscopic examination of each animal's lungs and LN tissues showed lesions consistent with Mtb infection. At necropsy,

pathologic evaluation of LN tissues from animal 31242 revealed an early granuloma (arrow, Figure 5A). Microscopic examination of lung tissue from animal 31338 showed interstitial epithelioid macrophage infiltrates in the pulmonary interstitium (Figure 5B). In newborns infected by the aerosol route, macrophages infiltration was observed in both right and left lungs.

Lymphoid and non-lymphoid tissues were collected at necropsy (see 2.5 section). In a Ghon focus, the organisms are transported to the associated lymph nodes in the hilum or mediastinum, referred to as a Ghon complex, with the lung lesion. In the majority of the newborns, the hilar cranial lymph nodes were enlarged (Table 2). These lesions were comprised of large areas with characteristic pathology (Table 2) revealing active lymph nodes with lymphoid hyperplasia (Figure 5C) as compared to a normal unstimulated lymphoid tissue (Figure 5D).

Overall, our results indicated that even low-dose Mtb-infection caused discernible pathological changes within a relatively short period of time. However, no clinical symptoms indicative of Mtb-infection (e.g., breathing difficulties, coughing) were observed at any time during the study period. Furthermore, all newborn/infant macaques showed a weight gain (Figure 3) similar to that of age-matched control animals.

3.6. IFN- γ and IL-12 responses

Enzyme-linked immunospot (ELISPOT) assays for both IFN- γ and IL-12 were performed to monitor cellular responsiveness over time in PBMC obtained from each blood samples and the various tissues/organs collected postmortem, according to schedule (Figure 2B). PBMC isolated from 10 additional uninfected newborn macaques blood samples (SNPRC breeding colony) were also processed and used as naïve unexposed controls.

Both Mtb-specific IFN- γ and IL-12 immune responses were detected by ELISPOT in all Mtb-infected macaques (Figure 6A, 6B) with variable SFC values depending on the antigen used (PPD or ESAT-6). Mtb-antigen specific SFC counts were higher for IL-12 response against PPD and ESAT-6 compared to INF- γ (Figure 6). As expected, newborn 31669 infected with high aerosol dose of Mtb Erdman (50CFU) had higher IL-12 specific responses to PPD and ESAT-6 that peaked between day 42 and 49 post-Mtb infection but significantly decreased by day 70 (Figure 6A). As expected, ELISPOT analysis of cellular responses in the lymph nodes of newborn/infant macaques euthanized at week 12 post-Mtb infection revealed strong responses to both INF- γ and IL-12 (Figure 6B).

4. Discussion

Pediatric tuberculosis continues to be a neglected disease in many endemic areas where limited resources restrict treatment to only the most infectious tuberculosis cases. However, recognition that children contribute to a significant proportion of the global TB disease burden and suffer severe TB related morbidity and mortality is growing^{22, 23}. Tuberculosis is the leading cause of death in AIDS patients worldwide and is one of the earliest opportunistic infections occurring in conjunction with HIV²⁴. Each year, one million children worldwide become newly infected with tuberculosis and 420,000 with HIV.

Animal studies can help address critical questions that cannot be answered in human studies, computer models or laboratory dishes. Nonhuman primates are the preferred animal models for pre-clinical research because they are physiologically and genetically closely related to humans compared to other animal models²⁵. Recent studies have shown that macaques infected with Mtb can develop pathology similar to human tuberculosis^{26, 27}, which make them an excellent animal model to assess critical early time-points post-Mtb infection.

We developed a neonatal macaque model to investigate clinical, bacteriological and immunological characteristics of early human pediatric tuberculosis. Mtb Erdman was chosen to optimize a best dose and route of infection to produce asymptomatic but active infection. Low dose (5CFU) of Mtb Erdman delivered directly into the bronchus resulted in a discrete nodule at day 42 post-infection that was clearly visible on the anteroposterior CXR, comparable to infants Mtb-infection. Histological changes consistent with early Mtb-infection were also observed in lungs and thoracic lymph nodes. Although neonatal macaques were monitored for less than 3 months and had no visible clinical sign of disease, early Mtb-specific cellular immune responses were detected. The combination of all clinical, radiologic, laboratory, and histopathologic findings were consistent with early TB diagnosis. Mtb-specific immune responses were detected within the first 3–5 weeks suggesting that newborns can generate an immune response as early as 28 days post-infection correlating with establishment of early TB.

We demonstrated that newborn macaques could develop true human-like early infection characterized by the absence of clinical symptom but presence of antigen-specific immunologic responses as measured by INF- γ and IL-12 release assays. We showed that Mtb-infection in neonatal macaques closely mimics Mtb-infection as observed in human newborns/infants. Neonatal macaques provide a unique opportunity to understand early immune responses, gain deeper insights into the immunopathogenesis of pediatric TB, and investigate the efficacy and safety of cocktail treatments and vaccine strategies.

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Figure 1.

(A) Each inoculum dose was confirmed by plating onto 7H10 agar plates. (B) An ultrasonic nebulizer was used for aerosol Mtb infection. The flow rate of the ultrasonic nebulizer was at 0.5 ml/minute. (C) Experimental design, animal procedures and assays performed

Figure 2.

Weight and Temperature charts with relevance to Erdman infected newborns. The growth curves of all Redman-infected newborns are comparable to that of naïve animal. Weight gain was gradual and temperature remained at a normal range for all animals. Each graph represents the weight in kilograms (Kg) and the temperature in Fahrenheit (F).

Figure 3.

Absolute frequency (number) of lymphocyte subsets in Mtb-infected newborn/infant macaques. Those included CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ T lymphocytes as well as CD3⁻CD8⁺ and CD3⁻CD8⁺CD16⁺ NK cells isolated from freshly collected blood samples over the 12 weeks study.

Figure 4. Chest radiograph of neonatal rhesus macaques infected with *M. tuberculosis*. Parenchymal lesions are circle.

Figure 5.

Representative microscopic examination of lung and lymph node tissues from a newborn macaque showing typical lesions consistent with *M. tuberculosis* infection. (A) Lymph node: epithelioid macrophages with early granuloma formation (arrow) 31242; and, (B) Lung infiltrates with interstitial epithelioid macrophages are shown. (C-D) Lymph node tissues from a neonatal macaque infected via the aerosol route with low dose *M. tuberculosis*. Panel C shows a low magnification (2× magnification) view of the lymph node with numerous active germinal centers. Panel D shows a normal lymph node tissue for comparison.

Figure 6.

Immune responses to *Mycobacterium tuberculosis* infection in newborn macaques. (A) Frequency of PPD and ESAT-6 specific IFN- γ and IL-12 secreting cells measured by ELISPOT assay. The secretion of IFN- γ and IL-12 was measured longitudinally in PBMC isolated from two newborn macaques (animals: 31338 and 31669) infected with aerosol Mtb Erdman at 10 and 50 CFU, respectively. (B) Frequency of PPD and ESAT-6 specific IFN- γ and IL-12 secreting cells isolated from multiple tissues/organs postmortem.

Table 1

Neonatal rhesus macaques infected with *M. tuberculosis* and disease outcome.

Newborn	31242	31338	31669
Weight at birth (Kg)	0.442	0.546	0.369
Age at infection (weeks)	7 weeks	6 weeks	6 weeks
<i>Mycobacterium tuberculosis</i> strain	Mtb Erdman	Mtb Erdman	Mtb Erdman
Inoculum dose (CFU/ml)	5 CFU	10 CFU	50 CFU
Route of inoculation	Broncho	Aerosol	Aerosol
Age at euthanasia (weeks)	13 weeks	12 weeks	12 weeks
Animal status at euthanasia	Alive No signs of disease	Alive No signs of disease	Alive No signs of disease

Table 2

Pathology Reports for animals inoculated with Mtb Erdman strain. CFU: Colony-Forming Units. LN: Lymph Node.

Newborn Mtb strain (Route/dose)	31242 Mtb Erdman (Broncho/5 CFU)	31338 Mtb Erdman (Aerosol/10 CFU)	31669 Mtb Erdman (Aerosol/50 CFU)
Hilar LN	Enlarged LN; Influx of macrophages; Giant cells infiltrated in cortex	Enlarged LN	No-significant lesion
Cranial-bronchial LN	Enlarged LN; Influx of macrophages; Giant cells infiltrated in cortex	Enlarged LN	No-significant lesion
Right Lung	Several macrophages, foamy in appearance	Increased number of Macrophages	Increased number of Macrophages
Left Lung	Several macrophages, foamy in appearance	Increased number of Macrophages	Increased number of Macrophages
Diagnosis	Slight Pneumonia: interstitial, perivascular, lymphocytic	Pneumonia: multifocal, histolytic and lymphocytic with type II pneumocystis hyperplasia	Excessive cells in alveoli associated with Broncho-alveolar mucus