Differential Virulence and Disease Progression following \textit{Mycobacterium tuberculosis} Complex Infection of the Common Marmoset (\textit{Callithrix jacchus})

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Existing small-animal models of tuberculosis (TB) rarely develop cavitory disease, limiting their value for assessing the biology and dynamics of this highly important feature of human disease. To develop a smaller primate model with pathology similar to that seen in humans, we experimentally infected the common marmoset (\textit{Callithrix jacchus}) with diverse strains of \textit{Mycobacterium tuberculosis} of various pathogenic potentials. These included recent isolates of the modern Beijing lineage, the Euro-American X lineage, and \textit{M. africanum}. All three strains produced fulminant disease in this animal with a spectrum of progression rates and clinical sequelae that could be monitored in real time using 2-deoxy-2-[\textsuperscript{18}F]fluoro-D-glucose (FDG) positron emission tomography (PET)/computed tomography (CT). Lesion pathology at sacrifice revealed the entire spectrum of lesions observed in human TB patients. The three strains produced different rates of progression to disease, various extents of extrapulmonary dissemination, and various degrees of cavitation. The majority of live births in this species are twins, and comparison of results from siblings with different infecting strains allowed us to establish that the infection was highly reproducible and that the differential virulence of strains was not simply host variation. Quantitative assessment of disease burden by FDG-PET/CT provided an accurate reflection of the pathology findings at necropsy. These results suggest that the marmoset offers an attractive small-animal model of human disease that recapitulates both the complex pathology and spectrum of disease observed in humans infected with various \textit{M. tuberculosis} strain clades.

The emergence of multidrug and extensively drug-resistant (MDR and XDR) tuberculosis (TB) has spurred global efforts to develop novel agents for treatment of patients infected with such strains. Agents that shorten the duration of chemotherapy for drug-sensitive TB could limit emergence of resistant strains by improving treatment completion rates (1). By some estimates there are as many as 14 new agents approaching clinical trials (2), and there are more than a dozen second-line agents in clinical use for MDR and XDR disease. Prioritizing drug combinations to test clinically based on a prediction of treatment duration is currently done using murine models that do not develop the hallmark pathologies of human disease (3). In many phase 3 studies, however, the presence of extensive pathology, particularly cavitary disease, is a significant risk factor for subsequent development of relapse disease after therapy is stopped (4–6). Treatment shortening predicted from murine chemotherapy studies has motivated recent phase 3 trials where the anticipated effect was not observed, causing skepticism to emerge regarding the predictive validity of this model (7). Therefore, there is an urgent need to develop additional animal models likely to be predictive of human therapeutic potential and to subject these models to careful validation studies using tools that are translatable into clinical trials.

Nonhuman primates (NHP), some species of which suffer from TB as a naturally occurring and highly transmissible zoonosis, develop disease that is virtually identical to that seen in humans (8). Despite this, they have been employed only sparingly as models of TB chemotherapy because of their size and expense. We have recently shown the feasibility of using nonhuman primates as a model for TB chemotherapy and prophylactic therapy using cynomolgus macaques (9). These are large, outbred animals, however, which limits the number of animals that can be studied, and they require large doses of prospective antibiotics, which limits the number of agents available to study. We therefore decided to explore the smaller New World primates and identified the common marmoset as a potential model for TB chemotherapy due to its small size (250 to 400 g as an adult), ease of husbandry, wide use in other disease models (10–14), and high incidence of dizygotic twinning (15). There was only a single case report of a zoonotic
infection in the published literature involving a marmoset companion animal infected by a human in South Africa (16).

Studies in mice, rabbits, and guinea pigs have shown that the lineage of *Mycobacterium tuberculosis* strains used to infect animals directly influences the rate of disease progression and the extent of pathology. The incidence of the Beijing lineage of the *M. tuberculosis* complex (lineage 2) (17) has dramatically increased in areas of the world where it was rare less than 50 years ago (17–20). This lineage has been associated with increased rates of extrapulmonary disease, multidrug resistance, treatment failure, and relapse (21–25). In contrast, TB caused by the more ancient *M. africanum* lineage (lineage 2) (17) has dramatically increased in areas of the world where it was rare less than 50 years ago (17–20). *M. africanum* has been associated with more disseminated disease in the complex, they have limitations in their recapitulation of human disease, especially in terms of the complex pathology typical of human tuberculosis (29–32). In these nonprimate models, strains of the Beijing lineage have been found to cause more rapid death in chronically infected mice and have been associated with more disseminated disease in rabbits.

As a first step toward developing a model of experimental chemotherapy in this primate species, we evaluated the impact of strain lineage on disease progression in common marmosets and demonstrated that we could monitor and quantify this progression using 2-deoxy-2-[18F]fluoro-D-glucose (FDG) positron emission tomography (PET)/computed tomography (CT). Our data demonstrate, for the first time, that marmosets are highly susceptible to infection with *M. tuberculosis* strains of diverse lineages, produce cavitary tuberculosis with the Euro-American lineage 4 strain CDC 1551, and produce extensive extrapulmonary disease when infected with an *M. africanum* or Beijing strain. Thus, marmosets represent a promising small nonhuman primate model that may be useful for future assessment of the impact of new drugs on all of the pathological manifestations of TB seen in patients.

**MATERIALS AND METHODS**

**Animals and ethics assurance.** This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Committee on the Ethics of Animal Experiments of the National Institute of Allergy and Infectious Disease approved the experiments described here under protocol LCID-9 (issued to the NIH Intramural Research Program as permit A-4149-01), and all efforts were made to provide intellectual and physical enrichment and minimize suffering. Once infected, marmosets were housed individually or paired in biocontainment cages in an animal biological safety level 3 (ABSL3) facility approved for the containment of *M. tuberculosis*. The University of Pittsburgh studies were approved by its Institutional Animal Care and Use Committee (IACUC). Animals were pair housed in an approved ABSL3 facility (regional biocontainment facility).

**Bacterial culture, infection, and clinical endpoint monitoring of marmosets.** *M. tuberculosis* cultures were grown to mid-log phase in plastic tissue culture roller bottles at 37°C in Middlebrook 7H9 liquid broth (Difco Laboratories) and frozen in aliquots for aerosol infection as previously described (33). Aerosols were generated using a BANG nebulizer delivering 5 liters/min of filtered (diluter) air and 6.0 liters/min (range, 5.9 to 6.2 liters/min) of aerosol through a CH Technologies inhalation system (Westwood, NJ) housed and operated in a dedicated class 2A biological safety cabinet within a dedicated ABSL3 laboratory. Aerosol infections were performed as previously described (34), except that mice were used to determine the dilution required for each frozen bacterial stock to deliver equal CFU prior to infecting marmosets. Mice were sequentially exposed to dilutions of each bacterial strain and then sacrificed 1 h later, and both lungs and all-glass impinger aerosol samples were plated on M7H11 agar containing albumin-dextrose complex and antimicrobial inhibitors for enumerating CFU (35). The respiratory minute volume for mice of a similar size and age range has been reported to be 0.02 liters/min (36, 37), while the respiratory minute volume for similarly sedated marmosets of the same weight and age range as those used here has been reported to be 0.07 liters/min (11). The CFU/mouse lung after each exposure was multiplied by the ratio of the marmoset to mouse respiratory minute volumes to calculate the dilution required to deliver 250 CFU (high dose) to the marmoset lung. Aerosol samples were also collected with an impinger during the actual aerosol exposures of the marmosets so that the actual delivered dose could be estimated (see Table S1 in the supplemental material). Sedated marmosets were fitted with small-animal anesthesia masks (Midmark Corporation, Versailles, OH) and exposed to infectious aerosol for 5 min, followed by clean air for 2 min. After infection, the animals were observed daily and given brief physical exams weekly, coinciding with PET/CT scans when scheduled. Animals were euthanized at the first sign that they were becoming clinically ill, as assessed by a combination of weight loss of >20% and/or any signs of disease. Potential signs included physical and behavioral changes due to disease, such as tachypnea, dyspnea, or lethargy.

**PET/CT scanning procedures.** Animals were anesthetized using atropine (0.04 mg/kg of body weight subcutaneously [s.c.]) followed by a cocktail of ketamine and xylazine (25 and 1 mg/kg, respectively, intramuscularly [i.m.]) prior to injecting FDG intravenously (2 mCi/kg). During scanning, 1 to 5% isoflurane was delivered using a face cone as necessary to maintain the anesthesia plane. Actual doses were determined by measuring the radioactivity content of the syringe with the FDG solution in a calibrated dose calibrator immediately before and after injection into the catheter and recorded along with the weight of the animal. FDG doses were matched within 5 min and 10% activity, respectively, to facilitate quantitative assessment of uptake parameters as recommended in the PERCIST criteria (38). During uptake and distribution of the FDGs, a 250-mm CT scan from the base of the skull spanning the lungs and the abdominal cavity was acquired in approximately 20 s using an 8-slice helical CT scanner (CereTom; NeuroLogica, Danvers, MA) using the following parameters: 120 kV, 5 mA/s, 1.25-mm slice thickness, and 0.4949-mm pixel spacing. The animal bed was then retracted into a microPET gantry (MicroPET Focus 220; Siemens Preclinical Solutions, Knoxville, TN), and 60 ± 5 min post-FDG injection, a series of 2 10-min emission scans with 75-mm-thick windows with a 25-mm overlap were acquired caudal to cranial. The emission data were processed and corrected as described previously (39).

**Necropsy and dissection.** A careful necropsy plan for each animal was made prior to euthanasia, guided by the PET/CT images, to locate and sample the major lesions for matching histology and bacterial burden with imaging data. Immediately prior to sacrifice, the animals were sedated, blood samples were collected, and the animals were euthanized with 200 mg/ml Beuthanasia-D injected intravenously (i.v.) through the femoral vein. The organs were removed and weighed, and the lung was dissected into lobes and photographed before recording the location, number, and diameter of lesions visible on the surface. Cavities identified in CT scans then were isolated during dissection. Tissue samples for bacterial burden were weighed and homogenized into single-cell suspensions and then plated as previously described (34, 35, 40). Bacterial burden in...
lungs, lymph nodes (LN), liver, and spleen were calculated as CFU per gram of tissue. Tissues were fixed by immersion in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy as described previously (34,40). Hypoxyprobe was administered and detected as described previously (34).

PET/CT data analysis. CT scans were exported from the Ceretom workstation into the Inveon Research Workstation software (IRW; Siemens Preclinical Solutions, Knoxville, TN) and coregistered as described previously (39). Further analysis used both IRW software and Osirix 3.8 64 bit (Pixmeo SARL, Bernex, Switzerland). Custom software (TBQuant, version 4; NET ESolutions Corporation, McLean, VA) was used to segment lungs and compute total volumes according to the Hounsfield unit (HU) intensity scale for radiodensity (41). The preinfection scan was used as the reference lung. The chest cavity was initially categorized as a potential lung region for voxels of ≥350 HU, bone region of ≥300 HU, and other. Based on anatomical position, voxels (a three-dimensional [3D] pixel or the smallest unit of volume computed in the scan) with ≥350 HU were then excluded if they mapped to the digestive system. The carina (the point corresponding to the bifurcation of the trachea into primary bronchial branches) was then identified, and a global registration of the lungs between scan 1 and subsequent scans was obtained. This globally optimal registration of lungs was used subsequently as an initial estimate in finding local matches across groupings of corresponding z-slices. Local matches that avoid transformations that mapped unlikely pairs of voxels to one another (for example, air in one scan being mapped into bone in another) were preferred (i.e., given greater weight in evaluating matches). To ensure that metrics across visits were meaningful, common volumes across scan series were then identified, isolated, and used for computation of volumes. For FDG glycolytic activity measurements, PET/CT images were loaded into MIM fusion software to create lung contours using the CT 3D region growing application with upper and lower voxel threshold settings of 2 and ≤1,024 HU, respectively, with hole filling and smoothing applied. Dense lesion centers were subsequently identified for inclusion in the lung region manually, and the program calculated the FDG signal parameters.

Statistical analysis. The statistical analyses were performed using a one-way analysis of variance (ANOVA) with Bonferroni’s multiple-comparison test for 3 or more groups in Prism 5.0 (GraphPad), and P values were calculated. Values were expressed as means ± standard deviations (SD). P values of less than 0.05 were considered significant, and the confidence intervals (CI) presented represent the 95% intervals unless otherwise noted. Comparison of survival of groups used the log-rank test, and tests for correlation used Pearson’s r.

RESULTS

Marmosets infected by aerosol with \textit{M. tuberculosis} complex develop rapidly progressing primary tuberculosis. Eighteen marmosets were infected by nose-only aerosol exposure with one of three \textit{M. tuberculosis} complex strains (Fig. 1). The \textit{M. tuberculosis} K04 strain was a drug-susceptible recent isolate of the Beijing lineage from a Korean subject with moderately advanced, noncavitary disease. \textit{M. tuberculosis} CDC 1551 originated in America, represents the Euro-American lineage 4 phylogenic strains (17, 42), and has been shown to be hyperimmunogenic in mice and
with the lower dose of high dose of as 2 weeks postinfection (p.i.) in the marmosets infected with the strain CDC 1551 or M. africanum-infected group. The slope of disease volume increase was not different between the two doses within each infection group, so the data are presented in aggregate. In addition, a slight increase in abnormal lung density in the animals given the higher infectious dose of K04 was observed (data not shown). The differences in mean lesion volume were significant at 4 weeks p.i. for K04 compared to N0091 and CDC 1551 and at 6 and 8 weeks between N0091 and CDC 1551 (P < 0.05).

Comparison of the dense lung volume observed in marmosets 4 weeks postinfection and the time to signs of clinical illness.

Animals were euthanized when they lost 20% of their starting weight or if any clinical signs of disease became manifest. All animals remained bright, alert, and responsive and were euthanized if any clinical signs of disease became manifest. Weight loss began. The median time to disease progression for the M. tuberculosis Beijing strain K04 was 36.5 days (range, 25 to 42 days) and was 59 and 57 days (range, 53 to 70 days) for CDC 1551 and N0091, respectively (Fig. 1C; P = 0.0001 by Mantel-Cox test). There was a significant impact on marmoset disease progression with the high-dose infection of K04 (hazard ratio, 15.34), while receiving the higher dose of the other two strains did not influence survival compared to that with the lower dose.

CT changes during disease development quantify differential pathogenicity. Disease development was monitored by PET/CT scanning prior to infection and then every 2 to 3 weeks after infection until necropsy. Strain K04 (see Fig. S1A in the supplemental material) progressed rapidly, with an increase in lung parenchymal radiodensity observable as early as 2 weeks p.i. (Fig. 2A and B), although at that time distinct lesions were not visible. By 4 weeks p.i., large lesions (see Fig. S1, arrows) were evident, and by 6 weeks, immediately before sacrifice, large lesions often involved the majority of lung lobes. For CDC 1551 (see Fig. S1B) disease progression was notably slower, with small lesions only evident about 1 month after infection, and large lesions were present only 2 months after infection. Infection with M. africanum N0091 produced abnormal density in the lung at a rate intermediate between those of the two M. tuberculosis strains (see Fig. S1C), despite the fact that the initial number of CT-visible lesions was less than that with either of the other two strains.

To quantify these changes in abnormal lung density, we applied a computational analysis program we previously developed for analysis of human CT scans (41). We determined quantitative CT changes in lung density by extracting the total three-dimensional lung volumes after aligning all scans in a series to the preinfection scan and summing the volume occurring at each density in each scan. The HU distributions of the serial scans were plotted and examined to identify the density of diseased lung (the data from one representative animal is shown in Fig. 2A). In the naive adult marmoset, tissue with a density greater than −400 HU (primarily the main bronchi and the vasculature) accounts for only...
about 7% of the total lung volume (1,200 mm$^3$; the average total lung volume was 18,000 mm$^3$). Upon infection, the volume of lung with a density of $>-400$ HU steadily increased on days 13, 27, and 41 (Fig. 2A). Lung voxels with density between -400 and +300 HU represent TB lesions, and quantifying this across all of the infected animals allows a direct measurement of the rate of disease progression (Fig. 2B). No significant difference was found between the high and low dose for the infecting strains, so the mean volumes were compared in aggregate. The average increase in lesion volume was most rapid for strain K04 (204 ± 43 mm$^3$/day) and slowest for CDC 1551 (71 ± 10 mm$^3$/day; $P < 0.01$), while the rate for N0091 was intermediate (139 ± 13 mm$^3$/day). For all three strains, the total number of lesions increased as infection progressed, suggesting dissemination from the initial sites of infection. The mean density of individual lesions that did not cavitate increased an average of 50 HU every 2 weeks (data not shown). The amount of high-density volume ($>-400$ HU) in the lung at 4 weeks postinfection was positively correlated with the time to clinical illness (and euthanasia) (Fig. 2C).

**FDG-PET uptake increases during disease development with increasing CT disease volume.** FDG-PET can add important functional data to the structural data provided by CT. Representative 3D images showing FDG-avid lesions as disease progressed are shown from animals infected with each strain (Fig. 3A to C). As with CT changes and weight loss, FDG-avid lesions were obvious at the earliest time point in animals infected with K04, and lesions could be observed in the lungs of all animals by 3 to 4 weeks after infection. Lesional FDG uptake was also apparent in scans taken 4 weeks after infection in the other two strains, but it was more modest in the CDC 1551-infected (Fig. 3B) and N0091-infected (Fig. 3C) animals. Heterogeneity in FDG uptake by individual lesions was observed in both CDC 1551- and N0091-infected animals (Fig. 3B, arrows). In some cases, larger lesions with less FDG-avid centers were present, possibly indicating the presence of large regions of necrosis. FDG uptake was also observed in lymph nodes in NHP (Fig. 3, arrowheads) as early as 14 days p.i. and increased over time. The 3D volume of the lung was isolated in each CT scan, and the total glycolytic activity (or FDG uptake) was determined. The glycolytic activity increased over time in each infectious group (Fig. 3D). In addition, there was higher FDG uptake in the lungs of animals infected with K04 (at 4 weeks; $P = 0.01$ by one-way ANOVA), consistent with the more rapid progression by CT. There were also more rapid increases in glycolytic activity in animals exposed to the higher dose of K04 than in animals exposed to the lower dose. Across all of the animals, the rate of increase in abnormal lung density correlated with the total glycolytic activity (Fig. 3E).

**M. tuberculosis strains replicates in the lung with strain-dependent spread to extrapulmonary organs.** Animals all were sampled at necropsy to determine the bacterial burden of lung and to examine extrapulmonary spread to liver, spleen, and lymph nodes (Fig. 4). The lung was processed according to a specific dissection plan to determine if there were differences in the bacterial burden of differentially affected lung tissue. As an estimate of overall lung burden, we homogenized the whole right middle lobe from these animals. In cases where the right middle lobe did not contain lesions representative of the entire lung (3 animals), another representative lobe or several slices of lobe with both lesions and normal tissue were substituted. The K04-infected animals averaged 1.4 log$_{10}$ CFU/g more bacteria than either the CDC 1551- or N0091-infected animals from whole middle lobe homogenates (Fig. 4A). From other lung lobes, we dissected grossly normal lung tissue away from visible lesions and analyzed the bacterial numbers per gram of tissue in these samples. The average CFU/g of normal tissue is presented in the graph, but it should be noted that not all lung samples from CDC 1551- and *M. africanaum*-infected animals had detectable bacterial loads. With all strains, the grossly normal lung CFU was at least 10-fold lower than that in middle lobe homogenates, suggesting that most bacteria were contained within visibly affected tissue. Microscopic examination of normal lung revealed the presence of small microscopic lesions and focal regions of alveolitis. The bacterial burden in grossly normal lung tissue of K04-infected animals was significantly higher than that in similar lung tissue from animals infected with either of the other two strains (Fig. 4A; $P < 0.0001$ by ANOVA). In isolated small nodular lesions, K04 had more bacteria per gram than the CDC 1551 or N0091 lesions; the same higher CFU burden was observed in the caecum of larger nodules (see Fig. S1A in the supplemental material) as well.

Extrapulmonary spread to spleen and liver was suspected due to higher FDG uptake in these organs during later scans, especially in the animals infected with strains K04 and *M. africanaum* N0091 (Fig. 3A and C). Accordingly, when these organs were analyzed, the bacterial burdens were found to be significantly higher than the burden in these organs from animals infected with CDC 1551 (Fig. 4B). K04-infected animals had a higher bacterial load in liver, spleen, and LN than those infected with either CDC 1551 or N0091. There was a positive correlation between survival postinfection and bacterial load of the liver (see Fig. S1B in the supplemental material) and spleen of the K04-infected marmosets, illustrating the increasing extrapulmonary burden in these animals with longer duration of infection. *M. africanaum* strain N0091 produced a higher bacterial burden in the liver and spleen than CDC 1551, although its lung burden was similar, suggesting enhanced dissemination to these sites (Fig. 4B). Finally, although the CFU/g in the lymph nodes of animals infected with CDC 1551 and the *M. africanaum* strain were similar, the average weight of the LNs after *M. africanaum* infection were 5 times higher than those after CDC 1551 infection (0.387 ± 0.302 g versus 0.084 ± 0.056 g; $P = 0.002$), increasing the overall bacterial burden in this compartment as well. It should be noted that the duration of infection for each animal was different. We performed comparisons of bacterial burden with time to necropsy for each group of animals and each tissue type and found no temporal relationship, except as indicated for the liver (see Fig. S1B). Likewise, the difference in bacterial burden with respect to infectious dose was examined and was not found to be significant at the time animals were euthanized, except as indicated for the liver and spleen (Fig. 4B).

**Histopathology at necropsy reveals a range of lesion types similar to those seen in human disease.** At necropsy, all of the animals showed evidence of pulmonary lesions consistent with active tuberculosis (Fig. 5; also see Table S2 in the supplemental material). All infected animals had solid cellular nonnecrotizing lesions (Fig. 5A), as well as lesions with a central area of necrosis surrounded by epithelioid macrophages and neutrophils, with more distal lymphocytes (Fig. 5B) usually embedded in regions of tuberculous pneumonia, as has been observed in other NHP models (40). In 3 of the 6 CDC 1551-infected animals (1 high dose and 2 low dose), 1 to 3 lesions with a cavitated necrotic core that was only partially filled with semiliquid caseum were observed at nec-
Histologically, these lesions demonstrated a suppurative central region, with infiltrating neutrophils and macrophages sometimes clearly connecting to a pulmonary airway with bordering fibrosis (Fig. 5C) or appearing adjacent to necrotic and fragmenting regions of infiltrative granulomatous pneumonia, which was recently described as characteristic of postprimary human disease in the preantibiotic era (46). Most granulomatous lesions in the CDC 1551-infected group had evidence of fibrosis and deposition of collagen exterior to the epithelioid macrophages (Fig. 5D and H), whereas lesions in animals infected with the K04 and N0091 strains showed more minimal marginal fibrosis and often had direct extension of inflammation into the adjacent alveolar airways (Fig. 5E, G, and I). The distribution pattern of lesions in the K04- and N0091-infected animals was primarily invasive granulomatous pneumonia, while in CDC 1551-infected animals the distribution was more heterogeneous, with multifocal and in-

FIG 3 Lesions show increased FDG-PET uptake during disease development and progression. The images show serial 3-dimensional projections of the anterior aspect of the chest of a single representative animal infected with a low dose of M. tuberculosis Beijing strain K04 (A), M. tuberculosis CDC 1551 (B), or M. africanum N0091 (C) scanned 4 or more times (labeled in days at the top). The blue signal forming the body is derived from the CT scan, with increased light blue intensity indicating tissue with a higher HU. All images were projected with a common [18F]FDG uptake scale, indicated at the bottom right. The heart (H) had variable [18F]FDG uptake and is marked in each scan where it was avid; labeling of the heart is variable even with fasted animals. G indicates the gallbladder position if [18F]FDG avid. [18F]FDG uptake in the lung was minimal prior to infection (0) and at 2 weeks, but as lesions expanded, focal areas of [18F]FDG activity appeared. [18F]FDG uptake in the lymph nodes (LN) was observed as early as 2 to 4 weeks in Beijing K04- and M. africanum-infected animals and increased over time (arrowheads in panels B and C). The FDG uptake of the CDC 1551-infected lungs showed more heterogeneity than that of the other strains, with some early lesions having very low uptake (arrows in panel B). As lesions became larger, the [18F]FDG uptake of the central part of the lesion often was lower. (D) The increase in [18F]FDG uptake, or total glycolytic activity of the lung and LNs for each animal, was determined, and the group means over time were determined. The K04-infected animals had higher glycolytic activity than either the CDC 1551- or N0091-infected animals (P = 0.0005 by one-way ANOVA). The increases in glycolytic activity for the K04 high- and low-dose groups were different and are shown separately but did not reach significance. (E) The increase in glycolytic activity of the lung correlated with the diseased lung volume (P = 0.004; \( r^2 = 0.44 \)) as measured by CT.
Beijing strain K04 was higher than that of animals infected with CDC 1551 or of bacteria administered were not significant. The bacterial burden of whole liver, spleen, and lymph nodes/g from marmosets infected with the occurred rapidly, with both liver and spleen having cultivatable receiving the low-dose aerosol of K04 (which survived longer) had significantly higher CFU (of marmosets infected with CDC 1551 were generally lower than those of animals infected with N0091 (P***, P***, P<0.001 by ANOVA). CDC 1551 normal lung and isolated lesions also had a significantly greater bacteria load than the samples from M. africanum (P<0.05). (B) Dissemination of the bacteria from the lung occurred rapidly, with both liver and spleen having cultivatable M. tuberculosis within 1 month. The extrapulmonary burden increased with time, so that animals receiving the low-dose aerosol of K04 (which survived longer) had significantly higher CFU (P = 0.008 by t test); otherwise, differences in CFU related to the dose of bacteria administered were not significant. The bacterial burden of whole liver, spleen, and lymph nodes/g from marmosets infected with the M. tuberculosis Beijing strain K04 was higher than that of animals infected with CDC 1551 or M. africanum N0091 (P<0.001 by ANOVA). Bacterial loads of the spleen and liver of marmosets infected with CDC 1551 were generally lower than those of animals infected with N0091 (P<0.05 by ANOVA posttest). *, P<0.05; **, P<0.01; ***, P<0.001. Bars indicate means.

Extrapulmonary sites of disease also contained granulomas with central necrosis and liquefaction surrounded by a rim of epithelioid macrophages, including in the thoracic, carinal, and mediastinal lymph nodes of K04- and N0091-infected marmosets (see Fig. S3 and Table S3 in the supplemental material), but these lymph nodes in CDC 1551-infected marmosets contained predominate hyperplasia with or without sinus histiocytosis (see Fig. S3B). The spleen and liver for 3 of the K04-infected animals and 5 of the N0091-infected animals had visible caseous lesions, but no grossly visible splenic or liver lesions were observed in the CDC 1551-infected animals (see Tables S4 and S5). K04- and N0091-infected animals had noncrotizing lesions in the liver and necrotizing lesions in the spleen more frequently than CDC 1551-infected animals (see Tables S4 and S5). Other extrapulmonary findings included granulomatous interstitial nephritis and extramedullary hematopoiesis in the adrenal glands, which also were more commonly seen in the N0091- and K04-infected animals. The pathology score of the groups of animals (see Fig. S3D) indicated that there was a difference in high-dose and low-dose animals, but because this score relies heavily on the number of lesions, this would be expected. However, the score did not distinguish among the different infectious strains.

Twin infections confirm reproducibility and strain-dependent disease features. Marmoset twins are identical or chimeric and provide an opportunity to reduce host variability within the context of an experimental animal model. Of the six groups of twins used in these studies, the first four were used to evaluate reproducibility of the overall infection process (Fig. 1A). Two twin animals infected with a high dose of M. tuberculosis K04 had 83 and 80 individual lesions at necropsy (Fig. 1A and B), respectively, and had very similar types of lesions and extents of extrapulmonary involvement (see Tables S3 to S5 in the supplemental material). Likewise, twins infected with high doses of CDC 1551 and N0091 showed nearly identical patterns of infection, as did twins infected with a low dose of K04. Infection outcomes in twins infected with different strains recapitulated the overall differences seen in the groups; for example, comparisons of members of one set of twins, where one sibling was infected with K04 and its littermate with CDC 1551, showed a very different rate of progression of disease (Fig. 6C and D). An animal infected with N0091, whose twin sibling was infected with CDC 1551, developed extensive extrapulmonary disease, including involvement of the lymph nodes, lung, spleen, and liver that was absent from its sibling, who was infected with CDC 1551 (see Tables S3 to S5).

DISCUSSION

Strains from the Beijing clade have consistently shown higher virulence in animal models of TB. Mice infected with most Beijing lineage strains have reduced survival, higher bacterial loads, greater volumes of lung disease, and, in some studies, lower production of gamma interferon (IFN-γ) compared to non-Beijing strains (29, 47–49). Guinea pigs are highly susceptible to experimental M. tuberculosis infection but can restrict the growth of M. tuberculosis under some exposure conditions (50–52). However, like mice, they are also highly susceptible to Beijing strains, with rapid and extensive primary lesion necrosis in the lung and extrapulmonary lesion necrosis, as well as higher bacterial burdens, compared to non-Beijing strains (32, 53). Even the New Zealand White rabbit, which is relatively resistant to M. tuberculosis (54–57), can develop chronic disease, including weight loss, lung consolidation, and frequent cavitary disease and dissemination from the site of infection, when infected with a Beijing strain (35, 45, 57). In contrast, low-dose infection with the immunostimulatory
lineage 4 strain CDC 1551 causes little mortality and no obvious signs of disease, despite bacterial burdens in the lungs similar to those of other strains early in infection (43). Be et al. observed that strain CDC 1551 disseminates to the central nervous system of guinea pigs more frequently than H37Rv (58). In the rabbit, *M. tuberculosis* strains CDC 1551 and H37Rv have been used to model latent disease because of their failure to maintain a measurable bacterial load past a few months (59,60).

All three *M. tuberculosis* complex strains tested in our study were capable of producing fulminant disease in the common marmoset after aerosol infection but showed different rates of disease progression. The first signs of disease were weight loss and lung abnormalities revealed by PET/CT, but the animals all ultimately lost enough weight to require euthanasia prior to 3 months. Early in the infection there were signs of disease differences between the high- and low-dose-exposed animals, including numbers of granulomas, initiation of weight loss, and even liver CFU, but with time these signs were lost, presumably because the bacteria disseminated from initial lesions into the adjacent lung tissue and to other organs of the body. At necropsy, each of the three strains ultimately had pulmonary pathology that was highly similar to that observed in untreated human TB, with some strain-dependent features. Animals infected with the K04 Beijing isolate developed invasive necrotic granulomatous disease, with extensive histiocytic infiltration into the adjacent alveoli and little fibrosis. This strain achieved the highest bacterial loads and caused the most rapid disease progression, with the NHP group having a median survival time of only 37 days, reminiscent of the disease observed in the models described above. *M. africanum* N0091 infection resulted in a lower rate of disease development but a similar histopathologic presentation, including extensive parenchymal consolidation and endobronchial disease. Although this group formed the fewest primary lesions, these animals had the largest lesions later in the disease process, and the disease spread from the lung and the LN into the chest cavity, causing adhesions to the plural surface and diaphragm. One interesting finding was the relatively large amount of disease volume (both by CT and by histology) observed in the animals infected with *M. africanum* for similar or lower levels of bacterial burden. This is consistent with the human data documenting more extensive pulmonary involve-

**FIG 5** Histopathology of marmoset lungs infected with the 3 *M. tuberculosis* complex strains is similar to that seen in human disease. Both nonnecrotizing lesions (A) and necrotizing lesions (B) were observed in each infection group and were characterized by neutrophil and epithelioid macrophage aggregates, often with central necrosis surrounded by concentric layers of lymphocytes and fibrosis. (C) Lesion erosion into airways forming cavities with variable amounts of liquefactive material with leukocytic infiltration (arrow) was observed in CDC 1551-infected animals. (D) Masson’s trichrome staining (D and E) revealed increased collagen (arrow, blue staining) in the lesions from CDC 1551 infections versus lower collagen deposition in granulomas in K04- and N0091-infected animals (E) at the same time p.i. (F) Staining with hypoxyprobe antibody revealed areas where *in vivo*-administered pimonidazole HCl was trapped in the lesion tissue, indicating areas of hypoxia (brown staining). Low-magnification images of lung sections from K04 (G)-, CDC 1551 (H)-, and N0091 (I)-infected animals are also shown. Alveolar pneumonia extending outward from granulomatous infiltrates occurred in all groups but was predominant in K04- and N0091-infected lungs. CDC 1551-infected lungs contained a more multifocal lesion distribution, but tuberculous pneumonia was also present (the arrow indicates adhesion to the lung surface). Grossly normal-appearing lung regions contained occasional small cellular lesions (arrowhead in panel I). Images shown in panels A to C and G to I were stained with hematoxylin and eosin. N, area of necrosis. Scale bars were 1 mm (G to I), 0.2 mm (A, B, and D to F), and 0.5 mm (C).
ment and extrapulmonary disease in patients infected with these strains, and there is precedent for this phenomenon in mice (61). Both the M. africanum and the Beijing strains showed increased extrapulmonary dissemination of disease to the spleen and liver, while NHPs infected with CDC 1551 showed the slowest rate of disease progression and limited extrapulmonary dissemination. Lung lesions in animals infected with CDC 1551 had more defined margins, with peripheral fibrosis, less loose histiocytic infiltration, and lower bacterial loads in apparently normal lung, all features that suggest partial immunological containment. Half of the CDC 1551-infected marmosets developed cavitary disease. Animal models that reproducibly develop caviarty disease are desirable for chemotherapy testing, because cavities are thought to be more difficult to sterilize and the presence of cavities is associated with relapse in human disease (5, 62).

Because of our previous experience with TB infections in an old world NHP (cynomolgus macaques) and the existence of only a very few previous reports suggesting TB as a naturally occurring zoonosis in marmosets (16, 63), the relatively high virulence of these TB strains for marmosets was somewhat unexpected. To explore whether this high virulence was simply a function of route of administration, we also performed infections on a small number of marmosets using intratracheal instillation of a low dose of M. tuberculosis. Lung lesions in animals infected with M. tuberculosis (K04 [C] and CDC 1551 [D]) demonstrated similar numbers of granulomas at 4 weeks (26 and 31), but the lesions in the CDC 1551-infected twins were much smaller at this time point.

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