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Increased Foxp3 expression in guinea pigs infected with W-Beijing strains of *M. tuberculosis*

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SUMMARY

There is increasing evidence that clinical isolates of *Mycobacterium tuberculosis* that belong to the W-Beijing genotype of newly emerging strains are often of very high virulence when tested in small animal models, including the mouse and guinea pig. In this report we provide further evidence to support this contention, and show that two W-Beijing strains are of very high virulence when introduced by low dose aerosol into out-bred guinea pigs. In addition to severe lung pathology, each of these infections was associated with large influxes of activated CD4 and CD8 T cells into the lungs. Large influxes of macrophages were also observed, but the fraction of these showing evidence of activation by Class-II expression was relatively low. A progressive increase in neutrophils was also seen, with highest levels accumulating in the lungs of the W-Beijing infected animals. In the case of these two infections mRNA levels for TH1 cytokines was elevated early, but these then declined, and were replaced by increasing levels of message encoding for Foxp3, IL-10, and TGF β . These observations support the hypothesis that W-Beijing strains are potent inducers of regulatory T cells, and that this event may enhance survival and transmission of these bacilli.

Keywords

Mycobacterium tuberculosis; Guinea pig; Foxp3+ regulatory T cells; clinical isolates; W-Beijing strains

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1. Introduction

The global epidemic caused by the bacterial pathogen *Mycobacterium tuberculosis* continues unabated, with the most recent figures in 2009 estimating 9.4 million incident cases of tuberculosis, with about 1.3 million deaths^{1, 2}. It is becoming evident that a significant percentage of new clinical isolates of *M. tuberculosis* are of extremely high virulence^{3–5}. Amongst these, the W-Beijing family of *M. tuberculosis* is globally distributed and is being increasingly documented as a cause of major outbreaks of infection worldwide that involve multidrug-resistant strains^{6–10}. Increasing evidence suggests that the Beijing genotype family can induce distinctly different host immune responses compared to other *M. tuberculosis* strains, and amongst these is the newly emerging idea that this family induces the generation of regulatory T cells¹¹; an event that could allow evasion of both innate¹² and acquired immunity^{11, 13}.

Despite the obvious high virulence of these newly emerging clinical strains, most work on screening new drugs and vaccines has used the “laboratory strains” H37Rv and Erdman^{14, 15}. This is of concern, because it has already been noted¹¹ in the mouse model that such strains are of far less potency in terms of their capacity to induce regulatory T cell responses. To date however it remains unknown if this caveat extends to the guinea pig animal model, which remains the gold standard for testing new vaccine candidates. To begin to address this question we compared three clinical isolates (two of them, W-Beijing strains) in parallel with the two laboratory strains for their ability to infect and grow in the lungs of guinea pigs after low dose aerosol infection. Using newly developed flow cytometry for this species¹⁶ we were further able to monitor the influx of several cell populations into the lungs, including activated T cell subsets. We are unable as yet to perform intracellular staining for cytokines in this species, but were able to track TH1 cytokines, as well as cytokines associated with negative regulation of immunity, using RT-PCR.

The results of this study show that the two W-Beijing strains, as well as a multidrug resistant “P family” isolate, grew to higher numbers in the lungs of these animals compared to the two laboratory strains. This was further associated with more severe lung pathology, and reduced survival. These events were associated with an initial higher expression of message encoding the TH1 cytokines IL-12p40 and gamma interferon (IFN γ) in animals infected with the clinical strains, but this was then followed by progressive increases in mRNA encoding the regulatory T cell markers Foxp3, IL-10, and TGF β in the animals exposed to the W-Beijing isolates. These data thus lead us to hypothesize that W-Beijing isolates of *M. tuberculosis* induce potent T regulatory cell responses in the guinea pig model, a finding that has the potential to seriously confound vaccine testing in this model which is routinely performed using the laboratory strains.

2. Methods

2.1. Guinea pigs

Female outbred Hartley guinea pigs (~500 g in weight) were purchased from the Charles River Laboratories (North Wilmington, MA, USA) and held under barrier conditions in a Biosafety Level III animal laboratory. The specific pathogen-free nature of the guinea pig colonies was demonstrated by testing sentinel animals. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University and comply with NIH guidelines.

2.2. Experimental infections

The laboratory strains *M. tuberculosis* H37Rv and Erdman were originally obtained from the Trudeau Institute collection, Saranac Lake, NY. The clinical isolates used in this study were

chosen specifically because they were all associated with outbreaks in the United States. Three strains from the Public Health Research Institute TB Center collection were selected in regard to their genetic backgrounds and their resistance phenotype. Strain TN14149 ("W10" DNA fingerprint) is a member of the W-Beijing strain family; it is drug susceptible, and has an identical fingerprint to the sequenced strain 210. Strain TN5904 is a "P" family cluster group VI MDR-TB isolate with resistance to isoniazid, rifampin, p-aminosalicylic acid, and streptomycin, that was isolated from HIV-positive patients that had undergone exogenous reinfection¹⁷. The W-Beijing strain SA161 is an isolate found within a cluster of cases in Arkansas.

All of the strains used in this study were grown in 7H9 broth containing 0.05% Tween-80. Thawed aliquots of frozen cultures were diluted in sterile water to the desired inoculum concentrations. A Madison chamber aerosol generation device was used to expose the animals to *M. tuberculosis*. This device was calibrated to deliver approximately 20 bacilli into the lungs. Lung bacterial counts on days 10, 30 and 60 were determined by plating serial dilutions of tissue homogenates on nutrient 7H11 agar and counting colony-forming units after 3 weeks incubation at 37° C. In survival studies, animals showing substantial weight loss with no evidence of weight rebound were euthanized. The results shown in the survival studies are based upon 8-10 guinea pigs per group.

2.3. Histological analysis

The lung lobes, spleen and mediastinal lymph nodes from each guinea pig were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). Sections from these tissues were stained using haematoxylin and eosin. The concurrent progression of lung and lymph node lesions was evaluated using a histological grading system¹⁸.

2.4. Organ digestion

To prepare single cell suspensions, the lungs, lymph nodes and spleens were perfused with 20ml of a solution containing PBS and heparin (50 U/ml; Sigma-Aldrich, St. Louis, MO) through the pulmonary artery and the caudal lobe aseptically removed from the pulmonary cavity, placed in media and dissected. The dissected lung tissue was incubated with complete DMEM (cDMEM media) containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 µg/ml; Sigma-Aldrich) for 30 minutes at 37°C. The digested lungs were further disrupted by gently pushing the tissue twice through a cell strainer (BD Biosciences, Lincoln Park, NJ). Red blood cells were lysed with ACK buffer, washed and resuspended in cDMEM. Total cell numbers were determined by flow cytometry using BD™ Liquid Counting Beads, as described by the manufacturer (BD Pharmingen, San Jose, CA USA 95131).

2.4. Flow cytometric analysis of cell surface markers

Single cell suspensions from each individual guinea pig were incubated first with antibodies as previously described^{16, 19} to CD4, CD8, pan T cell, CD45, MIL4, B cell, macrophage and class II antibodies at 4°C for 30 minutes in the dark after washing the cells with PBS containing 0.1% sodium azide (Sigma-Aldrich). The anti-guinea pig macrophage MR-1 antibody is an intracytoplasmic antigen and therefore cell membranes were permeabilized using Leucoperm (Serotec Inc, Raleigh, NC) according to the manufacturer's instructions prior to intracellular staining. Data acquisition and analysis were done using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences, San Jose, CA). Compensation of the spectral overlap for each fluorochrome was done using CD4 or MIL4 or CD3 antigens from cells gated in the FSC^{low} versus SSC^{low}; FSC^{mid/high} versus SSC^{mid/high}; SSC^{low} versus MIL4⁺; SSC^{high} versus MIL4^{neg}.

and SSC^{high} versus MIL4⁺ region respectively. Analyses were performed with an acquisition of at least 100,000 total events.

2.5. RT-PCR analysis

Expression of mRNA encoding the cytokines IFN γ , IL-12p40, TNF α , TGF β , IL-10, and the regulatory T cell associated intracellular marker Foxp3, was quantified using real-time reverse transcription-polymerase chain reactions (RT-PCR). One lobe from each guinea pig (n=5) lung was added to 1 ml of TRIzol RNA reagent (Invitrogen), homogenized, and frozen immediately. Total RNA was extracted according to the manufacturer's protocol. RNA samples from each group and each time point were reverse transcribed using the Reverse Transcriptase Enzyme (M-MLV RT- Invitrogene). Four μ l samples of cDNA were then amplified using the iQ SYBR Green Supermix (Bio-Rad) following the manufacturer's protocol on the iQ5 iCycler amplification detection system (Bio-Rad). A negative control using ultra pure Molecular Biology water as the template and a non-template control (NTC) were ran to confirm that the signals were derived from RNA and not due to contaminating genomic DNA. In order to ensure that only the correct gene was amplified, and was not the presence of primer-dimer or non-specific secondary products, a Melt Curve was performed for each run. Fold induction of mRNA was determined by analyzing cycle threshold (C_T) values normalized for HPRT (C_T) expression. The primer sequences for guinea pig IFN γ , TNF α , TGF β 1, IL-12p40 and 18S were previously published^{20,21}. The primer sequences for guinea pig Foxp3 and IL-10 were determined with assistance from Dr Anand Damodaran (Genotypic Technology, Bangalore, India). Primer sequences used for Foxp3 were forward: 5' AGAAAGCACCTTTCAAGCA 3'; reverse: 5' GAGGAAGTCCTCTGGCTCCT 3', and forward: 5' TTCTTCCAAACACAGGATCAGC 3'; reverse: 5' TCATTTCGATAGGGCTTGG 3' for IL-10.

2.6. Statistical analysis

Data is representative of two experiments. Mean values were calculated from results for individual guinea pigs within each group (n=5) $\pm$ standard error of the mean (SEM). One-way ANOVA was used to compare the statistical differences in numbers of bacilli, mean lesion scores and necrosis scores between different groups.

3. Results

3.1. Course of experimental infections

Guinea pigs were exposed to approximately 20 bacilli of the *M. tuberculosis* clinical strains TN14149, SA161 and TN5904, as well as the laboratory strains H37Rv and Erdman, and the bacterial loads in target organs followed versus time (Fig.1A). Both laboratory strains were contained in the lungs at a level of approximately 5.5-log, whereas the W-Beijing strain TN14149 grew to over 6.5-log, and W-Beijing strain SA161 exceeded 8-logs in the lungs. The MDR strain exhibited an intermediate pattern. The increased growth potential of both W-Beijing strains was also evident in the spleen and draining lymph nodes. In a parallel group of animals, these high bacterial burdens resulted in animal mortality, with animals beginning to die soon after day-60 (Fig.1B).

3.2. *M. tuberculosis* clinical strains of TN14149 and SA161 show increased organ tissue pathology

As anticipated, the five experimental infections resulted in a range of increasingly severe lung pathology over the first sixty days of the experiment (Fig.2). In all cases the infections induced extensive mixed inflammation and necrosis, but this was particularly severe in the case of the two W-Beijing infections (Fig.2G-J) compared to the two laboratory strains (Fig.

2A-D). Again, the MDR strain showed an intermediate pattern (Fig.2E&F). By day 60, lesions in animals infected with the clinical isolates showed severe increases in secondary lesion progression, characterized by multiple foci of extensive inflammation coalescing within the pulmonary parenchyma (Fig.2Q-T) whereas lung involvement in the cases of the two laboratory strains was more modest (Fig.2K-N). These progressive changes in tissue lesions were further reflected by lesion score analysis, revealing the extensive damage both in the lungs but also in the spleen and draining lymph nodes over the course of the infections (Fig.3).

3.3. Influx of defined cell populations using flow cytometric analysis

Our development of new gating strategies for flow cytometry¹⁶ now allows the analysis of the influx of T cell and other cell populations into infected organs over the course of the infection. Analysis of the T cell response still remains very limited (there are no antibodies to CD44, and guinea pigs lack CD62/L-selectin) but we have been able to define activated T cell subsets based on raised expression of the markers CD45 and CT-4¹⁶. As shown in Fig. 4, we observed substantially increased numbers of CD4⁺ CD45⁺ cells and CD4⁺ CT-4⁺ cells in the lungs and lymph nodes of guinea pigs infected with the two W-Beijing strains, and these raised levels were sustained out through day-60 of these infections. Similarly, increased numbers of CD8 T cells expressing (the putative selectin) CT-4 were also observed in response to these two infections.

We next analyzed the influx of macrophages and granulocytes (neutrophils) into the lungs and draining lymph nodes. Representative gating for two of the infections is shown in Fig. 5A. By combining MR-1 expression with high levels of MHC Class-II molecules we can distinguish total macrophage numbers and the numbers of these which are highly activated. Figure 5B shows that all five infections were associated with the progressive influx of large numbers of macrophages, as expected. As noted before¹⁹, only a small fraction of macrophages in animals infected with the two laboratory strains were activated, and whereas this fraction was higher for the two W-Beijing strains, this also waned with time. Finally, neutrophil influx is associated with progressive lung and lymph node damage, and numbers of these cells steadily increased in all five sets of animals with the highest numbers observed for the Beijing strains (Fig.5C).

3.4. Kinetics of emergence of messenger RNA encoding cytokines

As yet we cannot measure intracellular cytokine expression by flow cytometry in guinea pigs, but we can use RT-PCR methods to detect signals associated with specific T cell subsets. In Fig.6 we used RT-PCR to track the expression of the [Fig 6A] TH1 cytokines IL-12p40 and IFN γ , and compared this information to levels of [Fig 6B] Foxp3, IL-10, and TGF β , markers associated with down-regulation of immunity. The markers of TH1 immunity were substantially raised by day-30 of the infections, particularly in the case of the two W-Beijing strains, but these waned significantly by day-60. In contrast, in the case of the two W-Beijing infections, there were progressive increases in all three potential markers of regulatory T cell activity.

4. Discussion

Previous studies in our laboratory have shown that newly emerging clinical strains of *M. tuberculosis* cause progressive and highly inflammatory disease in both the mouse¹¹ and guinea pig^{4,5} models of the disease. Our studies in mice provided the new observation that such strains appear to be strong inducers of CD4⁺Foxp3⁺ regulatory T cells, thus explaining the “hypervirulence” of W-Beijing strain HN878 in the face of waning protective immunity¹¹, whereas the commonly used laboratory strains induce such cells weakly or not

at all. Hence, given our previous data regarding the extreme range of virulence of similar clinical strains in guinea pigs⁴ we decided to further investigate the cellular response to such strains in this relevant animal model, including whether these animals could express markers in the lungs that we believe to be associated with the emergence and accumulation of regulatory T cells.

The results of this simple descriptive study further illustrate the progressive and destructive nature of the W-Beijing clinical isolates when introduced into the lungs of guinea pigs. In addition this study provides the new information that this is associated with the influx of activated CD4 and CD8 T cells, but relatively poor numbers of Class-II activated macrophages. As lung damage continued, this was associated with a continued influx of neutrophils, which we postulate play a central role in driving the process of necrosis²², as well as playing a key role in bacterial persistence, even after chemotherapy²³. The most important new observation however was the demonstration that, despite evidence for a strong initial protective TH1 immune response, both W-Beijing strain infections potentially induced signals in the lungs that we can reasonably hypothesize represent the influx of CD4+Foxp3+ IL-10 secreting regulatory T cells.

Our data show that infection of guinea pigs with the *M. tuberculosis* W-Beijing strains TN14149 or SA161 resulted in significantly increased bacterial burden in the lungs, lymph nodes and spleens and reduced animal survival compared to the laboratory strains H37Rv and Erdman and the MDR-TB 5904 strain. In addition, the increased bacterial growth of the W-Beijing strains was associated with an increased granulomatous response and more severe tissue pathology. Coinciding with the increased bacterial burden in guinea pigs infected with the W-Beijing strains, there was increased influx of activated CD4+ and CD8+ T cells into the lungs and lymph nodes during the subacute phase of infection (Day 30) which was associated with increased IFN γ and IL-12p40 expression compared to the laboratory strains. Importantly, we show that during chronic infection (Day 60), this IFN γ response induced by the W-Beijing strains was replaced with an increased expression of Foxp3+ regulatory T cells.

These observations support the hypothesis that one element of the highly successful global spread of the W-Beijing family of isolates might be their ability to depress protective immunity by the induction of regulatory T cells. This could be due to very simple reasons, such as the fact that these isolates are highly inflammatory and virulent, or due to more subtle reasons such as the possession of molecules, such as lipids, that could specifically trigger regulatory T cell responses. Currently we favour the former possibility, given that we are seeing similar outcomes in our animal models using highly virulent but “non-W-Beijing” clinical isolates as well. Whichever is correct, we still have no way of distinguishing if this T cell subset is generated in response to the potent TH1 response in order to dampen it, or whether this subset is instead simply generated by the lung inflammation, with depression of the Th1 response an unfortunate side-effect.

In this regard the granulomatous response to the two W-Beijing strains was characterized in both cases by far greater amounts of primary lesion necrosis on day 30, more severe pulmonary inflammation, pyogranulomatous and necrotizing lymphadenitis of the draining lymph nodes, and increased numbers of secondary granulomas in comparison to the two laboratory strains. Secondary lesions caused by hematogenous dissemination developed at an increased rate in animals infected with the W-Beijing strains, compromising much of the healthy lung tissue as the disease progressed. It is tempting to hypothesize such rapid lung damage would be expected to result in increased clinical transmission of infection. However, multiple studies have demonstrated that *M. tuberculosis* strains responsible for major outbreaks have been shown to vary in virulence in animal models^{22, 23, 24}.

In addition, progression of the W-Beijing infections was associated with higher numbers of MIL4+ neutrophils being drawn into the lungs and draining lymph nodes. Our prior studies showed by immunohistochemical staining that MIL4+ neutrophils were primarily located within the prominent necrotic center of primary lesions in an area of acellular debris²⁵. This may suggest that the W-Beijing isolates are potent at inducing continuous degranulation of neutrophils coming into these structures, releasing proteolytic enzymes and thus further driving the necrotic process, thus in turn enhancing the likelihood of transmission to the next host.

The possibility that regulatory T cells are designed to depress these inflammatory processes, and yet also depress protective immunity, creates an obvious paradox. The apparent “success” of the newly emerging strains of tuberculosis may in fact reflect their ability to potentially drive both of these processes. Recent data²⁶ revealing the hyperconservation of certain epitopes across the entire family of bacterial strains raises the specter that this is a deliberate evolutionary act by *M. tuberculosis*, since potent expression of immunity in the lungs leads to tissue damage, necrosis, and subsequent escape. Hence, while it seems intuitive to regard regulatory T cell induction in the above disease process as a negative event, an alternative possibility is that it is a positive homeostatic response to a host protective process that is eventually detrimental.

A central conclusion of our observations here are much more practical however, because they directly relate to the current screening procedures used to test and prioritize new vaccine candidates which are to date almost exclusively based on the use of the “laboratory strains” H37Rv and Erdman. Not only are the W-Beijing strains far more virulent, but their induction of regulatory T cells may interfere with the vaccine candidates. These observations not only may explain why the W-Beijing strains remain prevalent in areas of the world that still apply routine BCG vaccination^{27, 28, 29, 30}, but may also constitute a serious impediment to the success of new tuberculosis vaccine candidates.

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References

1. Dye C, Espinal MA, Watt CJ, Mbiaga C, Williams BG. Worldwide incidence of multidrug-resistant tuberculosis. *J Infect Dis.* 2002; 185:1197–1202. [PubMed: 11930334]
2. WHO report. Global Tuberculosis control. 2010:1–205.
3. Zhang M, Gong J, Yang Z, Samten B, Cave MD, Barnes PF. Enhanced capacity of a widespread strain of *Mycobacterium tuberculosis* to grow in human macrophages. *J Infect Dis.* 1999; 179:1213–1217. [PubMed: 10191225]
4. Palanisamy GS, DuTeau N, Eisenach KD, Cave DM, Theus SA, Kreiswirth BN, et al. Clinical strains of *Mycobacterium tuberculosis* display a wide range of virulence in guinea pigs. *Tuberculosis (Edinb).* 2009; 89:203–209. [PubMed: 19251482]
5. Palanisamy GS, Smith EE, Shanley CA, Ordway DJ, Orme IM, Basaraba RJ. Disseminated disease severity as a measure of virulence of *Mycobacterium tuberculosis* in the guinea pig model. *Tuberculosis (Edinb).* 2008; 88:295–306. [PubMed: 18321783]
6. Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. *Trends Microbiol.* 2002; 10:45–52. [PubMed: 11755085]

7. Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, Ziazarifi AH, et al. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest*. 2009; 136:420–425. [PubMed: 19349380]
8. Wright A, Zignol M, Van Deun A, Falzon D, Gerdes SR, Feldman K, et al. Epidemiology of antituberculosis drug resistance 2002–07: an updated analysis of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance. *Lancet*. 2009; 373:1861–1873. [PubMed: 19375159]
9. Dye C. Doomsday postponed? Preventing and reversing epidemics of drug-resistant tuberculosis. *Nature Reviews*. 2009; 7:81–87.
10. Dye C, Espinal MA. Will tuberculosis become resistant to all antibiotics? *Proc Biol Sci*. 2001; 268:45–52. [PubMed: 12123297]
11. Ordway D, Henao-Tamayo M, Harton M, Palanisamy G, Troudt J, Shanley C, et al. The hypervirulent *Mycobacterium tuberculosis* strain HN878 induces a potent TH1 response followed by rapid down-regulation. *J Immunol*. 2007; 179:522–531. [PubMed: 17579073]
12. Shafiani S, Tucker-Heard G, Kariyone A, Takatsu K, Urdahl KB. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J Exp Med*. 2010; 207:1409–1420. [PubMed: 20547826]
13. Scott-Browne JP, Shafiani S, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, Rudensky AY, et al. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med*. 2007; 204:2159–2169. [PubMed: 17709423]
14. Orme IM. Preclinical testing of new vaccines for tuberculosis: a comprehensive review. *Vaccine*. 2006; 24:2–19. [PubMed: 16139397]
15. Lenaerts AJ, Degroote MA, Orme IM. Preclinical testing of new drugs for tuberculosis: current challenges. *Trends Microbiol*. 2008; 16:48–54. [PubMed: 18182291]
16. Ordway D, Palanisamy G, Henao-Tamayo M, Smith EE, Shanley C, Orme IM, et al. The cellular immune response to *Mycobacterium tuberculosis* infection in the guinea pig. *J Immunol*. 2007; 179:2532–2541. [PubMed: 17675515]
17. Small PM, Shafer RW, Hopewell PC, Singh SP, Murphy MJ, Desmond E, et al. Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. *N Engl J Med*. 1993; 328:1137–1144. [PubMed: 8096066]
18. Basaraba RJ, Dailey DD, McFarland CT, Shanley CA, Smith EE, McMurray DN, et al. Lymphadenitis as a major element of disease in the guinea pig model of tuberculosis. *Tuberculosis (Edinb)*. 2006; 86:386–394. [PubMed: 16473044]
19. Ordway D, Henao-Tamayo M, Shanley C, Smith EE, Palanisamy G, Wang B, et al. Influence of *Mycobacterium bovis* BCG vaccination on cellular immune response of guinea pigs challenged with *Mycobacterium tuberculosis*. *Clin Vaccine Immunol*. 2008; 15:1248–1258. [PubMed: 18508930]
20. Allen SS, Mackie JT, Russell K, Jeevan A, Skwor TA, McMurray DN. Altered inflammatory responses following transforming growth factor-beta neutralization in experimental guinea pig tuberculous pleurisy. *Tuberculosis (Edinb)*. 2008; 88:430–436. [PubMed: 18555747]
21. McMurray DN, Allen SS, Jeevan A, Lasco T, Cho H, Skwor T, Warren RM, et al. Vaccine-induced cytokine responses in a guinea pig model of pulmonary tuberculosis. *Tuberculosis (Edinb)*. 2005; 85:295–301. [PubMed: 16253558]
22. Aguilar D, Hanekom M, Mata D, Gey van Pittius NC, van Helden PD, et al. *Mycobacterium tuberculosis* strains with the Beijing genotype demonstrate variability in virulence associated with transmission. *Tuberculosis (Edinb)*. 2010; 90:319–325. [PubMed: 20832364]
23. Marquina-Castillo B, García-García L, Ponce-de-León A, Jimenez-Corona ME, Bobadilla-Del Valle M, Cano-Arellano B, et al. Virulence, immunopathology and transmissibility of selected strains of *Mycobacterium tuberculosis* in a murine model. *Immunology*. 2009; 128:123–133. [PubMed: 19191912]
24. Nicol MP, Wilkinson RJ. The clinical consequences of strain diversity in *Mycobacterium tuberculosis*. *Trans R Soc Trop Med Hyg*. 2008; 102:955–965. [PubMed: 18513773]
25. Basaraba RJ. Experimental tuberculosis: the role of comparative pathology in the discovery of improved tuberculosis treatment strategies. *Tuberculosis (Edinb)*. 2008; 88(Suppl 1):S35–47. [PubMed: 18762152]

26. Ordway DJ, Shanley CA, Caraway ML, Orme EA, Bucy DS, Hascall-Dove L, et al. Evaluation of standard chemotherapy in the guinea pig model of tuberculosis. *Antimicrob Agents Chemother*. 2010; 54:1820–1833. [PubMed: 20160055]
27. Comas I, Chakravarti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. *Nature Genetics*. 2010; 42:498–503. [PubMed: 20495566]
28. Sun R, Skeiky YA, Izzo A, Dheenadhyalan V, Imam Z, Penn E, et al. Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with *Mycobacterium tuberculosis*. *Vaccine*. 2009; 27:4412–4423. [PubMed: 19500523]
29. Abebe F, Bjune G. The emergence of Beijing family genotypes of *Mycobacterium tuberculosis* and low-level protection by bacille Calmette-Guerin (BCG) vaccines: is there a link? *Clin Exp Immunol*. 2006; 145:389–397. [PubMed: 16907905]
30. Kremer K, van-der-Werf MJ, Au BK, Anh DD, Kam KM, van-Doorn HR, et al. Vaccine-induced immunity circumvented by typical *Mycobacterium tuberculosis* Beijing strains. *Emerg Infect Dis*. 2009; 15:335–339. [PubMed: 19193289]

FIGURE 1.

Clinical strains of *M. tuberculosis* TN14149 and SA161 show increased organ bacterial loads. Panel A shows the bacterial growth in the lungs, spleens and lymph nodes from guinea pigs receiving a low dose aerosol of *Mycobacterium tuberculosis* laboratory strains H37RV (■), Erdman (●), and clinical strains TN5904 (▽), TN14149 (◇) and SA161 (□) was assayed on day 10, 30, and 60 after infection. Results are expressed logarithmically as the mean Log10 bacilli colony forming units (CFU) ($\pm$ SEM, n=5). Student t-test, TN14149, SA161, TN5904 compared to H37Rv and Erdman, *p<0.050, Panel B shows the median survival days of Hartley guinea pigs (n=10) infected with the clinical and laboratory and strains Kaplan Meier analysis, Student t-test, TN14149, SA161, TN5904 compared to H37Rv and Erdman, *p<0.050.

FIGURE 2.

Increased granulomatous responses from guinea pigs infected with the clinical strains of *M. tuberculosis* TN14149 and SA161. Panel A shows representative photomicrographs from sections of paraformaldehyde-fixed and paraffin embedded guinea pig tissues from lung (A-T) which were collected on day 30 and 60 after infection with the laboratory strains H37Rv (A, B, K, L), Erdman (C, D, M, N), MDR-TB strain 5904 (E, F, O, P), W-Beijing strains TN14149 (G, H, Q, R) and SA161 (I, J, S, T) of *M. tuberculosis*. By day 30, lesions in lung had extensive foci of mixed inflammation with central necrosis (G-J) compared to the laboratory strains (A-D). In addition the clinical strains showed by day 60, a severe increased in secondary lesion progression in which multiple foci of extensive inflammation coalesced within the pulmonary parenchyma. The increased rate of progression and involvement of large areas of lung correlated with reduce animal survival (Q-T) compared to the laboratory strains (K-N). The MDR TN5904 strain did not show substantial differences in bacterial load and pathology (E-F) during acute, subacute and chronic infection (O-P) compared to the laboratory strains (A-D; K-N). Hematoxylin and Eosin staining, total magnification=A, C, E, G, I, K, M, O, Q, S=10X and B, D, F, H, J, L, N, P, R, T=20X.

FIGURE 3.

Clinical strains of *M. tuberculosis* TN14149 and SA161 show increased lesion scores in the lungs, lymph nodes and spleens. Panel A shows the lesion scores of lungs, panel B lymph nodes and panel C, spleens for the guinea pigs on day 10, 30 and 60 after infection with the various strains of *M. tuberculosis*. The histopathology was characterized using a lesion scoring system that showed the significant extent of lung disease compared to the other organs during chronic infection (n=5, ANOVA, *p<0.05).

FIGURE 4.

Flow cytometric analysis of T cell subsets accumulating in the lungs and lymph nodes over the course of the infection with *Mycobacterium tuberculosis* laboratory strains H37Rv (■), Erdman (●), and clinical strains TN5904 (▽), TN14149 (◇) and SA161 (□). Cell numbers expressed as total cells ($\times 10^7$) expressing each phenotype per 1.0 gram of each tissue (n=4, mean values $\pm$ SEM). Cell numbers in all data sets after day-10 were significantly raised in the two W-Beijing infections compared to the two laboratory strains (Student *t*-test * $p < 0.01$).

FIGURE 5.

Flow cytometric analysis of macrophages and neutrophils accumulating in the lungs and lymph nodes over the course of the infection with *Mycobacterium tuberculosis* laboratory strains H37RV (■), Erdman (●), and clinical strains TN5904 (▽), TN14149 (◇) and SA161 (□). Panel A shows a representative example of macrophage gating strategy. Panel B shows numbers of total MR-1+ macrophages and MR-1+ cells expressing MHC Class-II molecules in the lungs and lymph nodes over the course of the infection. Data is shown as mean values (n=4, mean values ± SEM) for total cells ×10⁷ per 1.0 gram of tissue. MR-1+ MHC Class-II+ cell numbers on day-30 were significantly raised in the two W-Beijing infections compared to the two laboratory strains (Student *t*-test *p<0.01). Panel C shows numbers of MIL4+ neutrophils accumulating over the course of the infection (n=4, mean values ± SEM) for total cells ×10⁷ per 1.0 gram of lung tissue. Cell numbers were significantly raised in the two W-Beijing infections compared to the two laboratory strains from day-10 onwards (Student *t*-test *p<0.01).

FIGURE 6.

Increased TGF β , IL-10 and Fc γ messenger RNA (mRNA) expression in guinea pig infected with the clinical strains of *M. tuberculosis* TN14149 and SA161.

Panel A shows IFN- and IL-12p40 and panel B shows TGF β , Fc γ and IL-10 expression in the lungs on days 10, 30, and 60 from guinea pigs exposure to a low dose of with *Mycobacterium tuberculosis* laboratory strains H37RV (■), Erdman (●), and clinical strains TN5904 (▽), TN14149 (◇) and SA161 (□) were compared. Cytokine mRNA expression was quantified using real-time reverse transcription-polymerase chain reaction. Fold induction of mRNA was calculated from the threshold cycle (Ct) normalized to HPRT Ct values and then to uninfected guinea pig lung cells. Results are expressed as the average (n=4) of the fold induction in each group ($\pm$ SEM).