In vivo characteristics of Korean Beijing Mycobacterium tuberculosis strain K1 in an aerosol challenge model and in the Cornell latent tuberculosis model

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INTRODUCTION

The Beijing Mycobacterium tuberculosis family is widely distributed and is the most common M. tuberculosis strain in East Asia. The highly transmissible and predominant Beijing M. tuberculosis strain in Korea, M. tuberculosis K1, was characterized using an aerobic challenge mouse model and a latent tuberculosis model with M. tuberculosis H37Rv as a reference. M. tuberculosis K1 multiplied over ten times more rapidly than M. tuberculosis H37Rv during the early stage of infection and induced high levels of histopathology in the lung. Low levels of T helper cell (Th) Th1 [interferon (IFN)-γ], interleukin (IL)-12p40] and Th2 cytokines (IL-4, IL-10) were induced in the lungs of M. tuberculosis K1-infected mice. In the latent model, mice infected with M. tuberculosis K1 exhibited more frequent relapse from the latent state than did mice infected with M. tuberculosis H37Rv. In conclusion, M. tuberculosis K1, a prevalent Beijing strain in Korea, is expected to spread due to its rapid growth during the early stages of infection, low-level induction of the immune response and high relapse rates from a latent state.

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Abbreviations: H&E, haematoxylin and eosin; IFN, interferon; IL, interleukin; INH, isoniazid; PZA, pyrazinamide; TB, tuberculosis; Th, T helper cell; TNF, tumour necrosis factor.

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survey data by the CDC in Korea). Nearly 10% of Korean TB cases are drug-resistant (Hong et al., 1998) and even more concerning is that the proportion of extensively drug-resistant TB in all multidrug-resistant TB cases was found to be approximately 5–15% (Jain & Dixit, 2008; Kim et al., 2008). DNA fingerprinting of M. tuberculosis strains in Korea has revealed that a member of the Beijing M. tuberculosis family was predominant and this genotype accounted for 18.4% of the total M. tuberculosis isolates (Choi et al., 2010; Kim et al., 2001; Park et al., 2000). Kim et al. (2001) identified a highly transmissible M. tuberculosis strain, which caused an outbreak in high school students in Korea, which belongs to the K family; and this was named M. tuberculosis K1.

M. tuberculosis outbreak strains such as M. tuberculosis HN878 and CDC1551 have been characterized using animal models (Manca et al., 1999, 2001). Currently, the characteristics of predominant M. tuberculosis strains are being elucidated. Marquina-Castillo et al. (2009) reported that highly virulent M. tuberculosis 1020319, a predominant strain in the study population, could be transmitted to other animals. Relapse from latent infection as a novel virulence factor has been considered to contribute to the widespread prevalence of M. tuberculosis strains (Henao-Tamayo...
et al., 2009; Schulzer et al., 1987). However, relapse of the prevalent *M. tuberculosis* strains from a latent infection has not been explored in animal models.

In this study, the virulence and immunopathology of a prevalent *M. tuberculosis* K1 strain in Korea, which is highly transmissible and belongs to the Beijing *M. tuberculosis* family, were characterized using an aerobic infection mouse model. Moreover, the relapse from the latent infection of this strain was investigated using the Cornell model, a latent TB model, using *M. tuberculosis* H37Rv as a reference.

**METHODS**

**Animals.** Specific pathogen-free female C57BL/6 mice at 5–6 weeks of age were purchased from Japan SLC and maintained under barrier conditions in a biohazard animal room at Yonsei University Medical Research Center. All animal experiments were done according to the regulations of the Institutional Animal Care and Use Committee, Yonsei University Health System.

**Bacterial strains.** *M. tuberculosis* H37Rv (ATCC 27294) was purchased from the ATCC and *M. tuberculosis* K1 was obtained from the strain collections at the Korean Institute of Tuberculosis, Korean National Tuberculosis Association, Seoul, Republic of Korea. Each strain was prepared as previously described (Jeon et al., 2008). In brief, each strain was grown in Middlebrook 7H9 medium (Difco) supplemented with 10% Middlebrook OADC enrichment medium (BBL) until the late-exponential phase. The cells were frozen at −70 °C until used.

*M. tuberculosis* infection, bacterial counts and survival analysis in mice. *M. tuberculosis* challenge studies were performed as previously described (Jeon et al., 2008). For analysis of mycobacterial growth in mice, the C57BL/6 mice were challenged by aerosol exposure to *M. tuberculosis* H37Rv or K1 using an inhalation device (Glas-Col) calibrated to deliver approximately 200 bacteria into the lungs of each mouse. Five mice were sacrificed for bacterial counts at each time point for each strain and the numbers of viable bacteria in the lung and spleen were determined by plating serial dilutions of whole organ homogenates.

For survival analysis, 15 C57BL/6 mice per strain (total 30 mice) were aerobically challenged with a high dose (approx. 900 c.f.u.) of *M. tuberculosis* H37Rv or K1. Infected mice were monitored three times per week for survival.

**Analysis of relapse of *M. tuberculosis* strains using the Cornell model.** The relapse rates of *M. tuberculosis* H37Rv and K1 were analysed using the Cornell model, a latent TB infection model, as previously described with minor modification (see Fig. 5a) (Ha et al., 2003, 2005). Briefly, 30 mice per *M. tuberculosis* strain were aerobically challenged with a low dose (approx. 200 c.f.u. per each mouse) of *M. tuberculosis* H37Rv or K1. Mice were treated with isoniazid (INH) at 25 mg kg$^{-1}$ day$^{-1}$ and pyrazinamide (PZA) at 1000 mg kg$^{-1}$ day$^{-1}$, in the diet for 90 days, starting 30 days after challenge. Bacterial counts were taken from the lungs and spleens of mice at 10 days after the completion of 90 days of chemotherapy to confirm the clearance of viable bacteria. Viable bacteria in tissues from mice were counted at 200 or 300 days post-challenge.

**Fig. 1.** Growth of *M. tuberculosis* H37Rv and K1 in the lungs (a) and spleens (b) of C57BL/6 mice after aerosol challenge. The number of c.f.u. was measured in the lungs and spleens of five mice at 15, 30, 60, 90 and 150 days post-challenge. The experiment was repeated three times and data from one representative experiment are shown. Data are presented as mean c.f.u. ± SD from five mice at each time point. A *P*-value < 0.05 was considered to be significant and is represented as follows: *P* < 0.05, **P** < 0.01 and ***P*** < 0.001. ○, H37Rv; ●, K1.

**Fig. 2.** Survival rate of C57BL/6 mice after aerosol challenge with *M. tuberculosis* H37Rv or *M. tuberculosis* K1. Fifteen mice were aerobically challenged with a high dose (approx. 900 c.f.u.) of virulent *M. tuberculosis* H37Rv or *M. tuberculosis* K1 and infected mice were monitored three times per week. ●, H37Rv; ◆, K1.
**Quantitative RT-PCR analysis of cytokines in the lungs.** Right lung lobes from five mice per group at each time point were used to isolate mRNA. Lung tissues were homogenized in Trizol (Invitrogen) and total RNA was extracted using an RNeasy mini kit (Qiagen). Reverse transcription of mRNA was performed using a Superscript III first-strand synthesis kit (Invitrogen). RT-PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) and a DyNAmo HS SYBR Green qPCR kit (Finnzymes). Standard curves of quantified and diluted PCR product, as well as negative controls, were included in each PCR experiment. Specific primers were designed for the following targets: glyceraldehyde-3-phosphate dehydrogenase: 5’-ACAACATTGCCATTGGAA-3’, 5’-GATG-CAGGGATGATGTTCTG-3’; IFN-γ: 5’-AAATCTCGAGGCCAGAT-3’, 5’-CTTGCTGTTTGCAGAAAGG-3’; IL-12p40: 5’-ACTCAGTCTGCTCCAC-3’, 5’-GTCCGGAGTAATTGGTCT-3’; tumour necrosis factor (TNF)-α: 5’-CCAAAGGATGAGAAGTTCG-3’, 5’-TCACACTGGTGCTCCAC-3’, 5’-CTCGAGAAGGAGACTTCA-3’, 5’-ATTTCCACGATTTCCCAGAG-3’; IL-4: 5’-CCAGGATGAGAAGTTCG-3’, 5’-CCGAGGGAGGAGACTTCA-3’, 5’-ATTTCCACGATTTCCCAGAG-3’; IL-6: 5’-ACAACTTTGGCATTGTGGAA-3’, 5’-CTTGCTGTTGCTGAAGAAGG-3’; IL-10: 5’-CAAGGATTTCCACGATTTCCCAGAG-3’, 5’-ATTTCCACGATTTCCCAGAG-3’; IL-12p70: 5’-AAAATCCTGCAGAGCC-3’, 5’-AAAATCCTGCAGAGCC-3’. Quantities of the specific mRNA in the samples were measured according to the corresponding gene-specific standard. The mRNA copy number of each cytokine was analysed by the 2^-ΔΔCt method using the gene encoding glyceraldehyde-3-phosphate dehydrogenase as a reference.

**Histopathology and assessment of lung inflammation.** Lung sections were excised from five mice per group at each time point and stored in 10% formalin and then embedded and stained with haematoxylin and eosin (H&E) for pathological analysis. Evaluation of the levels of inflammation in the lungs of mice was performed as described previously (Leong et al., 2008). In brief, H&E-stained lung sections were photographed using a microscope (Olympus BX51) and the images were analysed using the ImageJ program (National Institutes of Health) to assess the level of lung inflammation. The resulting values are presented as the mean per cent of the inflamed area from lung sections of five mice per group.

**Statistical analysis.** Differences between experimental groups were analysed by Student’s t-test or the χ^2 test using R statistical software (version 2.6.2). A P-value <0.05 was considered statistically significant.

**RESULTS**

**Growth of *M. tuberculosis* strains in mice after aerosol challenge**

After challenging C57BL/6 mice with *M. tuberculosis* H37Rv or K1, the bacterial burdens were measured at 15, 30, 60, 90 and 150 days post-challenge (Fig. 1). Bacterial c.f.u. increased rapidly during the early stage of infection (3.9 and 5.4 log_{10} *M. tuberculosis* H37Rv and K1, respectively, at 15 days post-challenge, *P*<0.001). Bacterial c.f.u. in the lungs reached a peak at 30 days post-challenge in the lungs (5.1 and...
Survival rates of mice infected with *M. tuberculosis* H37Rv or K1

Fifteen mice per *M. tuberculosis* strain were challenged with a high dose of H37Rv or K1 and the survival periods after challenge were measured (Fig. 2). *M. tuberculosis* H37Rv-infected mice started to die approximately 200 days post-challenge and only six mice were alive 560 days post-challenge. In contrast, *M. tuberculosis* K1-infected mice started to die approximately 100 days post-challenge and all mice were dead by 350 days post-challenge. The mean survival periods were 443 ± 80 and 256 ± 65 days for the animals infected with *M. tuberculosis* H37Rv and K1, respectively (P<0.001).

Cytokine profiles in the lung after aerobic challenge

To examine the immune responses of the host after aerobic infection with *M. tuberculosis* H37Rv or K1, the expression levels of mRNA of cytokines such IFN-γ, IL-12p40, TNF-α, IL-4, IL-6 and IL-10 were measured in the lungs of *M. tuberculosis*-infected mice (Fig. 3). The expression levels of IFN-γ and IL-12p40, typical Th1 cytokines, increased after aerosol challenge with *M. tuberculosis* H37Rv until 60 days post-challenge, while the expression levels of these cytokines in the *M. tuberculosis* K1-infected mice increased until 30 days post-challenge and then decreased at 60 days post-challenge (P<0.001, H37Rv versus K1). TNF-α was induced in the lungs of both *M. tuberculosis* H37Rv- and K1-infected mice, and the expression levels of this cytokine in the *M. tuberculosis* K1-infected mice were slightly higher than those in the *M. tuberculosis* H37Rv-infected mice at 60 days post-challenge (P<0.05).

Low levels of IL-4 and IL-10, typical Th2 cytokines, were detected after challenge with *M. tuberculosis* strains and the expression levels of these cytokines increased dramatically at 60 days post-challenge in the *M. tuberculosis* strains while remaining at a very low level in the *M. tuberculosis* K1-infected mice at 60 days post-challenge (P<0.001). IL-6, a proinflammatory cytokine, was highly expressed at 30 days post-challenge, but then decreased according to the stage of infection both in the *M. tuberculosis* H37Rv- and in the K1-infected mice. The expression levels of IL-6 cytokine in the *M. tuberculosis* K1-infected mice were lower than those in the *M. tuberculosis* H37Rv-infected mice (P<0.05 and P<0.01, H37Rv versus K1 at 30 days and 60 days postchallenge, respectively).

Lung pathology after aerobic *M. tuberculosis* infections

The histopathology of lungs was compared after aerobic challenge with virulent *M. tuberculosis* strains (Fig. 4).
Granulomatory inflammations were clearly seen from 30 days post-challenge in both the M. tuberculosis H37Rv- and K1-infected mice, but there was no significant difference in the level of lung inflammation between the two groups. Then, the level of granulomatory inflammation in the M. tuberculosis K1-infected mice increased and was significantly higher than that in M. tuberculosis H37Rv-infected mice through 150 days post-challenge (P<0.001 at 60 days post-challenge, P<0.5 both at 90 and 150 days post-challenge).

**Relapse rates of M. tuberculosis H37Rv and K1 in the latent mouse model**

Relapses of M. tuberculosis strains from the latent infection were investigated using the Cornell model, a latent TB mouse model (Fig. 5a). At the start of drug treatment, bacterial c.f.u. were 5.21 and 5.79 log\(_{10}\) in the lungs of mice challenged with M. tuberculosis H37Rv and K1, respectively (Fig. 5b). After completion of chemotherapy, no viable M. tuberculosis was detected in the M. tuberculosis H37Rv- or K1-infected mice. Interestingly, viable M. tuberculosis was detected at 210 and 300 days post-challenge, that is, at 90 and 180 days after the completion of chemotherapy, respectively (Fig. 5, Table 1). No viable bacterium was detected in the M. tuberculosis H37Rv-infected mice at 210 days post-challenge, but bacteria were revived in two of the eight mice at 300 days post-challenge. In contrast, the percentages of M. tuberculosis K1-infected mice with bacterial revival were 27.3% (3/11) and 62.5% (5/8) at 210 and 300 days post-challenge, respectively. In summary, M. tuberculosis H37Rv was revived in 10.5% (2/19) of mice, whereas M. tuberculosis K1 was revived in 42.1% (8/19) of mice (P<0.05, \(\chi^2\) test).

**DISCUSSION**

We characterized the M. tuberculosis K1 strain, a predominant and highly transmissible Beijing M. tuberculosis genotype in Korea, by using an aerobic TB challenge model and a latent TB mouse model.

After aerosol challenge, M. tuberculosis K1 grew approximately tenfold faster in the early stage of infection and induced a higher level of lung inflammation than M. tuberculosis H37Rv. These results are consistent with previous reports that highly virulent M. tuberculosis strains such as M. tuberculosis HN878 and M. tuberculosis NY669 replicated rapidly during the early stages of infection and induced severe lung inflammation (Manca et al., 2001; Marquina-Castillo et al., 2009; Henao-Tamayo et al., 2009; Abebe & Bjune, 2006; Palanisamy et al., 2008).

Interestingly, the expression levels of Th1 as well as Th2 cytokines in the M. tuberculosis K1-infected mice were lower compared with expression levels in M. tuberculosis H37Rv-infected mice. These results are comparable with hypervirulent M. tuberculosis HN878, a Beijing genotype, which induced very low levels of Th1 cytokines IFN-\(\gamma\) and IL-12, while M. tuberculosis CDC1551, a low virulent M. tuberculosis strain, induced robust host immune responses (Manca et al., 1999). The cytokine expression pattern induced by M. tuberculosis K1 was also consistent with a highly transmissible

![Fig. 5. Relapse of M. tuberculosis H37Rv or K1 after the completion of 90 days of chemotherapy in the latent TB model.](image-url)
Table 1. Relapse rates of *M. tuberculosis* H37Rv and K1 in a latent TB model

Numbers indicate no. of mice with regrowth of *M. tuberculosis*/no. of mice infected with *M. tuberculosis*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Organ</th>
<th>210 days*</th>
<th>300 days†</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>Lung</td>
<td>0/11 (0.0%)</td>
<td>2/8 (25.0%)</td>
<td>2/19 (10.5%)</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>0/11 (0.0%)</td>
<td>1/8 (12.5%)</td>
<td>1/19 (5.3%)</td>
</tr>
<tr>
<td>K1</td>
<td>Lung</td>
<td>3/11 (27.3%)</td>
<td>5/8 (62.5%)</td>
<td>8/19 (42.1%)‡</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>2/11 (18.2%)</td>
<td>4/8 (50.0%)</td>
<td>6/19 (31.6%)§</td>
</tr>
</tbody>
</table>

*The regrowth of *M. tuberculosis* strains was observed at 210 days post-challenge (90 days after the completion of 90 days of chemotherapy).
†The regrowth of *M. tuberculosis* strains was observed at 300 days post-challenge (170 days after the completion of 90 days of chemotherapy).
‡ Relapse rate of *M. tuberculosis* K1 in the lung is significantly higher than that of *M. tuberculosis* H37Rv (*P*<0.05).
§ Relapse rate of *M. tuberculosis* K1 in the spleen is significantly higher than that of *M. tuberculosis* H37Rv (*P*<0.05).

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REFERENCES


