**INTRODUCTION**

The Beijing *Mycobacterium tuberculosis* family, the predominant genotype of *M. tuberculosis* strains, is distributed widely around the world (Glynn et al., 2002) and is highly prevalent in East Asia (European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis, 2006). The frequencies of Beijing *M. tuberculosis* genotypes are estimated at 92% in China, 72% in Korea and 40% in Vietnam (Glynn et al., 2002). Beijing genotypes are associated with drug resistance of *M. tuberculosis* strains including single-drug resistance and multidrug resistance (Buu et al., 2009). Moreover, Beijing genotypes are associated with the relapse of *M. tuberculosis* from the latent state (Burman et al., 2009).

In Korea, the overall prevalence of tuberculosis (TB) has decreased (prevalence of direct smear-positive TB, 686 per 100 000 in 1965 versus 93 per 100 000 in 1995) (Hong et al., 1998), but is still relatively high (0.11%) (unpublished survey data by the CDC in Korea).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 \textit{M. tuberculosis} strains from a latent infection has not been explored in animal models.

In this study, the virulence and immunopathology of a prevalent \textit{M. tuberculosis} K1 strain in Korea, which is highly transmissible and belongs to the Beijing \textit{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 \textit{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.** \textit{M. tuberculosis} H37Rv (ATCC 27294) was purchased from the ATCC and \textit{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\textdegree C until used.

\textit{M. tuberculosis} infection, bacterial counts and survival analysis in mice. \textit{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 \textit{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 \textit{M. tuberculosis} H37Rv or K1. Infected mice were monitored three times per week for survival.

**Analysis of relapse of \textit{M. tuberculosis} strains using the Cornell model.** The relapse rates of \textit{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 \textit{M. tuberculosis} strain were aerobically challenged with a low dose (approx. 200 c.f.u. per each mouse) of \textit{M. tuberculosis} H37Rv or K1. Mice were treated with isoniazid (INH) at 25 mg kg\textsuperscript{-1} day\textsuperscript{-1} and pyrazinamide (PZA) at 1000 mg kg\textsuperscript{-1} day\textsuperscript{-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 \textit{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 \textit{M. tuberculosis} H37Rv or \textit{M. tuberculosis} K1. Fifteen mice were aerobically challenged with a high dose (approx. 900 c.f.u.) of virulent \textit{M. tuberculosis} H37Rv or \textit{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 RNAeasy 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\'-ACAACCTTTGCAATGGAGA-3', 5\'-GATGCGGGTATGTTCTG-3'; IFN-\(\gamma\): 5\'-AAATTCTGCAAGGCCAGAT-3', 5\'-CTTGGTGTGGCAAGAACAG-3'; IL-12p40: 5\'-ACTCACATTCGTGCTCCAC-3', 5\'-GTCCGGAGATTTGATTCG-3'; tumour necrosis factor (TNF)-\(\alpha\): 5\'-CCCAAAGGAGAGACGATTCC-3', 5\'-CTCCACTTTGATGTTGGGTA-3'; IL-4: 5\'-CCAAAGGTGCTTGCA-3', 5\'-CCAAAGGAGAACATTGC-3'; IL-6: 5\'-AGTCCGAGAGGAAGCTCA-3', 5\'-ATTTCCAGATTTCCAGAG-3'; IL-10: 5\'-CCGAGAAATCAGGAGATT-3', 5\'-TCATCCTTCACCTGCTCCAC-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 RT-PCR 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 (Leon 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 \(F\)-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


5.7 log\textsubscript{10} \textit{M. tuberculosis} H37Rv and K1, respectively, \( P<0.05 \). The bacterial c.f.u. in the spleens of mice infected with \textit{M. tuberculosis} K1 were significantly higher than those from spleens of \textit{M. tuberculosis} H37Rv-infected mice (3.4 and 4.5 log\textsubscript{10} \textit{M. tuberculosis} H37Rv and K1, respectively, \( P<0.01 \)). After bacterial c.f.u. reached their peaks at 30 days post-challenge, the bacterial c.f.u. of both \textit{M. tuberculosis} strains were maintained through 150 days post-challenge, and the c.f.u. of \textit{M. tuberculosis} H37Rv and K1 approached similar levels according to the stage of infection.

**Survival rates of mice infected with \textit{M. tuberculosis} H37Rv or K1**

Fifteen mice per \textit{M. tuberculosis} strain were challenged with a high dose of H37Rv or K1 and the survival periods after challenge were measured (Fig. 2). \textit{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, \textit{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 \textit{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 \textit{M. tuberculosis} H37Rv or K1, the expression levels of mRNA of cytokines such as IFN-\( \gamma \), IL-12p40, TNF-\( \alpha \), IL-4, IL-6, and IL-10 were measured in the lungs of \textit{M. tuberculosis}-infected mice (Fig. 3). The expression levels of IFN-\( \gamma \) and IL-12p40, typical Th1 cytokines, increased after aerosol challenge with \textit{M. tuberculosis} H37Rv until 60 days post-challenge, while the expression levels of these cytokines in the \textit{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-\( \alpha \) was induced in the lungs of both \textit{M. tuberculosis} H37Rv- and K1-infected mice, and the expression levels of this cytokine in the \textit{M. tuberculosis} K1-infected mice were slightly higher than those in the \textit{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 \textit{M. tuberculosis} strains and the expression levels of these cytokines increased dramatically at 60 days post-challenge in the \textit{M. tuberculosis} H37Rv-infected mice while remaining at a very low level in the \textit{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 \textit{M. tuberculosis} H37Rv- and in the K1-infected mice. The expression levels of IL-6 cytokine in the \textit{M. tuberculosis} K1-infected mice were lower than those in the \textit{M. tuberculosis} H37Rv-infected mice (\( P<0.05 \) and \( P<0.01 \), H37Rv versus K1 at 30 days and 60 days post-challenge, respectively).

**Lung pathology after aerobic \textit{M. tuberculosis} infections**

The histopathology of lungs was compared after aerobic challenge with virulent \textit{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 log10 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, χ² 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 & Bjuene, 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-γ 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
Table 1. Relapse rates of M. tuberculosis H37Rv and K1 in a latent TB model

<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).

and predominant M. tuberculosis 1020319 strain that induced low levels of Th1 cytokines IFN-γ and IL-12, as well as Th2 cytokines IL-4 and IL-10 (Marquina-Castillo et al., 2009). These findings suggest that the M. tuberculosis K1 strain, like other highly virulent M. tuberculosis strains, might inhibit host immune responses to escape the immune system.

In a latent TB model, M. tuberculosis K1 was found to reactivate more frequently from the latent state than M. tuberculosis H37Rv. This is the first report, to our knowledge, on the comparison of relapse of M. tuberculosis strains using a latent animal model. This result is consistent with the report of Burman et al. (2009) that Beijing strains were strongly associated with relapse in the Asia-Pacific area. It is not clear how Beijing strains could efficiently reactivate from the latent state, but the high expression of latency-related genes, including the dormancy regulon and dormancy survival regulator (DosR), in the M. tuberculosis K1 strain in vitro latency model and in the non-replicating persistence oxygen depletion model compared with those of M. tuberculosis H37Rv might be related to the high reactivation of M. tuberculosis K1 from the latent state (Honaker et al., 2009; M.-Y. Hahn, unpublished data).

Future studies with more Beijing Korean strains, Beijing China strains and other non-Beijing strains using susceptible models and models showing human-like-pathology would confirm their characteristics and reveal more virulence factors.

In summary, the predominant and highly transmissible M. tuberculosis strain in Korea, M. tuberculosis K1 of the Beijing family, multiplied rapidly during the early stage of infection, but induced low levels of immune response. Moreover, M. tuberculosis K1 relapsed frequently from its latent state. These characteristics of M. tuberculosis K1 may enable it to spread and become predominant in a community.

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