Apoptosis, production of MMP9, VEGF, TNF-α and intracellular growth of *M. tuberculosis* for different genotypes and different *pks15/1* genes

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Summary

Background: **A previous study of IS*6110* RFLP and spoligotyping of *M. tuberculosis* isolates from 152 Thai patients with tuberculous meningitis revealed a significantly higher percentage (57%) of the Beijing genotype as compared to isolates obtained from pulmonary tuberculosis. We postulated that the *M. tuberculosis* Beijing genotype is likely to be more virulent than others.**

Objectives: **Ten *M. tuberculosis* cerebrospinal fluid (CSF) isolates from five RFLP groups, together with different characteristics of *pks15/1*, *M. tuberculosis* H37Rv and *M. bovis* BCG, were investigated for their virulence *in vitro*.**

Methods: **In this study, THP-1 cells were used as host cells to determine the intracellular growth and the induction of MMP9, VEGF, TNF-α and apoptosis. Determinations of the cytokine production and apoptosis were based on available commercial kits using ELISA techniques.**

Results: **No significant difference in intracellular multiplication was found between the *M. tuberculosis* CSF isolates. Three isolates, consisting of 2 Nonthaburi and 1 heterogeneous isolate, were found to stimulate high TNF-α and MMP-9 production during the early infection period.They were isolated from 3 different patients, 2 of whom died with initial stages II and III. This result suggested that there might be an association between TNF-α and MMP-9 production that could account for the specific virulent nature of Nonthaburi strains. VEGF production was determined and comparable levels were found in all isolates. No significant apoptosis was detected in *M. tuberculosis* CSF isolates. No significant differences suggesting that the 2 Beijing strains are more virulent than the others were observed.**

Conclusion: **The predominance of the Beijing strains in cases of tuberculous meningitis (TBM) in Thai patients is not a result of their hypervirulence.**
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Key words: *Apoptosis, MMP9, VEGF, TNF-α,* ***pks15/1* gene, *M. tuberculosis***

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Introduction

Tuberculous meningitis (TBM), the most severe form of extrapulmonary tuberculosis, is caused by *Mycobacterium tuberculosis*. The WHO estimated that 9.27 million new cases of tuberculosis (TB) occurred in 2007, most of which were in Asia[1]. The incidence of TBM has been calculated as a 1% annual risk of extrapulmonary tuberculosis[2]

M. tuberculosis has evolved multiple mechanisms to interfere with the host immune system. Many of these mechanisms are mediated by specific components of the mycobacterial cell wall, a highly complex structure consisting of various types of long-chain fatty acids, glycolipids and lipoproteins[3]. Phenolic glycolipids (PGL) produced by an intact *pks15/1* found in some isolates are related to a hypervirulent phenotype[4]. In H37Rv and CDC1551, the absence of PGL production has been confirmed to be associated with a 7-base-pair deletion resulting in a frame shift in what would otherwise be predicted to be a single gene encompassing *pks1* and

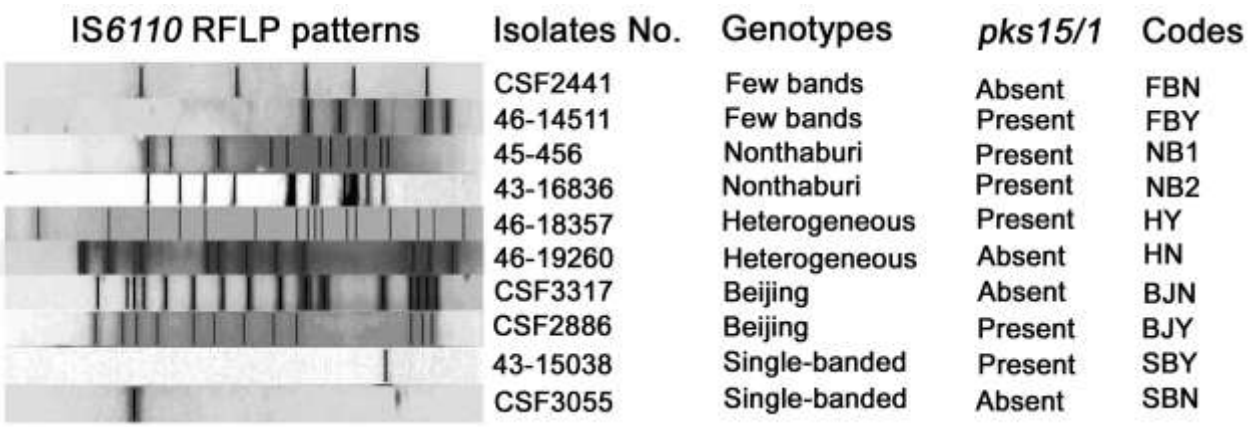

Figure 1. IS*6110* fingerprints of 10 *M. tuberculosis* CSF isolates for cell infection.[14]
Genomic DNA was digested with *Pvu*II and Southern-blot-hybridized with a fluorescein-labeled 326-bp fragment of an IS*6110* DNA probe. The IS*6110* hybridization patterns were analyzed using GelCompar II version 1.5 (Applied Maths, Kortrijk, Belgium). FBN, Few bands no insertion of *pks15/1;* FBY, Few bands 7-bp insertion of *pks15/1;* NB1, Nonthaburi 7-bp insertion of *pks15/1* no. isolate 45-456; NB2, Nonthaburi 7-bp insertion of *pks15/1* no. isolate 43-16836; HY, Heterogeneous with more than 5 bands 7-bp insertion of *pks15/1;* HN, Heterogeneous with more than 5 bands no insertion of *pks15/1*; BJN, Beijing no insertion of *pks15/1*; BJY, Beijing 7-bp insertion of *pks15/1;* SBY, Single-band 7-bp insertion of *pks15/1;* SBN, Single-band no insertion of *pks15/1*.

pks15[5]. An intact *pks1-15* gene (*pks15/1*) was identified in HN878, 210, W4 and W10, which are members of the W-Beijing group of strains; these strains can produce PGL and cause a high mortality rate in the rabbit meningitis model[4]. Furthermore, an intact *pks1-15* gene has been found in other clinical isolates (non-W-Beijing strains) obtained from cerebrospinal fluid (CSF) and sputum[6].

Some secreted molecules from the host, such as matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF) and tumour necrosis factor α (TNF-α), have been found to be involved in blood-brain barrier (BBB) disruption and related to the pathogenesis of TBM. The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play an essential role in the physiological and pathological remodelling of connective tissue by degrading extracellular macromolecules. MMP-9 is found in the basement membrane and plays a major role in the breakdown of the blood-brain barrier (BBB). Normally, MMP-9 is not present in CSF, but it has been detected in patients with viral, bacterial and tuberculous meningitis. MMP-9 has been detected at a high level in patients with TBM and at a low level in patients with meningitis resulting from other infections[7, 8]. VEGF is a potent, multifunctional cytokine that contributes to angiogenesis and inflammation. It has been found to be associated with inflammatory diseases and an increased VEGF serum level has been found in cases of pulmonary tuberculosis. The serum and CSF VEGF levels were significantly higher in TBM than in other forms of meningitis[9]. VEGF levels in the CSF were found to be correlated with BBB disruption[10]. In addition, a significant correlation was established between the level of VEGF and the level of MMP-9 in pleural effusions. VEGF was found to be able to stimulate MMP-9 secretion[11]. The level of TNF-α produced during mycobacterial CNS infection determines the extent of pathogenesis. High levels of TNF-α in the CSF are associated with high leukocytosis, protein accumulation and severe meningeal inflammation. Furthermore, TNF-α can stimulate secretion of both MMP-9 and VEGF, and this role may affect BBB disruption and cerebral oedema[12].

Another potential mechanism involved in the host response against *M. tuberculosis* is apoptosis, or programmed cell death. It has been shown that macrophage apoptosis results in reduced viability of mycobacteria. Attenuated *M. tuberculosis* H37Ra infection has been shown to induce a greater level of apoptosis in alveolar macrophages than infection by the virulent H37Rv strain[13]. Therefore, apoptosis in infected macrophages is considered part of the protective response.

Based on this information, it is interesting to examine *M. tuberculosis* isolated from the CSF of TBM patients. In this study, the host responses to infection by *M. tuberculosis* strains of various

genotypes namely Beijing, Nonthaburi, heterogenous group, few banders and single band IS*6110* RFLP with and without 7bp insertion in *pks15/1* genes were investigated in terms of cell-secreted molecules and the level of cell apoptosis in infected macrophages. A hypervirulent strain should be able to induce high level production of TNF-α, MMP-9, VEGF and induce less apoptosis.

Methods

Cell culture

The human monocytic cell line THP-1 (ATCC TIB-202) was purchased from the American Type Culture Collection (ATCC Manassas, VA). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 2 mM L-glutamine and incubated at 37°C and 5% CO_2.

M. tuberculosis ***isolates***

Ten *M. tuberculosis* strains isolated from CSF which had different genotypes and different *pks15/1* configurations (Figure 1.)[14] as well as *M. tuberculosis* strain H37Rv and *M. bovis* BCG were used in this study.

Preparation of mycobacterial inoculum

M. tuberculosis CSF isolates, *M. tuberculosis* strain H37Rv and *M. bovis* BCG were grown to late log phase (14 days) in Middlebrook 7H9 with 10% OADC. Aliquots were frozen at -70ºC. Representative samples were thawed and the colony forming units (CFUs) per ml were enumerated by plating on Middlebrook 7H10 with 10% OADC.

THP-1 cell infection with M. tuberculosis

The human monocytic cell line THP-1 was cultured in RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat-inactivated FBS. The cells were stained with trypan blue to determine the percentage of viable cells. Cells with more than 95% viability were used in subsequent steps. THP-1 (2×10^5 cells/ml) was seeded in a total volume of 500 µl per well in 24-well tissue culture plates and allowed to adhere and differentiate at 37°C in 5% CO_2 for 24 hr in the presence of 100 nM Phorbol 12-Myristate 13-Acetate (PMA). The cells were washed, the medium was replaced to remove the PMA and the cultures were further incubated for 24 hr before the addition of mycobacteria. The mycobacterial inoculum was passed 10 times through a 26-gauge needle before being added to THP-1 at a multiplicity of infection (MOI) of 10^{15}. The infection was allowed to take place for 4 hr at 37°C in 5% CO_2. The wells were washed 3 times with 500 µl of cold PBS, to remove extracellular bacteria, and the medium was replenished. *M. tuberculosis* H37Rv and *M. bovis* BCG were included as reference strains. All experiments were performed independently at least 3 times in duplicate.

Determination of the intracellular growth of M. tuberculosis in human macrophages

For counting intracellular bacteria, the supernatant in each well was removed and stored at -70°C for further cytokine analysis and the cells were washed 3 times with 500 µl of cold PBS. The bacilli were released from the cells by adding 100 µl of cold PBS/ 0.1% Triton X-100. The suspension was serially diluted 10-fold and the dilutions of 10^{-2} to 10^{-5} were plated in triplicate on M7H10 with 10% OADC and incubated at 37°C. Samples were collected 4 hr after infection (day 0) as well as at 1, 3, and 6 days post-infection. The CFUs were counted after 3-4 weeks of incubation and reported as mean values. The growth index of intracellular bacteria was determined by dividing intracellular bacterial CFUs at days 0, 1, 3 and 6 by intracellular bacterial CFUs at day 0.

Measurement of TNF-α and VEGF

Culture supernatants from the infected THP-1 cells were harvested 4 hr after infection (day 0) and on days 1, 2 and 3, frozen at -70°C, and assayed with the TNF-α Human Biotrak Easy Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (GE Healthcare, UK). The VEGF in the supernatant was measured at each time point using the Biotrak VEGF Human ELISA system (GE Healthcare, UK).

Matrix metalloproteinase-9 (MMP-9) detection

Culture supernatants from infected THP-1 at 4 hr, 1 day, 3 days and 6 days post-infection were collected and frozen at -70°C. MMP-9 was measured using the MMP-9 Biotrak activity assay system (GE Healthcare, UK).

Detection of apoptosis in human macrophages infected with M. tuberculosis

THP-1 cells (2×10^4 cells/ml) in a volume of 100 µl were seeded in each well of 96-well tissue culture plates and allowed to adhere and differentiate at 37°C in 5% CO_2 for 24 hr in the presence of 100 nM Phorbol 12-Myristate 13-Acetate (PMA). The cells were washed and the medium was replaced before adding bacteria. The mycobacterial inoculum was prepared as mentioned above. The infection was allowed to take place for 4 hr at 37°C in 5% CO_2.

Table 1. Intracellular bacterial colony-forming units (CFUs) and growth indices for *M. tuberculosis* strain H37Rv, *M. bovis* BCG and ten *M. tuberculosis* CSF isolates at day 0, day 1, day 3 and day 6 post infection are shown as mean values with standard deviations (SD) from three independent experiments carried out in duplicate.

Isolates	Mean CFUs $\log_{10}$ ± SD (Growth index)			
	Day 0	Day 1	Day 3	Day 6
H37Rv	3.76 ± 1.06	3.37 ± 1.09 (0.99)	4.03 ± 0.64 (1.24)	4.93 ± 0.55 (1.52)
BCG	5.10[a] ± 0.24	5.04 ± 0.33 (0.99)	5.03[b] ± 0.27 (0.99)	5.76[b] ± 0.51 (1.13)
BJ-N	5.05[a] ± 0.13	5.21 ± 0.08 (1.03)	5.16 ± 0.22 (1.02)	5.78 ± 0.43 (1.15)
BJ-Y	4.64 ± 0.54	4.80 ± 0.43 (1.04)	4.95 ± 0.32 (1.07)	4.91[b] ± 0.42 (1.06)
FB-N	4.33 ± 0.71	4.08 ± 0.73 (1.08)	4.63 ± 0.49 (1.25)	5.12 ± 0.22 (1.45)
FB-Y	4.55 ± 0.52	4.77 ± 0.49 (1.05)	4.95 ± 0.37 (1.08)	4.79[b] ± 0.44 (1.06)
H-N	4.01 ± 0.86	3.88 ± 0.75 (1.08)	4.32 ± 0.54 (1.21)	4.92 ± 0.20 (1.37)
H-Y	4.77[a] ± 0.22	4.95 ± 0.12 (1.04)	5.39 ± 0.22 (1.13)	5.57 ± 0.15 (1.17)
NB-1	4.99[a] ± 0.24	5.20 ± 0.11 (1.04)	5.42 ± 0.13 (1.09)	5.46[b] ± 0.32 (1.10)
NB-2	4.79[a] ± 0.22	5.10 ± 0.23 (1.06)	5.44 ± 0.08 (1.14)	5.57 ± 0.15 (1.16)
SB-N	4.54 ± 0.50	4.65 ± 0.67 (1.02)	4.95 ± 0.37 (1.09)	4.86[b] ± 0.57 (1.07)
SB-Y	5.16[a] ± 0.29	5.21 ± 0.12 (1.01)	5.12[b] ± 0.15 (0.99)	5.89 ± 0.52 (1.14)

[a] Intracellular bacterial CFUs were higher than H37Rv with statistical significance ($p < 0.05$).
[b] Intracellular growth indexes were lower than H37Rv with statistical significance ($p < 0.05$).

The wells were washed 3 times with 100 µl cold PBS to remove extracellular bacteria and the medium was replenished. *M. tuberculosis* H37Rv and *M. bovis* BCG were included as reference strains. All experiments were performed independently at least 3 times in duplicate.

The amount of apoptosis was detected in infected THP-1 cells at 1, 3, and 5 days post-infection by determination of the mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates using the Cell Death Detection ELISA^{PLUS} (Roche, Germany) according to the manufacturer's protocol and was shown as the enrichment factor.

Data analysis

The results of the cell infection experiments were presented as the mean ± SD of three independent experiments. The significance of the differences between the experimental and control groups was analysed by Student's *t*-test using SPSS® ver.13.0. *p* -values less than 0.05 were considered statistically significant.

Results

Determination of the intracellular growth of *M. tuberculosis* in human macrophages

The intracellular bacteria in THP-1 cells were observed 4 hr after the start of infection (day 0) and at 1, 3, and 6 days post-infection (Table 1.). At day 0, the *M. tuberculosis* strains with the highest and lowest intracellular bacterial CFUs were SBY and H37Rv, respectively.

At 6 days post-infection, the *M. tuberculosis* strain H37Rv showed the highest bacterial growth index, whereas the *M. tuberculosis* strains "few bands with insertion" (FBY), "single band with no insertion" (SBN) and "Beijing with insertion" (BJY) showed the lowest intracellular growth index. When the amounts of intracellular bacteria were compared between *M. tuberculosis* strains containing a 7-bp insertion (intact *pks15/1*) and no insertion of *pks15/1*, most of the intact *pks15/1*-containing strains were more efficient at invading cells on day 0 but had lower intracellular multiplicities (on days 3 and 6) than strains with the deletion; however, these differences were not statistically significant. All TBM strains were better able to invade THP-1 cell than *M. tuberculosis* H37Rv, but had multiplied less at day 6. It was not possible to demonstrate a difference in virulence of the various strains, based on this characteristic.

Tumour necrosis factor-alpha (TNF-α) production of human macrophages infected with *M. tuberculosis*

The levels of TNF-α produced by infected THP-1 cells were elevated, starting at day 0. They peaked at day 2 and decreased at day 3 after infection for all but one *Mycobacterium* isolate, BJY. THP-1 cells

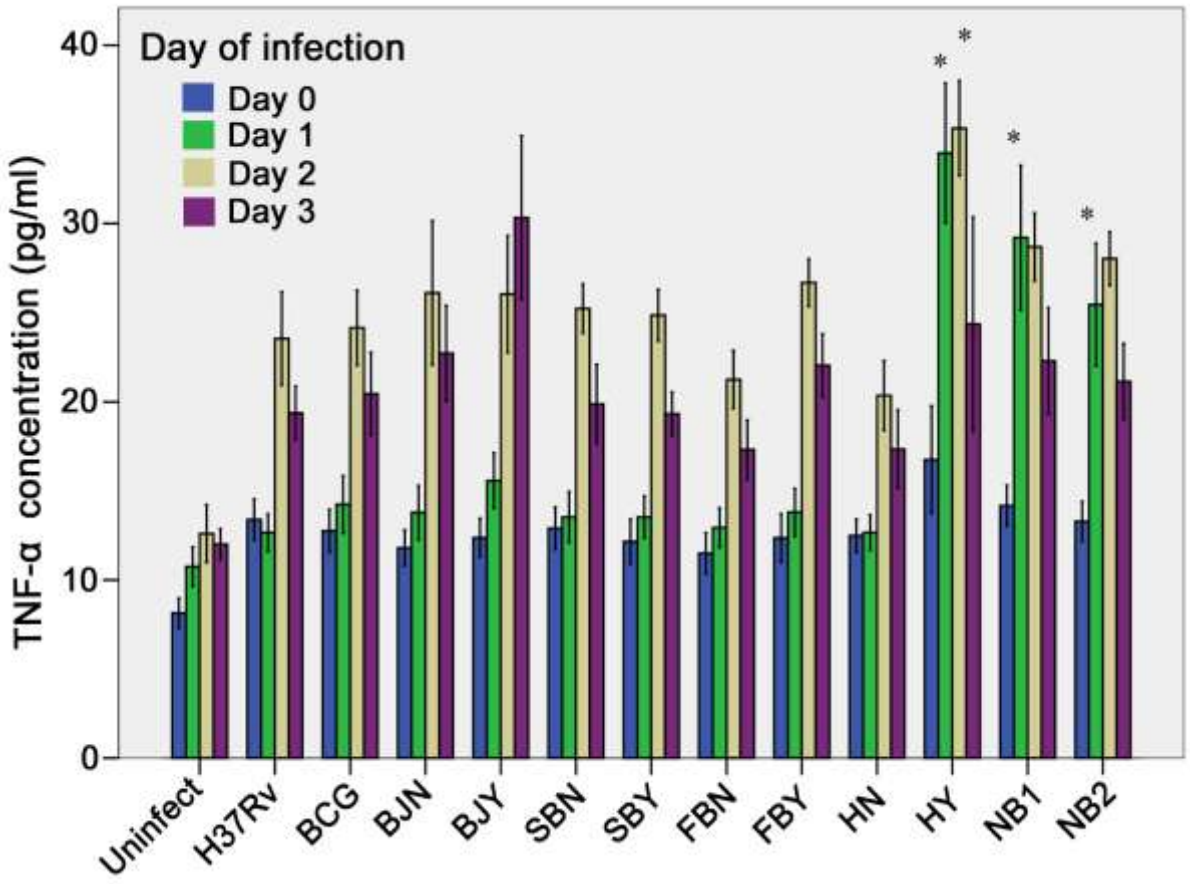

Figure 2. TNF-α production from THP-1 cells infected with 12 mycobacterial strains. Each bar represents a day of infection; day 0 (4 hr), day 1, day 2 and day 3. TNF-α concentrations were measured by ELISA and shown as mean values from three independent experiments carried out in duplicate. The error bars represent standard errors (SE) and * indicates a statistically significant difference ($p < 0.05$), as compared with H37Rv using Student's t-test.

infected with *M. tuberculosis* strain BJY produced the highest TNF-α at 3 days post-infection (Figure 2.).

All mycobacterial isolates stimulated macrophages to produce TNF-α in higher amounts than control, uninfected, cells.

When compared with *M. tuberculosis* H37Rv at 1 day post-infection, *M. tuberculosis* strains HY, NB1 and NB2 stimulated THP-1 cells to produce significantly higher amounts of TNF-α ($p < 0.05$). At day 2, the highest and lowest amounts of TNF-α were secreted in THP-1 cells infected with *M. tuberculosis* strains HY and HN, respectively. In addition, cells infected with *M. tuberculosis* strain HY produced more TNF-α than those infected with strain H37Rv ($p < 0.05$). At 3 days post-infection, the highest level of TNF-α was found in *M. tuberculosis* strain BJ-Y-infected THP-1 cells, but the difference in levels was not statistically significant. When TNF-α production at days 1 and 2 was compared between *M. tuberculosis* strains with and without a 7-bp insertion in *pks15/1*, most of the macrophages infected with strains containing the intact *pks15/1* gene (BJY, FBY, HY, NB1 and NB2) produced more TNF-α than those infected with no-insertion strains, including H37Rv and *M. bovis* BCG. Furthermore, cells infected with *M. tuberculosis* strain HY produced more TNF-α than strain HN ($p < 0.05$) at days 1 and 2 and cells infected with *M. tuberculosis* strain FBY also produced more TNF-α than strain FBN ($p < 0.05$) at day 2. The results suggested that strains containing 7-bp insertion in *pks15/1* should be more virulent.

Vascular endothelial growth factor (VEGF) production of THP-1 cells infected with *M. tuberculosis*

VEGF production by mycobacterium-infected THP-1 cells was elevated from 0 to 3 days post-infection (Figure 3.). On day 0, macrophages infected with *M. tuberculosis* strain NB1 produced the most VEGF, whereas those infected with *M. tuberculosis* strain H37Rv produced the least. *M. tuberculosis* strains NB1 and NB2 stimulated macrophages

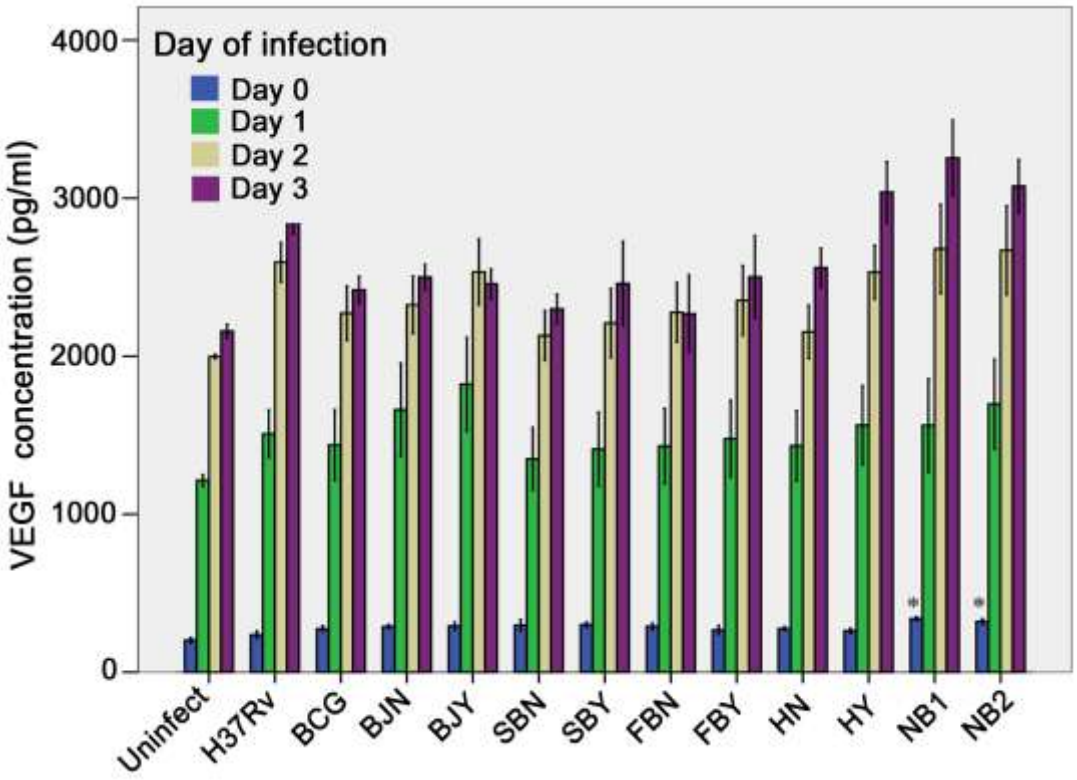

Figure 3. VEGF production of activated THP-1 cells infected with 12 mycobacterial strains. Each bar represented day of infection; day 0 (4 hr), day 1, day 2 and day 3. VEGF concentrations were measured by ELISA and showed in mean values from three independent experiments carried out in duplicate. The error bars represent standard errors (SE), * indicates a statistically significant difference (p <0.05) as compared with H37Rv using Student's t-test.

to produce significantly higher amounts of VEGF than H37Rv (p <0.05). Additionally, macrophages infected with *M. tuberculosis* strain NB1 generated VEGF in the highest quantity at day 2 and day 3 post-infection.

No statistically significant difference in VEGF production was observed between THP-1 cells infected with different genotypes of *M. tuberculosis* strains containing the 7-bp insertion and those with no insertion of *pks15/1*.

Matrix metalloproteinase-9 (MMP-9) production of human macrophages infected with *M. Tuberculosis*

MMP-9 produced by infected THP-1 cells increased from 0 to 6 days after infection in all mycobacterial strains tested (Figure 4.). When compared with *M. tuberculosis* H37Rv, *M. tuberculosis* strains H-Y, NB-1 and NB-2 stimulated macrophages to produce significantly higher amounts of MMP-9 at days 0, 3 and 6 (p < 0.05). Macrophages infected with *M. tuberculosis* strain H-Y produced the highest level of MMP-9 at days 0, 1 and 3, and, furthermore, those infected with strain NB-1 produced the highest amount of MMP-9 at day 6 post-infection.

MMP-9 production was compared between cells infected with *M. tuberculosis* strains containing the 7-bp insertion and those without an insertion of *pks15/1*. *M. tuberculosis* strain H-Y stimulated THP-1 cells to secrete MMP-9 at higher levels than strain HN. In addition, *M. tuberculosis* strain FBY stimulated cells to produce more MMP-9 than cells infected with *M. tuberculosis* strain FBN. The production of MMP-9 revealed that some strains, such as HY, NB1 and NB2, should be more capable of crossing the BBB or more virulent.

Apoptosis of THP-1 cells infected with *M. Tuberculosis*

The amounts of mono- and oligonucleosomes released from macrophages infected with most of the mycobacterial isolates tested were increased when the incubation time was extended. *M. bovis* BCG, which produced the highest apoptosis level at day 1 and then showed lower levels of apoptosis at days 3 and 5, was the exception. At 1 day post-infection, the highest rate of apoptosis was found in cells

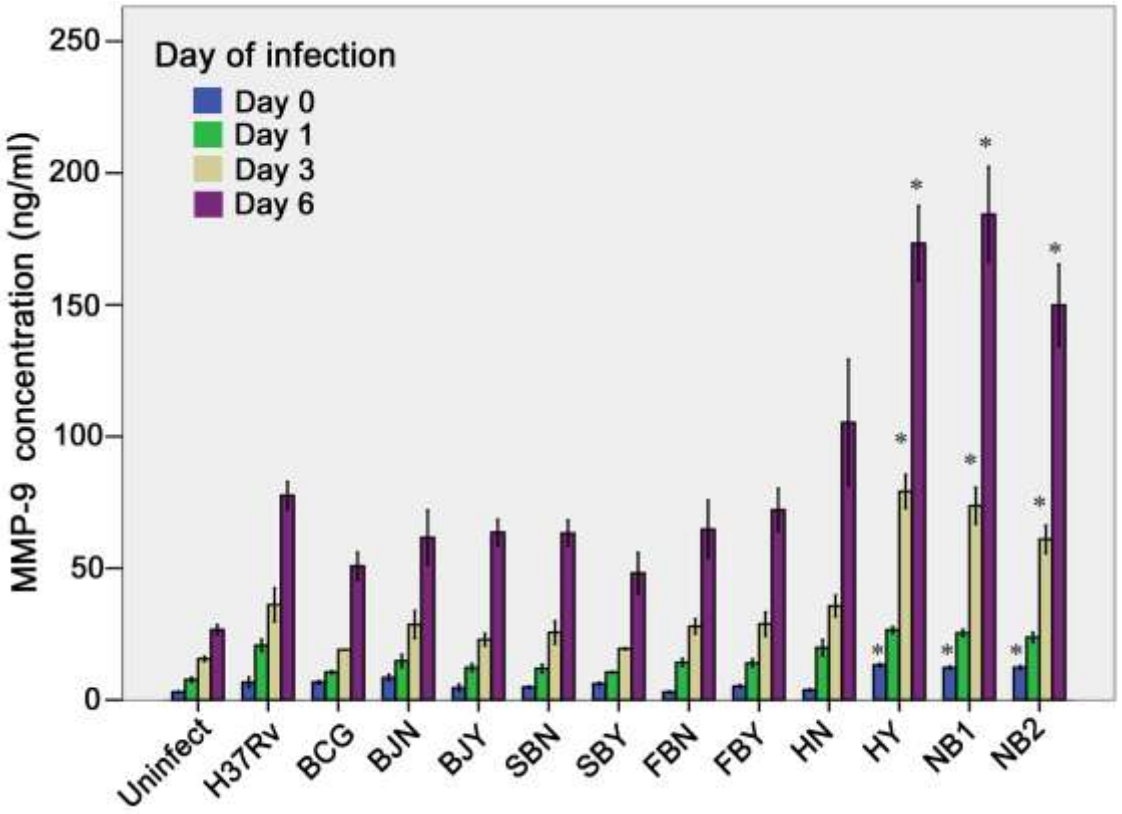

Figure 4. MMP-9 production of activated THP-1 cells infected with 12 mycobacterial strains. Each bar represents a, day of infection; day 0 (4 hr), day 1, day 3 and day 6. MMP-9 concentrations were measured by ELISA and showed in mean values from three independent experiments carried out in duplicate. The error bars represent standard errors (SE), * indicates aa statistically significant difference (p <0.05) as compared with H37Rv, at the same indicated time, using student's t-test.

infected with *M. bovis* BCG and the mean value of enrichment for this strain was 22.58, while the mean values of enrichment for the other strains were between 1.45 and 5.46. When compared with strain H37Rv, many mycobacterial isolates, such as *M. bovis* BCG and *M. tuberculosis* strains BJN, BJY, SBY, FBN, FBY and NB2, induced more apoptosis (p < 0.05). The *M. tuberculosis* Beijing strain that contained the *pks15/1* 7-bp insertion (BJY) induced less apoptosis than the Beijing strain with no insertion (BJN) at day 1 (p < 0.05). At 3 days post- to induce significantly higher rates of apoptosis in cells than strain H37Rv, which induced the lowest apoptosis (enrichment factor of 1.70). At 5 days post-infection, the highest apoptosis level was found in macrophages infected with *M. bovis* BCG (enrichment factor of 4.59). Additionally, *M. bovis* BCG as well as *M. tuberculosis* strains FBN, NB1 and NB2 were significantly more potent inducers of apoptosis than *M. tuberculosis* strain H37Rv (p <0.05). These three strains should showed less virulence, based on apoptosis induction.

Discussion

Early reports showed that mycobacterial PGL was related to the virulence of *M. tuberculosis* and that intact *pks15/1* was associated with PGL production. *M. tuberculosis* strains containing intact *pks15/1* were assumed to be more virulent than those containing the 7-bp deletion in *pks15/1*. In this study, activated THP-1 cells were employed and observed under an inverted microscope 24 hours after incubation with 100 nM PMA[16-18]. The intracellular bacterial CFUs of all mycobacterial strains were elevated from 0 to 6 days post-infection and all isolates exhibited growth indexes of >1 at day 6. *M. tuberculosis* strain H37Rv had the least potential to invade cells. This poor cell invasion could be one of the protective mechanisms with which macrophages are equipped within the early

Table 2. Apoptosis of infected THP-1 cells as measured by the enrichment of nucleosome in the cytoplasm. The enrichment of nucleosomes of macrophages infected with 12 mycobacterial strains at day 1, 3 and day 5 post-infection, are shown as mean ± SD.

Isolates	Enrichment day 1 $\overline{X}$ ± SD	Enrichment day 3 $\overline{X}$ ± SD	Enrichment day 5 $\overline{X}$ ± SD
H37Rv	1.47 ± 0.40	1.70 ± 1.10	1.83 ± 0.50
BCG	22.58 ± 9.69[a]	2.73 ± 1.49	4.59 ± 0.58[a]
BJN	5.46 ± 2.21[a]	2.37 ± 1.07	2.77 ± 1.62
BJY	2.89 ± 1.14[a]	1.91 ± 0.77	2.20 ± 0.77
SBN	1.45 ± 0.68	1.76 ± 0.58	2.06 ± 1.16
SBY	2.20 ± 0.63[a]	2.54 ± 0.82	3.08 ± 1.26
FBN	2.59 ± 0.77[a]	4.02 ± 1.12[a]	3.15 ± 0.85[a]
FBY	2.92 ± 0.77[a]	4.40 ± 1.08[a]	1.62 ± 0.74
HN	2.06 ± 0.82	4.05 ± 1.65[a]	2.84 ± 1.64
HY	2.17 ± 1.18	3.28 ± 1.84	4.32 ± 2.78
NB1	2.47 ± 1.13	2.36 ± 1.19	4.10 ± 1.19[a]
NB2	3.04 ± 1.38[a]	3.69 ± 1.50[a]	4.19 ± 1.18[a]

[a] Enrichment factor levels were higher than those for H37Rv, with statistically significance ($p < 0.05$).

stage of infection. The intracellular growth of *M. tuberculosis* in macrophages has previously been investigated in several studies. Rapid intracellular growth was found in strains isolated from epidemiologic sites, the so-called cluster strains, whereas strains isolated from a single case, or "unique strains", had low an intracellular growth rate[18, 19]. Another study observed the intracellular growth of 125 *M. tuberculosis* clinical isolates (Beijing strains were 57% of all isolates), including 13 isolates from CSF in human peripheral blood monocyte-derived macrophages[20]. Most of the clinical isolates and H37Ra exhibited no intracellular survival on day 10, while 3 clinical isolates from CSF strains that all belong to a non-Beijing family together with H37Rv showed a 2-fold to 4-fold rise in CFU, on average. Wong *et al* showed that strain H37Rv exhibited a growth index of 1.5 at day 6, which was comparable with the results of our study. In addition, the rapidly growing strains (growth index of 1.37-1.45 at day 6) found in this study were non-Beijing strains. Intracellular growth in IFN-γ-activated THP-1 cells was determined for 18 *M. tuberculosis* isolates from the Beijing family[21]. It was found that rapid growth in macrophages is not a common characteristic of all Beijing strains. Only a few Beijing strains grew as rapidly as strain 210, which was responsible for many outbreaks in the USA. The results of our study further support this evidence. No significant difference in intracellular multiplication within *M. tuberculosis* CSF isolates was found in this study.

TNF-α is known to play an essential role in preventing the extension and dissemination of mycobacterial infection. In this study, three isolates were found to stimulate high TNF-α production during the early infection period (day 1). These isolates all contained the intact *pks15/1* gene and did not belong to the Beijing family (2 isolates with the Nonthaburi genotype and 1 isolate with a heterogeneous genotype). An inverse relationship between the TNF-α concentration and mycobacterial intracellular growth rate has been observed in some studies[18, 21]. Three CSF isolates in our study namely, HY, NB1 and NB2, stimulated high levels of TNF-α and had lower intracellular multiplication than H37Rv, which stimulated a low level of TNF-α. In those studies, THP-1 cells were differentiated and activated by the addition of PMA and recombinant human interferon gamma (IFN-γ) and infected at a ratio of 50 mycobacteria to 1 macrophage. This treatment may account for the higher levels of TNF-α secretion than those observed in our study. PGL production was found to be associated with the loss of Th-1 immunity, including pro-inflammatory cytokines such as TNF-α in some studies[4,22]. However, persistent levels of TNF-α were found in CNS infection of rabbits with PGL-producing *M. tuberculosis* strains[23]. In this study, high levels and early production of TNF-α were found in 3 strains containing the intact *pks15/1* gene, namely, HY, NB1 and NB2 (Figure 2.). Therefore, this characteristic could be strain dependent.

In the present study, mycobacterium-infected THP-1 macrophages were found to produce VEGF and higher levels of this factor were found with a longer infection period. This finding was consistent with reports that PMA-stimulated THP-1 produced higher amounts of some factors than untreated THP-1 monocytes[24, 25]. More importantly, our study was the first to determine VEGF production in THP-1 infected with different isolates of *M. tuberculosis*. All mycobacterial isolates tested in this study

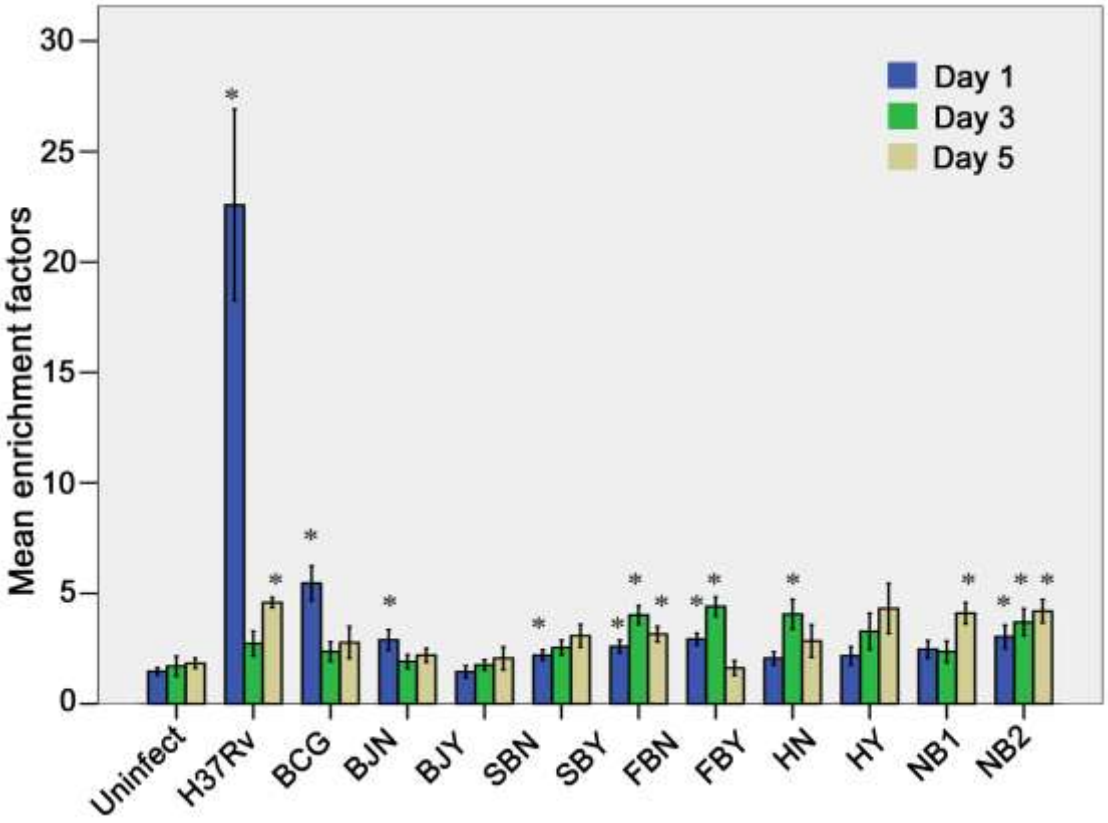

Figure 5. Apoptosis of infected THP-1 cells as measured by the enrichment of nuclosome in the cytoplasm. The enrichment of nucleosomes of macrophages infected with 12 mycobacterial strains at day 1, 3 and day 5 post-infection, are shown. The error bars represent standard errors (SE), * indicates a statistically significant difference (p <0.05), as compared with H37Rv at the same indicated time, using student's t-test.

stimulated cells to produce VEGF at a comparable level, except for the 2 Nonthaburi isolates. These 2 isolates stimulated a high level of TNF-α and higher VEGF levels at 4 hours. TNF-α-stimulated THP-1 can produce VEGF, and both cytokines are important factors in BBB disruption[10, 12]. It was shown that *M. tuberculosis* can stimulate VEGF production independent of different characteristics of strains, such as their genotypes and the DNA sequence of their *pks15/1* genes. In a previous study, THP-1 cells infected with *M. tuberculosis* were shown to upregulate many genes; TNF-α and VEGF were highly expressed early in infection (6 hours), and MMP-9 was highly up-regulated later (12 hours)[26]. The stimulation of VEGF production by *M. tuberculosis* was previously studied in monocytes as well[10,27]. Both *M. Tuberculosis* culture supernatant and sonicated cells can strongly stimulate VEGF production by THP-1 monocytes[10]. In addition, the expression of VEGF was elevated in human peripheral blood mononuclear cells infected with *M. tuberculosis*[27]. In our study, VEGF production was produced at the comparable levels in all isolates and didn't depend on genotypes and the intact *pks15/1* gene.

MMP-9 has been detected in TBM patients and this protein is known to play a major role in the breakdown of the BBB that results in meningitis[7, 8]. The current study is the first to show that activated THP-1 cells infected with several isolates of *M. tuberculosis* secreted MMP-9 at different levels and that the amount of MMP-9 increased with the period of infection. In this study, 3 *M. tuberculosis* isolates were found to stimulate high levels of MMP-9 production at all time points tested. Interestingly, these isolates also induced TNF-α and VEGF production at high levels; all of them were non-Beijing isolates (2 Nonthaburi isolates and 1 heterogeneous isolate) and all contained intact *pks15/1*. Our results showed a positive relationship between TNF-α and MMP-9. This finding was concordant with a previous study that showed that MMP-9 production was regulated by TNF-α, IL-18, and IFN-γ[28,29]. Neutralisation of TNF-α substantially

reduced the MMP-9 production of murine peritoneal macrophages infected with mycobacteria. The addition of exogenous TNF-α or IL-18 induced macrophages to express MMP-9, even in the absence of mycobacteria. In contrast, the immunoregulatory cytokines IFN-γ, IL-4 and IL-10 all suppressed MMP-9 production[28]. *M. tuberculosis*-induced MMP-9 production was found in cells other than macrophages, such as monocytes[7, 30, 31], multinucleated giant cells[32] and astrocytes[32]. It was suggested that monocyte-astrocyte networks are important in regulating MMP-9 activity in tuberculosis and that astrocytes are a major source of MMP-9 in CNS-TB[33]. Cell-wall components of mycobacteria were found to induce the expression and secretion of MMP-9. Lipomannans (LM) from various mycobacterial species and lipoarabinomannan (LAM) of *M. tuberculosis* were found to stimulate MMP-9 production from both monocytes and macrophages[29, 31, 34]. In this study, 3 high-MMP-9-inducing strains were isolated from 3 different patients. Two of the patients, who initially presented stage II and III disease died.

Apoptosis of phagocytes has been proposed as a host mechanism to prevent the progression of infection by causing death of the intracellular microorganisms[35]. *M. bovis* BCG was found to induce higher apoptosis at day 1 compared to the *M. tuberculosis* isolates. Apoptotic cells were detected at a comparable level to others with longer infection periods. These findings were consistent with previous studies showing that the infection of macrophages with the virulent *M. tuberculosis* H37Rv and Erdman strains produced a less significant effect on cell apoptosis than infection with the attenuated strain H37Ra or with *M. bovis* BCG[15, 36, 37]. It has been suggested that virulent *M. tuberculosis* strains may be able to induce the release of soluble TNF-receptor 2 molecules, which bind TNF-α, leading to its inactivation and reduced apoptosis of infected cells[38]. Moreover, the mannosylated lipoarabinomannan (ManLAM) of *M. tuberculosis* has been found to reduce apoptosis in cells infected with *M. bovis* BCG[39]. Unlike macrophages, monocytes are less prone to undergo apoptosis when infected *in vitro* with mycobacteria [(40)]. In a similar way, *M. bovis* BCG increases the viability of infected monocytes[41]. The high apoptosis level in differentiated THP-1 induced by *M. bovis* BCG was detected only at 1 day post-infection. Many clinical *M. tuberculosis* isolates induced more apoptotic cell death than H37Rv; however, comparable levels of apoptosis were detected among clinical isolates.

In conclusion, multiple factors were shown to be involved in the virulence of *M. tuberculosis* strains isolated from CSF. These factors were dependent on the mycobacterial strains and related genes. Ten *M. tuberculosis* CSF isolates, representing 5 RFLP groups together with 2 different *pks15/1* characteristics, were investigated for their virulence *in vitro* in THP-1 cells model and compared with *M. tuberculosis* strain H37Rv and *M. bovis* BCG and no significant difference in intracellular multiplication was found among them in this study. Our results also showed that *M. tuberculosis* stimulates VEGF production independently of its genotype and the presence of the intact *pks15/1* gene. Regarding the induction of apoptosis in this study, *M. bovis* BCG could induce the highest apoptosis at the early stage of infection. No significant apoptosis levels were detected among *M. tuberculosis* CSF isolates.

In conclusion, two isolates of the most prevalent genotype, the Beijing strains, didn't show higher virulence than other studied genotypes, in respect to intracellular growth, production of TNF-α, VEGF, MMP9 and apoptosis *in vitro*. Therefore, the predominance of the Beijing strains in TBM may result from their superior ability to spread and survive in the environment, a possibility that awaits further clarification. The unexpected interesting results were the abilities of 2 Nonthaburi isolates and 1 heterogeneous isolate to stimulate high TNF-α production in the early infection period. These strains all contained the intact *pks15/1* gene and were found to induce VEGF and MMP-9 production at high levels. This high induction may be a specific virulent nature of Nonthaburi strains which allow them to cross BBB and cause severe TBM which lead to death in two of these three patients.

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