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Two genetically-related multidrug-resistant *Mycobacterium tuberculosis* strains induce divergent outcomes of infection in two human macrophage models

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ABSTRACT

Mycobacterium tuberculosis has a considerable degree of genetic variability resulting in different epidemiology and disease outcomes. We evaluated the pathogen-host cell interaction of two genetically closely-related multidrug-resistant *M. tuberculosis* strains of the Haarlem family, namely the strain M, responsible for an extensive multidrug-resistant tuberculosis outbreak, and its kin strain 410 which caused a single case in two decades. Intracellular growth and cytokine responses were evaluated in human monocyte-derived macrophages and dU937 macrophage-like cells. In monocyte-derived macrophages, strain M grew more slowly and induced lower levels of TNF-α and IL-10 than 410, contrasting with previous studies with other strains, where a direct correlation was observed between increased intracellular growth and epidemiological success. On the other hand, in dU937 cells, no difference in growth was observed between both strains, and strain M induced significantly higher TNF-α levels than strain 410. We found that both cell models differed critically in the expression of receptors for *M. tuberculosis* entry, which might explain the different infection outcomes. Our results in monocyte-derived macrophages suggest that strain M relies on a modest replication rate and cytokine induction, keeping a state of quiescence and remaining rather unnoticed by the host. Collectively, our results underscore the impact of *M. tuberculosis* intra-species variations on the outcome of host cell infection and show that results can differ depending on the *in vitro* infection model.

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1. Introduction

Tuberculosis (TB) remains a major cause of suffering and death worldwide. The HIV/AIDS pandemic, the decline of public health systems and the emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains have contributed to the global upsurge of TB observed towards the turn of the millennium. This unexpected re-emergence of the disease, which until then had been deemed to be close to elimination (Paolo and Nosanchuk, 2004), prompted a strengthening of control policies and also boosted research on TB.

In the early '90s, a large AIDS-related MDR-TB outbreak occurred in hospitals located in Buenos Aires, Argentina (Ritacco et al., 1997), which thereafter disseminated to immunocompetent individuals (Palmero et al., 2003). Epidemiological, bacteriological and genotyping data have allowed the identification of the so-called strain M, which belongs to the Haarlem family (subfamily H2) and is responsible for the largest reported MDR-TB cluster in Latin America (Ritacco et al., 2012a). This highly successful genotype has been able to prevail over other MDR *M. tuberculosis* strains and to persist in the community. It still accounts for one in every 3.5 new MDR-TB cases in Argentina (Ritacco et al., 2012b). Another MDR strain closely related to strain M, the strain 410, was first identified at the early epidemic as having a single band difference in the IS*6110* RFLP pattern (Geffner et al., 2009, Supplementary Fig. 1). Strain 410 caused one MDR-TB case which has remained unique, suggesting that it has an impaired ability to cause active disease in new hosts.

Molecular epidemiology studies revealed that *M. tuberculosis*, previously regarded as a highly conserved species, has a considerable degree of genetic variability, and mounting evidence suggests that intrinsic properties of certain *M. tuberculosis* genotypes might

Abbreviations: MDM, monocyte-derived macrophages; MDR, multidrug-resistant; TB, tuberculosis; RFLP, restriction fragment length polymorphism; dU937, PMA-differentiated U937 cells; CFU, colony forming units; AFB, acid-fast bacilli; MR, mannose receptor; MOI, multiplicity of infection.

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influence epidemiological and clinical outcomes of the disease (Caws et al., 2008; Parwati et al., 2010; Valway et al., 1998). Strains with diverse epidemiological and/or genotypic backgrounds were found to induce different infection outcomes and cytokine profiles in several *in vitro* (Manca et al., 2004; Mathema et al., 2012; Theus et al., 2005) and *in vivo* (Dormans et al., 2004; López et al., 2003; Mathema et al., 2012; Tsenova et al., 2005) infection models, clearly indicating that the genetic intra-species variability is linked to phenotypic and functional variability. However, the experimental parameters that accurately reflect the epidemiological success of different *M. tuberculosis* strains remain undefined. Herein we aim to investigate whether the differences in virulence, regarded as epidemiological success, of two closely related Haarlem MDR *M. tuberculosis* strains, namely strains M and 410, could be associated with their ability to grow and elicit cytokine production in two *in vitro* models of human macrophage infection.

2. Materials and methods

2.1. *M. tuberculosis* strains and epidemiological background

Three *M. tuberculosis* strains were used: MDR strains M and 410 from the collection kept at the Reference Laboratory for Mycobacteria at the INEI-ANLIS "Carlos G. Malbrán" in Buenos Aires, and the laboratory strain H37Rv, which was included as reference. Isolate 6548 representative of strain M had been obtained in 1998 from a male HIV positive patient hospitalized at the epicenter of the outbreak. Isolate 410 was obtained earlier in the MDR-TB epidemic from a female patient who was HIV negative and had no other known co-morbidity. She remained infectious and was hospitalized in several health centers in the outbreak area between diagnosis and death. All four isolates from this patient available to genotyping (1992, 1995, 1998, 1999) had the same unique IS*6110* RFLP pattern, which was not detected again by the national MDR-TB surveillance system (Yokobori et al., 2012). Strain H37Rv was kindly provided by de Kantor (former head, TB laboratory, INPPAZ PAHO/WHO). Strains were grown in Middlebrook 7H9 broth (Becton Dickinson, USA) with ADC enrichment supplemented with 100 U/ml penicillin with agitation for 15–21 days. Clumps were disaggregated mechanically using glass beads, and after 2 h of settling, the supernatant was harvested, aliquoted and stored at −80 °C until use. To minimize virulence loss, we only used stock cultures which had undergone three or less recultures after primary isolation. Bacterial concentration of each strain was determined by serial dilution and plating for each strain. Recultures used in this study were re-submitted to standard IS*6110* DNA fingerprinting (van Embden et al., 1993) and spoligotyping (Kamerbeek et al., 1997) (Supplementary Fig. 1). Additional information related to the selected strains is resumed in Supplementary Table I.

2.2. Cell culture and differentiation

Human monocyte-derived macrophages (MDM) were obtained from buffy coats of six healthy volunteers who gave written informed consent (Servicio de Hemoterapia, Hospital Fernández, Buenos Aires). All donors were seronegative for HIV, hepatitis B, syphilis and Chagas disease; all received BCG vaccination in childhood but PPD skin test status was unknown. The Ethics Committee of the Academia Nacional de Medicina approved all experimental procedures. Peripheral blood mononuclear cells were purified in a Ficoll-Hypaque gradient, plated at 5×10^6 per well in 24-well plates (GBO, Germany) and allowed to adhere for 2 h at 37 °C. After removal of nonadherent cells by washing with warm saline solution (SS), monocytes were allowed to differentiate in a 5% CO_2 humidified atmosphere at 37 °C for 5 days in RPMI 1640 medium (HyClone, Thermo Scientific, USA) supplemented with 10% fetal calf serum (Natocor, Argentina), hereafter mentioned as complete medium. Around 5×10^5 MDM per well were obtained.

The human myelomonocytic leukemia cell line was used as secondary model of *in vitro* infection. Unlike the more widely used THP-1 line, these monoblasts remain in a pliant state of maturation and, upon standard induction, they promptly differentiate into a macrophage-like, cytokine producing, phenotype (dU937) (Harris and Ralph, 1985). U937 were grown in complete medium supplemented with 100 U/ml penicillin. Cultures were started at a density of 10^5 cells/ml every 3–4 days. For differentiation, cells were harvested at exponential growth phase, washed and suspended in complete medium containing 100nM phorbol 12-myristate 13-acetate (PMA, Sigma, USA). 2×10^5 cells were seeded into culture plates and allowed to differentiate for 3 days before use. Cell viability of MDM and dU937 cells was determined by 0.2% trypan blue exclusion, which routinely exceeded 95%.

2.3. Macrophage infection

Adherent MDM or dU937 cells were infected with *M. tuberculosis* strains H37Rv, M or 410 on day 0. Bacterial stocks were spun down at low speed to remove clumps before preparing the infecting suspensions in complete medium. Strains were added into cell culture plates containing adherent macrophages at a multiplicity of infection (MOI) of 5 viable bacilli per cell in triplicate. After incubation at 37 °C for 3 h, infected cells were washed three times with warm RPMI medium to eliminate free bacteria and were cultured in complete medium.

2.4. Intracellular bacilli replication

Intracellular replication of each *M. tuberculosis* strain was measured by counting colony forming units (CFU) at days 0, 2 and 5 after MDM infection. To this end, ice-cold SS was vigorously pipetted into the wells and the plates were incubated for 10 min at 4 °C to further facilitate detachment of adherent cells. Then, cells from each well were transferred into microtubes, pelleted and resuspended in SS. An aliquot of this suspension was used to determine the cell-associated CFU by lysing MDM with 0.05% Triton X-100 in PBS and plating serial dilutions on 7H10/OADC agar plates. Plates were incubated at 37 °C 5% CO_2 for 3–5 weeks until *M. tuberculosis* colonies became visible and countable. At the same time points MDM were placed onto glass slides, fixed by heat and acid-fast bacilli (AFB) were stained by Ziehl–Neelsen method. The slides were examined at 1000× magnification in an optical microscope. The percentage of infection was calculated as the number of MDM containing at least one AFB among 100 cells per slide. Simultaneously, the number of bacilli inside each infected MDM was counted, and the median bacillary load per MDM was calculated based on the frequency distribution of number of bacilli per infected cell. Samples were assessed in duplicate. dU937 cells were similarly processed to determine CFU counts at days 0, 3 and 7 post-infection.

2.5. Cytokine detection

Culture supernatants of infected and uninfected control MDM and dU937 cells were harvested at different time points (after 4 h of culture for TNF-α and 24 h for IL-10 and IL12p70) and were frozen at −70 °C until assayed with commercial ELISA kits (Ready-SET-Go!, eBioscience, USA) for human TNF-α, IL-10 and IL-12p70 according to the manufacturer's instructions. Sensitivities of the assays were 4.7 pg/ml for IL-10 and 7.8 pg/ml for TNF-α and IL-12p70.

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2.6. Flow cytometry

The following fluorochrome conjugated anti-human monoclonal antibodies and their corresponding isotypes were used: fluorescein isothiocyanate-anti-Mannose Receptor (MR, BD), phycoerythrin-anti-CD11b and PerCP/Cy5.5- anti-CD14 (Biolegend, USA). Harvested cells were incubated with the antibodies for 20 min at 4 °C, washed, fixed with 0.5% paraformaldehyde and suspended in Isoflow™ (BD). Fluorescence-labeled cells were measured in a FACScan cytometer (BD) and data were analyzed with FCS Express software (De Novo Software, USA). 20,000 specific events were acquired according to forward and side scatter properties.

2.7. Statistical analysis

Nonparametric Friedman test was performed to compare MDM responses to the different strains within each experimental group, followed by Wilcoxon paired test to compare two groups. Results from dU937 cells were analyzed by ANOVA, followed by paired *t* test. Comparisons among cell types were performed by Mann Whitney test. A value of $P < 0.05$ was assumed as significant.

3. Results

3.1. Strain M grows less than strain 410 in MDM

First, we studied the intracellular growth of the selected strains using human MDM as host cells. No significant differences in MDM-associated CFUs were observed on day 0 (Fig. 1A). Growth lag was observed with both MDR strains until day 2, but by day 5 intracellular growth was observed for all three *M. tuberculosis* strains (day 0 vs. day 5: $P < 0{,}05$). Strain M showed the lowest median CFU counts at days 2 and 5 but this difference was not statistically significant when compared with strain H37Rv. At day 5 post-infection, strain 410 showed significantly higher CFU counts compared with M. The lower replication rate of the outbreak strain M had also been observed in axenic culture (Supplementary Table I).

Having observed that the growth of strains M and 410 were different, we determined the percentage of infected MDM and the bacillary load for each strain. The lower replication of strain M in MDM was also reflected in the percentage of infected cells and intracellular bacillary loads. In contrast with CFU counts, at day 0 both the percentages of MDM containing AFB and the bacillary loads were significantly lower for strains M and 410 than for H37Rv (Fig. 2A and B), probably owing to differences in bacterial viability or staining pattern. While strain M showed the lowest percentage of infection and number of bacilli per cell throughout the experiment, strain 410 equaled the percentage of infection and surpassed the intracellular bacterial burden of H37Rv at day 2 post-infection.

Given the high degree of variability in the growth of the strains among donors (Supplementary Fig. 2) we proceeded to confirm MDM results in a model with higher reproducibility such as the cell line dU937. CFU were recovered at 0, 3 and 7 days post-infection. Surprisingly, the differences in growth rate between M and 410 were abolished, and both MDR strains grew significantly slower than H37Rv (Fig. 1B).

3.2. Strain M elicits less IL-10 and early TNF-α release than strain 410 in MDM

Next, we evaluated the ability of the selected strains to induce the production of cytokines in MDM. Infection with strain M elicited lower amounts of TNF-α at 4 h post infection than strain 410 and similar to those induced by H37Rv (Fig. 3A). After this time point, TNF-α levels remained high and no significant differences among strains were observed (data not shown). IL-10 was induced after infection with all three strains (Fig. 3B), and again, strain M induced lower amounts than strain 410 and similar to those

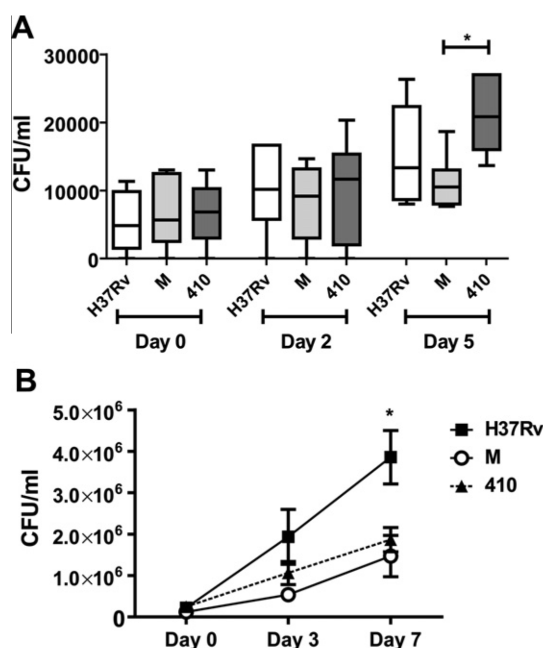

Fig. 1. (A) Intracellular growth of strains H37Rv, M and 410 on days 0, 2 and 5 post-infection in human MDM infected at a MOI of 5. Data are represented as the median and 25th–75th percentiles (box & whiskers plot) of CFU/ml of lysate. (*$P < 0.05$; paired Wilcoxon test). $n = 6$. (B) Intracellular growth of strains H37Rv, M and 410 on days 0, 3 and 7 post-infection in dU937 cells. Data are represented as mean ± SEM of CFU/ml of lysate. Statistical significance: H37Rv vs. M/410: *$P < 0.05$; paired *t* test. $n = 6$.

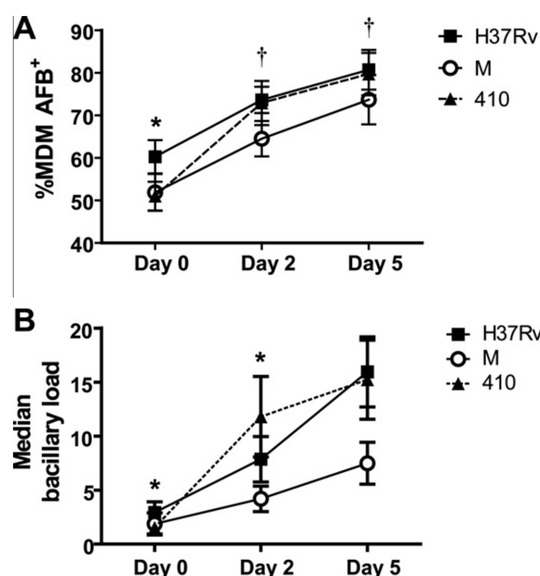

Fig. 2. (A) Percentage of MDM infected with strains H37Rv, M or 410 on day 0, 2 and 5 post-infection was determined by Ziehl–Neelsen staining. Statistical significances: H37Rv vs. M/410: *$P < 0.05$, H37Rv/410 vs. M: †$P < 0.05$; paired Wilcoxon test. $n = 6$. (B) Intracellular bacterial burden of MDM infected with strains H37Rv, M and 410 on days 0, 2 and 5 post-infection. Data are represented as the mean ± SEM of the median bacillary load from each donor assessed by Ziehl–Neelsen staining. Statistical significance: H37Rv vs. M/410: *$P < 0.05$; M vs. 410: †$P < 0.05$; H37Rv vs. M: §$P < 0.05$; paired Wilcoxon test. $n = 6$.

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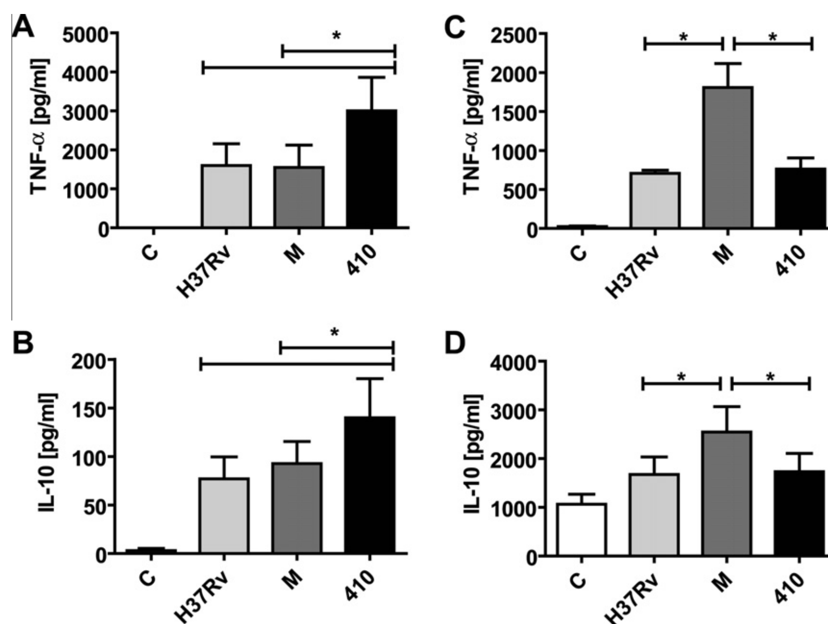

Fig. 3. Cytokine induction by strains H37Rv, M and 410 on MDM (A, B) and dU937 (C, D). TNF-α release at 4 h (A, C) and IL-10 at 24 h post infection (B, D) are shown. Data are represented as pg/ml for each strain assessed by ELISA. Statistical significance: H37Rv/410 vs. M in MDM: $^{*}P < 0.05$; paired Wilcoxon test. $n = 6$. In dU937 cells: $^{*}P < 0.05$; paired *t* test. $n = 3$.

induced by strain H37Rv. No spontaneous release of TNF-α or IL-10 to culture supernatants was detected in the uninfected controls.

In dU937 cells, strain M was the strongest inducer of both TNF-α and IL-10 (Fig. 3C and D), and a high spontaneous release of IL-10 was observed (Fig. 3D).

None of the strains induced detectable levels of IL-12p70 at any time point either in MDM or in dU937 cells (data not shown).

3.3. MDM and dU937differ in critical receptors for M. tuberculosis uptake

Given the discrepancy in growth rates observed between MDM and dU937, we speculated that the differences could be due to critical surface receptors for *M. tuberculosis* uptake. Thus, the expression of CD11b, CD14 and MR was determined. A higher CD11b and CD14 expression was observed in MDM than in dU973 and, while MR was strongly expressed in MDM it was undetectable in dU937 (Supplementary Fig. 3).

4. Discussion

It has been proposed that the most accurate measure of virulence of a given *M. tuberculosis* strain is the extent to which it is able to cause active disease cases in a given period of time, rather than enhanced mortality and disease severity (Brites and Gagneux, 2011). However, experimental parameters such as mortality in animal models are usually assumed to reflect epidemiological success (Dormans et al., 2004; Manca et al., 1999). In line with this, the epidemiological virulence of different strains and lineages is usually expected to correlate with bacterial growth in experimental *in vitro* and *in vivo* infection models. Indeed, highly transmitted strains were shown to grow more rapidly than sporadic strains in human macrophages (Theus et al., 2004; Theus et al., 2006). In particular, the remarkably successful Beijing family strains (Parwati et al., 2010) have shown enhanced ability to grow *in vitro* as well as in animal infection (López et al., 2003; Li et al., 2002; Manca et al., 1999; Theus et al., 2007; Tsenova et al., 2005).

Paradoxically, from an evolutionary point of view, enhanced host mortality might represent a dead end for *M. tuberculosis* (Brites and Gagneux, 2011). In fact, our results challenge the notion that the epidemiological success of a given strain always correlates with a high *in vitro* intracellular growth rate, as we found the opposite in the MDM model presented herein. Results of a recent study by Mathema et al. (2012) endorse our finding. These authors showed that, within a selection of closely related strains of the Euro-American lineage from New York–New Jersey, the strains with slower replication, weaker pro-inflammatory response in primary human monocytes, and milder disease in guinea pigs were the most prevalent in the area. Several other reports failed to prove a definite correlation between enhanced growth rate and epidemiological virulence (Alonso et al., 2010; Hoal-van Helden et al., 2001; Marquina-Castillo et al., 2009; Zhang et al., 1999).

Herein, we also show that, in the MDM model, the successful strain M is a poor inducer of TNF-α and IL-10 compared to 410, critical cytokines for the immune response against *M. tuberculosis* (Flynn and Chan, 2005). In a previous work, we demonstrated that these differences were not related to the antigenic load but were abolished upon heat inactivation, showing that production of these cytokines is dependent on heat labile, probably actively secreted antigens (Yokobori et al., 2012). While a depressed induction of TNF-α has been reported for the extensively studied HN878 Beijing strain (Krishnan et al., 2011; Manca et al., 2004) and other prevalent strains (Mathema et al., 2012; Theus et al., 2005), contrasting results regarding IL-10 induction and its role in *M. tuberculosis* virulence have been reported. Some studies found that clustered strains induce high levels of IL-10 and low levels of TNF-α at early time points favoring an anti-inflammatory profile (Theus et al., 2005). Other studies showed that successful strains induce lower levels of both cytokines (Krishnan et al., 2011). Indeed, our results obtained with MDM are in line with these latter findings. Thus, it is not possible to find a distinctive innate cytokine induction pattern of epidemiologically successful strains as a whole (Alonso et al., 2010; Chacón-Salinas et al., 2005; Manca et al., 2004; Theus et al., 2005; Zhang et al., 1999), and intra-lineage variability could account for this failure (Krishnan et al., 2011; Wang et al., 2010). Herein we also showed that, both strain M and 410 were fully able

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to inhibit the macrophage production of IL-12, a trait which is considered a virulence mechanism (Dao et al., 2008; Henao et al., 2007; Nau et al., 2002).

Another important outcome of the current work is that the performance of clinical isolates can differ critically between experimental models. The discrepancies between results in MDM and dU937 cells are probably due to the differential expression of critical surface receptors related to *M. tuberculosis* recognition and uptake (Schäfer et al., 2009). The enhanced replication rate of strain 410 was reestablished when plated on 7H10 agar after dU937 cell lysis, giving rise to countable colonies earlier than strain M (data not shown), also suggesting that the intracellular milieu was determinant. It is known that MR induces a strong inhibitory signal (Kang et al., 2005; Rajaram et al., 2010) and it would be interesting to determine if our strains differ in the usage of this and other receptors.

Although macrophage-like cell lines are widely used in models of infection, in our case dU937 cells fail to show the growth differences between the epidemiologically successful strain and the sporadic. Other limitations of the dU937 model are the high spontaneous release of IL-10 and the null expression of MR. In contrast, macrophages obtained from primary cells did reveal intriguing differences between both *M. tuberculosis* strains, making MDM the choice model despite high variability observed among donors. Differentiated THP-1 cells might constitute an alternative model as these cells can recognize mycobacteria through MR (Diaz-Silvestre et al., 2005).

The higher intracellular growth rate and the cytokine profile induced by 410 in MDM recalls the behavior of strain CDC1551 (Manca et al., 2004; Manca et al., 1999). This strain induced an unusually high rate of skin test conversion (Valway et al., 1998). In line with this, rather than being unable to cause disease in new hosts, strain 410 might have an enhanced ability to go into latency. Evidence supporting this hypothesis is currently lacking, since PPD skin test status among contacts is unknown, and we consider that this is an interesting issue for future research.

We have previously shown that, compared to strain 410, strain M is a poor inducer of CD8 T lymphocyte mediated cytotoxicity (Geffner et al., 2009) and macrophage cell death (Yokobori et al., 2012). Therefore, we speculate that the epidemiologic success of the non-Beijing strain M actually relies on its modest replication rate and cytokine induction, keeping a state of quiescence and remaining rather unnoticed by the host at early stages of infection, as it has recently been described for other successful genotypes (Mathema et al., 2012).

In summary, our study highlights the importance of defining an accurate selection criterion when comparing performance of clinical isolates in experimental models of infection. In addition to lineage specific characteristics, substantial intra-lineage variations have also been reported (Alonso et al., 2010; Homolka et al., 2010; Portevin et al., 2011; Wang et al., 2010). In most publications, strains were selected based on epidemiological features or family/lineage markers but not on both. During the course of *M. tuberculosis* diversification, different strategies might have been developed, and micro-variations in critical functions might further determine the epidemiological fate of a certain genotype (Reed et al., 2004). In fact, in addition to the IS*6110* RFLP pattern, strains M and 410 differ in other genotypic markers like one VNTR-MIRU 15 locus, presence of strain specific mutations and rifampin resistance-conferring mutation in *rpoB* gene (Supplementary Table I). This latter difference shows that the strains diverged from their ancestor before becoming MDR. Further studies are needed to determine the exact relationship between these differences, the results obtained in the current study and the epidemiological divergence of these strains. Even though our study is limited by the number of clinical isolates tested, it underscores the importance of studying *M. tuberculosis* subpopulations taking into account the different levels of variation, from lineage and family to micro-variations in a certain community, which were previously underestimated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.01.007.

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