Infection, Genetics and Evolution 16 (2013) 151–156

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

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

Noemí Yokobori [a,*], Beatriz López [b], Laura Geffner [a], Carmen Sabio y García [a], Pablo Schierloh [a], Lucía Barrera [b], Silvia de la Barrera [a], Shunsuke Sakai [c], Ikuo Kawamura [c], Masao Mitsuyama [c], Viviana Ritacco [b], María del Carmen Sasiain [a]

[a] *Instituto de Medicina Experimental (IMEX) – CONICET, Academia Nacional de Medicina, Pacheco de Melo 3081, (C1425ASU) Buenos Aires, Argentina*
[b] *Instituto Nacional de Enfermedades Infecciosas ANLIS "Carlos G. Malbrán", Vélez Sarsfield 563, (C1282AFR) Buenos Aires, Argentina*
[c] *Graduate School of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, (606-8501) Kyoto, Japan*

ARTICLE INFO

Article history:
Received 20 September 2012
Received in revised form 4 January 2013
Accepted 9 January 2013
Available online 24 January 2013

Keywords:
Multidrug resistance
Mycobacterium tuberculosis
Monocyte derived macrophages
U937
Virulence
Cytokines

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.

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.

* Corresponding author. Tel.: +54 11 4805 5695x240; fax: +54 11 4805 0712.
E-mail address: kaoru.noemi@gmail.com (N. Yokobori).

1567-1348/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.meegid.2013.01.007

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.