Infection, Genetics and Evolution 73 (2019) 248–254

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Research paper

Survival of an epidemic MDR strain of *Mycobacterium tuberculosis* and its non-prosperous variant within activated macrophages

Noemí Yokobori[a,b,*], Johana Monteserin[b,c], Bárbara Rearte[a,b], Roxana Paul[c], Norberto Símboli[c], Beatriz López[c], Viviana Ritacco[b,c], María del Carmen Sasiain[a,b]

[a] Instituto de Medicina Experimental (IMEX)-CONICET-Academia Nacional de Medicina, Pacheco de Melo 3081, C1425AUM Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290, C1425FQB Buenos Aires, Argentina
[c] Instituto Nacional de Enfermedades Infecciosas (INEI), ANLIS "Carlos G. Malbrán", Vélez Sarsfield 563, C1282AFF Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Mycobacterium tuberculosis
Macrophage
Genetic fitness
Apoptosis
Interferon-gamma
Vitamin D

ABSTRACT

The fitness of a pathogen results from the interaction of multiple factors favoring either epidemiological success or failure. Herein, we studied the performance of the M strain, a highly successful multidrug resistant *Mycobacterium tuberculosis* genotype, and its non-prosperous variant, the 410 strain, in activated human monocyte-derived macrophages. Both strains showed comparable ability to induce necrotic cell death and to survive in apoptotic macrophages. Of the various macrophage activation conditions tested, none led to an enhanced control of the outbreak strain. The combination of $1,25(OH)_2$ vitaminD3 and IFN-γ favored significantly the control of the non-prosperous 410 strain. These observations indicate that the ability of the M strain to survive within the hostile intracellular milieu is conserved, and the overall fitness cost paid by this genotype would be low. Our results provide additional evidence on bacterial traits that may have contributed to the epidemiological success of the M strain.

1. Introduction

Mycobacterium tuberculosis is an ancient obligate human pathogen and its evolution was shaped by this interaction with the host. Recently, antibiotics became a major selective pressure, and the emergence of highly transmissible multidrug-resistant (MDR) clones showed the plasticity of this species to adapt and survive. The acquisition of drug resistance-conferring mutations has long been assumed to impose severe fitness costs, but ample epidemiological evidence shows that this is not always the case (Borrell and Gagneux, 2009). The fitness of a pathogen results from the interaction of multiple factors favoring either epidemiological success or failure. This is illustrated by the contrasting fate of the outbreak MDR strain M and its closely related, non-prosperous variant 410. We have recently shown that the M strain, responsible for the largest MDR-tuberculosis cluster in Latin America (Ritacco et al., 2012), paid a partial fitness cost in its way to multidrug-resistance (Yokobori et al., 2018). However, a more extended analysis showed that the M strain has an unaltered axenic replication in the presence of rifampicin, outgrows the non-prosperous strain 410, and induces a much fainter immune response *in vivo* (Yokobori et al., 2018). Altogether, these observations support the idea that the epidemiologically successful M strain has paid a lower fitness cost than its abortive variant 410.

Being professional phagocytes, macrophages are highly efficient at killing and digesting numerous pathogens (Jordao et al., 2008). Paradoxically, they are also the preferred replication niche of *M. tuberculosis*, which manages to escape phagosome-lysosome fusion. Moreover, the activation by IFN-γ, a canonical enhancer of macrophage microbicidal activity, was reported to be insufficient to activate mycobacterial clearance in human cells (Rook et al., 1986a), which was only achieved in the presence of vitamin D3 (VitD) (Fabri et al., 2011; Liu et al., 2006). In addition, this pathogen manipulates survival and death of macrophages for its own benefit, favoring necrotic cell death (Divangahi et al., 2009; Lerner et al., 2017; Mahamed et al., 2017) over apoptosis (Arcila et al., 2007; Molloy et al., 1994; Velmurugan et al., 2007).

The immunological responses induced by the M strain further suggest its adaptation to the host. Antigens from inactivated

Abbreviations: CFU, colony forming units; IS*6110*-RFLP, restriction fragment length polymorphism based on insertion sequence *6110*; MDM, human monocyte-derived macrophages; MDR, multidrug-resistant; MOI, multiplicity of infection; ST, staurosporine; VitD, $1,25(OH)_2$ vitamin D3

* Corresponding author at: Instituto de Medicina Experimental-CONICET-Academia Nacional de Medicina, Pacheco de Melo 3081, C1425AUM Buenos Aires, Argentina.
E-mail address: nyokobori@anlis.gov.ar (N. Yokobori).

https://doi.org/10.1016/j.meegid.2019.05.005
Received 26 December 2018; Received in revised form 6 May 2019; Accepted 7 May 2019
Available online 08 May 2019

representatives of the M strain were able to manipulate macrophage cell death and innate immunity (Yokobori et al., 2013; Yokobori et al., 2012), as well as adaptive immune responses and T cell-mediated cytotoxicity (Basile et al., 2011; Geffner et al., 2009; Geffner et al., 2014; Sabio y García et al., 2017) suggesting that it efficiently evades host defenses. Moreover, despite its delayed growth in axenic culture, the M strain has no disadvantage in intracellular replication (Yokobori et al., 2018).

Differential abilities to induce host cell death (Aporta et al., 2012; Divangahi et al., 2009; Keane et al., 2000) and resistance to macrophage effector mechanisms (Estrella et al., 2011; Homolka et al., 2010; Loeuillet et al., 2006) have been related to *M. tuberculosis* virulence. Taking these observations into account, we question whether despite the comparable intracellular replication of the M strain and the 410 variant in resting macrophages, the former had a better resistance to mycobactericidal mechanisms upon activation of the host cell.

The objective of this study was to further characterize the interaction of the outbreak strain M with human monocyte-derived macrophages (MDM). Two main aspects were evaluated: (1) the induction of cell death by two M strain representatives (isolates 6548 and 15526), the non-prosperous M strain-variant 410, and the laboratory strain H37Rv in resting and activated MDM, and (2) the intracellular replication of these strains upon MDM challenge with agents reported to enhance macrophage microbicidal activity, namely the apoptosis inducer staurosporine (ST) and the macrophage activators IFN-γ and VitD alone or combined with each other. We analysed the results taking into account available data on host-pathogen interaction in order to shed light on the bacterial traits that may have contributed to the epidemiological success of the M strain.

2. Materials and methods

2.1. Clinical isolates

The isolates 6548 and 15,526, representative of different time points of the M outbreak, and the non-prosperous M strain variant named 410 were selected from the collection belonging to the Mycobacteriology Laboratory, INEI-ANLIS "C. G. Malbrán". The isolates had been recovered from bacilliferous patients with pulmonary tuberculosis in Buenos Aires city, Argentina, at the peak of the outbreak (isolates 6548 and 410) or at the late phase of the outbreak (isolate 15,526). They were identified by IS*6110*-based restriction fragment length polymorphism (RFLP) as belonging to the M strain (isolates 6548 and 15,526) or as a closely related variant with a single additional IS*6110* band (strain 410). The particular RFLP pattern of 410 strain was found only once in more than two decades of MDR tuberculosis surveillance in the country. Additional genotypic markers, drug resistance profiles and phenotypes of the strains/isolates included in this study were extensively characterized in a previous work (Yokobori et al., 2018). The laboratory strain H37Rv was used as control (kindly provided by I. Kantor, former head of the Tuberculosis Laboratory, INPPAZ PAHO/WHO). Frozen stocks of each strain were recovered in Middlebrook 7H11 (BD, USA) plates with albumin-dextrose enrichment for 14–21 days. Reculture was kept to a minimum to avoid loss of virulence.

2.2. Blood donors and human macrophage differentiation

To obtain human macrophages, monocytes were purified from buffy coats of healthy volunteer blood donors (Regional Center of Hemotherapy, Garrahan Hospital, Buenos Aires, Argentina). Exclusion criteria included positive serology for HIV, hepatitis B, syphilis and Chagas disease. A total of 30 donors were recruited after giving their written informed consent. The Ethics Committee of the Academia Nacional de Medicina approved all experimental procedures in accordance with the Helsinki Declaration. The PPD status and other medical information of these donors were not available. Briefly, peripheral blood monocytes were purified in Ficoll-Hypaque and Percoll (GE Healthcare, Sweden) gradients as described elsewhere (Hardin and Downs, 1981). Monocytes were plated in 96- or 48-well plates and allowed to differentiate to monocyte-derived macrophages (MDM) in 5% CO_2 at 37 °C for 7 days in RPMI 1640 medium (EMEVE, Argentina) supplemented with 10% heat-inactivated fetal calf serum (Natocor, Argentina) and 2 mM L-glutamine (EMEVE), hereafter mentioned as complete medium, and 2 ng/ml GM-CSF (Peprotech, USA). Purity and viability were routinely checked.

2.3. Macrophage infection, stimulation and CFU count

For MDM infection, a fresh culture of each strain in 7H9-albumin-dextrose-0.05% Tween 80 was harvested in late exponential phase. Clumps were removed by water bath sonication and low speed centrifugation. Concentration was adjusted according to the 600 nm optical density (OD). An OD_{600nm} of 0.1 was considered as equivalent to 10^7 colony forming units (CFU)/ml and concentrations were checked by serial dilution and plating onto 7H11-albumin-dextrose. For an estimated *M. tuberculosis* multiplicity of infection (MOI) of 1, real MOI ranged from 0.5 to 2. Depending on the assay, MDM were infected in triplicate with a MOI of 1 to 20 bacteria per cell for 1 h. Extracellular bacilli were washed twice with warm saline, and complete medium was added. No significant differences in the CFU/ml recovered at this point were observed among the strains (Yokobori et al., 2018). MDM were either left untreated or immediately stimulated with the apoptosis inducer staurosporine (ST; Invitrogen, USA), recombinant human IFN-γ (Peptrotech, USA), 1,25$(OH)_2$ vitamin D3 (VitD; Santa Cruz Biotechnology, USA) or the combination of IFN-γ and VitD. Working concentrations were 1 μM for ST, 100 U/ml for IFN-γ, and 10 nM for VitD. The effect of IFN-γ and VitD on MDM microbicidal activity was confirmed in an *Escherichia coli* (ATCC 25922 strain) killing assay in similar conditions (data not shown). Intracellular replication was determined by counting CFU at 2 (for ST) or 24 h (for IFN-γ and VitD) after infection. MDM were lysed with a 0.05% Triton X-100 solution in phosphate buffered saline (PBS) and pooled with their respective cell culture supernatants. CFU were determined by serial dilution and plating. Plates were incubated at 37 °C in 5% CO_2 for 2–4 weeks until colonies became visible and countable. In some experiments the percentual effect of the treatment (%E; based on Rook et al., 1986a) was calculated as follows:

$$\%E = (CFU\ per\ ml\ in\ infected\ MDM\ with\ treatment) / (CFU\ per\ ml\ in\ infected\ MDM\ without\ treatment) \times 100\% - 100\%$$

2.4. Vital stain and flow cytometry

M. tuberculosis-induced cell death involves a unique signalling pathway with multiple characteristics including loss of integrity of the host cell plasma membrane (Divangahi et al., 2009; Lee et al., 2006; Lerner et al., 2017; Mahamed et al., 2017), hereafter referred to as necrosis. For the assessment of MDM viability, cells were infected as described. After 2 h, 24 h, 48 h or 96 h of infection, depending on the assay, MDM were detached from the culture plate by incubation at 4 °C with PBS-8 mM EDTA for 15 min. MDM were gently harvested in microcentrifuge tubes, washed with PBS, and stained with the vital dye LIVE/DEAD Fixable Dead Cell Stain Kit, Green fluorescent reactive dye (Invitrogen, USA) for 30 min following the instructions from the manufacturer. Prior to acquisition, MDM were fixed with 4% paraformaldehyde for 20 min. Cells were acquired in a Becton Dickinson FACScan flow cytometer (Becton Dickinson, USA) and analysed using FCS express software (*De Novo* Software, USA). ST-treated MDM were analysed with LIVE/DEAD stain in combination with alterations in cell

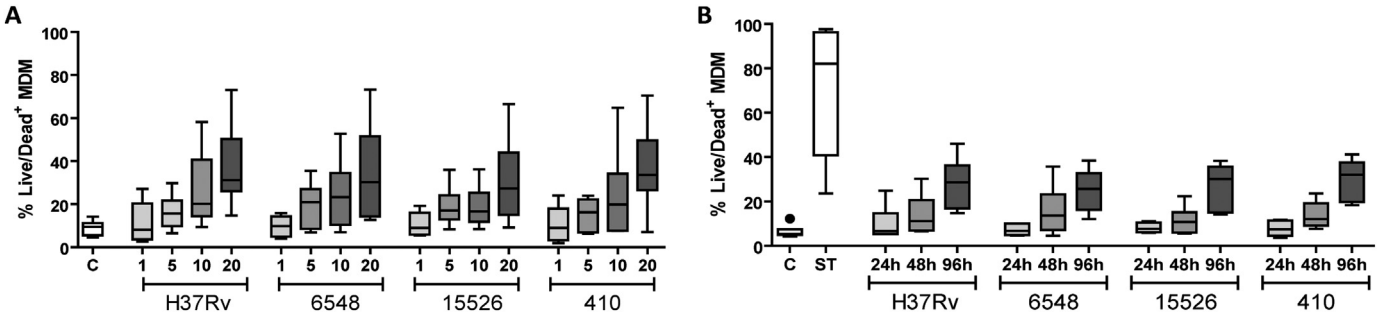

Fig. 1. Human monocyte-derived macrophage (MDM) death induction. MDM were infected with (A) increasing MOI for 24 h or for (B) different times with a MOI of 1 with the reference strain H37Rv, isolates 6548 and 15,526 of the outbreak M strain and the non-prosperous M-variant 410. Induction of cell death was determined by Live/Dead vital stain and analysed by flow cytometry. Results are shown as Tukey box and whiskers plots (description in Materials and methods). Detailed description of statistical analysis as well as raw data are included in Suppl. Data 1. A. Significant levels of MDM necrosis were observed for MOI 20 (Friedman test: $p < .05$; Wilcoxon test: Bonferroni-adjusted $\alpha = 0.01$, $p > .01$ for all the comparisons; $n = 6$; Suppl. Data 1A). B. Significant levels of MDM death 48 h after infection (Friedman test: $p < .05$; Wilcoxon test: Bonferroni-adjusted $\alpha = 0.01$, $p > .01$ for all the comparisons; $n = 6$; Suppl. Data 1B). Staurosporine (ST; 1 μM) evaluated after 24 h was used as a positive control for necrosis.

size (Munoz et al., 2013) as described in Supplementary Fig. 1.

2.5. Statistics

Data were represented using Tukey box and whisker plots (the central line represents the median, box hinges represent 25% and 75% percentiles respectively, whiskers extends to the last value not further than 1.5 times the interquartile range and outliers are represented as circles) (Chambers, 1983). Results were evaluated with the nonparametric Friedman test (Daniel, 1990) to detect differences among treatments ($\alpha = 0.05$). When Friedman test was significant, Wilcoxon signed-rank test was used as *post-hoc* test to compare pairs of treatments, applying the Bonferroni correction to adjust the α level of 0.05 to the number of evaluated conditions. Data represented in Fig. 1 were evaluated by MOI/time points or by the different strains as treatments in separate analyses. Detailed results of statistical analyses along with raw data represented in the figures were included as supplementary material (Supplementary Data 1, 2, 3 and 4). Categorical variables were evaluated with the Fisher's exact test ($\alpha = 0.05$).

3. Results

3.1. Cell death induction

Macrophage death modality influences the intracellular fate of *M. tuberculosis*. Viable bacilli mainly induce a type of programmed necrotic death in macrophages (Butler et al., 2012; Divangahi et al., 2009; Lee et al., 2006), which boosts bacilli replication (Lerner et al., 2017; Mahamed et al., 2017) to ultimately induce cell lysis and escape to the extracellular medium. In previous experiments with inactivated bacilli, we showed that the M strain (isolate 6548), the non-prosperous 410 strain, and the H37Rv strain differed in the ability to induce MDM cell death (Yokobori et al., 2012).

Considering these antecedents, we first studied the induction of necrosis by live bacilli of the M strain (isolates 6548 and 15,526, corresponding to the peak and the late phase of the outbreak, respectively), the 410 strain (non-prosperous; closely related and contemporaneous to the outbreak peak), and the laboratory strain H37Rv (Yokobori et al., 2018). MDM were either infected with increasing MOIs (1, 5, 10, 20) for 24 h or for different times (24 h, 48 h and 96 h) with a MOI of 1. Necrosis was assessed as loss of plasma membrane integrity by LIVE/DEAD stain and flow cytometry. It resulted both time- (Fig. 1A, Suppl. Data 1A) and dose-dependent (Fig. 1B, Suppl. Data 1B), and no significant differences were observed among the strains. Based on these results, MDM were infected with a MOI of 1 for up to 24 h in the rest of the experiments.

3.2. Effect of staurosporine on intracellular bacilli survival

In contrast to the boosting effect of necrosis on bacilli replication, apoptotic cell death was reported to induce a mycobactericidal mechanism in macrophages (Herbst et al., 2011; Loeuillet et al., 2006; Molloy et al., 1994; Oddo et al., 1998). We have previously observed that heat-resistant antigens of the M strain (isolate 6548) and the 410 strain differed in their ability to interfere with a pro-apoptotic stimulus (Yokobori et al., 2012). However, the impact of this difference on the fate of live intracellular bacilli was not clear. Herein, we evaluated the effect on bacilli viability of ST-induced apoptosis of MDM infected with the selected isolates/strains (Fig. 2). CFU were determined after ST stimulation for 2 h, when most cells were in the early apoptotic phase. Treatment with ST led to a reduction in intracellular CFU (percentual effect, %E < 0) in some donor/strain combinations but the results were heterogeneous (Fig. 2A, Suppl. Data 2A). No significant differences among strains were observed. Next, we evaluated the induction of cell death by ST in the *M. tuberculosis*-infected cells (Fig. 2B, Suppl. Fig. 1). As expected, ST uniformly induced apoptosis rather than necrosis. In MDM infected with the different strains, ST was equally effective, and no significant differences among strains were observed (Fig. 2B, Suppl. Data 2B and 2C).

3.3. Effect of IFN-γ on intracellular bacilli

IFN-γ is a canonical activator of macrophage microbicidal activity, and we evaluated the effect of this cytokine on the viability of intracellular bacilli after 24 h stimulation. The effect of IFN-γ was highly variable, leading to enhancement or reduction of intracellular CFU depending on the donor (Fig. 3A, Suppl. Data 3A). No significant differences in IFN-γ %E were observed among strains. IFN-γ *per se* had a negligible effect on MDM death with no significant differences among strains (Fig. 3B, Suppl. Data 3B).

3.4. Effect of 1,25(OH)$_2$ vitamin D3

VitD has long been considered to boost favorable outcomes in tuberculosis, and this is supported by its *in vitro* ability to enhance mycobactericidal activity of human macrophages (Fabri et al., 2011). Moreover, to reach full activation, *M. tuberculosis* infected macrophages would require both IFN-γ and VitD triggered signals (Crowle et al., 1987; Fabri et al., 2011; Rook et al., 1986b). Our results were also highly donor-dependent, and VitD *per se* did not lead to a uniform control of intracellular *M. tuberculosis* in the tested conditions (Fig. 4A, Suppl. Data 4A). Interestingly, the combined treatment with IFN-γ led to a significant reduction of the %E of 410 strain compared to the M

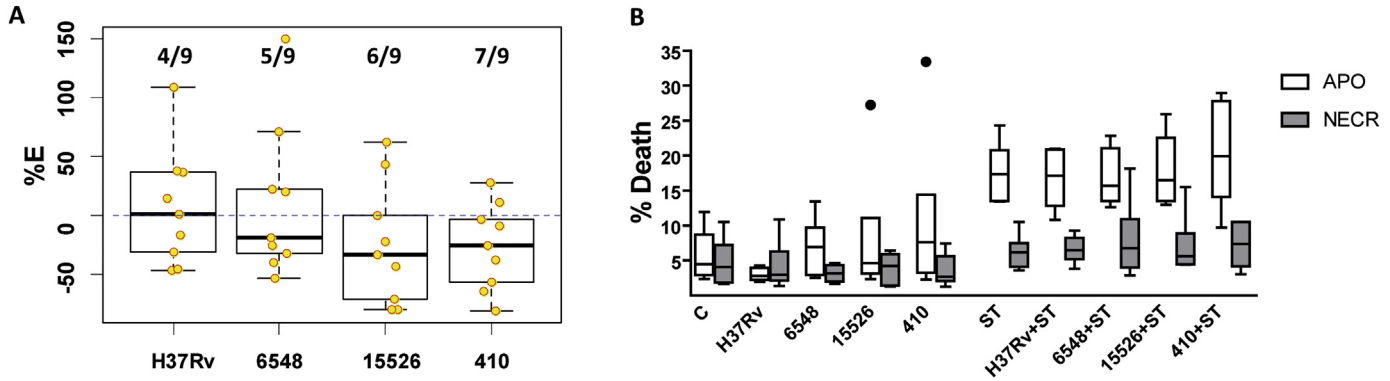

Fig. 2. Effect of the induction of MDM apoptosis with staurosporine (ST). A. Human MDM were infected with the different strains/isolates with a MOI of 1 and were treated or not with 1 μM ST for 2 h and intracellular CFU were determined. Results are shown as box and whiskers plots of the percentual effect of the treatment (%E; Materials and methods). Results from individual donors represented as circles were overlaid. Numbers over the boxes represent the numbers of donors with %E < 0 over the total tested donors. No significant differences among strains were observed (Friedman test: $p > .05$; $n = 9$; Suppl. Data 2A). B. Induction of MDM death in the same conditions. Cells were differentiated in early apoptotic (APO) and late apoptotic/necrotic (NECR) as described in the Suppl. Fig. 1. Circles over the box and whiskers represent outliers. Treatment with ST uniformly augmented APO and no significant differences were observed between ST and *Mtb* + ST in APO (Friedman test: $p < .0001$; Wilcoxon test: Bonferroni-adjusted $\alpha = 0.005$, $p > .005$ for all the comparisons; n = 6; Suppl. Data 1B) or NECR (Friedman test: $p < .01$; Wilcoxon test: Bonferroni-adjusted $\alpha = 0.005$, $p > .005$ for all the comparisons; n = 6; Suppl. Data 1C).

strain isolate 6548 (Bonferroni-adjusted $\alpha = 0.0125$, $p = .0061$; Fig. 4B, Suppl. Data 4B). In addition, the number of donors that controlled isolate 6548 compared to 410 strain was significantly different (Fisher's exact test, $p < .05$). These differences were not paralleled by divergent induction of necrosis (Fig. 4C, Suppl. Data 4C).

4. Discussion

M. tuberculosis has to sort numerous challenges to persist as a pathogen. One of them is the innate control mediated by macrophages, and attenuated strains as well as vaccine strains usually show an impaired or reduced intracellular replication (Herbst et al., 2011; Keane et al., 2000; Lerner et al., 2017; Loeuillet et al., 2006; Perez et al., 2001; Welin et al., 2011). Despite its epidemiological failure, the disadvantage of 410 strain was not evident at first glance, as its axenic and intracellular replication rates were comparable to those of the reference strain (Yokobori et al., 2018). This prompted us to deepen our studies.

In contrast with our previous observations with inactivated strains (Yokobori et al., 2012), in the present study we found no significant differences in the ability of M and 410 to induce macrophage death. Moreover, infection of MDM with representatives of the M strain and the non-prosperous variant 410 had a negligible effect on ST-induced cell death, in line with a previous report (Loeuillet et al., 2006). The discrepancies with our previous work can be explained, at least in part, by the fact that the programmed necrosis induced by this pathogen depends on bacilli viability (Danilchanka et al., 2014; Lee et al., 2006) and the active secretion of antigens (Danelishvili et al., 2016; Danilchanka et al., 2014; Derrick and Morris, 2007; Welin et al., 2011). Furthermore, purified *M. tuberculosis* antigens have been reported to induce divergent outcomes regarding macrophage cell death (Ciaramella et al., 2004; Danelishvili et al., 2016; Danilchanka et al., 2014; Dao et al., 2004; Loeuillet et al., 2006; Singh et al., 2018). The strains selected in this study probably harbor antigenic differences, as supported by whole genome sequencing (WGS) analysis (Bigi et al., 2017) and our previous works (Geffner et al., 2014; Yokobori et al., 2012). However, in the conditions tested in the current study, these variations did not translate into differences in the ability of viable bacilli to manipulate MDM cell death. The low specificity of the method

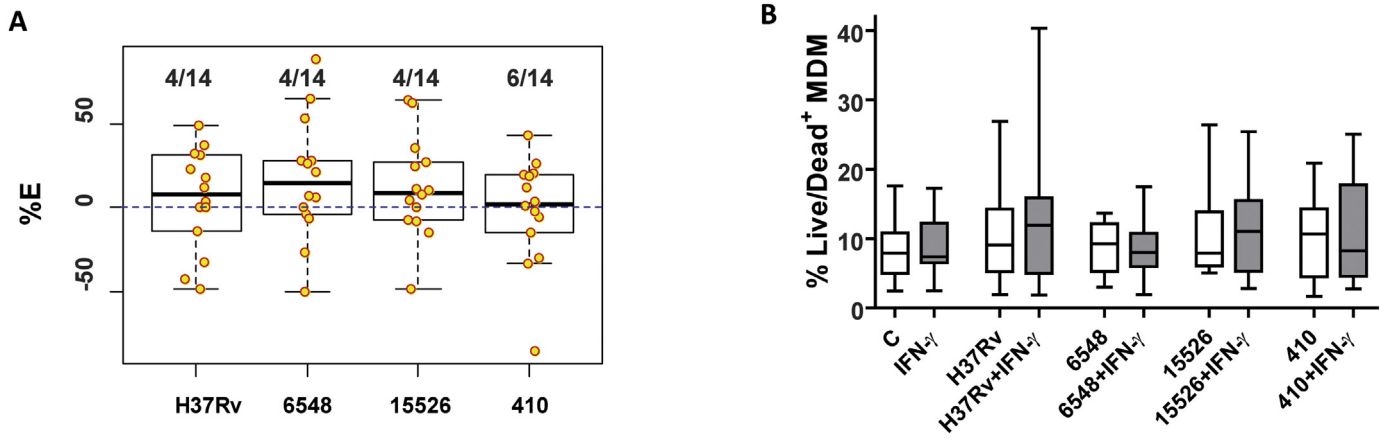

Fig. 3. Effect of MDM stimulation with IFN-γ. A. Human MDM were infected with the different strains/isolates with a MOI of 1, treated or not with 100 U/ml of recombinant IFN-γ and intracellular CFU were determined at 24 h post infection. Results are shown as box and whiskers plots of the percentual effect of the treatment (%E). Results from individual donors represented as circles were overlaid. Numbers over the boxes represent the numbers of donors with %E < 0 over the total number of tested donors, and indicates that IFN-γ was both able to enhance of reduce intracellular growth depending on the donor. No significant differences among strains were found (Friedman test: $p > .05$, $n = 14$; Suppl. Data 3A). B. Induction of MDM death in the same conditions. Results are shown as box and whiskers plots of the % of Live/Dead$^+$ MDM determined by flow cytometry. The effect of IFN-γ in *Mtb*-infected MDM was variable, and no significant differences among strains were found (Friedman test: $p > .05$; n = 14; Suppl. Data 3B).

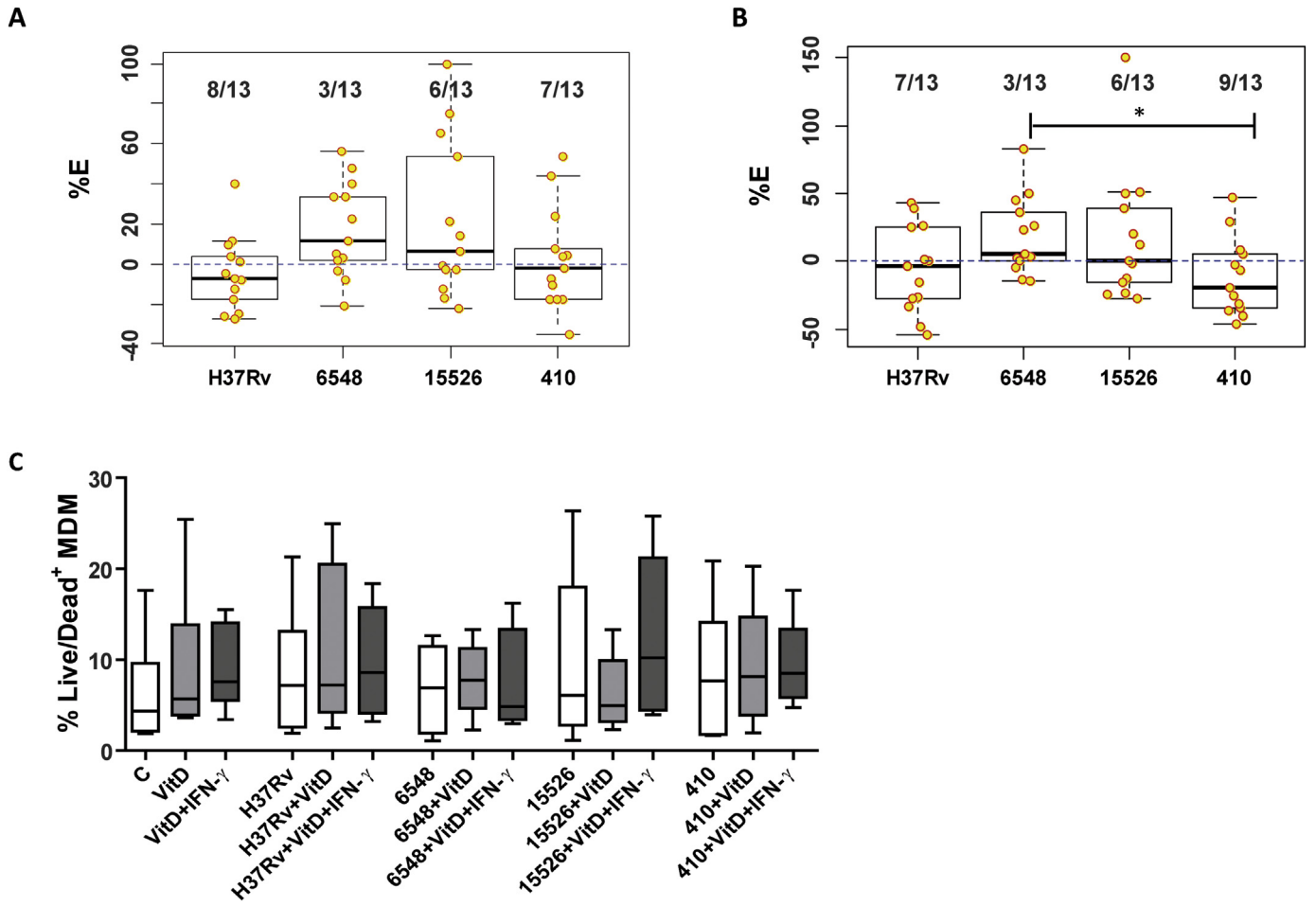

Fig. 4. Effect of stimulation of MDM with 1α,25-dihydroxyvitamin D3 (VitD) alone or in combination with IFN-γ. MDM were infected with the different strains/isolates with a MOI of 1 and treated or not with 10 nM of VitD alone (A) or in combination with 100 U/ml IFN-γ (B) and intracellular CFU were determined 24 h post-infection. Results are shown as box and whiskers plots of the percentual effect of the treatment (%E). Results from individual donors represented as circles were overlaid. Numbers over the boxes represent the numbers of donors with %E $<$ 0 over the total number of tested donors. A. No significant differences among strains were found (Friedman test: $p > .05$; $n = 13$; Suppl. Data 4A). B. %E of VitD + IFN-γ on the 410 strain was significantly lower than on isolate 6548 of the M strain (Friedman test: $p < .01$; Wilcoxon test: Bonferroni-adjusted $\alpha = 0.0125$, $^{*}p < .0125$ for 6548 *vs.* 410; n = 13; Suppl. Data 4B). C. Induction of MDM death in the same conditions. Results are shown as box and whiskers plots of the % of Live/Dead^{+} MDM determined by flow cytometry. No significant differences among treated and untreated cells or among strains were found (Friedman test: $p < .05$; Wilcoxon test: Bonferroni-adjusted $\alpha = 0.0033$, $p > .0033$ for all comparisons; $n = 8$; Suppl. Data 4C).

used to screen early apoptotic death could be pointed as a limitation of the current work, but our results did not unveil differences worthy of in-depth analysis. Treatment with ST uniformly induced macrophage apoptosis, and this contrasted with the variable effect on intracellular bacilli count. Although the identity of the effector molecules involved in apoptosis-induced mycobactericidal activity is still unclear (Lam et al., 2017), our observation suggests that the ability to survive in apoptotic host cells may vary depending on a combination of host and strain factors.

In a murine model, IFN-γ-mediated activation was found to disclose differences among *M. tuberculosis* lineages regarding their ability to replicate within macrophages (Homolka et al., 2010). Noticeably, in our human macrophage model, IFN-γ failed to enhance mycobactericidal activity, a finding which is at odds with its widely accepted protective effect in human tuberculosis (Flynn, 1999), as described in pioneering works (Denis, 1991; Douvas et al., 1985; Robertson and Andrew, 1991; Rook et al., 1986a). Our results show that the ability to resist IFN-γ activation is highly donor-dependent, which is in accordance with previous works (Bonay et al., 1999; Rook et al., 1986a). This resistance however, was conserved in the M and 410 strains. In this line, we have observed that these strains uniformly induced the down regulation of IFN-γ receptor expression in MDM (Yokobori et al., unpublished results). Interestingly, we observed a similar donor-dependent response in the induction of cell death, but unfortunately those experiments were not performed pair-wise, which precluded correlation analysis.

The most interesting result was observed in MDM simultaneously stimulated with IFN-γ and VitD. The intracellular survival of the M strain isolate 6548 contrasted with the poor resistance of the 410 strain, as evidenced by both the number of donors that controlled multiplication of intracellular bacilli as well as the magnitude of CFU reduction. Induction of necrosis by *M. tuberculosis* is considered a virulence mechanism that boosts intracellular replication (Lerner et al., 2017; Mahamed et al., 2017). However, the poor performance of 410 strain in MDM activated by VitD + IFN-γ did not correlate with a lower induction of necrosis or an enhanced ability to induce apoptosis, suggesting that that the non-prosperous strain resists poorly the macrophage effector mechanisms. It would be interesting to study the *in vitro* effect of recombinant LL37, the anti-mycobacterial effector molecule downstream VitD + IFN-γ activation (Liu et al., 2006; Martineau et al., 2007), to test this hypothesis. Conversely, the M strain isolate 6548 had the best performance in VitD and VitD + IFN-γ MDM. This observation

reinforces our previous results indicating that, despite its poor replication in axenic culture, isolate 6548 has found a unique path to success (Yokobori et al., 2018).

A limitation of the current study is the variability in the results, associated to studies with human monocyte-derived macrophages, which conditioned the statistical power of the analysis. In addition, the Bonferroni correction was applied to the α level of the *post hoc* Wilcoxon signed rank test, and set a very stringent threshold for the rejection of the null hypothesis. This was reflected in those experiments in which some differences were detected by the Friedman test but could not be confirmed by the pairwise *post hoc* test.

The novelty of our approach is the selection of closely related *M. tuberculosis* strains displaying sharp epidemiological differences. Although the study of host-pathogen interactions and virulence of clinical isolates has increased in the last years, most works focused in the effect of lineage, or clinical isolates with phenotypic/epidemiological differences that also had divergent genetic background (Homolka et al., 2010; López et al., 2003; Manca et al., 2004; Reiling et al., 2013). Only a few studies focused on closely related or clonal variants of the same strain (Navarro et al., 2013). It is interesting to note that some differences, although not statistically significant, could be appreciated between the early and late representative of the M strain (isolates 6548 and 15,526) including the effect of VitD + IFN-γ on CFU counts. This is not surprising if we take into account that the prototype M strain cluster has evolved continuously from the late 1970's until the onset of the outbreak and beyond (Eldholm et al., 2015). This is supported by the presence of polymorphisms unique to isolate 6548 compared to selected M strain representatives as described by Bigi et al. (Bigi et al., 2017).

The 410 strain diverged from the M strain cluster two decades before the onset of the outbreak in the 1990's (Eldholm et al., 2015; Yokobori et al., 2018) and its evolution has been strongly shaped by antibiotic pressure. WGS analysis showed that isolates 6548 and 410 have 11 non-synonymous SNPs in coding regions and 3 differences in intergenic regions that led to transcriptional alterations in their downstream genes, some of which have been related to the synthesis of mycolic acids and other cell wall constituents (Bigi et al., 2017). In addition, drug-resistance conferring mutations have also been related to physiological alterations that can modify their interaction with the host immune system (Koch et al., 2014; Rifat et al., 2017). The impact of these polymorphisms on the poor resistance of the 410 strain in a hostile macrophage environment should be weighted in future studies.

5. Conclusions

Collectively, our results suggest that microevolution events can lead to divergent host-pathogen interactions that ultimately can determine the epidemiological fate of a certain genotype. The complexity behind this apparently linear inference is illustrated by the M strain isolate 6548, which has a clear disadvantage in comfortable culture conditions but performs well in the stressful intracellular milieu of an activated macrophage.

Potential conflicts of interest

All authors declare no conflicts of interest.

Funding

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica [PICT-2013-0050], and Fundación A. J. Roemmers [Subsidio para Investigación Médica Básica, 2013]. The funders had no role in study design, data collection, analysis or interpretation of the data.

Author contributions statement

N.Y., V.R and M.C.S. conceived and designed the experiments; N.Y., B.L., R. P. and J. M. performed the experiments and provided technical support. N.Y. and B.L. analysed the results. N.Y., B. R., N.S., B. L. and M.C.S. provided resources and materials. N.Y., V. R. and M.C.S. wrote the manuscript. All authors reviewed and approved the final manuscript.

Acknowledgements

We thank the valuable assistance of Unidad Operativa Centro de Contención Biológica (UOCCB) - ANLIS personnel.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.05.005.

References

Aporta, A., Arbues, A., Aguilo, J.I., Monzon, M., Badiola, J.J., de Martino, A., Ferrer, N., Marinova, D., Anel, A., Martin, C., Pardo, J., 2012. Attenuated *Mycobacterium tuberculosis* SO2 vaccine candidate is unable to induce cell death. PLoS One 7, e45213.

Arcila, M.L., Sanchez, M.D., Ortiz, B., Barrera, L.F., Garcia, L.F., Rojas, M., 2007. Activation of apoptosis, but not necrosis, during *Mycobacterium tuberculosis* infection correlated with decreased bacterial growth: role of TNF-alpha, IL-10, caspases and phospholipase A2. Cell. Immunol. 249, 80–93.

Basile, J.I., Geffner, L.J., Romero, M.M., Balboa, L., Sabio, Y.G.C., Ritacco, V., Garcia, A., Cuffre, M., Abbate, E., Lopez, B., Barrera, L., Ambroggi, M., Aleman, M., Sasiain, M.C., de la Barrera, S.S., 2011. Outbreaks of *Mycobacterium tuberculosis* MDR strains induce high IL-17 T-cell response in patients with MDR tuberculosis that is closely associated with high antigen load. J. Infect. Dis. 204, 1054–1064.

Bigi, M.M., Lopez, B., Blanco, F.C., Sasiain, M.D., De la Barrera, S., Marti, M.A., Sosa, E.J., Fernandez Do Porto, D.A., Ritacco, V., Bigi, F., Soria, M.A., 2017. Single nucleotide polymorphisms may explain the contrasting phenotypes of two variants of a multidrug-resistant *Mycobacterium tuberculosis* strain. Tuberculosis (Edinb) 103, 28–36.

Bonay, M., Bouchonnet, F., Pelicic, V., Lagier, B., Grandsaigne, M., Lecossier, D., Grodet, A., Vokurka, M., Gicquel, B., Hance, A.J., 1999. Effect of stimulation of human macrophages on intracellular survival of *Mycobacterium bovis* Bacillus Calmette-Guerin. Evaluation with a mycobacterial reporter strain. Am. J. Respir. Crit. Care Med. 159, 1629–1637.

Borrell, S., Gagneux, S., 2009. Infectiousness, reproductive fitness and evolution of drug-resistant *Mycobacterium tuberculosis*. Int. J. Tuberc. Lung Dis. 13, 1456–1466.

Butler, R.E., Brodin, P., Jang, J., Jang, M.S., Robertson, B.D., Gicquel, B., Stewart, G.R., 2012. The balance of apoptotic and necrotic cell death in *Mycobacterium tuberculosis* infected macrophages is not dependent on bacterial virulence. PLoS One 7, e47573.

Chambers, J.M., 1983. Graphical Methods for Data Analysis. Wadsworth International Group; Duxbury Press, Belmont, Calif. Boston.

Ciaramella, A., Cavone, A., Santucci, M.B., Garg, S.K., Sanarico, N., Bocchino, M., Galati, D., Martino, A., Auricchio, G., D'Orazio, M., Stewart, G.R., Neyrolles, O., Young, D.B., Colizzi, V., Fraziano, M., 2004. Induction of apoptosis and release of interleukin-1 beta by cell wall-associated 19-kDa lipoprotein during the course of mycobacterial infection. J. Infect. Dis. 190, 1167–1176.

Crowle, A.J., Ross, E.J., May, M.H., 1987. Inhibition by 1,25(OH)2-vitamin D3 of the multiplication of virulent tubercle bacilli in cultured human macrophages. Infect. Immun. 55, 2945–2950.

Danelishvili, L., Everman, J., Bermudez, L.E., 2016. *Mycobacterium tuberculosis* PPE68 and Rv2626c genes contribute to the host cell necrosis and bacterial escape from macrophages. Virulence 7, 23–32.

Daniel, W.W., 1990. Applied Nonparametric Statistics, 2nd ed. PWS-KENT Pub, Boston.

Danilchanka, O., Sun, J., Pavlenok, M., Maueroder, C., Speer, A., Siroy, A., Marrero, J., Trujillo, C., Mayhew, D.L., Doornbos, K.S., Munoz, L.E., Herrmann, M., Ehrt, S., Berens, C., Niederweis, M., 2014. An outer membrane channel protein of *Mycobacterium tuberculosis* with exotoxin activity. Proc. Natl. Acad. Sci. U. S. A. 111, 6750–6755.

Dao, D.N., Kremer, L., Guerardel, Y., Molano, A., Jacobs Jr., W.R., Porcelli, S.A., Briken, V., 2004. *Mycobacterium tuberculosis* lipomannan induces apoptosis and interleukin-12 production in macrophages. Infect. Immun. 72, 2067–2074.

Denis, M., 1991. Killing of *Mycobacterium tuberculosis* within human monocytes: activation by cytokines and calcitriol. Clin. Exp. Immunol. 84, 200–206.

Derrick, S.C., Morris, S.L., 2007. The ESAT6 protein of *Mycobacterium tuberculosis* induces apoptosis of macrophages by activating caspase expression. Cell. Microbiol. 9, 1547–1555.

Divangahi, M., Chen, M., Gan, H., Desjardins, D., Hickman, T.T., Lee, D.M., Fortune, S., Behar, S.M., Remold, H.G., 2009. *Mycobacterium tuberculosis* evades macrophage defenses by inhibiting plasma membrane repair. Nat. Immunol. 10, 899–906.

Douvas, G.S., Looker, D.L., Vatter, A.E., Crowle, A.J., 1985. Gamma interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances

replication of macrophage-associated mycobacteria. Infect. Immun. 50, 1–8.
Eldholm, V., Monteserin, J., Rieux, A., Lopez, B., Sobkowiak, B., Ritacco, V., Balloux, F., 2015. Four decades of transmission of a multidrug-resistant *Mycobacterium tuberculosis* outbreak strain. Nat. Commun. 6, 7119.
Estrella, J.L., Kan-Sutton, C., Gong, X., Rajagopalan, M., Lewis, D.E., Hunter, R.L., Eissa, N.T., Jagannath, C., 2011. A novel in vitro human macrophage model to study the persistence of *Mycobacterium tuberculosis* using vitamin D(3) and retinoic acid activated THP-1 macrophages. Front. Microbiol. 2, 67.
Fabri, M., Stenger, S., Shin, D.M., Yuk, J.M., Liu, P.T., Realegeno, S., Lee, H.M., Krutzik, S.R., Schenk, M., Sieling, P.A., Teles, R., Montoya, D., Iyer, S.S., Bruns, H., Lewinsohn, D.M., Hollis, B.W., Hewison, M., Adams, J.S., Steinmeyer, A., Zugel, U., Cheng, G., Jo, E.K., Bloom, B.R., Modlin, R.L., 2011. Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages. Sci. Transl. Med. 3, 104ra102.
Flynn, J.L., 1999. Why is IFN-gamma insufficient to control tuberculosis? Trends Microbiol. 7, 477–478 (author reply 478-479).
Geffner, L., Yokobori, N., Basile, J., Schierloh, P., Balboa, L., Romero, M.M., Ritacco, V., Vescovo, M., Gonzalez Montaner, P., Lopez, B., Barrera, L., Aleman, M., Abatte, E., Sasiain, M.C., de la Barrera, S., 2009. Patients with multidrug-resistant tuberculosis display impaired Th1 responses and enhanced regulatory T-cell levels in response to an outbreak of multidrug-resistant *Mycobacterium tuberculosis* M and Ra strains. Infect. Immun. 77, 5025–5034.
Geffner, L., Basile, J.I., Yokobori, N., Kviatcovsky, D., Sabio y Garcia, C., Ritacco, V., Lopez, B., Sasiain Mdel, C., de la Barrera, S., 2014. *Mycobacterium tuberculosis* multidrug resistant strain M induces an altered activation of cytotoxic CD8+ T cells. PLoS One 9, e97837.
Hardin, J.A., Downs, J.T., 1981. Isolation of human monocytes on re-orienting gradients of Percoll. J. Immunol. Methods 40, 1–6.
Herbst, S., Schaible, U.E., Schneider, B.E., 2011. Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. PLoS One 6, e19105.
Homolka, S., Niemann, S., Russell, D.G., Rohde, K.H., 2010. Functional genetic diversity among *Mycobacterium tuberculosis* complex clinical isolates: delineation of conserved core and lineage-specific transcriptomes during intracellular survival. PLoS Pathog. 6, e1000988.
Jordao, L., Bleck, C.K., Mayorga, L., Griffiths, G., Anes, E., 2008. On the killing of mycobacteria by macrophages. Cell. Microbiol. 10, 529–548.
Keane, J., Remold, H.G., Kornfeld, H., 2000. Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. J. Immunol. 164, 2016–2020.
Koch, A., Mizrahi, V., Warner, D.F., 2014. The impact of drug resistance on *Mycobacterium tuberculosis* physiology: what can we learn from rifampicin? Emerg. Microb. Infect. 3, e17.
Lam, A., Prabhu, R., Gross, C.M., Riesenberg, L.A., Singh, V., Aggarwal, S., 2017. Role of apoptosis and autophagy in tuberculosis. Am. J. Physiol. Lung Cell Mol. Physiol. 313, L218–L229.
Lee, J., Remold, H.G., Ieong, M.H., Kornfeld, H., 2006. Macrophage apoptosis in response to high intracellular burden of *Mycobacterium tuberculosis* is mediated by a novel caspase-independent pathway. J. Immunol. 176, 4267–4274.
Lerner, T.R., Borel, S., Greenwood, D.J., Repnik, U., Russell, M.R., Herbst, S., Jones, M.L., Collinson, L.M., Griffiths, G., Gutierrez, M.G., 2017. *Mycobacterium tuberculosis* replicates within necrotic human macrophages. J. Cell Biol. 216, 583–594.
Liu, P.T., Stenger, S., Li, H., Wenzel, L., Tan, B.H., Krutzik, S.R., Ochoa, M.T., Schauber, J., Wu, K., Meinken, C., Kamen, D.L., Wagner, M., Bals, R., Steinmeyer, A., Zugel, U., Gallo, R.L., Eisenberg, D., Hewison, M., Hollis, B.W., Adams, J.S., Bloom, B.R., Modlin, R.L., 2006. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 311, 1770–1773.
Loeuillet, C., Martinon, F., Perez, C., Munoz, M., Thome, M., Meylan, P.R., 2006. *Mycobacterium tuberculosis* subverts innate immunity to evade specific effectors. J. Immunol. 177, 6245–6255.
López, B., Aguilar, D., Orozco, H., Burger, M., Espitia, C., Ritacco, V., Barrera, L., Kremer, K., Hernandez-Pando, R., Huygen, K., van Soolingen, D., 2003. A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin. Exp. Immunol. 133, 30–37.
Mahamed, D., Boulle, M., Ganga, Y., Mc Arthur, C., Skroch, S., Oom, L., Catinas, O., Pillay, K., Naicker, M., Rampersad, S., Mathonsi, C., Hunter, J., Wong, E.B., Suleman, M., Sreejit, G., Pym, A.S., Lustig, G., Sigal, A., 2017. Intracellular growth of *Mycobacterium tuberculosis* after macrophage cell death leads to serial killing of host cells. eLife 6.
Manca, C., Reed, M.B., Freeman, S., Mathema, B., Kreiswirth, B., Barry 3rd, C.E., Kaplan, G., 2004. Differential monocyte activation underlies strain-specific *Mycobacterium tuberculosis* pathogenesis. Infect. Immun. 72, 5511–5514.
Martineau, A.R., Wilkinson, K.A., Newton, S.M., Floto, R.A., Norman, A.W., Skolimowska, K., Davidson, R.N., Sorensen, O.E., Kampmann, B., Griffiths, C.J., Wilkinson, R.J., 2007. IFN-gamma- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. J. Immunol. 178, 7190–7198.
Molloy, A., Laochumroonvorapong, P., Kaplan, G., 1994. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. J. Exp. Med. 180, 1499–1509.
Munoz, L.E., Maueroder, C., Chaurio, R., Berens, C., Herrmann, M., Janko, C., 2013. Colourful death: six-parameter classification of cell death by flow cytometry–dead cells tell tales. Autoimmunity 46, 336–341.
Navarro, Y., Perez-Lago, L., Sislema, F., Herranz, M., de Juan, L., Bouza, E., Garcia-de-Viedma, D., 2013. Unmasking subtle differences in the infectivity of microevolved *Mycobacterium tuberculosis* variants coinfecting the same patient. Int. J. Med. Microbiol. 303, 693–696.
Oddo, M., Renno, T., Attinger, A., Bakker, T., MacDonald, H.R., Meylan, P.R., 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. J. Immunol. 160, 5448–5454.
Perez, E., Samper, S., Bordas, Y., Guilhot, C., Gicquel, B., Martin, C., 2001. An essential role for phoP in *Mycobacterium tuberculosis* virulence. Mol. Microbiol. 41, 179–187.
Reiling, N., Homolka, S., Walter, K., Brandenburg, J., Niwinski, L., Ernst, M., Herzmann, C., Lange, C., Diel, R., Ehlers, S., Niemann, S., 2013. Clade-specific virulence patterns of *Mycobacterium tuberculosis* complex strains in human primary macrophages and aerogenically infected mice. mBio 4.
Rifat, D., Campodonico, V.L., Tao, J., Miller, J.A., Alp, A., Yao, Y., Karakousis, P.C., 2017. In vitro and in vivo fitness costs associated with *Mycobacterium tuberculosis* RpoB mutation H526D. Future Microbiol 12, 753–765.
Ritacco, V., Iglesias, M.J., Ferrazoli, L., Monteserin, J., Dalla Costa, E.R., Cebollada, A., Morcillo, N., Robledo, J., de Waard, J.H., Araya, P., Aristimuno, L., Diaz, R., Gavin, P., Imperiale, B., Simonsen, V., Zapata, E.M., Jimenez, M.S., Rossetti, M.L., Martin, C., Barrera, L., Samper, S., 2012. Conspicuous multidrug-resistant *Mycobacterium tuberculosis* cluster strains do not trespass country borders in Latin America and Spain. Infect. Genet. Evol. 12, 711–717.
Robertson, A.K., Andrew, P.W., 1991. Interferon gamma fails to activate human monocyte-derived macrophages to kill or inhibit the replication of a non-pathogenic mycobacterial species. Microb. Pathog. 11, 283–288.
Rook, G.A., Steele, J., Ainsworth, M., Champion, B.R., 1986a. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. Immunology 59, 333–338.
Rook, G.A., Steele, J., Fraher, L., Barker, S., Karmali, R., O'Riordan, J., Stanford, J., 1986b. Vitamin D3, gamma interferon, and control of proliferation of *Mycobacterium tuberculosis* by human monocytes. Immunology 57, 159–163.
Sabio y García, C.A., Yokobori, N., Basile, J.I., Balboa, L., Gonzalez, A., Lopez, B., Ritacco, V., Barrera, S., Sasiain, M.D., 2017. C5aR contributes to the weak Th1 profile induced by an outbreak strain of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 103, 16–23.
Singh, P., Rameshwaram, N.R., Ghosh, S., Mukhopadhyay, S., 2018. Cell envelope lipids in the pathophysiology of *Mycobacterium tuberculosis*. Future Microbiol 13, 689–710.
Velmurugan, K., Chen, B., Miller, J.L., Azogue, S., Gurses, S., Hsu, T., Glickman, M., Jacobs Jr., W.R., Porcelli, S.A., Briken, V., 2007. *Mycobacterium tuberculosis* nuoG is a virulence gene that inhibits apoptosis of infected host cells. PLoS Pathog. 3, e110.
Welin, A., Raffetseder, J., Eklund, D., Stendahl, O., Lerm, M., 2011. Importance of phagosomal functionality for growth restriction of *Mycobacterium tuberculosis* in primary human macrophages. J. Innate Immun. 3, 508–518.
Yokobori, N., Sabio y Garcia, C.A., Geffner, L., Schierloh, P., Lopez, B., Ritacco, V., Barrera, L., de la Barrera, S., del Carmen Saisiain, M., 2012. Differential induction of macrophage cell death by antigens of a clustered and a non-clustered multidrug-resistant *Mycobacterium tuberculosis* strain from Haarlem family. FEMS Immunol. Med. Microbiol. 66, 363–371.
Yokobori, N., Lopez, B., Geffner, L., Sabio y Garcia, C., Schierloh, P., Barrera, L., de la Barrera, S., Sakai, S., Kawamura, I., Mitsuyama, M., Ritacco, V., Sasiain Mdel, C., 2013. Two genetically-related multidrug-resistant *Mycobacterium tuberculosis* strains induce divergent outcomes of infection in two human macrophage models. Infect. Genet. Evol. 16, 151–156.
Yokobori, N., Lopez, B., Monteserin, J., Paul, R., Von Groll, A., Martin, A., Marquina-Castillo, B., Palomino, J.C., Hernandez-Pando, R., Sasiain, M.D.C., Ritacco, V., 2018. Performance of a highly successful outbreak strain of *Mycobacterium tuberculosis* in a multifaceted approach to bacterial fitness assessment. Int. J. Med. Microbiol. 308, 349–357.