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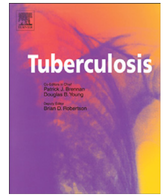

Cholesterol plays a larger role during *Mycobacterium tuberculosis in vitro* dormancy and reactivation than previously suspected

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ABSTRACT

It is known that cholesterol plays a key role for *Mycobacterium tuberculosis* (*Mtb*) adaptation and survival within the host, thus contributing to the establishment of dormancy. It has been extensively demonstrated that fatty acids are the main energy source of *Mtb* during infection and dormancy, and it has been proposed that these molecules are implicated in reactivation of bacilli from a dormant state. We used *in vitro* models to analyze *Mtb* gene expression during dormancy and reactivation when fatty acids and cholesterol are the unique carbon source in the media. Our results suggest that cholesterol might function as a signal to trigger *Mtb* expression of some genes required for stress protection earlier than the one induced by fatty acids alone, indicating that cholesterol is very favorable for its development. This process is so conducive that cholesterol-adapted bacilli can reactivate their growth after NRP2 dormancy state even 10 min post ventilation. Thus, we hypothesize that cholesterol is not only involved in *Mtb* dormancy but that it also plays a critical role for favorable and almost immediate reactivation from an *in vitro* long-lasting dormant state induced by hypoxia.

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1. Introduction

Tuberculosis is still one of the leading causes of mortality throughout the world. It is estimated that 2 billion people are latently infected, representing potential cases of reactivation and transmission of the disease [1]. During infection, *Mycobacterium tuberculosis* (*Mtb*) encounters a variety of host pressures, requiring adaptation to these stress conditions in order to survive within the host. These adaptation processes involve the induction of a regulatory network led by a transcriptional profile characterized by the expression of some specific genes, for example, 1) the *dos*R hypoxia regulator [2], that ensures survival of the bacillus during hypoxia-induced *in vitro* dormancy, and controls reversal to replication upon re-exposure to oxygen [3,4], and 2) the *pho*P virulence regulator [5], involved in the control of ESAT-6 ("6 KDa early secreted antigenic target") secretion [6] and in the control of different set of genes involved in the plasticity of mycobacteria for its use of different carbon sources [7].

We know that pathogenic mycobacteria switch from carbohydrates to lipids as their main carbon and energy sources once inside the host [7] and it has been extensively described that *Mtb* even prefer to consume fatty acids and lipids rather than carbohydrates [8–12]. One of the vital enzymes involved in the persistence of *Mtb* inside its host is isocitrate lyase (*icl*). This is a key enzyme of the glyoxylate cycle, the pathway that is used by organisms that live on

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fatty acids, demonstrating the critical role of fatty acids as an energy source during long-term infection and persistence of *Mtb* [9]. Another phenomenon that has been reported during dormancy is the accumulation of triacylglycerol (TAG) [13], and it was proposed that triacylglycerol synthase 1 (coded by *tgs*1) constitutes one of major components during TAG synthesis and accumulation throughout dormancy [14].

Rodriguez et al. (2014) have suggested that a lipid-rich environment at the stationary phase changes the *Mtb* global expression profile by overexpressing genes required for stress protection (*ie*, *whi*B3), as well as genes required for their survival during dormancy (*ie, dos*R) supporting the hypothesis of the critical role of lipids during *Mtb* host adaptation and during its persistence [15].

Fatty acids are not the only lipidic molecules involved in *Mtb* long term-infection. Cholesterol, one of the main components found in human membrane cells, has been described as an important nutrient source for *Mtb* intracellular survival [16] and persistence [17]. Cholesterol has also been implicated in the effective phagocytosis of mycobacteria [18,19] and in the inhibition of phagosomes maturation [19].

Since the genetic repertoire of *Mtb* contributes to its survival and persistence inside the host, as well as to its reactivation after long time dormancy, the further elucidation of *Mtb* transcriptional changes during both latent tuberculosis and reactivation are important for a better understanding of *Mtb* pathogenesis [20].

Mtb reactivation or resuscitation is the reestablishment of metabolic and replicative activity [21] of the bacilli. Its initial steps are of particular interest because they trigger a cascade of enzymatic processes, ending with the establishment of fully active and viable cells [22]. Some of the proteins involved in the reactivation induction are the Clp proteases [20,23] and the Rpf proteins [22,24]. Rpf proteins are believed to have muralytic activity and it is reported that they release muropeptides that play a signaling role for the reactivation process. In addition, it was demonstrated that certain fatty acids, such as oleic acid, induce *Mtb* reactivation in an Rpf-dependent fashion [22]. Therefore, lipids appear to be involved in dormancy, persistence and reactivation of *Mtb*.

In this study, we have used two *in vitro* models to analyze the role of fatty acids and cholesterol during active growth, dormancy and reactivation of *Mtb*. We have confirmed the role of cholesterol during *Mtb* dormancy and shown that this molecule is involved in the rapid *in vitro* reactivation of *Mtb*.

2. Methods

2.1. Bacterial strain and growth conditions

Mtb H37Rv was grown in Dubos medium (Difco™, USA) supplemented independently with either: a) 0.2% dextrose, b) lipid mixture, FA + Cho (oleic acid, palmitic acid, stearic acid, at a final concentration of 0.001% each, plus 0.01% cholesterol), c) fatty acids FA (oleic acid, palmitic acid, stearic acid, at a final concentration of 0.001% each) or, d) 0.01% cholesterol, Cho. Each culture was followed by CFU/ml to obtain corresponding growing curves and identify exponential and stationary phases; bacterial generation time was calculated from exponential phases using ($g = \frac{t_1 - t_0}{(3.3)(logN_1 - logN_0)}$). Then, cultures at exponential phase were subjected to hypoxia, essentially as described by Wayne and Hayes [25], and NRP phases (NRP1 and NRP2) were determined for each lipid condition (b, c and d). Parallel cultures for "b", "c" and "d" conditions were supplemented with methylene blue (MB) at 1.5 µg/ml as an indicator of oxygen depletion, in order to establish NRP phases. NRP1 phase (initial fading of MB and bacterial stop growing time) was determined at day 4 after exponential phase for conditions "a", "c" and "d". NRP2 phase (complete discoloration of MB) was determined at day eight. Regarding condition "b", NRP1 was determined at day six (stop growing time) and NRP2 at day 14. In all cases, RNA isolation and CFU/ml quantification were carried out 24 h after the corresponding phase (Exp, NRP1 or NRP2) was reached.

NRP1 and NRP2 cultures were harvested for RNA isolation or reactivated by ventilation (see below). Total RNA was isolated from cultures at NRP1 and NRP2 as previously described [26] and purified with Trizol™ reagent (Invitrogen). RNA integrity was analyzed with the bioanalyzer (Agilent technologies), quantified by spectrophotometry with the NanoDrop ND-1000 (Thermo Scientific) and DNA-absence was determined by qPCR, using ribosomal protein S12 and *rrs* genes.

2.2. In vitro reactivation of Mtb

Cultures in NRP2 from the Wayne *in vitro* model using dextrose as carbon source were reactivated by ventilation as described by Gopinath et al. [27] as follows: air/medium ratio of NRP2 cultures (1:2) was changed to a 2:1 ratio using 500 ml flasks and then incubated at 37 °C and at 200 rpm orbital agitation (aerobic conditions). To confirm that mycobacteria were reactivated, cultures were harvested at 10 min, 1 h, 24 h, 48 h and 6 days and RNA isolation was performed. Some control genes were also quantified: *fts*Z and *dna*A as cell-cycle genes, and *rpf*B as reactivation control gene. Two reactivation times (10 min and 24 h) were chosen to reactivate *Mtb* using FA or Cho as carbon sources. RNA isolation, CFU/ml and RT-qPCR quantitation were also carried out (see below). Three independent experiments were performed in order to obtain the standard deviation and to conduct the pertinent statistical analysis.

2.3. RNA isolation and cDNA synthesis

Total RNA from each culture condition (exponential phase, NRP2 and reactivation) was isolated as reported previously [28] and purified by the Trizol™ (Invitrogen, USA) method informed by Shi [29]. cDNA was prepared using 1 µg of RNA, random hexamers (0.5 µg/µl), and the SuperScript first Strand cDNA synthesis kit (Invitrogen, USA) following manufacturer instructions.

2.4. Quantitative real-time PCR

Twelve genes involved in cell cycle, dormancy and stress, antigen synthesis and lipid metabolism were quantified by real-time PCR using gene-specific primers (designed by the Primer Premier Designer program V.3.0 based on the genome sequence for *Mtb* H37Rv; GenBank accession no. NC_000962) and SYBR green reporter using the FastStart DNA master SybrGreen I kit (life Science, Roche) and the LightCycler 2.0 instrument (life Science, Roche). Primer sequences and products are shown in Table 1. Quantification was carried out five times, using RNA isolated from three different culture batches of each condition. Absolute mRNA levels for target genes were normalized using the 16S rRNA expression. To analyze quantitatively gene expression differences between the exponential phase and the NRP2, logarithmic graphics of the expression for each gene (reported as copies of the gene/ug RNA) were constructed. Additionally, to compare the expression obtained between different carbon sources or to compare expression obtained from reactivation to the one from NRP2, we performed a relative expression analysis by calculating the ratio of the gene expression of the condition to be analyzed to the gene expression of the control condition. Then, logarithmic graphics of this ratio were created showing how many times each gene is overexpressed or down-

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