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Original article

Survival of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG in lysosomes *in vivo*

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Abstract

Mycobacterium tuberculosis is one of the most successful pathogens known, having infected more than a third of the global population. An important strategy for intracellular survival of pathogenic mycobacteria relies on their capacity to resist delivery to lysosomes, instead surviving within macrophage phagosomes. Several factors of both mycobacterial and host origin have been implicated in this process. However, whether or not this strategy is employed *in vivo* is not clear. Here we show that *in vivo,* following intravenous infection, *M. tuberculosis* and *Mycobacterium bovis* BCG initially survived by resisting lysosomal transfer. However, after prolonged infection the bacteria were transferred to lysosomes yet continued to proliferate. A *M. bovis* BCG mutant lacking protein kinase G (PknG), that cannot avoid lysosomal transfer and is readily cleared *in vitro*, was found to survive and proliferate *in vivo.* The ability to survive and proliferate in lysosomal organelles *in vivo* was found to be due to an altered host environment rather than changes in the inherent ability of the bacteria to arrest phagosome maturation. Thus, within an infected host, both *M. tuberculosis* and *M. bovis* BCG adapts to infection-specific host responses. These results are important to understand the pathology of tuberculosis and may have implications for the development of effective strategies to combat tuberculosis.

Keywords: Tuberculosis; *In vivo*; Pathogenesis; Lysosomes; Survival; PknG

1. Introduction

Cells of the innate immune system are among the first lines of host defense against invading pathogens. Pathogens such as *Mycobacterium tuberculosis* have evolved active mechanisms to not only resist innate immune defense mechanisms, but also to actively co-opt innate immune cells for their survival and dissemination [1,2]. At a cellular level, several mechanisms have been elucidated that allow mycobacteria to survive inside innate immune cells, specifically within macrophages [3,4]. It has long been appreciated that *M. tuberculosis* actively manipulates phago-lysosomal trafficking of the host cell by blocking the maturation of their phagosomes and eventual fusion with lysosomes [5]. Avoiding delivery to lysosomes and exposure to its hostile environment is thus considered an important survival strategy for *M. tuberculosis*. Several factors of both host and bacterial origin that play important roles in the arrest of mycobacterial phagosome maturation These include

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the lipid phosphatase SapM [6], the lipoamide dehydrogenase LpdC [7], the metalloprotease Zmp1 [8], the tyrosine phosphatase PtpA [9] and the Ser/Thr protein kinase G (PknG) [10,11]. From the host side, several molecules have been shown to play key a role in phagosome maturation arrest, including coronin 1 [12,13], calcineurin [13], vps33 [9], WASH [14] and v-ATPase [15,16] Given the multitude of factors involved, it is likely that multiple mechanisms are involved in arresting the maturation of mycobacterial phagosomes.

Most studies on the role of mycobacterial phagosome maturation arrest in mycobacterial pathogenesis are performed with controlled infections of macrophages using *in vitro* culture systems. However, the environment that is encountered by *M. tuberculosis in vivo* is more complex. First, *in vitro* experiments are usually short term, whereas *in vivo* infections have a far longer time course. Second, most *in vitro* experiments are performed using a single type of host cells such as macrophages, whereas *in vivo*, *M. tuberculosis* experiences influences from multiple cell types. Third, *in vivo* infection involves a distinct sequence of events such as the initial encounter with innate immune cells and subsequent onset of adaptive immunity. Thus, the environment encountered by *M. tuberculosis* in the course of *in vivo* infections is likely to be significantly different and more complex than during *in vitro* experiments. While avoiding delivery to lysosomes is important for mycobacterial survival in macrophages *in vitro*, the relevance of phagosome maturation arrest *in vivo* is not clear. Several lines of evidence suggest that mycobacteria may have evolved mechanisms to adapt to a lysosomal or acidic environment. First, mycobacteria have been observed in lysosomes under specific conditions such as opsonization, cholesterol depletion or co-infection with other pathogens [17–21]. Second, although most mycobacterial mutants generated through transposon-mediated gene inactivation that are unable to arrest phagosome maturation fail to survive within macrophages, some mutants survive in an acidified phagolysosome [22,23]. Finally, acid resistance genes have been shown to play a crucial role in mycobacterial survival [21].

In order to better understand the role of phagosome maturation arrest during *in vivo* infections, we infected mice with *M. tuberculosis*, and analyzed infectivity as well as intracellular localization *in vivo* using quantitative image analysis. In addition, we analyzed the course of infection of *Mycobacterium bovis* BCG, an attenuated strain that equally resist lysosomal delivery upon entry into macrophages in Rag2−/− mice. Strikingly, we found that both *M. tuberculosis* as well as *M. bovis* BCG, while initially residing within non-lysosomal phagosomes, were subsequently delivered to lysosomes. In accordance with the capacity of *M. tuberculosis* or *M. bovis* BCG to replicate at the times at which the majority of the bacilli reside in lysosomes, mycobacterial mutants lacking the mycobacterial Ser/Thr kinase Protein kinase G (PknG), essential for phagosome maturation arrest [10], were able to replicate at later times of infection. Interestingly, the innate capacity of mycobacteria to withstand lysosomal delivery was not affected; our data rather suggest that the alterations in the host environment result in mycobacterial delivery to lysosomes. These results suggest that the strategy of *M. tuberculosis* to avoid lysosomal delivery is operational in a limited time window *in vivo*, and highlight the versatile mechanisms utilized by *M. tuberculosis* to survive within infected hosts.

2. Materials and methods

2.1. Mice and infection model

Rag2−/− mice were kept under specific pathogen-free conditions in the Animal House of the Department of Biomedicine, University Hospital, Basel according to the regulations of Swiss veterinary law, mice were kept in Scientific Institute of Public Health, Brussels according to the regulation of Belgian veterinary law. Rag2$^{-/-}$ mice were infected with 10^6 CFU of either *M. bovis* BCG or *M. bovis BCGΔpkng;* DBA/2 mice with 10^6 CFU *M. tuberculosis* (H37Rv) or *M. tuberculosisΔpkng*, respectively by tail vein injection. Animals were monitored twice weekly for lethargy and weight loss and euthanized by i.p. injection of 150 mg/kg Pentothal when body weight had decreased more than 20% of the initial body weight [24]. Kaplan–Meier analysis was applied to compare death rates of both the bacterial strains.

2.2. Bacteria

M. tuberculosis expressing GFP was a kind gift from Dr. Jean-Louis Herrmann (Hopital Pointcarre, Garches, France). *M. bovis* BCG and the derived *M. bovis BCGΔpknG* mutant have been described [10]. Construction of the *ΔpknG* mutant from *M. tuberculosis* H37Rv was carried out by transduction using the recombinant phage phLN5 as described [10]. Gene deletion was confirmed by polymerase chain reaction (PCR) and immune blotting using anti-PknG antibody [10]. *M. bovis* BCG was isolated from infected liver by homogenization of liver in 10 ml ice cold PBS and incubating with buffer containing 1% Triton ×100, 150 mM NaCl and 10 mM Tris-Cl pH 7.5 on ice for 10 min followed by centrifugation at 4000×*g* for 20 min. The bacteria present in the pellet were washed 3 times in PBS by centrifugation at 4000×*g* for 20 min. The final pellet was resuspended in 2.5 ml DMEM and used for infections of bone marrow derived macrophages *in vitro*. For the analysis of bacterial viability by CFU enumeration, infected liver and spleen were homogenized in saline and serial dilutions were plated on Middlebrook 7H11-OADC agar plates, incubated at 37 °C for 3 weeks and the resulting colonies were counted after three weeks [25].

2.3. Histology

For analyzing mycobacterial localization *in vivo*, the procedure was followed as previously described [13]. Briefly, animals were sacrificed at different time points after infection and 8 μm thin sections of infected organs were prepared using a Leica Cryostat CM1100 set to −17 °C. Slides were air dried and fixed with ice-cold methanol at −20 °C for 4 min and

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