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Inhibition of apoptosis by Rv2456c through Nuclear factor-κB extends the survival of *Mycobacterium tuberculosis*

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ABSTRACT

Mycobacterium tuberculosis, the causative agent of tuberculosis, is an intracellular pathogen with several survival mechanisms aimed at subverting the host immune system. Apoptosis has been shown to be mycobactericidal, to activate $CD8^+$ T cells, and to be modulated by mycobacterial proteins. Since few mycobacterial proteins have so far been directly implicated in the interactions between *M. tuberculosis* and host cell apoptosis, we screened *M. tuberculosis* H37Rv transposon mutants to identify mutants that fail to inhibit cell death (FID). One of these FID mutants, FID19, had a transposon insertion in Rv2456c and is important for survival in host cells. The lack of the protein resulted in enhanced caspase-3 mediated apoptosis, which is probably due to an inability to activate nuclear factor-κB. Additionally, FID19 infection enhanced polyfunctional $CD8^+$ T cells and induced a higher frequency of interferon-γ secreting immune cells in a murine model. Taken together, our data suggest that Rv2456c is important for the survival of H37Rv by subduing the innate and ultimately adaptive immune responses of its host by preventing apoptosis of the infected cell. Better understanding of the host-mycobacterial interactions may be beneficial to develop novel drug targets and engineer more efficacious vaccine strains against tuberculosis.

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Introduction

Tuberculosis is a serious global health threat, infecting 9 million new individuals and causing 1.5 million deaths each year [1]. The prime etiological agent of this disease is *Mycobacterium tuberculosis*, an aerobic, acid-fast bacillus that dwells inside host cells after infection [2,3]. The pathogen is transmitted via aerosolized particles from an actively infected individual [4], and it primarily resides inside host alveolar macrophages [5]. Once inside its targeted cell, the

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mycobacterium is able to modulate its surroundings in order to prevent immune detection and ensure survival.

As apoptosis is bactericidal to *M. tuberculosis*, it is not surprising that the bacterium has found ways to inhibit this pathway [6–11]. Cysteine proteases called caspases mediate apoptosis and are found in the cytoplasm until they are activated via cleavage at their active site [12]. Varieties of stimuli are able to induce cell death and can be found either internal or external to the cell. The Bcl-2 protein family regulates intrinsic apoptosis via their interactions with the mitochondrial membrane and can cause activation of caspase 9 [13]. Conversely, external stimuli such as tumor necrosis factor-α (TNFα) bind to death receptors on the exterior surface of the cell to initiate a cascade that activates caspase 8 [14]. These initiator caspases then activate caspase 3, which subsequently executes events that lead to apoptosis of the cell.

M. tuberculosis is able to block both the intrinsic and extrinsic apoptotic pathways. The bacterium reduces extrinsic apoptosis by preventing the secretion of Fas [6] and TNFα [8,15], as well as by interacting with host proteins [9]. Intrinsic apoptosis is abrogated through the upregulation of a prosurvival Bcl-2 protein, Mcl-1 [10]. Other genes have been identified as being important in the inhibition of apoptosis [8,16], but the specific mechanisms remain elusive. Thus, additional study in this area could uncover novel ways to prevent the survival of *M. tuberculosis* within host cells. It was reported that inhibition of apoptosis by *M. tuberculosis* could prevent cross presentation and activation of $CD8^+$ T cells [17,18], minimizing the cytotoxic adaptive immune response. By identifying how the bacterium is able to subvert host immune responses in macrophages, it may be possible to engineer a nonpathogenic strain of *M. tuberculosis* that enhances immune responses for use as a vaccine. This is urgently needed, as there is no efficacious vaccine against tuberculosis [19,20].

To identify genes that are important in the inhibition of apoptosis, a transposon mutant library of *M. tuberculosis* H37Rv was generated and screened for mutants unable to inhibit cell death. Mutants were further screened for heightened immunogenicity in comparison to the parental strain. Based on its failure to inhibit apoptosis and to induce an immune response, fails to inhibit cell death mutant 19 (FID19), a transposon insertion in Rv2456c, was selected for further study. Our studies aimed to determine how this protein inhibits apoptosis in the wild type bacterium, if it is important for survival, and if a mutant lacking this protein has enhanced immunogenicity *in vivo*.

Materials and methods

Bacterial strains and culture conditions

M. tuberculosis H37Rv was obtained through the National Institute of Health Biodefense and Emerging Infectious Research Resources Repository (BIH-*M. tuberculosis*, strains H37Rv and NR-123). An arrayed transposon library containing 9400 mutants was generated through Mariner transposon mutagenesis as described previously [21]. The flanking genomic region of the transposon insertion site was sequenced and the disrupted gene was complemented by either a plasmid expressing the single target gene with its promoter or a cosmid encoding the gene. A cosmid used for the complementation was generously provided by Dr. William R. Jacobs, Jr. (Albert Einstein School of Medicine, New York, NY, USA). To reduce variability between experiments, freshly frozen bacterial stocks were used for infection. To prepare the stocks, bacteria were grown until they reached an optical density between 0.6 and 0.8. At this point, the cells were washed twice in phosphate buffered saline (PBS; Gibco, Invitrogen, Carlsbad, CA) with 0.05% tyloxapol (Sigma–Aldrich, Hamburg, Germany), passed through a sterile 40 μM filter, and finally through a sterile 20 μM filter. The optical density of the culture was then adjusted to 1.2, aliquoted into 1-mL vials, and quickly frozen using dry ice and ethanol. Mycobacterial liquid growth media consisted of Middlebrook 7H9 media (Difco, BD) supplemented with 0.5% glycerol (Sigma–Aldrich), 0.05% tyloxapol (Sigma–Aldrich), and 10% of a solution of oleic acid, albumin, dextrose, and catalase supplement (BD Diagnostics, Sparks, MD). For growth plates, Middlebrook 7H10 media was supplemented with the same components as above. Plates were grown in incubators at 37 °C with 5% CO_2 for 3 weeks or until colonies were visible.

Cell death assay

Human epithelial cells (A549: American Type Culture Collection, CCL-185) were infected at a multiplicity of infection (MOI) of 10 for 3 h with the arrayed transposon mutant library. After this time, cells were washed twice with PBS and fresh media containing 50 μg/mL of gentamycin was added. The experiment was allowed to continue for 3 days and the supernatant was assayed for lactate dehydrogenase (LDH; Cytotoxicity Detection Kit, Roche, Mannheim, Germany) to measure membrane damage and cell death. Mutants that induced significant LDH release were further tested for their ability to induce death in human monocyte-derived macrophages (THP-1: TIB-202, American Type Culture Collection, Manassas, VA). To measure cell death, THP-1 cells were differentiated with 10 ng/mL of phorbol myristate acetate overnight, washed twice with PBS, and then infected with an MOI of 10. THP-1 cells were infected as described above for 4 days after which cells were fixed with 4% formaldehyde and stained with terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick-end labeling (TUNEL) as described in the package insert (*In-situ* Cell Death Detection Kit; Roche). Cells were analyzed on a BD FACS Canto or BD LSRII (Becton Dickinson Biosciences, Rutherford, NJ) after staining. All flow cytometry data was analyzed using Flowjo software (TreeStar, Ashland, OR).

Measurement of pyroptosis induction

Small hairpin RNA (shRNA) knockdowns in *PYCARD* and *NLRP3* in THP-1 cells were generously provided by Dr. Jenny Ting (University of North Carolina, Chapel Hill, NC, USA) and are described elsewhere [22]. Cell death was measured by TUNEL staining and interleukin (IL)-1β secretion was measured by enzyme-linked immunosorbent assay (ELISA; eBiosciences, San Diego, USA).

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