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MECHANISMS OF PATHOGENESIS

Mycobacterium tuberculosis EsxO (Rv2346c) promotes bacillary survival by inducing oxidative stress mediated genomic instability in macrophages

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SUMMARY

Mycobacterium tuberculosis (*Mtb*) survives inside the macrophages by modulating the host immune responses in its favor. The 6-kDa early secretory antigenic target (ESAT-6; esxA) of *Mtb* is known as a potent virulence and T-cell antigenic determinant. At least 23 such ESAT-6 family proteins are encoded in the genome of *Mtb*; however, the function of many of them is still unknown. We herein report that ectopic expression of *Mtb* Rv2346c (esxO), a member of ESAT-6 family proteins, in non-pathogenic *Mycobacterium smegmatis* strain (*Msm*Rv2346c) aids host cell invasion and intracellular bacillary persistence. Further mechanistic studies revealed that *Msm*Rv2346c infection abated macrophage immunity by inducing host cell death and genomic instability as evident from the appearance of several DNA damage markers. We further report that the induction of genomic instability in infected cells was due to increase in the hosts oxidative stress responses. *Msm*Rv2346c infection was also found to induce autophagy and modulate the immune function of macrophages. In contrast, blockade of Rv2346c induced oxidative stress by treatment with ROS inhibitor N-acetyl-L-cysteine prevented the host cell death, autophagy induction and genomic instability in infected macrophages. Conversely, *Mtb*ΔRv2346c mutant did not show any difference in intracellular survival and oxidative stress responses. We envision that *Mtb* ESAT-6 family protein Rv2346c dampens antibacterial effector functions namely by inducing oxidative stress mediated genomic instability in infected macrophages, while loss of Rv2346c gene function may be compensated by other redundant ESAT-6 family proteins. Thus EsxO plays an important role in mycobacterial pathogenesis in the context of innate immunity.

1. Introduction

After inhalation *Mycobacterium tuberculosis (Mtb)*, causative agent of tuberculosis (TB), infects the alveolar macrophages and deploys various strategies to subvert the host immunity to create a responsive environment for its proliferation [1]. Inside macrophages mycobacteria are enclosed into phagosomes where they are exposed to antibacterial effector molecules such as nitric oxide (NO), reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS). Numerous studies have demonstrated the importance of NO and ROS in control of *Mtb* growth [2–4]. Although the

Abbreviations: WT, Wild Type; *Msm*, *Mycobacterium smegmatis*; *Mtb*, *Mycobacterium tuberculosis*; *Msm*Rv2346c, *Mycobacterium smegmatis* expressing Rv2346c; MOI, multiplicity of infection; CFU, Colony forming unit; O.D, Optical Density; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, Nitric Oxide; INOS, Inducible Nitric Oxide Synthase; NAC, N-Acetyl Cysteine; ROS, Reactive Oxygen Species; RNS, Reactive nitrogen species; FBS, Fetal Bovine Serum; SOD, Superoxide dismutase; LC3 II, Microtubule-associated protein 1A/1B-light chain 3 Subunit II; NF-κB, Nuclear Factor Kappa B-chain; TNF-α, Tumor Necrosis Factor alpha; IL-12, Interleukin 12; IL-10, Interleukin 10; PCD, Programmed Cell death.

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role of NO in human TB remains unsettled, several previous studies have shown that inducible nitric oxide synthase (iNOS) deficient mice, which lack inducible NO production, are highly susceptible to *Mtb* infection [5]. Additionally, the production of ROS was found to protect the host by controlling the *Mtb* growth during the early infection process [6]. However during the course of infection, *Mtb* copes with these host-mediated oxidative stresses by a range of mechanisms including scavenging of the reactive species and the repair and protection of DNA and proteins [7]. In addition to oxidative stress, autophagy plays a crucial role in defense against *Mtb* [8]. Induction of autophagy in macrophages results in an increased mycobactericidal activity. However, under certain circumstances, excessive activation of autophagic processes in response to cellular stress, such as oxidative stress results in autophagy-related host cell death [9].

Upon activation, macrophages produce NO and ROS, which at lower concentrations act as signaling molecules and regulators of the immune system [10]. However, increased production of these molecules by macrophages is considered to be pathological because of interference with signaling pathways related to apoptosis, cell cycle arrest and, the ability to cause severe damage to cellular constituents, such as DNA and proteins [11]. Indeed, several studies have reported that high levels of NO and ROS production in response to bacterial infections weaken cellular immunity by inducing genomic instability due to DNA damage [12].

In the context of *Mtb*, the thick cell wall containing lipoarabinomannan (LAM), phenolic glycolipids and mycolic acids, which act as potent scavengers of oxygen radicals confer resistance to ROS [13]. Moreover, *Mtb* exploits more peculiar survival strategies, including ESX secretion systems, which are responsible for exporting virulence factors and immunomodulators across the cell envelope [14]. These ESX substrates are known to dampen the host immune responses to aid bacillary persistence in macrophages. Early secreted antigen target-6 (ESAT-6; EsxA) is one of such proteins secreted by ESX-1 secretion system that plays a critical role in persistence of *Mtb* by modulating the host immune responses. *Mtb* displays membrane-perforating activity due to ESAT-6 secretion [15], which is responsible for the phagosomal membrane rupture and the subsequent escape of *Mtb* into cytoplasm [16]. Genome sequencing suggested that there are 23 ESAT-6 family members present in the *Mtb* H37Rv genome [17]. However, role of the majority of these proteins in *Mtb* pathogenesis and host immunity is still not defined.

Here we demonstrate a novel mechanism by which *Mtb* Rv2346c, a member of ESAT-6 like family proteins, endow bacterial persistence inside the macrophages by dampening their antibacterial effector functions by inducing genomic instability and autophagy, thereby transforming them into immune-compromised host cells. *Msm*Rv2346c showed enhanced survival in human and mouse macrophages. Further, we proved that genomic instability and autophagy death pathways were rendered due to augmented oxidative stress responses in *Msm*Rv2346c infected macrophages. Surprisingly, deletion of Rv2346c in *Mtb* did not affect intracellular bacterial survival and oxidative stress responses when compared with *Mtb* H37Rv infected macrophages.

2. Results

2.1. Characterization of Rv2346c in M. tuberculosis H37Rv

EsxO (Rv2346c) has been assigned to a member of Rv1793, Rv1198 and Rv1037c, all belonging to the ESAT-6 family proteins [18]. Epitope mapping of Rv2346c, Rv1793, Rv1198 and Rv1037c using Netmhc3.0 predicted several conserved high affinity peptides to human HLA class I. Comparative proteome analysis of culture supernatant from virulent *Mtb* H37Rv and attenuated *Mycobacterium bovis* BCG identified 22 novel *Mtb* specific proteins, which include Rv1198 and Rv1793 as potential vaccine candidates [19]. Figure 1A shows schematic genetic organization of *esxO* in *Mtb* H37Rv genome. Upstream of this gene is Rv2345, encoding a possible transmembrane protein. Multiple sequence alignment and BLAST results revealed more than 90% protein sequence homology of Rv2346c with other ESAT-6 family proteins Rv1198, Rv1037c, Rv3619c and Rv1793 (Figure 1B).

2.2. M. smegmatis strain expressing Rv2346c showed increased invasion in human epithelial cells

Many cell membrane associated proteins are known to play an important role in initial infection process [20]. We, therefore, investigated the role of Rv2346c in host cell invasion by expressing it in non-pathogenic *Mycobacterium smegmatis* strain (*Msm*Rv2346c) using pSMT3 shuttle vector. Previously several studies have used *M. smegmatis* as a surrogate host to study the function of *Mtb* proteins due to close homology between genomic architecture of these two strains [21–23]. To check invasion rate, we used non-phagocytic

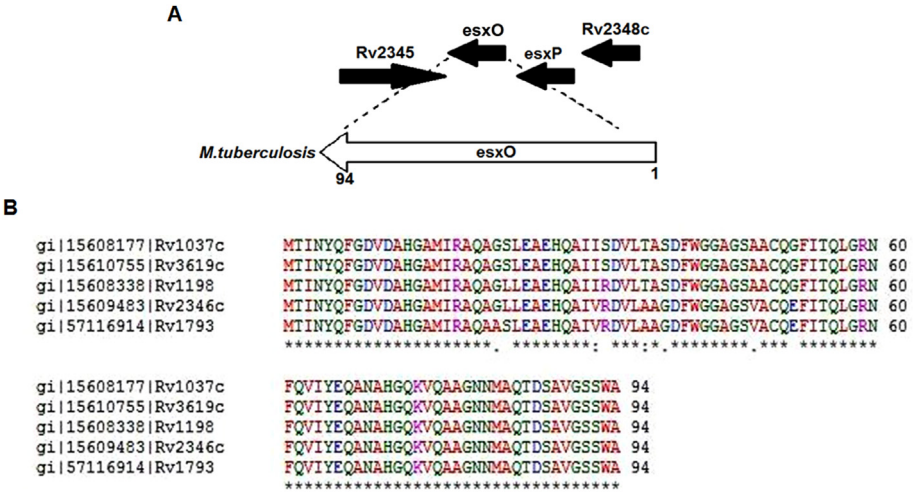

Figure 1. Genetic organization of *Mtb* H37Rv Rv2346c. (A) Schematic representation of Rv2346c in *Mtb* H37Rv genome. (B) ClustalW multiple sequence alignment of Rv 2346c with ESAT-6 like proteins.