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Original Article

Virulent Strain of *Mycobacterium tuberculosis* Causes Necrosis in Murine Macrophage and rmIFN-γ Promotes Necrotic Death of Macrophage

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Macrophage is known to be a major immune barrier in preventing the spread of MTB after its entry into host. Fate of parasitized macrophage may be important in shaping the events after infection. In the present study, the effect of virulent H37Rv strain of MTB on viability of murine macrophage-like cell RAW 264.7 (ATCC) over a range of multiplicity of infections (MOIs) has been investigated. We have performed lactate dehydrogenase (LDH) release assay for determining the extent of LDH release from murine macrophage-like cell RAW264.7 infected with virulent H37Rv strain of *Mycobacterium tuberculosis* (MTB) at different multiplicity of infections (MOIs) in the presence or absence of rmIFN-γ. Our data has demonstrated that virulent H37Rv strain of MTB induces significant level of necrosis in RAW264.7 cells over a range of MOIs (10, 15 and 20) in time-dependent manner. Furthermore, exogenous addition of rmIFN-γ to infected cells promotes necrosis of macrophages. This information has relevance in improving our understanding of immunopathology of disease.

Keywords: *Mycobacterium tuberculosis*; rmIFN-; Murine macrophage; Necrosis

Introduction

Mycobacterium tuberculosis (MTB), which is an etiological agent of tuberculosis, is still a major public health threat despite good available therapeutic and preventive measures. It annually claims 1.5 million lives and about 6 million new cases of tuberculosis are reported worldwide annually (WHO 2015). It is estimated that 2 billion

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persons, one third of the world's population, has latent MTB infection, out of which 10% develop active tuberculosis during their entire life span (Dye et al 1999). The major cause of this high burden of tuberculosis, as well as mortality is contributed by the evolution of various kinds of drug-resistant strains of MTB, co-infection with HIV & other diseases, malnutrition and poor hygienic and living conditions.

Macrophage is a major immune barrier in restricting the spread of MTB after its entry into host. After engulfment by macrophages, MTB tries to escape the killing by the host by manipulating the intracellular hostile environment(s) including prevention of acidification of phagosomes and fusion of phagosomes with lysosomes (Xu et al 2014). As a consequence of interaction between MTB and macrophages, either or a combination of the three major kinds of outcomes are observed in the host. These are (i) necrosis (ii) apoptosis and (iii) survival of infected macrophages. The outcome of this interaction dictates the fate of both macrophages and MTB (Divangahi et al 2013).

Cellular death of the parasitized host cell provides the door/opportunity to MTB to expand its infection by further intracellular generation and infecting new host cells. In the defense mechanisms of the host, apoptosis largely benefits the host in clearing the intracellular MTB from host cells by packaging MTB antigens in apoptotic vesicles and its cross-presentation to dendritic cells (Schaible et al 2003). Necrosis, however, may be helping the MTB in its unrestricted replication in pockets where the antimicrobials as well as the host effector mechanisms have limited accessibility (Lee et al 2006) and may be contributing to its survival.

In the present study, the effect of virulent H37Rv strain of MTB on viability of murine macrophage-like cell RAW264.7 (ATCC) over a range of multiplicity of infections (MOIs) has been investigated.

Materials and Methods

Culture of MTB and macrophage

Reference strain (H37Rv) of MTB (ATCC, USA) was cultured and maintained in Middlebrook 7H9 medium supplemented with 10% ADC (BD), 0.2% glycerol and 0.05% Tween-80. Murine macrophage like cells RAW264.7 (ATCC) was cultured in DMEM (Gibco) supplemented with 10% (v/v) heat inactivated FBS (Hyclone), 1.46 g/l L-glutamine (Sigma), 2.3 g/l HEPES (Gibco), 3.7 g/l $NaHCO_3$ (Sigma) and 10 ml/l of Pen Strep (Gibco) and continuously maintained at 37^0C in a 5% CO_2 humidified incubator.

MTB infection to macrophage

A total of 1.5 × 106 RAW264.7 cells were infected with an exponentially grown culture of MTB (OD_{600} 0.6) at various multiplicity of infection (MOIs) i.e. 10,15 and 20 in 90 × 100 mm Cell Culture Dish (Corning) containing antibiotic free complete DMEM. Briefly, after 8 hrs of infection, infected cells were washed twice with warm antibiotic free DMEM followed by treatment with 0.2 mg/ml amikacin sulfate (Sigma) for an additional 2 h to kill any extracellular bacteria. Finally, infected cells were incubated for the various time-points (1-4 days) with or without three increasing concentrations (i.e 0.5, 1 and 2 ng/μl) of rmIFN-γ (R&D Systems).

Cytotoxicity assessment by LDH release

The level of necrosis in cells was assessed by the measurement of lactate dehydrogenase (LDH) release from MTB infected RAW264.7 using the CytoTox^{96} non-radioactive cytotoxicity assay (Promega). Percentage cytotoxicity was determined using the following formula: [experimental LDH release (OD490)/maximum LDH release (OD490)] × 100.

Statistical analysis

Non-parametric Mann-Whitney and Kruskal-Wallis tests were applied to determine the level of significance between various experimental groups. Data were reported as two-tailed 'p' values, where $p < 0.05$ was considered statistically significant.

Results and Discussion

Mycobacterium tuberculosis is a successful pathogen in terms of its ability to survive inside host cells for a long time by impairing host's killing mechanisms. There are different as well as several hypothesis on the outcome of interaction of MTB and macrophages. It is widely believed that bacillary load and infectivity of MTB strains influences the fate of both infecting MTB and host macrophages (de Chastellier 2009).

Necrosis or type-III cell death is thought to be non-programmed, accidental and unregulated form of cell demise caused due to defect in the pathological process, however, others opine that it is simply a traumatic death of cells while other workers consider it as a physiological event as a secondary apoptosis to (Challa and Chan 2010).

It is morphologically characterized by cell swelling, plasma membrane disruption and loss of cellular integrity (Kitanaka and Kuchino 1999). A long debate is ongoing on the mechanism(s) of facilitation of this form of cell death. A group of workers believed that necrosis is a well orchestrated molecular event involving the intrinsic signal transduction mechanisms (Festjens et al 2006). Involvement of caspase in necrotic cell death is, however, not clear leaving many outcomes unsolved and gaps in the knowledge (Kuranaga 2012). However, receptor interacting protein 1 (RIP1) has emerged as a one of the key mediators of necrosis (Holler et al 2000).

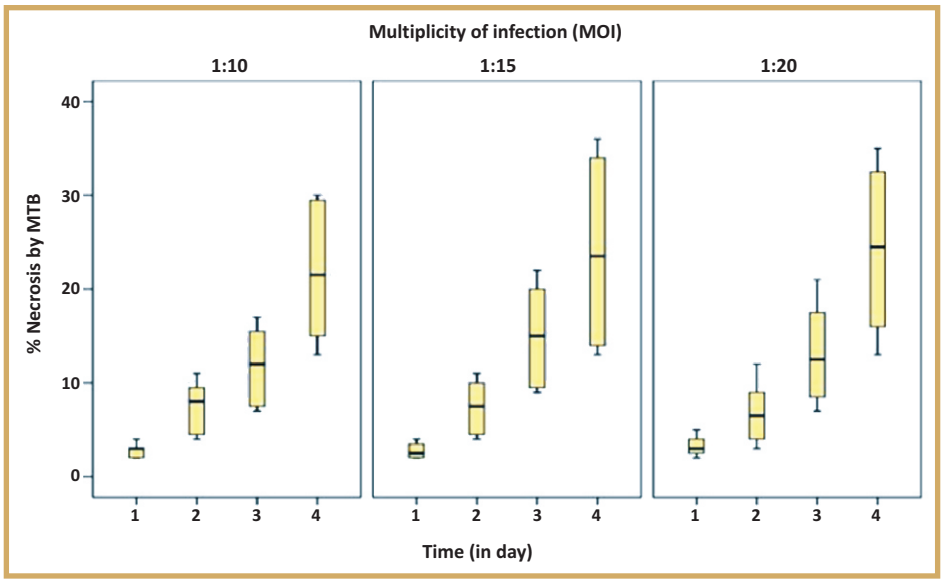

Fig 1 : Effect of *Mycobacterium tuberculosis* (H37Rv) on the necrosis of murine macrophage-like cell RAW264.7 at different MOIs (10, 15 and 20) and time-points (1-4 days). The level of necrosis was expressed in percentage.

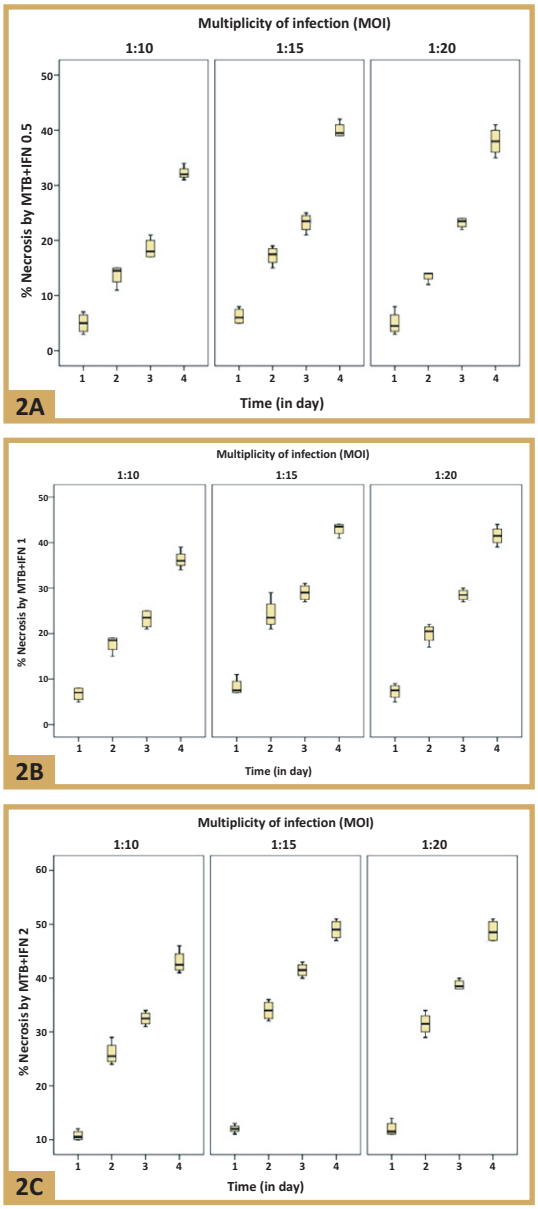

Fig 2 : Effect of three increasing concentrations (2A) 0.5 ng/µl, (2B) 1 ng/µl and (2C) 2 ng/µl C of rmIFN-γ on the necrosis of murine macrophage-like cell RAW264.7 infected with *Mycobacterium tuberculosis* (H37Rv) at different MOIs (10, 15 and 20) and time-points (1-4 days). The level of necrosis was expressed in percentage. IFN-Interferon gamma.

In the present study, we have observed that virulent H37Rv strain of MTB induces significant level of necrosis in RAW264.7 cells over a range of MOIs (10, 15 and 20) in time-dependent manner. The data (mean ± SD) derived from three separate LDH release assays were significantly different at p=0.023 between infected versus uninfected untreated controls at all the time - points (1-4 days) and is seen in Fig 1. It was also observed that exogenous addition of three increasing concentrations (0.5, 1 and 2 ng/µl) of rmIFN-to MTB infected RAW264.7 cells significantly promotes necrotic death of macrophages at three different MOIs (10, 15 and 20). The data (mean ± SD) obtained from three separate LDH release assays were found significant ($p < 0.001$) between infected versus uninfected untreated controls at all the time - points (1-4 days) (Fig 2 A-C). The similar results were also obtained by Lee and Kornfeld (2010) previously. In contrast, it was also observed that infection of macrophages with high number of virulent MTB Erdman strain (≥ 25 bacilli per macrophage) predominantly induces apoptosis in caspases, TNF-α, TLR-independent manner leading to necrosis (Lee et al 2006). Other workers observed that virulent H37Rv strain leads to necrosis of macrophage at low intracellular bacillary load due to disruption of inner membrane of mitochondria of macrophages (Chen et al 2006).

To summarize, our data showed that virulent strain of MTB causes necrosis in murine macrophages and MOIs play a key role in necrosis of macrophages. Our data also showed that rmIFN-γ promotes necrotic death of MTB infected murine macrophages. The study needs to be undertaken in experimental animal models with TB infection to see if similar results are obtained in them and also if these can be extrapolated in humans.

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