MECHANISMS OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS (MAP) INDUCED APOPTOSIS AND NECROSIS IN BOVINE MACROPHAGES

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Abstract
Bovine monocyte-derived macrophages infected by equal number (MOI=1) of Mycobacterium avium subsp. paratuberculosis (Map) were not affected, but Map at MOI=10 caused macrophage apoptosis. Map at higher bacterial burden (MOI=50 or greater) induced both apoptosis and necrosis in macrophages. Activation of caspase-3 and alterations in membrane potential/membrane permeability transition of mitochondria were observed in Map-infected macrophages. Map at lower bacterial burden induced caspase-dependent and mitochondrial pathway of apoptosis, while Map at higher burden induced caspase-independent and nitric oxide-independent apoptosis and mitochondrial damage-associated necrosis in macrophages. It is concluded that under higher bacterial burden and spatial stress, Map induced apoptosis and necrosis of macrophages by complex mechanisms as to find a new niche for survival and replication.

Introduction
Little is known about the host-pathogen interactions that regulate the pathogenesis of paratuberculosis (Johne’s disease), particularly host cell death or survival of bacteria within infected cells. The interaction of Mycobacterium avium subsp. paratuberculosis (Map) with bovine macrophages was found to be a complex processes involving strategies for survival of bacteria or host cell death depending upon the number of bacteria infected per macrophage (multiplicity of infection, MOI). In the present study, we report that Map at equal bacterial burden per cell (MOI=1) was not harmful for macrophages, but at MOI of 10 induced apoptosis. Interestingly, Map at higher bacterial burden (MOI=50 or greater) induced both apoptosis and necrosis in macrophages.

Materials and methods
A characterized mycobactin dependent bovine strain (C-123/IVRI) of Map isolated form clinically infected cattle was grown in the Middlebrook-7H9 broth (10^9 CFU/ml) for infection of bovine blood monocyte-derived macrophages. Bovine macrophages (2 to 2.5 x 10^5/wells) were infected with Map at different multiplicity of infection (MOI, ranging from 1 to 100). The infected cells were cultured at 37°C for 4 to 48 h post-infection (hpi) depending on the experimental design. Apoptotic changes of macrophages were assessed by nuclear morphology and cellular permeability to fluorescence dyes (DAPI/PI and AO/EB). For detection of necrosis in macrophages, the release of lactate dehydrogenase (LDH) was estimated in cultured cells. TUNEL assay and electron microscopic evaluation of apoptosis were performed to confirm apoptotic and necrotic changes. Pro-apoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl2) gene expressions were analyzed by qPCR assay. Caspase-3 activity was measured using CaspACE™ colorimetric assay system. Nitrite was measured using quantitative assay based on Griess reaction. The change in mitochondrial inner membrane potential (ΔΨm) was measured using DiOC6(3). Data were analyzed in GraphPad Prizm for statistical significance.

Results and Discussion
Map at lower bacterial burden (MOI=1) did not induce cell death, but Map at MOI=10 induced macrophage apoptosis (Fig A, B, C, D). Strikingly, Map at higher bacterial burden (MOI=50 or 100) induced both apoptosis and necrosis in macrophages. Thus Map induced apoptosis in macrophages is dose dependent as reported previously for M. tuberculosis (Lee et al., 2006). LDH assay suggested that Map at low level was non-cytotoxic but was cytotoxic at higher bacterial burden (Allen et al., 2001; Weiss et al., 2004; Lee et al., 2006). On fluorescence microscopy, cells undergoing apoptosis had condensed and fragmented nuclei, whereas necrotic cells showed higher cellular permeability for impermeant dyes. At ultrastuctural level, apoptotic cells had chromatin condensation and fragmentation, while necrotic cells showed breaks in cell membrane and loss of cytoplasmic and nuclear contents. Apoptotic and necrotic changes were mostly observed in those cells having intracellular bacteria and occasionally were seen in
bystander cells without intracellular bacteria. Activation of caspases, induction of mitochondrial permeability transition (MPT), and structural changes of mitochondria were observed in apoptotic macrophages. Change in mitochondrial outer membrane permeability during apoptosis has been reported previously (Chipuk et al., 2006). Addition of caspase inhibitors (Z-VAD-fmk or Z-DEVD-fmk) (Fig. E, F) or MPT inhibitor (cyclosporine A) (Fig G, H) significantly reduced in numbers of apoptotic macrophages infected with Map indicating that caspases and mitochondria were involved in induction of apoptosis. In contrast, Map at higher burden (MOI=50 or 100) induced macrophage apoptosis in caspase-independent pathway since caspase inhibitors or MPT inhibitor did not reduce apoptotic changes in macrophages. Macrophage apoptosis in response to high intracellular burden of M. tuberculosis was mediated by a novel caspase-independent pathway (Lee et al., 2006). Similarly, NO-dependent and independent mechanisms have been implicated in apoptosis and necrosis at different MOI. Nitric-oxide mediated apoptosis in murine peritoneal macrophages has been reported by Albina et al. (1993). Thus, it is concluded that under higher bacterial burden and spatial stress, Map induced apoptosis and necrosis of macrophages by unknown mechanism as to find a new niche for survival. It was further supported by the observation of more number of cells infected with Map at 48 h (55%) when compared to 4 h (41%) post-infection at MOI=50 or 100 (data not shown).

References