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Mycobacterium bovis BCG Inhibits Spontaneous Apoptosis in Human Monocytes via a Phosphatidylinositol (PI)-3 Kinase Dependent Pathway

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Mycobacterium attachment and uptake into monocytes has previously been shown to involve Protein Tyrosine Kinase (PTK), mitogen activated protein kinase (MAPK) and phosphatidylinositol (PI)-3 kinase pathways. Human monocytes were infected with BCG Copenhagen vaccine strain and host cell apoptosis was monitored over a time course using Annexin-V staining of cells. Monocytes were pretreated with pharmacological inhibitors to PTK, MAPK and PI-3 kinase pathways to investigate their role in BCG-induced apoptosis. BCG infection of monocytes increased within 24 h of stimulation at infection doses of 0.5, 1 and 2 per cell. BCG-induced inhibition of monocyte apoptosis was not affected by PTK inhibitor genistein (10 and 40 μM) or the MAPK inhibitor PD98059 (10 and 40 μM). However, when cells were pretreated with the PI 3-kinase inhibitor LY25009 (25 μM) BCG-induced monocyte survival was no longer observed. Our results suggest a role for the PI-3 kinase pathway in BCG-induced monocyte survival.

Key words: Apoptosis, mycobacterium, phosphatidyl-inositol 3-kinase, monocyte, BCG

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INTRODUCTION

Constitutive spontaneous apoptosis is responsible for the homeostatic regulation of cells and is the mechanism by which cells respond to stress conditions such as, infection by pathogenic organisms. Apoptosis can occur either via the intrinsic pathway, involving mitochondrial release of cytochrome c and activation of caspase 9, or the extrinsic pathway, involving the stimulation of death receptors expression on the cells surface and activation of caspase 8. The Bcl-2 family of apoptotic proteins regulate the mitochondrial pathway of apoptosis and therefore activation or modification of these anti-, or pro-apoptotic proteins has been shown to determine the outcome of host cell viability.

Apoptotic signaling mechanisms have been studied in relation to cell survival with the help of apoptosis inhibiting or inducing factors. Growth factors such as GMCSF and cytokines such as IL-3 are survival factors and inhibit apoptosis. While, the cytokine TNFα can mediate apoptosis via the death receptor signaling pathway (Salamone *et al*., 2001) but has also been shown to induce survival in cells. Reports show GMCSF mediated survival of cells to require the extra cellular signal regulated (ERK) 1/2 Mitogen Activated Protein (MAP) kinase pathway (Klein *et al*., 2000), while IL-3 functions by inducing the phosphatidyl inositol (PI)3-kinase/Akt pathway (Craddock *et al*., 1999). The PI3 kinase pathway has also been shown to play a role in TNFα mediated survival in neutrophils (Cowburn *et al*., 2002) and melanocytes (Larribere *et al*. 2004).

Mycobacteria activate and modulate host signaling pathways in order to create an environment conducive to their own persistence (Reiner, 1994). Host macrophage apoptosis, but not necrosis, is linked to killing of intracellular mycobacteria (Molloy *et al*., 1994). Virulent *M. tuberculosis* H37Rv can downregulate apoptosis in host macrophages, while non-pathogenic *M. bovis* BCG and avirulent *M. tuberculosis* H37Ra induce greater levels of apoptosis (Klingler *et al*., 1997; Riendeau and Kornfeld, 2003; Sly *et al*., 2003). In contrast, other studies have shown that the BCG vaccine strain is able to inhibit spontaneous apoptosis in both human monocytes (Kremer *et al*., 1997), peripheral blood neutrophils (Suttmann *et al*., 2003) and murine macrophages (Kausalya *et al*., 2001). Variation between studies can be attributed to differences in host cells employed and in the mycobacterial strains used.

Attachment and uptake of mycobacteria into host cells has been shown to activate a number of host cellular signaling pathways (Nandan *et al*., 2000). Mycobacteria trigger protein tyrosine kinase (PTK) (Mendez-Samperio *et al*., 1996) and MAPK (Hasan *et al*., 2003) activation in cells. The PTK signaling pathway is shown to be involved in Fas-mediated apoptosis in monocytes (Mendez-Samperio *et al*., 2001) while there is a role for PI-3 kinase in the biogenesis of mycobacterial phagosomes within macrophages (Fratti *et al*., 2001). *M. avium* has been shown to induce apoptosis in macrophages via the activation of caspases involving ASK1/p38 mitogen activated protein kinase (MAPK) (Bhattacharyya *et al*., 2003). Mycobacterial cell wall component lipoarabinomannan (LAM) from *M. tuberculosis* has been shown to inhibit apoptosis by phosphorylation of the pro-apoptotic protein Bad involves the PI3-kinase/Akt pathway (Maiti *et al*., 2001).

The ability of BCG vaccine strain to replicate and persist within host cells will determine the efficacy of immune response generated by the organism. BCG has been previously shown to persist within host monocytes but by the mechanism by which BCG inhibits apoptosis is as yet unclear. We investigated cell signaling pathways involved in BCG induced monocyte survival with the use of pharmacological inhibitors to these pathways. We found BCG to inhibit spontaneous apoptosis in monocytes within 24 h of infection. We found that pre-treatment of monocytes with PTK and ERK 1/2 inhibitors did not have any effect on the ability of BCG to inhibit spontaneous apoptosis. However, BCG infection of cells pre-treated with the PI3-kinase inhibitor, LY294002 did not result in increased viability of monocytes. Therefore, we propose a role for the PI3-kinase pathway in BCG-induced inhibition of monocyte apoptosis.

MATERIALS AND METHODS

***Mycobacterium* culture:** The *M. bovis* BCG Copenhagen vaccine strain was grown to obtain logarithmic growth in Sauton medium. Mycobacterial culture was washed in PBS by centrifugation at 4000 rpm. The bacterial suspension was vortexed very briefly with glass beads (3 bursts of 1 s each) to break up any bacterial clumps, followed by sonication in a water bath for 60 sec. large bacterial clumps were allowed to settle via gradient sedimentation, leaving behind a mainly single cell suspension. This was not destructive to the mycobacterial cell wall as evidenced by non-reduced viability (confirmed by fluorescein diacetate/ethidium bromide staining and cultures). The concentration of the cell suspension was adjusted using OD 600 nm readings and BCG diluted as required to obtain an inoculum of 0.5 and 1 per monocyte. Mycobacterial inoculum was plated out on growth medium to obtain viable colony forming units for each assay.

Isolation and infection of monocytes: Buffy coats from healthy PPD skin test negative donors were obtained from the blood bank of Karolinska Hospital, Solna, Sweden. Ethical consent for use of fresh buffy coats was as obtained as per requirements of Swedish Institute of Infectious Diseases Control and Karolinska Institute, Sweden. Blood was diluted in RPMI and separated using Ficoll centrifugation as described previously (Hasan *et al*., 2004). PBMCs were plated on tissue culture dishes and incubated for 2 h at 37°C for purification of monocytes. Adherent cells were harvested using Cell Dissociation Solution (Sigma, USA) and plated at 10^6 per well in 6 well plates. Cells were subsequently infected with BCG at multiplicity of infection (MOI) of 0.5, 1 and 2 as required. Infected monocytes were harvested at 6, 18 and 48 h for flow cytometry staining and analysis.

Annexin V-FITC propidium iodide staining: Cells were stained for apoptotic markers using Annexin V-FITC (BD Pharmingen, USA) and Propidium Iodide (PI) (BD Pharmingen, USA) as per manufacturer's instructions. Briefly, cells were washed in PBS, re-suspended in binding buffer, stained with Annexin-V-FITC and PI solution and incubated for 15 min at RT in dark on ice. The cell suspension was diluted appropriately in binding buffer for measurement performed by flow cytometry analysis of 5000 cells using CellQuest Software (BD Sciences, USA). An-/Pi- cell populations were determined as live; An+/PI- as early apoptotic population; while, An+/PI+ represented late stage apoptotic or necrotic cells. Controls included in each assay included; unstained cells, unstimulated (spontaneous) cells and cells treated with 5 mM H_2O_2 as a positive stimulator of apoptosis (Laochumroonvorapong *et al*., 2005). Monocytes were also labeled with anti-CD14/CD45 FITC labeled (BD Pharmingen, USA) to confirm the monocyte population, which was found to be >95% amongst the cells studied.

Treatment with cell signaling inhibitors: The pharmacological inhibitors used included genistein, a broad based inhibitor for protein tyrosine kinases and PD98059, a MEK inhibitor which blocks phospho-activation of extracellular signaling regulated kinase (ERK) and the PI 3-kinase inhibitor LY294002. Cell monolayers were pre-treated for 30 min with the appropriate inhibitor prior to infection with BCG in each case. Optimal dosage was obtained for each inhibitor; genistein was tested at 1 and 40 μM, PDD98059 at 10 and 40 μM and LY294002 at 5 and 25 μM. Cells treated with 0.1% DMSO were also included amongst controls and were found to have no significant effect on monocyte viability under the conditions studied.

RESULTS

***M. bovis* BCG inhibits spontaneous apoptosis in human monocytes:** Cells purified from the PBMCs population were stained with antibody to CD14-CD45 in order to determine the percentage monocyte present. As shown in Fig. 1 the cells were 95% positive for CD14 indicating the predominance of monocytes in the population studied.

We investigated the effect of BCG on monocytes after 24 h of *in vitro* culture. Spontaneous apoptosis occurred during culture of monocytes and as shown in Fig. 2a, monocyte viability was reduced to 58% with 22% early apoptotic (An^-/PI^-) and 10% late apoptotic (An^-/PI^+) cells. When cells were infected with BCG at a multiplicity of infection (MOI) of 0.5, 1 and 2 per cell for 24 h, BCG-induced a decrease in monocyte apoptosis at each MOI as observed by an increase in the number of live (An^-/PI^-) cells together with a subsequent decrease in An^+ cells. BCG induced viability was; 79% at MOI-0.5, 84% at MOI-1 and 87% at MOI-2 (Fig. 2b-d). This was accompanied by a concomitant decrease in total apoptotic non-viable (An^+) cells; 18.2% at MOI-0.5; 12% at MOI-1 and 11.9% at MOI-2. However, there was no significant difference between BCG-induced at these low infection doses.

BCG induced cellular activation and cytokine secretion has been shown be detectable between 6 and 24 h post-infection of monocytes (Hasan *et al*., 2003). We next investigated the time course effect of BCG infection on monocyte apoptosis at 6, 24 and 48 h post-infection.

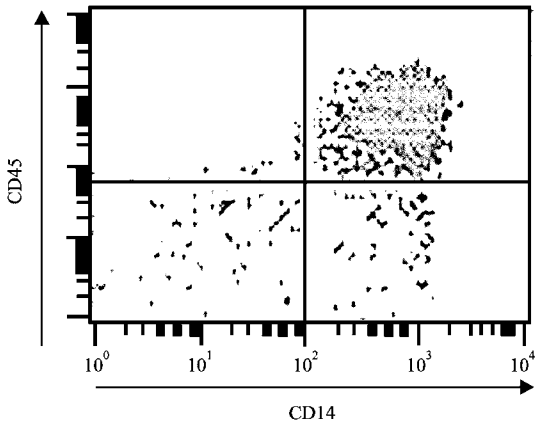

Fig. 1: Selection of monocytes in adherent cell population. Adherent cells were stained with the CD45-CD14 FITC and analysis of cells carried out using CellQuest software, BD Sciences, USA. The scatter plot of cells is illustrates a gated populated comprising of 95-96% monocytes

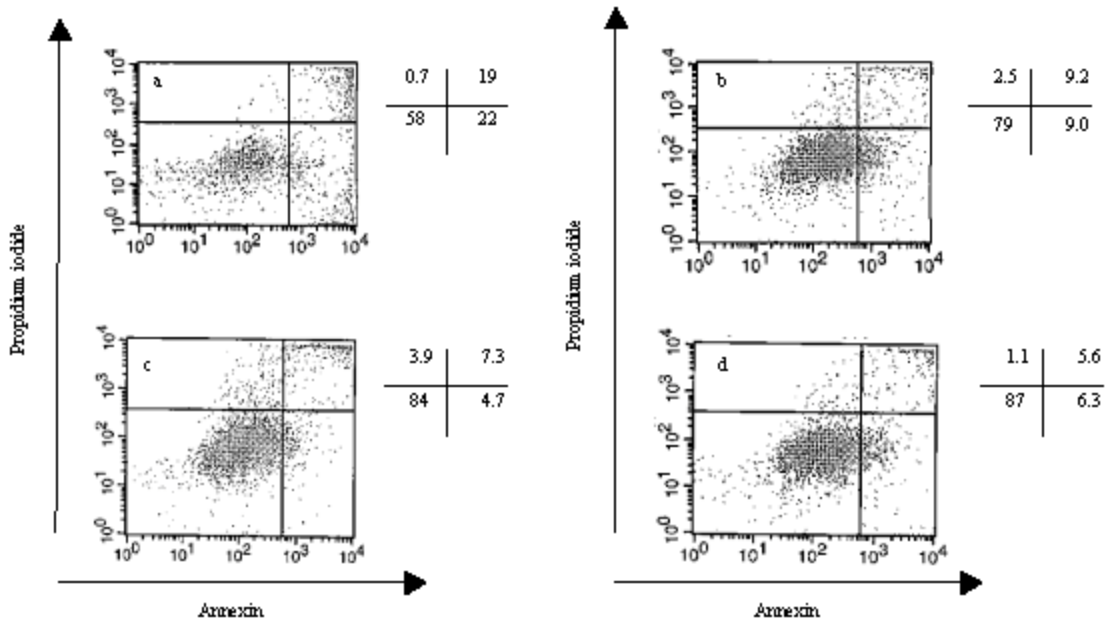

Fig. 2: BCG inhibits apoptosis in monocytes. Monocytes isolated from peripheral blood were infected with BCG at MOIs of 0.5, 1 and 2 for 24 h. Cells were harvested and stained with Annexin-V-FITC and PI and analyzed by flow cytometry. Quadrant stats indicate percentage of cells in the gated population from a total of 5000 events counted. Viable population is defined by cells which are AnV$^-$/PI$^-$ LL (lower left) quadrant, early apoptotic cells are AnV$^+$/PI$^-$ LR (lower right), while late apoptotic/necrotic cells are AnV$^+$/PI$^+$ UR (upper right) quadrant. Graphs illustrate data from one representative experiment. A. spontaneous, B. BCG MOI-0.5, C. BCG MOI-1 and D. BCG MOI-2

Cells were infected with BCG at a multiplicity of infection (MOI) of 1 per cell and subsequently harvested for staining with Annexin V. Figure 3 shows the Annexin V positive population which comprises both early and late apoptotic cells. At the earlier 6 h time point, no difference was observed between apoptosis in spontaneous and BCG infected cells. However, after at 24 and also at 48 h post-infection, BCG induced a significant decrease in monocyte apoptosis ($p \leq 0.05$). Monocyte viability measured at 6 h was comparable in unstimulated and BCG infected cells (83±2%). Infection of cells with BCG for 24 h resulted in a significant increase in spontaneous viability of 56±15 to 80±3% ($p \leq 0.05$). Monocytes cultured for 48 h displayed a viability of 55±15 while BCG-infection of cells resulted in an increase to 74±10% ($p \leq 0.05$). Cells treated with H_2O_2 (5 mM), a positive inducer of apoptosis were found to contain only 40% viable cells while the remaining was Annexin V positive (data not shown).

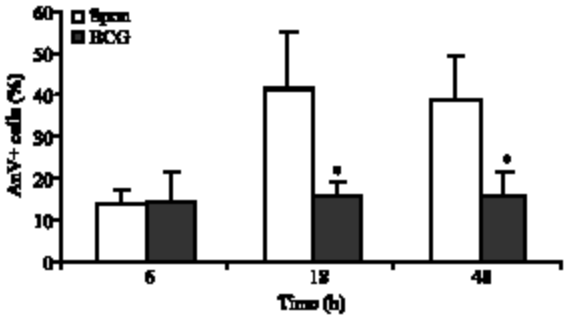

Fig. 3: Time course effect of BCG infection on human monocytes. Monocytes were infected with BCG at MOI of 1 for 6, 24 and 48 h. Cells were harvested and stained with Annexin V/ propidium iodide and subsequently analyzed by flow cytometry. The figure shows the percentage of annexin V positive cells in each case. Data shown is mean of 3 independent experiments with SD as y error bars, * denotes significantly different values $p<0.05$

Role of PTK, MAPK and PI 3-kinase pathways in BCG mediated apoptosis inhibition: Having established that infection of monocytes with BCG resulted in the inhibition of apoptosis after 24 h post-stimulation we investigated the cell signaling mechanisms involved. Cells were pre-treated cells with pharmacological inhibitors prior to

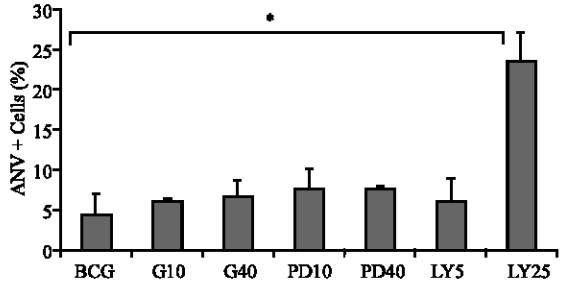

Fig. 4: Inhibition of PI-3 kinase prevents BCG-induced reduction in monocyte apoptosis. Monocytes were pretreated with genistein at 10 and 40 μM, PD98059 at 10 and 40 μM and LY294002 at 5 and 25 μM for 30 min prior to infection with BCG (MOI 1). Infected cells were harvested at 24 h post-stimulation and analyzed by Annexin V/PI staining as described above. The graph illustrates the effect of inhibitor pretreatment on AnV^+/PI^+ cells as percentage of total during BCG infection of cells

infection with BCG. The inhibitors used were the PTK inhibitor, genistein; ERK 1/2 inhibitor, PD98059 and PI-3 kinase inhibitor, LY294002. Inhibitors were pre-tested as described in methods and used at previously established doses which did not cause any cell cytotoxicity (Hasan *et al*., 2003). Therefore, cells were incubated with genistein and PD98059 at 10 and 40 μM and LY294002 used at 5 and 25 μM. The effect of inhibitor pretreatment of monocytes on BCG-induced cell survival is shown in Fig. 4. Pre-treatment of monocytes with either genistein or PD98059 did not result in any change on BCG-induced viability of monocytes as determined by number of An^+/PI^+ cells present. However, pretreatment on monocytes with the PI3-kinase inhibitor, LY294002 at 25 μM prior to infection with BCG resulted in a significant increase in monocyte apoptosis as compared with control cells infected with BCG only. This suggests a role for the PI3-kinase pathway in the BCG-mediated inhibition of spontaneous apoptosis in monocytes.

DISCUSSION

Present study shows that the BCG Copenhagen vaccine strain inhibits apoptosis in monocytes and that this involves a role for the PI3-kinase pathway. There is conflicting literature available as the effect of BCG on monocytes. It has previously been demonstrated that BCG inhibits apoptosis in human monoytes (Kremer *et al*., 1997) and in neutrophils (Suttmann *et al*., 2003). In addition, BCG mediates protection of J774 murine macrophages against NO-induced apoptosis (Kausalya *et al*., 2001). However, other studies show that BCG induces apoptosis in the THP-1 human monocytic cell line (Riendeau and Kornfeld, 2003) and increased apoptosis as compared with *M. tuberculosis* in human alveolar macrophages (Keane *et al*., 2000).

Variation between studies can be attributed to a variety of factors including differences in host cells employed in the assays, between mycobacterial strains used and also the infection doses used in mycobacterium: macrophage assays. The effect of *Mycobacterium* on host cell apoptosis shows variability between cell type and *M. tuberculosis* induces increased apoptosis in alveolar epithelial cells as compared with monocytes (Danelishvili *et al*., 2003). The THP-1 monocytic cell line has been shown to be a good model for studying mycobacterial infections (Theus *et al*., 2004) and is thought to be comparable with human alveolar macrophages. Keane *et al*. (2000) employing alveolar macrophages have shown that BCG-infection of cells induces apoptosis. Additional variability between studies can be attributed to the source of BCG strain as there is a great deal of variability between the efficacy and immunogenicity of different BCG vaccine strains (Corbel *et al*., 2004; Lagranderie, 1996).

Mycobacterium: host cell infection dose also plays a role in outcome and increasing mycobacterial dose can result in increased pathology and cytokine activation (Bekker *et al*., 2000; Scott and Flynn, 2002). We used the BCG Copenhagen vaccine strain at bacteria: monocyte ratios of 0.5, 1 and 2 and observed no significant difference in inhibition of apoptosis at these low doses. A recent study by Lee *et al*. (2006) show that the mycobacterial load determines the apoptotic response to *M. tuberculosis* and even in the case of BCG, at MOI of 5 or below there was limited apoptosis observed in murine macrophages.

Previously, phosphorylation of Bad has been shown to be a mechanism for *M. tuberculosis* (Maiti *et al*., 2001) mediated downregulation of apoptosis and Bad phosphorylation can be catalysed by MAPK-activated kinases. In addition, the protein tyrosine kinase pathway has been shown to be involved in Fas-mediated apoptosis (Mendez-Samperio *et al*., 2001). The ERK 1/2 kinase has also been shown to be involved in growth factor induced cell survival via the Ras-mitogen-activated protein kinase (MAPK) signaling pathway (von Gise *et al*., 2001). However, although BCG-induced TNFα production has been shown to involve the ERK 1/2 pathway (Hasan *et al*., 2003), we did not find a role for the ERK 1/2 or PTK pathway in BCG induced survival of primary human monocytes.

From this study, it is not possible to ascertain whether it is infected cells or bystander cells which are prevented from undergoing spontaneous apoptosis in the presence of BCG. Cell survival is also induced by IL-3 and GMCSF by inhibition of apoptosis. GMCSF can promote cell survival by activating the phosphatidylinositide-3'-OH kinase and its downstream target, the serine-threonine kinase Akt. A role for the ERK 1/2 and PI3 kinase pathways are shown in response to both IL-3 and GMSCF (Craddock *et al*., 1999; Klein *et al*., 2000). Present results suggest a role for PI3 kinases in inhibition of apoptosis by the mycobacteria. The PI3-kinase/Akt pathway has also been identified as a survival pathway downstream of extracellular stimuli. Present results also correlate with previous reports that apoptosis inhibition by lipoarabinomannan from *M. tuberculosis* via phosphorylation Bad from the Bcl-2 family of proteins involves the PI 3-kinase pathway (Maiti *et al*., 2001).

The efficacy of vaccination with the *M. bovis* BCG strain is dependent on the ability of the organism to replicate and persist within monocytes. Increased persistence of the mycobacteria would lead to great immune activation in the host. Further understanding of increased survival of host cells may in turn shed light on the variability in host protection generated by different BCG vaccine strains.

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