

Inhalable Microparticles Containing Isoniazid and Rifabutin Target Macrophages and “Stimulate the Phagocyte” to Achieve High Efficacy

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Abstract

Macrophage responses to infection with *Mycobacterium tuberculosis* (MTB) and treatment with soluble isoniazid (INH) plus rifabutin (RFB) versus microparticles containing equivalent amounts of drugs were compared. It was investigated whether macrophages driven to alternative activation upon infection with MTB could be rescued to display the classical activation phenotype. It was established that microparticles sustain high levels of drugs in the cytosol of macrophages for longer periods as compared to soluble drugs. Microparticles co-localized with intracellular bacteria, and induced a variety of innate bactericidal responses, including induction of free radicals, alteration of mitochondrial membrane potential and apoptosis. The data strongly suggest that additional benefit may be derived from the nature of the drug delivery system, which fulfils Koch's dictum for curing tuberculosis: “*stimulate the phagocyte*”.

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Introduction

Innate responses of the host macrophage infected with *Mycobacterium tuberculosis* MTB have not been traditionally considered as a possible component of chemotherapeutic strategy against the infection. In recent years, we and others 1-12 have proposed that alveolar macrophage-resident MTB may be targeted by inhaled drug delivery systems, and have reported extremely encouraging results.

The present report is intended to highlight two aspects of inhaled therapies in TB: first, the delivery of a large amount of anti-TB drugs to the macrophage where MTB could be resident; and second, the possibility that phagocytosis of particulate material may activate "classical" bactericidal macrophage responses that the cell type has evolved against generic particulate matter inhaled in physiological breathing.

Materials and Methods

The anti-TB drugs isoniazid (INH) and rifabutin (RFB), as well as inhalable microparticles containing these 1 were provided by Lupin Research Park. MTB was grown in Sauton's medium to log phase, harvested and dispersed in RPMI-1640 by sonication for 20 sec and OD at 600 nm was used as a measure of CFU, employing a standard curve prepared in advance. THP-1 human monocytic cells were induced to differentiate to the macrophage phenotype by treatment with phorbol ester and infected at a MOI of 10 for 3 hrs. Extracellular bacteria were washed off with 3 portions of RPMI and the infected cells treated with drugs and/or microparticles 2, 4. Uninfected cells were used to establish the time-course of intracellular drug concentrations following administration of drugs in solution or in microparticles.

HPLC on a c-18 column was used to estimate drug concentrations in cell lysates prepared at 24, 48, 72 and 96 h after treatment of 2×10^6 cells with $3 \mu\text{g/ml}$ each of INH and RFB in solution in the culture medium, or in microparticles 1, 5.

The following experimental groups were formed to evaluate cellular and biochemical phenomena associated with microparticle phagocytosis: Normal (uninfected) cells (N), uninfected cells treated with drug-containing microparticles (NMP), infected cells receiving no treatment (I), infected cells treated with $3 \mu\text{g/ml}$ each of INH and RFB (ISD), or with an equal amount of the two drugs in inhalable microparticles (IMP), or an equivalent amount of drug-free (blank) microparticles (IBMP).

Reactive oxygen species (ROS) induced in each group were estimated by flow cytometry. The indicator 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) was used for the estimation of ROS, particularly peroxides 13. DCHF-DA solution was prepared by diluting a 20mM DMSO stock solution kept in dark to $25 \mu\text{M}$ DCHF-DA with RPMI. For equal loading of all cells with the dye, cells were pelleted by centrifugation ($400 \times g$ for 10 min at 20°C), resuspended in RPMI and incubated with $25 \mu\text{M}$ DCHF-DA solution at 37°C for 1 h. DCHF-DA-loaded cells were used in the experimental groups described above. To estimate intracellular peroxide production, fluorescence emissions at 520 nm were recorded after 4 h. Flow cytometric analysis was carried out with a Becton-Dickinson FACS Calibur equipped with an argon laser using an excitation wavelength of 488 nm.

Mitochondrial membrane potential potential was estimated by flow cytometry following loading cells with the fluorescent dye Rhodamine 123. A 96-well flat-bottom culture plate was seeded with 0.2×10^6 cells/well and the cells differentiated, infected and treated as described for 14, 24 and 48 hrs. The lipophilic, cationic dye Rhodamine 123 (Sigma) was then added to wells ($1 \mu\text{L}$ of a 0.5 mg/mL soln.) for 15 min at 37°C in complete medium. Cells were washed twice with PBS, re-suspended in $300 \mu\text{L}$ of PBS and stained with PI 14. Samples were analyzed on the flow cytometer with the CellQuest software. The intensity of staining was used as a measure of $\Delta\psi$ 15.

DNA fragmentation was studied as a measure of apoptosis of infected cells subjected to different modes of treatment. DNA was extracted according to the procedure of Miller *et al.* 16. Briefly, DNA was extracted from $1-3 \times 10^7$ cells by adding 3 mL of nuclei lysis buffer (10 mM Tris-Cl, 400 mM NaCl, and 2 mM Na₂EDTA at pH 8.2), 0.2 mL of 10% SDS and 0.5 mL proteinase K solution (1 mg proteinase K, 2 mM Na₂EDTA, and 1% SDS) and incubated at 37 °C overnight. 6 M NaCl was added and the supernatant collected by spinning at $1300 \times g$ for 15 min. Two volumes of 95% ethanol were added to the supernatant and gently mixed. The DNA precipitate was dissolved in 200 μ L of Tris-EDTA and quantitated spectrophotometrically. Electrophoresis was performed with equal amounts of DNA on a 1.4% agarose gel.

Results

Concentrations of RFB observed from 0-96 hrs after administration of microparticles or drugs in solution to uninfected THP-1-derived macrophages are depicted in Fig 1. Very similar intracellular concentrations were observed immediately after adding the drug, either in soluble or microparticulate form, as well as when sampled 24 h later. Significant ($P < 0.01$, ANOVA, $n=2$) differences were discernible from 48 h onwards.

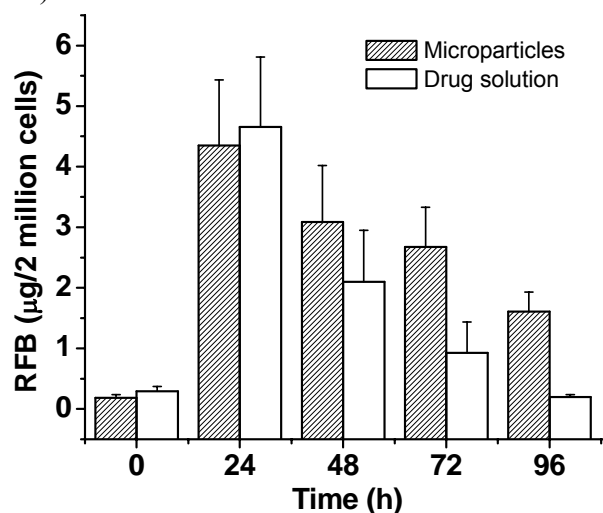


Figure 1: Intracellular concentrations of RFB resulting from treatment of cultured THP-1-derived macrophages with equivalent amounts of RFB in solution (open bars) or in microparticles (hatched bars).

Intracellular reactive oxygen species were observed in flow cytometry using DCHF-DA (Fig. 2). Even uninfected cells apparently comprised two distinct sub-populations, differing in their intracellular ROS. This observation is probably due to phorbol treatment of THP-1 cells in order to induce differentiation. Thus, whereas the ratio of ROS^{hi} to ROS^{lo} cells was 5.7 in uninfected cells (N), infection (I) brought this ratio down to 3.5. Treatment of infected macrophages with soluble drugs (ISD) resulted in marginal increase in the high:low ratio to 4.0, microparticles significantly ($P < 0.001$, ANOVA, $n=3$) enhanced ROS to yield a ratio of 7.9.

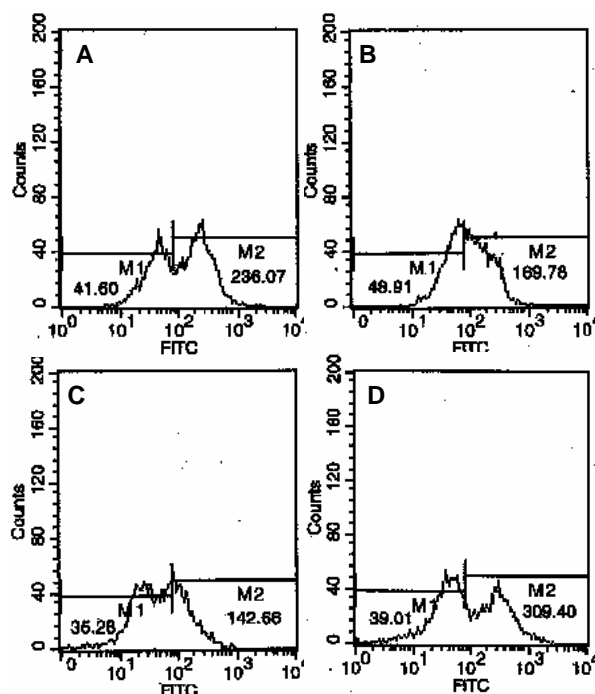


Figure 2: Representative data demonstrating that intracellular ROS is enhanced by microparticle treatment. (A): Two sub-populations of THP-1-derived macrophages, that differ in intracellular O_2^- levels as revealed by incubation with DCHF-DA. The mean fluorescence intensity (MFI) of the ROS^{lo} population encompassed by Marker 1 (M1) was 41.60, while that of the ROS^{hi} population (M2) was 236.07. (B): Infection with *M. tb.* H37Ra at a MOI of 20 resulted in reduction of the MFI of the population under M2 to 169.78, while the M1 population showed a MFI of 48.91. (C): The MFI of populations M1 and M2 were 35.28 and 142.66 respectively when infected cells were administered drugs in solution. (D): MFI values for M1 and M2 populations were 39.01 and 309.40 respectively, when infected cells were treated with MP, indicating enhancement of ROS production.

It was investigated whether mitochondrial integrity would correlate with microparticle treatment. The lipophilic cationic dye rhodamine 123 was used as an indicator of $\Delta\psi_m$ in flow cytometry experiments. Figure 3 shows data from one of three experiments, where forward light scatter (cell size) is plotted *versus* rhodamine fluorescence intensity on the abscissa ($\Delta\psi_m$) observed 12 hrs after treatment. After 6 or 18 hrs of treatment, no differences were discernible between groups.

Reduction in $\Delta\psi_m$ was observed at 12 hrs following uptake of either drug-containing microparticles or *M. tuberculosis* H37Ra. The drop in $\Delta\psi_m$ after treatment with drug-free microparticles was maximal, with drugs containing microparticles and dissolved drugs showing progressively smaller effects, though the difference between drug-containing and drug-free microparticles was not statistically significant.

Disruption of mitochondrial membrane integrity is likely to result in apoptosis of the infected cell. Flow cytometric analysis of DNA damage, such as that induced in late apoptosis, showed minor differences between the proportions of apoptotic cells in various treatment groups (data not shown). However, DNA fragmentation was clearly visible when DNA isolated from various groups after 48 hrs of treatment was resolved on a 1.4% agarose gel. As shown in Figure 4, a ladder pattern was observed in case of infected cells. Densitometric comparison of band intensities within a window on the gel indicated that drug-free microparticles induced the highest fragmentation (IBMP), followed by drug-

containing microparticles (IMP) and dissolved drugs (ISD) respectively. Normal controls (N and NMP) did not yield a ladder pattern.

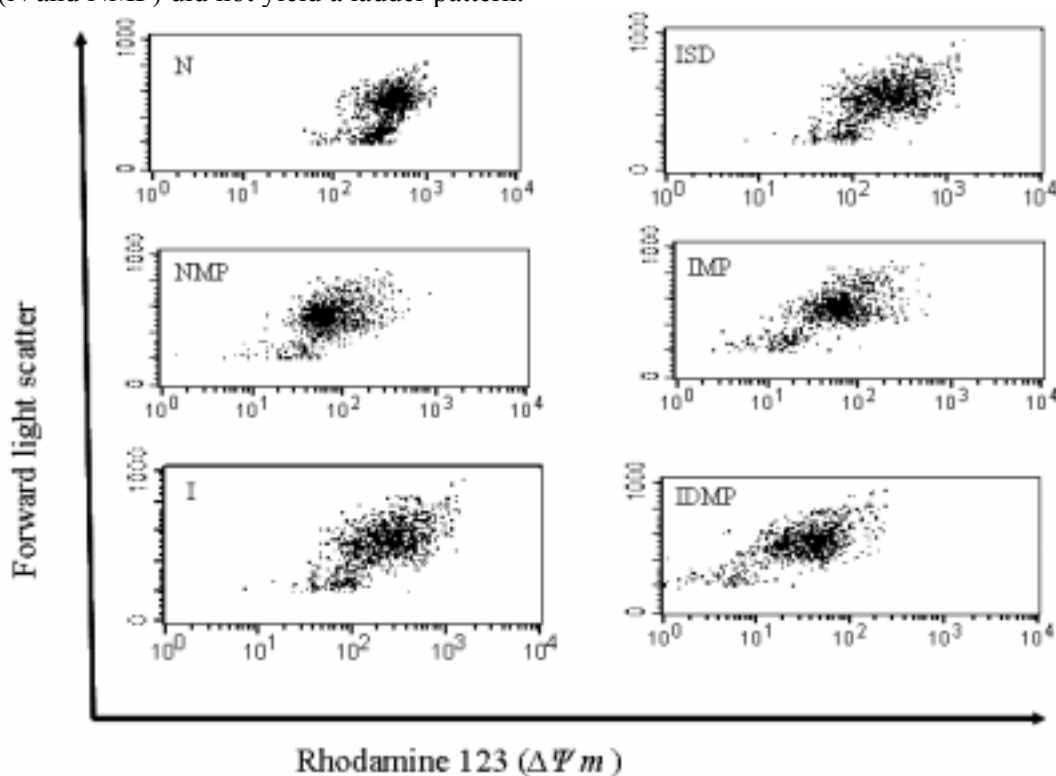
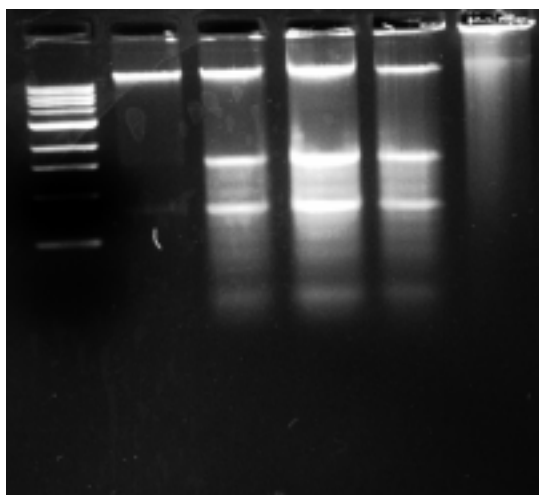


Figure 3. Reduction in mitochondrial membrane potential ($\Delta\psi_m$) at 12 hrs after infection. Significant drop in potential was observed after infection, which was maintained by treatment with dissolved drugs. Drug-containing as well as drug-free microparticles induced significantly higher decrease, but the difference between the two kinds of microparticles was significant only at $P < 0.1$. Group nomenclature is the same as in text and legend to Figures 1-3 ($n=2$; $P < 0.05$ for NMP, IMP and DMP compared to I or N).

M I ISD IMP IDMP N



Groups	I	ISD	IMP	IDMP
Genomic DNA	30670	33438	37579	17625
Fragments	5111	2394	59029	34297
	17012	2448	21976	49693
		23601	58726	7043
		7982	16433	
		11896	14045	
		12443		
	10356			
Total Fragmented	22123	71120	170209	91033
GDNA/Fragments	1.386	0.470	0.220*	0.193*

M:

cells treated with soluble drugs. *IMP*: treated with microparticles. *IDMP*: treated with dummy microparticles. *N*: uninfected cells. Densitometry results are shown to the right of the gel image, with an asterisk to denote significant ($P < 0.05$) differences between the ratios of genomic to fragmented DNA.

Discussion

Virtually all forms of TB begin with pulmonary infection, when droplet nuclei are inhaled into the lungs and phagocytosed by alveolar macrophages. These cells usually serve as the first line of defense against invading microorganisms, but several intracellular parasites including MTB have evolved to survive and replicate within maturation-arrested phagosomes that do not fuse with lysosomes. The ability of the macrophage to mount an appropriate response has long been known to affect the course of TB, as enunciated in Koch's dictum: "*Stimulate the Phagocyte*" 17. Effective host response to infection, including responses to the survival strategies of the pathogen, is mediated by several mechanisms. Early induction of innate responses such as ROS and reactive nitrogen intermediated are now recognized as examples of innate mechanisms. When the host defense fails to counter the infection, disease ensues and chemotherapy is required. Prolonged multi-drug regimens, however, have several well-known drawbacks. The present data illustrates how host innate responses may be recruited to synergise with targeted chemotherapy against macrophage-resident MTB.

Maintenance of high intracellular drug concentrations over long periods following a single exposure to particles (Fig. 1) promises direct benefits related to killing intracellular MTB. Targeting the alveolar macrophage upon inhalation of these particles has been reported several times elsewhere 1, 3-5. It is possible that administration of inhalable microparticles to patients harbouring MTB in lung macrophages would show similar results.

The consequences of targeting alveolar macrophages by inhaled particles, however, are not limited to sustaining drug concentrations in the cytosol. As demonstrated in Fig. 2, microparticles sustain the production of ROS, whereas untreated infection serves to deplete intracellular ROS. While the ability to scavenge ROS produced by the host macrophage is an important aspect of pathogenicity 18, the sustained induction of ROS in an infected macrophage could prove inimical to bacterial survival.

Apart from induction of ROS, we have demonstrated elsewhere that phagocytosed microparticles induce Th1 cytokines, predominantly, TNF, in murine macrophages 1. TNF and ROS are both pro-apoptotic, whereas pathogenic MTB induces necrotic or caspase-independent death of the infected macrophage 19-21. There is reason to believe that *M. tuberculosis*-induced cell death correlates not only with virulence, but also with the microbial burden in the macrophage 22. At a low MOI, virulent strains inhibit host macrophage apoptosis while attenuated strains such as H37Ra or Bacille Calmette-Guerin induce it 15, 20, 23-25. Molecular mechanisms of such host-pathogen interaction continue to be worked out, and yield interesting information 23. The pathogen is known, for instance to neutralize TNF signalling by enhancing host IL-10; which leads to shedding of TNF receptor proteins from the infected cell surface 20, 26. Induction of host Bcl-2 25 and congener anti-apoptotic proteins such as Mcl-1 27 is another documented strategem. At the transcript level, anti-apoptotic *bcl-w* is upregulated by virulent bacteria, whereas attenuated bacteria show upregulation of proapoptotic genes designated as "upregulated during camptothecin-induced apoptosis of U937 cells." The clearest instance of mutually-opposed gene regulation by virulent and avirulent MTB is demonstrated in the case of mitochondrial superoxide dismutase-2. This gene is downregulated upon infection with virulent bacteria, whereas it is upregulated when the infectious strain is avirulent. This distinction, too, hinges on TNF signalling, such that treatment of cells infected with avirulent bacteria with anti-TNF- α antibodies results in downregulation of mitochondrial superoxide dismutase-2 23.

At higher MOI and early in infection, however, cell death induced by both strains H37Rv and H37Ra shows features associated with necrosis. Lee *et al.* recently demonstrated that a MOI ≥ 25 does not inhibit apoptosis, regardless of the virulence of the infecting strain. Further, cells carrying a large bacillary load progress rapidly from apoptosis to necrosis 21.

Cell death under these circumstances is not a defense response, therefore, and rather indicates that the host macrophage has finally succumbed to infection 27. In contrast, inhibition of host macrophages apoptosis by stabilization of mitochondrial membrane potential promotes innate macrophage bactericidal activity against *M. tuberculosis* 15.

Fig 3. demonstrates that microparticles, regardless of drug content, reduce mitochondrial membrane potential. Thus, microparticle treatment may be expected to induce apoptosis as a defense response. Figure 4 bears out this expectation, demonstrating the greatest amount of DNA fragmentation in cells treated with blank microparticles. Although we have not observed bactericidal effects of microparticle-induced apoptosis in the cell culture model 2, it is possible that apoptotic bodies containing MTB would contribute to the generation of a bactericidal acquired immune response, whereas the entry of naked or opsonized MTB into bystander macrophages is a step in pathogenesis. We therefore conclude that the proposed drug delivery system has a role in stimulating the phagocyte to mount innate defense responses, and possesses potential in contributing to the chemotherapy of TB.

The indicators of macrophage activation outlined in the present report have prompted us to undertake a genome-wide transcription analysis of gene expression induced by MTB H37Rv in the THP-1-derived macrophage. These studies have been carried out on a microarray platform using Affymetrix chips. The data is currently under analysis and shall shortly be available openly for mining at the website of CSIR's OSDD programme (<http://sysborgtb.osdd.net/bin/view/OpenLabNotebook/WebHome>).

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References

1. Sharma R, Muttill P, Yadav A B, et al. Uptake of Inhalable Microparticles Affects Defence Responses of Macrophages Infected with Mycobacterium Tuberculosis H37ra. *J Antimicrob Chemother*, 59 (3) (2007) 499.
2. Muttill P, Kaur J, Kumar K, et al. Inhalable Microparticles Containing Large Payload of Anti-Tuberculosis Drugs. *Eur J Pharm Sci*, 32 (2) (2007) 140.
3. Sharma R, Saxena D, Dwivedi A K, et al. Inhalable Microparticles Containing Drug Combinations to Target Alveolar Macrophages for Treatment of Pulmonary Tuberculosis. *Pharm Res*, 18 (10) (2001) 1405.
4. Verma R K, Kaur J, Kumar K, et al. Intracellular Time Course, Pharmacokinetics, and Biodistribution of Isoniazid and Rifabutin Following Pulmonary Delivery of Inhalable Microparticles to Mice. *Antimicrob Agents Chemother*, 52 (9) (2008) 3195.
5. Kaur J, Muttill P, Verma R K, et al. A Hand-Held Apparatus For "Nose-Only" Exposure of Mice to Inhalable Microparticles as a Dry Powder Inhalation Targeting Lung and Airway Macrophages. *Eur J Pharm Sci*, 34 (1) (2008) 56.
6. O'Hara P & Hickey A J Respirable Plga Microspheres Containing Rifampicin for the Treatment of Tuberculosis: Manufacture and Characterization. *Pharm Res*, 17 (8) (2000) 955.
7. Suarez S, O'Hara P, Kazantseva M, et al. Airways Delivery of Rifampicin Microparticles for the Treatment of Tuberculosis. *J Antimicrob Chemother*, 48 (3) (2001) 431.
8. Lu D, Garcia-Contreras L, Xu D, et al. Poly (Lactide-Co-Glycolide) Microspheres in Respirable Sizes Enhance an in Vitro T Cell Response to Recombinant Mycobacterium Tuberculosis Antigen 85b. *Pharm Res*, 24 (10) (2007) 1834.

9. Suarez S, O'Hara P, Kazantseva M, et al. Respirable Plga Microspheres Containing Rifampicin for the Treatment of Tuberculosis: Screening in an Infectious Disease Model. *Pharm Res*, 18 (9) (2001) 1315.
10. Makino K, Nakajima T, Shikamura M, et al. Efficient Intracellular Delivery of Rifampicin to Alveolar Macrophages Using Rifampicin-Loaded Plga Microspheres: Effects of Molecular Weight and Composition of Plga on Release of Rifampicin. *Colloids Surf B Biointerfaces*, 36 (1) (2004) 35.
11. Pandey R, Sharma A, Zahoor A, et al. Poly (DL-Lactide-Co-Glycolide) Nanoparticle-Based Inhalable Sustained Drug Delivery System for Experimental Tuberculosis. *J Antimicrob Chemother*, 52 (6) (2003) 981.
12. Pandey R & Khuller G K Antitubercular Inhaled Therapy: Opportunities, Progress and Challenges. *J Antimicrob Chemother*, 55 (4) (2005) 430.
13. Bass D A, Parce J W, Dechatelet L R, et al. Flow Cytometric Studies of Oxidative Product Formation by Neutrophils: A Graded Response to Membrane Stimulation. *J Immunol*, 130 (4) (1983) 1910.
14. Zamzami N, Metivier D & Kroemer G Quantitation of Mitochondrial Transmembrane Potential in Cells and in Isolated Mitochondria. *Methods Enzymol*, 322 (2000) 208.
15. Gan H, He X, Duan L, et al. Enhancement of Antimycobacterial Activity of Macrophages by Stabilization of Inner Mitochondrial Membrane Potential. *J Infect Dis*, 191 (8) (2005) 1292.
16. Ma J, Chen T, Mandelin J, et al. Regulation of Macrophage Activation. *Cell Mol Life Sci*, 60 (11) (2003) 2334.
17. Young D B Stimulate the Phagocytes. *Tuberculosis (Edinb)*, 81 (4) (2001) 257.
18. Ng V H, Cox J S, Sousa A O, et al. Role of Katg Catalase-Peroxidase in Mycobacterial Pathogenesis: Countering the Phagocyte Oxidative Burst. *Mol Microbiol*, 52 (5) (2004) 1291.
19. Barber G N Host Defense, Viruses and Apoptosis. *Cell Death Differ*, 8 (2) (2001) 113.
20. Riendeau C J & Kornfeld H Thp-1 Cell Apoptosis in Response to Mycobacterial Infection. *Infect Immun*, 71 (1) (2003) 254.
21. Lee J, Remold H G, Jeong M H, et al. Macrophage Apoptosis in Response to High Intracellular Burden of Mycobacterium Tuberculosis Is Mediated by a Novel Caspase-Independent Pathway. *J Immunol*, 176 (7) (2006) 4267.
22. Rajavelu P & Das S D A Correlation between Phagocytosis and Apoptosis in Thp-1 Cells Infected with Prevalent Strains of *Mycobacterium Tuberculosis*. *Microbiol Immunol.*, 51 (2) (2007) 201.
23. Spira A, Carroll J D, Liu G, et al. Apoptosis Genes in Human Alveolar Macrophages Infected with Virulent or Attenuated Mycobacterium Tuberculosis: A Pivotal Role for Tumor Necrosis Factor. *Am J Respir Cell Mol Biol*, 29 (5) (2003) 545.
24. Keane J, Remold H G & Kornfeld H Virulent Mycobacterium Tuberculosis Strains Evade Apoptosis of Infected Alveolar Macrophages. *J Immunol*, 164 (4) (2000) 2016.
25. Zhang J, Jiang R, Takayama H, et al. Survival of Virulent Mycobacterium Tuberculosis Involves Preventing Apoptosis Induced by Bcl-2 Upregulation and Release Resulting from Necrosis in J774 Macrophages. *Microbiol Immunol*, 49 (9) (2005) 845.
26. Balcewicz-Sablinska M K, Keane J, Kornfeld H, et al. Pathogenic Mycobacterium Tuberculosis Evades Apoptosis of Host Macrophages by Release of Tnf-R2, Resulting in Inactivation of Tnf-Alpha. *J Immunol*, 161 (5) (1998) 2636.
27. Sly L M, Hingley-Wilson S M, Reiner N E, et al. Survival of Mycobacterium Tuberculosis in Host Macrophages Involves Resistance to Apoptosis Dependent Upon Induction of Antiapoptotic Bcl-2 Family Member Mcl-1. *J Immunol*, 170 (1) (2003) 430.