EFFECT OF SIO_2, *M. BOVIS* BCG, *M. KANSASII* AND γ-RADIATION ON U-937 AND THP-1 CELLS *IN VITRO*

A thesis submitted to the Faculty of Health Sciences, University of Johannesburg, in fulfilment of the requirement for the degree of Doctor of Technology: Biomedical Technology

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"...our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light not our darkness that most frightens us. We ask ourselves, who am I to be brilliant, gorgeous, talented, and fabulous? Actually, who are you not to be? You are a child of God. Your playing small doesn't serve the world. There is nothing enlightened about shrinking so that other people won't feel insecure around you. We are born to make manifest the glory of God within us..."

Nelson Mandela
(1994 Presidential Inaugural Speech)

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Technology at the University of Johannesburg, Johannesburg, South Africa. It has not been submitted before for any other degree in any other University.

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(André Phillip Trollip)

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ABSTRACT

About one third of the world's population, or 1.8 billion people, are infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the organism that causes tuberculosis. The disease is spread by the inhalation of droplet-nuclei that forms when infectious material is discharged into the air by coughing or other means. The major site of initial infection is the lung. The incidence of tuberculosis on the mines in South Africa is approximately three times the national average. In addition to the high background incidence of tuberculosis, and the predominantly poor socio-economic living conditions of 65% of the workforce, extremes of temperatures, long hours of shift work and smoking also contribute to the reason why pulmonary tuberculosis is the most common non-occupational lung disease in South African mines. In addition to tuberculosis, miners are also uniquely susceptible to pulmonary disease due to non-tuberculous mycobacteria, specifically *Mycobacterium kansasii* (*M. kansasii*).

Underground miners are exposed to radiation and silica. Considering the high prevalence of pulmonary disease associated with *M. tuberculosis* and *M. kansasii* in South African miners, it is hypothesized that silica and radiation exposure may both be involved in predisposing gold mine workers to pulmonary tuberculosis by their effects on the macrophage's ability to contain mycobacterial infection.

U-937 and THP-1 are promonocytic cell types that, upon differentiation, can be used as surrogates for alveolar macrophages. The homogenous nature of these two cell lines makes them ideal to study the interaction between promonocytes/macrophages and mycobacteria. The objective of this study was to establish if a synergistic relationship exists between the effects of silica and low-dose γ-radiation in impairing the response of U-937 and THP-1 cells to mycobacterial infection. The mechanism for impairment of monocyte function could involve (i) impairment of cell viability, leading to apoptosis or necrosis and (ii) impairment of cell function, as exemplified by TNF-α secretion. Both of these mechanisms could theoretically impair the ability of monocyte/macrophages to contain mycobacteria infection. Therefore, cell viability was evaluated using MTT assay, and cytotoxic effects by a combination of MTT, Annexin PI, Acridine Orange/Ethidium Bromide and Comet assays, while TNF-α

levels were measured in differentiated cells following exposure to silicon dioxide (SiO_2), and/or low-dose γ-radiation, and/or infection with *Mycobacterium bovis* Bacille Calmette-Guérin (*M. bovis* BCG) and *M. kansasii*.

It was established that neither γ-radiation nor infection with either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on U-937. It was also established that γ-radiation does not have a significant cytotoxic effect on THP-1. However, infection by either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on THP-1, reducing the metabolic activity below that of the control. Results from cytotoxicity studies on SiO_2 were inconclusive for both U-937 and THP-1. No synergism between SiO_2 and γ-radiation that resulted in an increase in cytotoxic effect could be determined in either cell type.

The study also examined the mechanisms of cell death of U-937 and THP-1 following SiO_2 and low-dose γ-radiation before and after infection with *M. bovis* BCG and *M. kansasii*. It was established that in U-937 there is no significant increase in apoptosis in cells exposed to γ-radiation or infected by *M. bovis* BCG or *M. kansasii*. In THP-1, there is no significant increase in apoptosis in cells exposed to γ-radiation. However, there is a significant increase in apoptosis in cells exposed to SiO_2, and cells infected by *M. bovis* BCG or *M. kansasii*. No synergism between SiO_2 and γ-radiation that resulted in an increase in apoptotic effect could be determined in either cell type. Furthermore, it was established that neither SiO_2, γ-radiation nor infection by *M. bovis* BCG nor *M. kansasii* are genotoxic to U-937. In THP-1, it was found that exposure to γ-radiation, exposure to SiO_2 and infection with either *M. bovis* BCG or *M. kansasii* were genotoxic to THP-1. No synergism between SiO_2 and γ-radiation that resulted in an increase in DNA damage could be determined in either cell type.

The study examined the ability of U-937 and THP-1 cell lines to control the intracellular growth of *M. bovis* BCG and *M. kansasii* after exposure to SiO_2 and/or low-dose γ-radiation. It was found that U-937 was permissive for the growth of *M. bovis* BCG and *M. kansasii in vitro*. U-937 was also permissive for the growth of *M. bovis* BCG and *M. kansasii* in cells exposed to SiO_2 and/or γ-radiation. In contrast, THP-1 controlled the growth of *M. bovis* BCG and *M. kansasii in vitro*.

TNF-α is involved in macrophage activation and can induce apoptosis, thus the study examined the TNF-α production caused by SiO_2 and/or low-dose γ-radiation in U-937 and THP-1 before and after infection with *M. bovis* BCG and *M. kansasii*. It was concluded that TNF-α release in U-937 is suppressed by exposure to SiO_2, and stimulated in THP-1. TNF-α release is increased in response to γ-radiation exposure in U-937, but remained unaltered in THP-1 cells. There is no significant differences in TNF-α release in infected U-937 or THP-1. A combination of SiO_2 and γ-radiation caused a decrease release of TNF-α in U-937, that was more pronounced in cells infected by *M. bovis* BCG than cells infected by *M. kansasii.* In THP-1, combinations of SiO_2, γ-radiation and infection with *M. bovis* BCG or *M. kansasii* does not stimulate or suppress the secretion of TNF-α more than SiO_2, γ-radiation or infection with *M. bovis* BCG or *M. kansasii* alone.

It was the primary objective of this study, to establish if a synergistic relationship exists between the effects of SiO_2 and low-dose γ-radiation in impairing promonocytic cells, U-937 and THP-1, to mycobacterial infection. In U-937, no synergistic relationship could be determined between the effects of SiO_2 and/or γ-radiation and the potentiation of mycobacterial growth by U-937. Using U-937 as a model, the results may be reflective of immature monocyte/macrophage function allowing permissive growth of mycobacteria.

In THP-1, *M. bovis* BCG, *M. kansasii*, SiO_2 and γ-radiation were associated with apoptosis or DNA damage. No synergistic effect of SiO_2 and γ-radiation could be determined in THP-1 infected with *M. kansasii*. However, the growth of *M. bovis* BCG was potentiated by SiO_2 and/or γ-radiation, despite apoptosis, which supported the hypothesis that SiO_2 and radiation exposure may both be involved in predisposing gold mine workers to pulmonary tuberculosis by their effects on the macrophage's ability to contain mycobacterial infection.

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To God be the glory- Mmoloki Warona

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PUBLICATIONS

- Intracellular mycobacterial proliferation in THP-1 and U-937 cells after silicon dioxide and γ-radiation exposure. To be submitted to South African Journal of Epidemiology and Infection.

- Cytotoxicity and apoptosis in THP-1 exposed to silicon dioxide, γ-radiation, *M. bovis* BCG and *M. kansasii*. To be submitted to Environmental Science & Technology.

CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

About one third of the world's population, or 1.8 billion people, are infected with *M. tuberculosis*, the organism that causes tuberculosis. Active disease developed in 8.9 million people in 2004. Tuberculosis is responsible for 1.7 million deaths in 2004, making it the leading infectious cause of death in the world (Snider *et al*., 1994; World Health Organisation, 2006). The disease is spread by the inhalation of droplet-nuclei that forms when infectious material is discharged into the air by coughing or other means. The major site of initial infection is the lung.

The beginning of the tuberculosis epidemic in South Africa is intimately linked with the rapid industrialisation that occurred after the discovery of gold on the Witwatersrand in 1886 (Packard, 1989). It would appear that tuberculosis was introduced into South Africa by mineworkers from Cornwall, England. The discovery of gold created a demand for cheap labour. The labour for the mines was drawn from South African rural homelands. The poor working conditions, especially underground, provided ideal conditions for the rapid spread of the disease amongst mineworkers (Lalloo & Mets, 1991). Workers who contracted tuberculosis had their contracts terminated and were repatriated from the mines as soon as possible. The practice of repatriation not only led to an underestimation of the severity of tuberculosis on the mines, but also played a key role in disseminating the disease to rurally impoverished areas.

Today, in addition to the high background incidence of tuberculosis, and the predominantly poor socio-economic living conditions of 65% of the workforce; extremes of temperatures, long hours of shift work, smoking and exposure to silica also contribute to the reason why pulmonary tuberculosis is the most common non-occupational lung disease in South African mines (Cowie *et al*., 1989; Leon *et al*., 1995). In addition to tuberculosis, miners are also uniquely susceptible to pulmonary disease due to non-tuberculous mycobacteria, specifically *M. kansasii* (Nel *et al*., 1977).

Silicosis is a fibrosing disease of the lung that is caused by the intensity of exposure to respirable particles of silica containing dust (Davis, 1986). Extended exposure to silica leads to progressive massive fibrosis that is characterized by silicotic nodules and the replacement of lung tissue by scar tissue with the subsequent loss of lung function. The association of silicosis and tuberculosis is devastating. In South Africa, the risk of tuberculosis among miners with silicosis is approximately three-fold higher than among non-silicotic workers (Cowie, 1994). Silicotic miners are also susceptible to pulmonary disease due to non-tuberculous mycobacteria (Corbett *et al*., 1999a).

Radon is a colourless, odourless, and almost chemically inert radioactive gas that is a decay product of radium and uranium (Cothern, 1985). Radium and uranium are ubiquitous in rocks and soils. In mines, radon is released in the atmosphere mainly by diffusion through the ore. The radiation hazard associated with underground atmosphere depends on the amount of radon progeny in the atmosphere of the mine, and the age of the ventilating atmosphere in the mine (Wymer, 2001).

Circulating mononuclear phagocytes, called monocytes, differentiate into macrophages and settle in different tissues. In the lung, they are termed alveolar macrophages, and are the initial cells that defend against infection from mycobacteria, silica and radiation. A cell line is a population of cells that has been cloned from a single cell, and has acquired the capacity for an unrestricted number of cell divisions. U-937 and THP-1 are promonocytic continuous cell lines *i.e.* they are precursors of the monocyte/macrophage lineage (Hass *et al*., 1989; Tsuchiya *et al*., 1980). The homogenous nature of these two cell lines make them ideal to study the interaction between promonocytes/macrophages and mycobacteria, silica and low-dose ionizing radiation. Considering the high prevalence of pulmonary disease associated with *M. tuberculosis* and *M. kansasii* in South African miners, it is hypothesized that silica and radiation exposure may both be involved in predisposing mine workers to pulmonary tuberculosis (or pulmonary disease due to *M. kansasii*) by their effects on the macrophage's ability to contain mycobacterial proliferation.

The objective of this study was to establish if a synergistic relationship exists between the effects of silicosis and low-level ionizing radiation in impairing promonocytic cells, U-937 and THP-1, to mycobacterial infection. The study:

- examined the cytotoxicity of silica or low-dose γ-radiation exposure or infection by *M. bovis* BCG and *M. kansasii* on U-937 and THP-1, and examined the cytotoxicity of silica and/or low-dose γ-radiation exposure on U-937 and THP-1 infected with *M. bovis* BCG or *M. kansasii,*

- examined the mechanisms of death (apoptosis/necrosis) in U-937 or THP-1 after silica or low-dose γ-radiation exposure or infection by *M. bovis* BCG and *M. kansasii*, and examined the the mechanisms of death in U-937 and THP-1 after silica and/or low-dose γ-radiation exposure on U-937 and THP-1 infected with *M. bovis* BCG or *M. kansasii,*

- examined the DNA damage caused by silica or low-dose γ-radiation exposure or infection by *M. bovis* BCG and *M. kansasii* on U-937 and THP-1, and examined the DNA damage caused by silica and/or low-dose γ-radiation exposure on U-937 and THP-1 infected with *M. bovis* BCG or *M. kansasii,*

- examined the ability of U-937 and THP-1 cell lines to control the intracellular growth of *M. bovis* BCG and *M. kansasii* after exposure to silica and/or low-dose γ-radiation,

- examined the TNF-α production of U-937 and THP-1 following exposure to silica or low-dose γ-radiation exposure or infection by *M. bovis* BCG and *M. kansasii*, and examined the TNF-α production following exposure to silica and/or low-dose γ-radiation exposure in U-937 and THP-1 infected with *M. bovis* BCG or *M. kansasii.*

CHAPTER 2: LITERATURE REVIEW

2.1 MYCOBACTERIA

2.1.1 *Mycobacterium tuberculosis*

The genus *Mycobacterium* (Gr. n. *Myces* a fungus; Gr. neut, dim.n. *bakterion* a small rod; M.L. neut.n. *Mycobacterium* a fungus rodlet) is descended from a family of saprophytes, the Mycobacteraceae, of the Order Actinomycetales.

M. tuberculosis was discovered by Robert Koch in 1882. It is a member of the "Mycobacterium complex", which consists of four species viz. *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*. The "Mycobacterium complex" lacks inter-strain genetic diversity with >85% DNA-DNA relatedness between the four species. Systematic sequence analysis by Sreevatsan (1997) showed that the genome of *M. tuberculosis* is unusually inert, suggesting that the organism is relatively young in evolutionary terms.

Pulmonary tuberculosis

Tuberculosis is caused by *M. tuberculosis*. "Tuberculosis" is derived from the word “tubercle”, which means small lump or nodule. Histologically the tubercle consists of a discrete focus of granulomatous inflammation consisting of lymphocytes, epitheloid cells, macrophages and giant cells. About one third of the world's population, or 1.8 billion people, are infected with *M. tuberculosis*, the organism that causes tuberculosis. Active disease developed in 8.9 million people in 2004. Most disease results from infections that occur in the developing world. Tuberculosis is responsible for 1.7 million deaths in 2004, making it the leading infectious cause of death in the world (Snider *et al.*, 1994; World Health Organisation, 2006).

Events in the pathogenesis of tuberculosis

Our understanding of the pathogenesis of tuberculosis is based on studies performed on experimentally infected rabbits (Dannenberg & Rook, 1994). The first stage of infection begins with the inhalation of droplet nuclei containing viable *M. tuberculosis*. The droplet nuclei may contain as few as one to two bacilli, and are

small enough (1-10 µm) to evade the physical barriers of the innate immune system. Organisms that are able to reach the lower respiratory tract face four possible fates (Schluger & Rom, 1998):

- they may be effectively removed from the lung, without the disease progressing,
- they may multiply within macrophages and cause primary tuberculosis,
- they may become dormant, and never cause disease (latent infection),
- they may become dormant, reactivate, and cause disease (adult tuberculosis) months to years later.

Organisms which escape the initial intracellular destruction (by their own virulence, or inability to be killed by the host), will multiply and destroy the alveolar macrophage. The pathogenesis of tuberculosis is dependent on previous infection. In a person, that has no history of a previous tubercle infection (*i.e.* Purified Protein Derivative (PPD) negative), the tubercle bacilli are able to increase in numbers in the alveolar macrophages almost as rapidly as they do in culture media (Youmans, 1985). In primary disease, when the bacillary load becomes significant, there is a cellular reaction followed by widespread dissemination of the infecting organisms. Disseminated organisms can lodge in almost any organ or tissue.

In the majority of persons, following dissemination, the rate of multiplication of the tubercle bacilli is halted. The two immunological events that characterize the halt of the spread of infection, is the development of a cutaneous reaction of delayed hypersensitivity to certain low molecular weight proteins and peptides that are found in the tubercle bacillus; and the activation of macrophages. Activated macrophages are more capable mycobacterial killers, and they halt the logarithmic growth of the tubercle bacilli (van Crevel *et al.*, 2002). In approximately 5%-10% of infected individuals, the infection is not halted, and it develops into tuberculosis within a year (Schluger & Rom, 1998). During latency, tubercle bacilli may remain viable but dormant within tissues for many months or years (Gangadharam, 1995; Parrish *et al.*, 1998). The organisms are prevented from active multiplication by the acquired cellular immune response.

Secondary tuberculosis (adult, re-infection or reactivation tuberculosis) occurs in person who has previously been infected by *M. tuberculosis*. These persons have tuberculin hypersensitivity (*i.e*. PPD positive) and have an acquired cellular immune response to the tubercle bacillus. Infection may be either by the inhalation of viable tubercle bacilli from an active case (exogenous re-infection), or through the reactivation of dormant bacilli previously inhaled (endogenous re-infection). In either case, tuberculosis develops despite acquired cellular immunity. In endogenous re-infection, the exact reason for the local breakdown of resistance that initiates secondary disease is not known (Parrish *et al.,* 1998). It is known, however, that many factors (e.g. age, degenerative disease, HIV infection) that lower resistance to infection, promote the development of tuberculous disease. Adult tuberculosis is characterized by chronicity, caseation, sloughing of liquefied caseous materials, necrosis and cavity formation (Youmans, 1985). It is primarily liquefaction that perpetuates the disease (Dannenberg & Rook, 1994).

Macrophages and the arrest of the spread of tubercle bacilli

Circulating mononuclear phagocytes, called monocytes, differentiate into macrophages and settle in different tissues. In the lung, they are termed alveolar macrophages, and are the initial cells that defend against infection from *M. tuberculosis* (Schluger, 2001). Macrophages are actively phagocytic. They contain digestive enzymes, and form an important link between the innate and acquired cellular immunity. Macrophages can be activated by cytokines (Goerdt, 1999) or by the phagocytosis of certain particles (Schnyder & Baggiolini, 1978). Upon activation, macrophages show increased metabolism, and phagocytic ability; all which facilitate tubercle killing (Ando *et al.*, 1977). They also produce a number of cytokines that regulate immune function (Romagnani, 1997). Macrophages can initiate a cell-mediated response, and act as antigen presenting cells (APC's) or effector cells. Resident alveolar macrophages are the primary cell type involved in the initial uptake of tubercle bacilli. After the first encounter, dendritic cells (DC) and monocyte-derived macrophages also take part in the phagocytic process (Henderson *et al.*, 1997). Phagocytosis of *M. tuberculosis* generally begins with the activated macrophage engulfing the invading organism into a membrane-bound vacuole that is created by pseudopods that surround the bacterium and fuse distally (Schlesinger, 1996a). Surface binding of tubercle bacilli (or its constituents) takes place through

complement receptors (CR) (*viz.* CR1, CR3 and CR4), mannose receptors, surfactant protein receptors, cluster differentiation (CD)-14 or scavenger receptors (Schlesinger, 1996b).

Also associated with tubercle binding are the toll-like receptors (TLRs). Brightbill *et al.* (1999) demonstrated that interleukin (IL)-12 is up-regulated in THP-1 cells when TLR's are activated by lipoproteins from tubercle bacilli. To date, ten TLRs have been identified; of which TLR2, TLR4 and TLR9 seem most associated with immune recognition in tuberculosis. It would appear that TLR2 and TLR4 subsets of receptors are activated by different mycobacterial components (Means *et al.*, 1999). *M. tuberculosis* uptake and immune recognition shows that TLRs are expressed on both the cell surface and the phagosome, thus immune activation can occur with or without phagocytosis (Figure 2-1). TLR binding results in IL-12 production through the signalling compound Myeloid Differentiation Protein 88 (MyD88) and IL-1R-associated Kinase (IRAK) (Oddo *et al.*, 1998). After *M. tuberculosis* is engulfed and incorporated in to a phagosome, it is subject to killing by a variety of mechanisms including phagosome-lysosome fusion, generation of reactive oxygen and nitrogen intermediates (Schluger & Rom, 1998). Evidence suggests that survival of the tubercle bacilli in the macrophage is crucial for the pathogenesis of tuberculosis (Hsu, 1971):

- *M. tuberculosis* can escape phagosome-lysosome fusion by the production of ammonia (Gordon *et al.*, 1980),
- *M. tuberculosis* can escape phagosome-lysosome fusion by the production of sulfatides (Goren *et al.*, 1976),
- *M. tuberculosis* may be able to evade reactive oxygen and nitrogen intermediates produced by macrophages (the latter being the most important in human disease) (Chan, *et al.*, 1992).

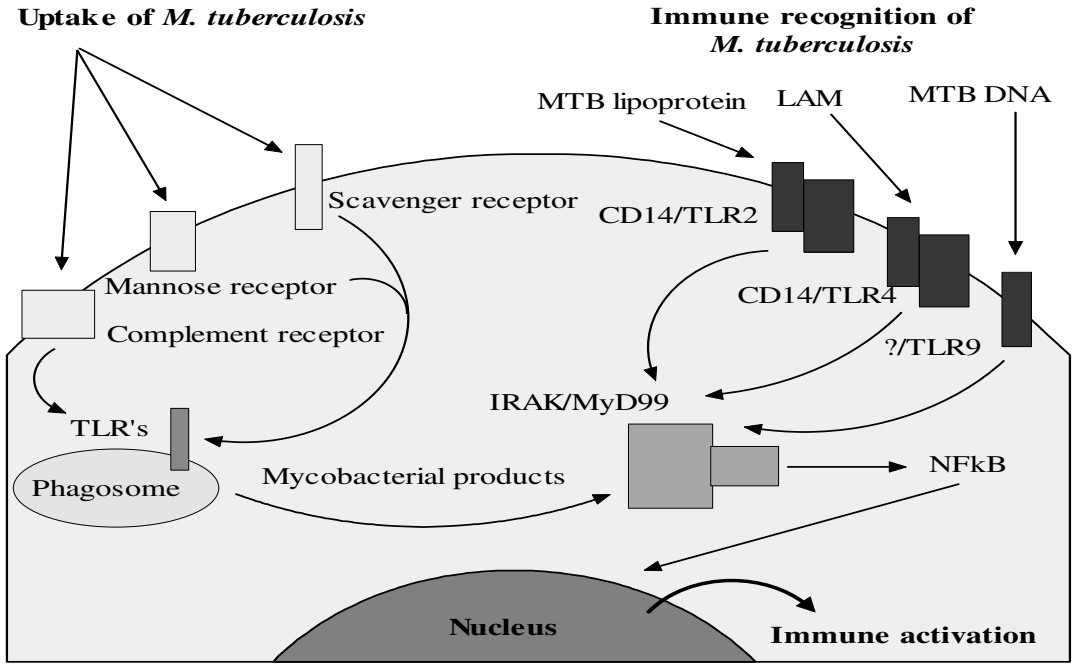

Figure 2-1 **Uptake and immune recognition of *M. tuberculosis*. Immune activation results from the uptake via complement, mannose and scavenger receptors, while immune recognition is achieved through Toll-like receptors (van Crevel *et al*., 2002).**

Cytokines in host response to tuberculosis

Cytokines are small soluble molecules secreted by one cell that can alter the behaviour or properties of the cell itself, and in turn of another cell. Recognition of *M. tuberculosis* by phagocytic cells leads to cell activation and the production of cytokines. This internalisation induces further activation and cytokine production in a process of regulation and cross-regulation. The pro-inflammatory cytokines include Interferon gamma (IFN-γ), Tumour necrosis factor alpha (TNF-α), IL-1β, IL-6, IL-12 and IL-18. Several cytokines produced by activated macrophages and DC's are essential for the stimulation of T-lymphocytes by the production of the Type 1 (Th1) cytokines IL-12, IL-18 and IL-23 (van Crevel *et al.*, 2002). The stimulation of the T-lymphocytes is dependant on the capacity of the macrophages and DC's to produce or react to Th1 cytokines.

The anti-inflammatory cytokines regulate the secretion of pro-inflammatory cytokines, preventing massive tissue damage. However, delicate balances between pro- and anti-inflammatory processes are required, since overproduction of anti-

inflammatory cytokines would favour the outgrowth of *M. tuberculosis*. The pro-inflammatory responses are antagonized by anti-inflammatory mechanisms specifically by soluble cytokine receptors that prevent binding of pro-inflammatory cytokines to cellular receptors. Chemokines are chemotactic cytokines that are largely responsible for inflammatory cell recruitment. Chemokines that have been investigated in tuberculosis include IL-8, monocyte chemoattractant protein 1 (MCP-1) and Regulated on Activation Normal T cell Expressed and Secreted (RANTES).

2.1.2 *Mycobacterium kansasii*

M. kansasii is a non-tuberculous mycobacteria (NTM) belonging to Runyon Group I. In clinical samples, individual bacilli are long, thick, and often appear cross-barred (Wolinsky, 1979).

Pulmonary disease associated with *M. kansasii*

M. kansasii is well associated with pulmonary disease (Falkinham, 1996). It commonly causes a chronic lung infection which closely resembles pulmonary tuberculosis. Pulmonary disease follows a somewhat milder and chronic course than tuberculosis infection (Youmans, 1985). Most often, the disease is progressive, but in occasional patients, it remains stable. A comprehensive epidemiological survey in the USA (O'Brien *et al.*, 1987), estimated that 75% of *M. kansasii* isolation represents pathogenic infections. Host factors are important, as pulmonary disease occurs mainly in people with identifiable predisposing factors such as silicosis, previous tuberculosis, chronic bronchitis, chronic obstructive lung disease, bronchiectasis, malignant disease, chronic aspiration from oesophageal disease and HIV (Rigsby & Curtis, 1994; Wolinsky, 1984).

Host immune response to *M. kansasii* infection

Studies that have examined the host immune response to *M. kansasii* have identified many of the characteristics associated with the host immune response to *M. tuberculosis*. These include uptake by complement receptors (Lé Cabec *et al*., 2000), nonopsonic phagocytosis (Peyron *et al.*, 2000), the activation of the phagocyte oxidative respiratory burst (Launois *et al.*, 1989), granulocyte participation (Silva *et*

al., 1989) and granuloma formation (Narayanan *et al.*, 1981). However, there are many important differences between the host responses to these mycobacteria.

Experimental infections with NTMs, including *M. kansasii*, have identified the ability of these organisms to persist in target organs of susceptible hosts (Collins & Watson, 1981). Collins & Cunningham, (1981), found differences in the kinetics of helper T-lymphocyte production within the spleens of mice heavily infected with different *M. kansasii* strains. Furthermore, there were a number of variations in the suppressor and helper T-lymphocyte populations as infection progressed. They propose that such cellular interactions could well be responsible for the observed persistence of some strains of *M. kansasii*. Hepper & Collins, (1984), showed that mice heavily infected with *M. kansasii* are not immunosuppressed, as earlier thought, but that they fully capable of responding to appropriate *in vivo* stimuli.

Keane *et al.*, (2000) compared human alveolar macrophage apoptosis between different strains of *M. tuberculosis* and *M. kansasii*, and found that both species induce apoptosis, but that 'avirulent' strains such as *M. kansasii* and *M. tuberculosis* H37Ra, induced significantly more apoptosis than virulent strains (*M. tuberculosis* H37Rv) . The findings suggest that mycobacterial virulence determinants modulate the apoptotic response of macrophages to intracellular infection (Keane *et al*., 2000).

Cytokine production in *M. kansasii* infection

The interaction of *M. tuberculosis* and *M. kansasii* with human macrophages at the level of macrophage monokine expression was investigated by Beltan *et al.* (2000). The results from this study indicate that the levels of TNF-α, Granulocyte monocyte colony stimulating factor (GM-CSF), IL-6 and IL-1 expression differ only slightly between these two species. A comparison between viable and heat-killed bacilli as initiators of monokine expression indicate that viable *M. kansasii* elicits a stronger response to heat-killed bacilli. Heat-killed *M. tuberculosis* however, elicits strong responses of TNF-α and IL-6. It is thought that this latter observation indicates that once killed, *M. tuberculosis* loses some of the heat-unstable virulence components and is unable to inhibit phagosome-lysosome fusion (Hart *et al.*, 1987). A comparison of TNF-α and IL-6 expression in human monocytes infected with either *M. avium* or *M.*

kansasii showed that *M. kansasii* elicited the production of significantly lower amounts of TNF-α and IL-6 than *M. avium*. The authors hypothesize that the extent of cytokine production might be relevant for the clinical manifestation of mycobacterial disease (Stauffer *et al.*, 1994).

The significance of GM-CSF and TNF-α expression in *M. kansasii* infection is highlighted by a case of persistent disseminated *M. kansasii* infection in a 27-year-old HIV negative woman. It was found that the patient's peripheral blood monocytes were abnormally permissive to the intracellular growth of *M. kansasii*. Extensive investigation yielded a normal number of interferon receptors, but reduced numbers of receptors to GM-CSF and TNF-α (Bermudez *et al.*, 1994).

2.2 SILICOSIS

Silicosis is a fibrosing disease of the lung that is caused by the intensity of exposure to respirable particles of silica-containing dust (Cowie, 1991). The name is derived from the Latin, silex, which means flint. Silicosis forms part of a larger category of diseases, the pneumoconiosis, that are all characterized by pulmonary reactions to bioactive dusts. Pneumoconiosis ranges from diffuse collagenous pulmonary reactions caused by small lung burdens (e.g. silica and asbestos) to largely non-collagenous reactions caused by heavy lung dust burdens (e.g. coal workers pneumoconiosis) (Becklake, 1992).

Most of the earth's crust consists of compounds containing silicon and oxygen. The compound that is responsible for silicosis is silica. It occurs in nature in three crystalline forms: viz. quartz, cristobalite and tridymite. Quartz is a hard colourless substance, and is the most common of the three forms. It is a common constituent of many rocks, granite and sandstone. The mining of gold, tin, copper and mica produces dust that contains high concentrations of free silica.

The development of silicosis is dependant on the inhalation of respirable free silica particles that are <10 µm in diameter. The size of the particle required for minimal alveolar deposition is 1-3 µm. Watkins-Pitchford, (1927), and Irvine, (1932) both

recognized the importance of the small silica particles from their studies of lungs from deceased miners. The intensity and duration of exposure to silica containing dust is important to the progression of the disease (Cowie, 1988). Another factor that determines the prevalence and severity of silicosis is the proportion of free silica in the respirable fraction of dust (Middleton, 1936).

In a recent study to determine the prevalence of silicosis in black workers on mines, Churchyard *et al.* (2003) examined the 520 chest X-rays of in-service miners, and classified these using International Labour Organisation (ILO) profusion category criteria. Only 226 chest x-rays (43.5%) were read as completely normal. One hundred and fifty (28.9%) were read as abnormal but as having no parenchymal abnormalities on the ILO scale (*i.e.* were equivalent to 0/0). It was found that among in-service black gold miners over 38 years of age, almost one in five have evidence of silicosis at the 1/1 level of profusion, and almost a quarter at the 1/0 level. The authors suggest that the study under-estimates the overall prevalence due to their sampling technique, and they point to the fact that silica exposure results in a life-long risk (over half of the radiological silicosis cases occur after the worker has left the exposure).

2.2.1 Host immune response in silicosis

Pathogenesis

In silicosis, the silica particles are deposited in the small airways and alveoli, and are rapidly phagocytosed by alveolar macrophages. Silica particles can also penetrate the respiratory epithelium and interact with interstitial macrophages and tissues (Brody *et al.*, 1980). Most dust is cleared soon after deposition by macrophages, but the small fraction that remains is responsible for silicosis (Davis, 1986). Brody *et al.*, (1982) found that silica particles are not translocated from their initial position of deposition until 24 hours after exposure.

Alveolar macrophages are rapidly drawn to the site of silica deposition following inhalation. It is thought that activation of complement C5a (Brody *et al.*, 1981; Reynolds, 1983) and the release of chemotactins by macrophages that have ingested

particles (Reynolds, 1983) may be the triggers for this cell recruitment. The *in vitro* activation of C5a is described by Governa *et al.*, (2002). The hypothesis is that the redox-active iron, which is possibly present on the silica surface, catalyses (via the Haber-Weiss cycles) the production of hydroxyl radicals. These convert native C5 to an oxidized C5-like form. This product is then cleaved by kallikrein, which has been activated by the same silica particles, yielding an oxidized C5a-type molecule. The chemoattractant cytokine, osteopontin (also known as early T-lymphocyte activation protein 1), has also shown to be unregulated in silicosis (Nau *et al.*, 1997).

Histologically, silicosis is a localized disease in which lesions develop in close proximity to the silica that causes it. The minimum lesion is an accumulation of macrophages with silica particles concentrated within it. Smaller macrophages (or perhaps monocytes), lymphocytes and occasionally neutrophils surround the central cells (Davis, 1986). Fibroblasts and connective tissue surround the small macrophages and lymphocytes, and interact with them. Larger and older silicotic nodules show concentric layers of fibrosis, with most of the silica distributed at the periphery. These nodules appear to collapse in the centres, with new layers of silica and cells being formed around them. As they enlarge, neighbouring nodules coalesce and collapse into one another, progressively distorting the surrounding lung tissue.

Effects of silica on the macrophage

Resident alveolar and recruited pulmonary macrophages demonstrate intimate contact with silica for the entire time it remains in the lung. Early studies stressed the toxicity of silica for macrophages *in vitro* (Allison, 1971; Allison *et al*., 1966). Allison postulated that injury, and ultimately silicosis developed as a response to toxic proteolytic enzymes and lysosomal hydrolyses released by dying macrophages that released their contents into lung. This model has been outmoded by more recent evidence presented by deShazo, (1982), and Lowrie, (1982), that suggests that the disease in caused by the secretion of synthesized mediators by macrophages.

Silica particles demonstrate two forms of cytotoxicity on macrophages *in vitro*. A rapid cytotoxicity is produced when relatively large amounts of silica is added to serum-free medium; and a delayed cytotoxicity that occurs in serum-containing medium after cells have digested the protein coat on the silica particle (deShazo,

1982). Silica reacts with the plasma membrane resulting in increased permeability and lysis. It has been suggested that the cytotoxicity may be related to the negative charge of the silica surface (O'Rourke *et al.*, 1978).

Ghio *et al.*, (1990) hypothesized that silica cytotoxicity is caused by oxidants generated from accumulated iron in the silicotic lung. The mechanism by which oxidants lead to apoptosis can differ from substance to substance (Figure 2-2) (Kim *et al.*, 1999). Apoptosis is induced via stimulation of several different cell surface receptors e.g. Scavenger receptor (SR), FAS (CD95) and TNF receptor. Cleavage of these receptors leads to caspase activation. Internal and external signals set off apoptosis in different ways. The mitochondria of cells that receive internal signals express a series of reactions, which result in the release of Cytochrome C and caspase 9 activation. External signals activate caspase 8 and initiate cell death. Both these pathways lead to the degradation of DNA in the nucleus, which is the hallmark of apoptosis (Cohen, 2000).

A number of studies have examined the importance of oxidants in the development of silicosis. Chen *et al.*, (1996), showed that there was a pattern of apoptotic events in silica-treated alveolar macrophages, starting with reactive oxygen species (ROS) formation, followed by caspase 9 and caspase 3 activation, poly-(ADP-ribose) polymerase cleavage and DNA fragmentation. Silica-induced apoptosis was significantly attenuated by a caspase 3 inhibitor, N-acetyl-Asp-Glu-Val-Asp aldehyde, and ebselen (a potent antioxidant) (Chen *et al.*, 1996).

Rojanasakul *et al.*, (1999) investigated the role of Nuclear Factor (NF)-kappa or ß and oxygen free radicals in silica-induced poly-(ADP-ribose) polymerase production in primary alveolar macrophages and RAW 264.7 cells. It was shown that silica could induce NF-kappaß activation and TNF-α expression in a dose-dependent manner, indicating that silica-mediated free radical generation and NF-kappaß activation play important roles in silica-induced TNF-α gene expression (Rojanasakul *et al.*, 1999).

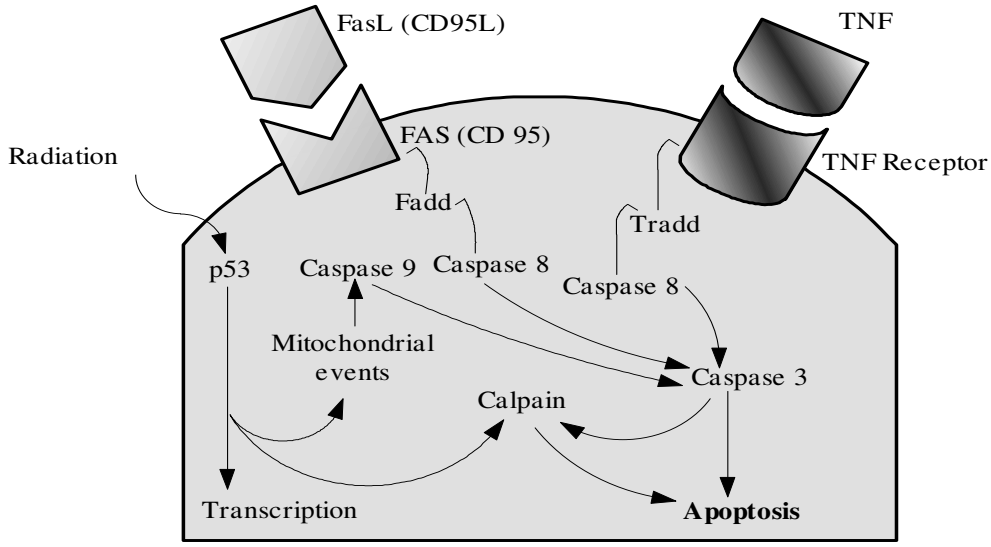

Figure 2-2 Common apoptotic pathways of cells. Internal (via p53), and external signals (via FAS and TNF receptors), set off apoptosis in different ways. Internal signalling leads to cytochrome C release and caspase 9 activation. External signals activate caspase 8 and initiate cell death via caspase 3 (Kim *et al.*, 1999).

Kang *et al.*, (2000a), investigated the involvement of ROS in the silicotic apoptotic process by examining the role of protein tyrosine phosphatase in NF-kappaß activation in mouse peritoneal macrophages (RAW 264.7 cells). It was found that a specific inhibitor of protein tyrosine kinase, genistein, prevented the NF-kappaß activation induced by pervanadate in the presence of silica. Inhibitors of protein kinase A or C, such as staurosporine or H7, had no inhibitory effect on NF-kappaß activation. A variety of antioxidants, such as catalase, superoxide dismutase, N-acetyl cysteine and pyrrolidine dithiocarbamate, inhibited NF-kappaß activation induced by pervanadate in the presence of silica. The results suggest that protein tyrosine phosphatase plays a crucial role in the negative regulation of silica-signaling pathways leading to NF-kappaß activation in macrophages (Kang *et al.*, 2000a). Further studies were performed to determine whether protein tyrosine kinase stimulation and NF-kappaß activation in silica-stimulated macrophages are directly connected through tyrosine phosphorylation of I kappaß-alpha. Specific inhibitors of protein tyrosine kinase, such as genistein and tyrophostin AG126, prevented tyrosine phosphorylation of I kappaß-alpha in response to silica. Antioxidants, such as superoxide dismutase, N-acetylcysteine and pyrrolidine dithiocarbamate, blocked tyrosine phosphorylation of I kappaß-alpha induced by silica, suggesting that ROS may be important regulatory molecules in NF-kappaß activation through tyrosine phosphorylation of I kappaß-alpha (Kang *et al.*, 2000b).

Glutathione is a tripeptide that is found in abundance in lung tissue. Glutathione is a potent antioxidant, and appears to play a critical role in protecting against silica-induced cell injury. Zang *et al.*, (1999) showed that that silica led to a dose- and time-dependent decrease in glutathione content in alveolar macrophages. N-acetylcysteine increased intracellular glutathione levels and protected the macrophages against silica-induced ROS species formation, lactate dehydrogenase leakage, and DNA strand breaks. In contrast, buthionine sulfoximine pre-treatment depleted cellular glutathione and enhanced the susceptibility of alveolar macrophages to the cytotoxic and genotoxic effects of silica. Similar to glutathione, the mucolytic ambroxol decreased the silica-induced cell viability loss in rat alveolar macrophages *in vitro*. Ambroxol attenuated the production of superoxide, hydrogen peroxide, and nitric oxide (NO) and the release of acid phosphatase and lysozyme in macrophages activated by silica. It is suggested that ambroxol may provide a protective effect on pulmonary tissues against the toxic action of silica (Kim *et al.*, 2002).

Borges, (2001), investigated the role of FAS ligand in murine silicosis. Activated cytotoxic T-lymphocytes (CTL's) express FAS ligand, which binds FAS, an apoptosis–inducing cell surface molecule on target cells. It was found that wild-type mice instilled with silica developed severe pulmonary inflammation, with a local production of TNF-α, and interstitial neutrophil and macrophage infiltration in the lungs. FAS ligand-deficient generalized lymphoproliferative disease mutant mice did not develop silicosis, leading the investigators to conclude that FAS ligand plays a central role in induction of pulmonary silicosis.

SRs are responsible for recognition of endogenous and exogenous ligands such as FAS ligand. Using a murine alveolar macrophage cell line (MH-S), the role of SR-A in apoptosis was examined. Silica stimulated significant caspase activity and apoptosis of MH-S cells, which was attenuated by 2F8 (a blocking antibody) and polyinosinic acid (a non-specific SR antagonist). The results suggest that the SR's are necessary for caspase activation, and subsequent apoptosis, caused by silica in macrophages (Chao *et al.*, 2001).

In addition to the ROS pathway to cell death through apoptosis, studies suggest that NF-kappaß activation in macrophages can, under certain conditions, be induced by

NO (Kim *et al.,* 2002). NO produced by phagocytes exposed to silica up-regulates the activation of NF-kappaß (Kang *et al.*, 2000a), which in turn leads to apoptosis.

Effect of silica on acquired and cellular immunity

Silica dust is not only a macrophage toxin, but has also been shown to cause changes in T- and B-lymphocytes (Miller & Zarkower, 1974). The various substances secreted by cells during the development of silicosis are summarized in Table 2-1. The complex interaction of cells involved in the development of silicosis can be simplified as the interactions between macrophages and lymphocytes, the recruitment of neutrophils and the interaction between macrophages and fibroblasts.

Table 2-1 Summary of up-regulated molecules following silica exposure.

Cell-mediated immunity	***Humoral immunity***
Macrophage-lymphocyte interaction	RF
MAF	Auto-antibodies
IFN-γ	IgG
IL-12	IgM
IL-18	
Osteopontin	
Neutrophil recruitment	
ICAM-1	
TNF-α	
L-selectin	
Macrophage-fibroblast interaction	
MDGF	
IL-1	
Fibronectin	
TNF-α	
TGF-β	
IL-6	

Macrophage and lymphocyte interactions

T-lymphocytes either directly or indirectly influence the maintenance of the macrophage infiltrate after silica exposure (Hubbard, 1989). In both human and animal studies with silicosis, it has been shown that lymphocytes accumulate in the lung tissue, bronchoalveolar lavages and bronchial-associated lymphoid tissue. The cells are mostly $CD4^+$, and to a lesser degree $CD8^+$ phenotypes (Davis *et al*., 1993; Kumar, 1989; Struhar *et al*., 1989). Macrophages that have ingested silica are stimulated to release biologic response modifiers that influence lymphocytes. Kumar, (1990) found that in silicosis, the T-lymphocytes appeared to be activated, and were able to express the IL-2 receptor. Activated T-lymphocytes secrete monocyte

chemotactins, macrophage activating factor (MAF), macrophage fusion factor and IFN-γ. Macrophages recovered from animals exposed to silica show evidence of some of these activating effects (Davis *et al.*, 1981).

The increased production of IFN-γ in silicotic mice is described by Davis *et al.* (1999). The investigators hypothesized that the increased IFN-γ production might be due to selective enrichment of one of the lymphocyte phenotypes. Further studies revealed that that IFN-γ is produced by CD4+ T-lymphocytes, NK cells and γδ-T-lymphocyte receptor. This increase is not due to changes in the proportions of cells, but rather an increase in the total number of cells in the lung (Davis *et al.*, 2000). In further studies in mice, Davis, (2001), found that messenger ribonucleic acid (mRNA) transcripts for the macrophage-derived cytokines IL-12 and IL-18 were increased. He concludes that silica induces a Th-1 type response in mouse lung tissue.

Garn, (2000), also reports on a shift toward an IFN-γ dominated Th-1 reaction patterns within the thoracic lymph nodes of silicotic mice. IFN-γ mRNA expression increased, whereas IL-2 expression remained unchanged. Gene transcription for IL-4 and IL-10 was diminished. With regard to IFN-γ cytokines, IL-12 mRNA expression increased *in vivo*, whereas IL-18 gene expression was slightly decreased.

Silicosis is associated with humoral immunological abnormalities (Doll *et al.*, 1981). Although investigators have demonstrated an increased incidence of silicosis with autoimmune disease, the exact role of the humoral response in silicosis is not clear (Ziskind *et al.*, 1976). Sandblasters with silicosis were shown to have had elevated serum levels of IgG and IgM, and a high frequency of antinuclear antibodies, rheumatoid factor, immunoglobulins and immune complexes (Doll *et al.*, 1981). Healthy Vermont granite workers were shown to have increased immunoglobulin concentrations in bronchoalveolar lavages. These workers had normal serum immunoglobulin concentrations, suggesting that the production is localized to the lung (Christman *et al.*, 1985). The mechanism of antibody production is likely to be via activated helper T-lymphocytes. The specific contact between a helper T-lymphocyte and a B-lymphocyte initiates IL-4 transfer, this activates the B-lymphocyte. IL-5

transfer stimulates activated B-lymphocytes to proliferate, and IL-6 transfer induces maturation into an antibody-secreting cell (Roitt, 1977).

Subra *et al.*, (2001), assessed the immune status of 58 silicotic slate workers, and compared these to 41 healthy case-matched controls. There was a significant decrease in the total lymphocyte count ($p < 0.001$) involving B- and T-lymphocytes and NK cells in silicotic patients as compared with healthy controls. A significant increase in the percentage of activated T-lymphocytes (12.3%) was observed in the silicotic group as compared to 6.5% in the control group. They suggest that these findings may predispose to the development of autoimmune disorders in these workers as the silicotic patients had a higher prevalence of autoimmune diseases (6/58 vs. 0/41: $p < 0.05$) and of elevated antinuclear antibody titres compared to the control group in this study.

Neutrophil recruitment

Neutrophils, although abundant in the lungs and lavage fluids of animal silicotic models, are less prominent in human disease. They may however be important because of their potential to cause tissue damage in acute silicosis. Acute silicosis is characterized by extensive epithelial damage, and abundant airspace debris (mainly serum and lung proteins, surfactant lipids and Type II epithelial cells) and neutrophilia (Davis, 1986).

Absher, (1989), identified a biphasic recruitment of neutrophils, macrophages and lymphocytes to the alveolar spaces in rats. The first recruitment took place between the eighth day and the eighth week following exposure; and the second took place 24 weeks after the end of exposure. A number of chemotactic substances that are released from macrophages may be responsible for the recruitment of neutrophils.

Intercellular adhesion molecule-1 (ICAM-1) is expressed on a variety of cells including vascular endothelial cells, fibroblasts, types I and II alveolar epithelial cells, some lymphocytes, and monocytes/macrophages (Hubbard *et al.*, 2001). Intratracheal exposure of mice to silica, elicits increased expression of ICAM-1 on pulmonary macrophages and type II epithelial cells within the lung parenchyma. ICAM-1 is also

expressed on BAL macrophages, and as a soluble protein in lavage fluid. Hubbard hypothesizes that increased ICAM-1 expression may participate in silica-induced neutrophil influx into the alveoli, as well as in the macrophage signalling that is responsible for migration of the neutrophils. ICAM-1 expression on silica-exposed mouse macrophages are enhanced by ROS and TNF-α, and appears to be regulated through specific sequence elements within the ICAM-1 promoter region (Hubbard *et al.*, 2001). L-selectin (previously known as lymph node homing receptor leukocyte adhesion molecule 1 (or leu8) is also a cell adhesion molecule that is expressed on lymphocytes, neutrophils and monocytes. L-selectin is associated with the recruitment of leukocytes to sites of inflammation (Clements & Gearing, 1997). Ishihara *et al.* (2001) described the role of L-selectin in silica induced lung injury. It was found that the early phase of leukocyte activation was diminished by blocking L-selectin, but that treatment with anti-L-selectin increased the formation of granulomas in the rats.

Macrophage fibroblast interaction

Lung fibrosis is an important feature of chronic and advanced silicosis. An increase in lung collagen may be detected as early as 14 days after silica exposure in rats and guinea pigs (Lugano *et al.*, 1984). Although the mechanism of collagen production in silicosis is not fully understood, it is thought that the interaction between macrophages and fibroblasts is a key event (Schmidt *et al*., 1984; Sjöstrand *et al*., 1991). The release of fibroblast proliferation factors is specific to silica, as nonfibrogenic dusts do not stimulate its release (Schmidt *et al.,* 1984). It has been noted that silica particles released by dead macrophages have diminished cytotoxicity when added to fresh cultures of macrophages (Allison *et al.*, 1966). This was taken to mean that repeated cycles of macrophage killing *in vivo* leads to the activation of fibroblasts and the deposition of excess connective tissue. Subsequent studies have shown that silica damaged macrophage release soluble factors that stimulate collagen production by cultured fibroblasts (Heppleston & Styles, 1967). Kilroe-Smith classified these factors further by finding that the substances responsible for fibrogenicity of quartz was not the quartz itself, but an insoluble substances produced by the action of quartz on the macrophage (Kilroe-Smith *et al.*, 1972).

Substances that are associated with stimulating fibroblast replication include macrophage derived growth factor (MDGF), IL-1 and fibronectin. MDGF is a potent stimulator of fibroblasts, and is secreted from activated T-lymphocytes (Kovacs & Kelley, 1985). Schmidt, (1984), found that *in vitro* stimulated monocytes, released fibroblast proliferation factors that were identical to IL-1.

Fibronectin is a product of macrophages and fibroblasts, and may enhance fibroblast adhesion and growth (Davis, 1986). Macrophages produce increased amounts of TNF-α and TGF-β in response to silica. Arcangeli *et al.* (2001) showed that both TNF-α and TGF-β are able to stimulate the proliferation of human lung fibroblasts in culture. They are also able to increase the collagen production of the cells and increase IL-6 production by lung fibroblasts of patients with silicosis. Bodo *et al.*, (2001), found that airway epithelial cells also played a part in the pathogenesis of silicosis. It was found that silica induced changes in T antigen-transformed human airway epithelial cell line by increasing production of collagen, and fibronectin.

2.2.2 Clinical silicosis

Cowie, (1991), describes three forms of simple silicosis; acute silicosis (arises in response to an intense exposure to free silica, with lung disease developing in as short as six months after the exposure), accelerated silicosis (follows four to fourteen years of moderate to high exposure of silica dust) and chronic silicosis (develops after many years exposure to low intensity silica dust).

Tuberculosis and silicosis

The association of silicosis and tuberculosis is devastating. In South Africa, the risk of tuberculosis among miners with silicosis is approximately three-fold higher than among non-silicotic workers. Furthermore, one quarter of miners with silicosis will develop tuberculosis by the age of 60 (Cowie, 1994). There is a higher incidence of tuberculosis among miners with more severe silicosis. The results of two studies, in which silicosis was graded using the ILO classification, are shown in Table 2-2. These findings were confirmed by Hnizdo *et al.*, (1998), who showed that the

adjusted rate ratio of pulmonary tuberculosis increased with the severity of silicosis as found at autopsy.

Table 2-2 Incidence of tuberculosis among gold miners by ILO silicotic grade (White, 2001).

Silicosis category	*Tuberculosis incidence rate ratio (unadjusted)*	*Tuberculosis incidence rate (per 100 000 p.a.)*
Cowie 1994 (older silicotics)	*1*	*1 000*
ILO category 1	*2.2*	*2 200*
ILO category 2	*2.9*	*2 900*
ILO category 3	*6.3*	*6 600*
Corbett 2000 (miners with normal X-rays, HIV negative)	*1*	*700*
ILO grade 0/1	*1.8*	*1 400*
ILO grade 1/0	*1.8*	*1 400*
ILO grade 1/1	*2.6*	*1 900*
ILO grade 2/2 to 3/3	*5.3*	*4 000*

Chang *et al.*, (2001), examined the records of 718 subjects who had been diagnosed with silicosis between 1988 and 1993. The study identified four risk factors associated with the development of tuberculosis in this cohort that had a relative risk of tuberculosis nine times higher than the general population adjusted for age and sex:

- no prior antimycobacterial treatment (relative risk 4.5)
- progressive massive fibrosis (relative risk 3.78)
- small opacities exceeding 1.5mm (relative risk 2.17)
- caisson work (relative risk 1.56)

The confirmation of the diagnosis of tuberculosis and silicosis is often difficult to establish due to the difficulty in isolating *M. tuberculosis* from sputum (Cowie, 1988). Despite early reports to the contrary, Watkins-Pitchford (1927) proved that *M. tuberculosis* isolated from silicotic patients did not lack virulence. Lundborg *et al.*, (2001) demonstrated that the phagocytic ability of human alveolar macrophages is impaired after silica phagocytosis. The authors suggest that the decreased capacity of macrophages to phagocytose may predispose silicotics to infection (Lundborg *et al.*, 2001).

Early reports dismissed the role NTM have played in silicotic patients. However, reporters such as Snider, (1978), showed that silicotics were highly susceptible to NTM infections. This opinion was refuted by Morgan, (1979), who suggests that the increased prevalence of NTM infections is due to the presence of NTM's in dust inhaled by miners. Policard *et al.* (1967) showed that in an *in vitro* guinea pig model, dust aggravates experimental infection with *M. kansasii*. While the exact source of NTM's in mines is uncertain, their role in disease has been clearly established (Corbett *et* al.,1999d).

2.3 RADIATION AND MINING

2.3.1 Radium, Radon and radiation in mining

Approximately 82% of our exposure to ionizing radiation is from natural sources in the environment. The largest contributor (47%) to this total is radon (Axelson, 1995). The three important natural occurring radioactive series are uranium, thorium and actinium. Each of these three series decays to a stable isotope of lead. Approximately halfway through each series, an isotope of radon is formed. Radon is a colourless, odourless, and almost chemically, inert gas that is a decay product of radium and uranium. Radium and uranium are ubiquitous in rocks and soils, and as a result can be found both indoors and outdoors. In mines, radon is released in the atmosphere mainly by diffusion through the ore. It may also be present in water that percolates from the ore body. Radon has 27 known isotopes ranging from 200Rn to 226Rn. The half lives of all the isotopes except 222Rn (3.823 d), 210Rn (2.5 h) and 211Rn (14.7 h), are less than one hour. On decay, radon emits α-, β- and γ-particles (Cothern, 1985).

In South African gold mines, the concentration of uranium and/or thorium may be as much as 25 times higher than in the earth's crust (Wymer, 2001). The distribution of radiation doses received by South African miners in 1999 is shown in Table 2-3. The range of radiation doses received is widely distributed amongst the workforce, with only a small percentage of workers (2.6%) receiving more than 20 mSv p.a..

Table 2-3 Radiation doses received by gold miners in 1999, showing that most miners receive less than 5 mSv of radiation per annum (Wymer, 2001).

	Number of workers					
	0-5 mSv	5-10 mSv	10-15 mSV	15-20 mSv	>20 mSv	Total
Gold miners	130 986	48 365	14 998	2 296	5 230	201 875

2.3.2 Effects of radiation exposure

Effects of radon exposure

The radon-related radiation dose to respiratory tissue and the resulting biological effect comes from the inhalation of radon progeny in the atmosphere, rather than inhaled radon and its decay products (Cross, 1985). There are several reasons for this:

- once inhaled, radon is quickly exhaled,
- the half-life of radon is relatively long in comparison to breathing, so only a small portion of deposited radon decays in the lung,
- the short half-lives of the immediate radon progeny preclude any appreciable radiation damage,
- the longer lived progeny (e.g. ^{210}Pb) are likely to be cleared by the body before they accrue to appreciable amounts.

The estimated dose of radon progeny to the peripheral lung has been determined to be 9.8 ±1.2 mGy/Working level month (WLM) (Johnson & Newton, 1994). Johnson & Newton, (1994), found that there was no reduction in phagocytic capability of macrophages isolated from exposed rats as compared to non-exposed controls. However, an exposure-dependent relationship between bi- or multinucleated macrophages was noted. The presence of nuclear aberrations in macrophages isolated from radon-exposed rats is also described by Taya *et al.*, (1994).

The radiation hazard associated with underground atmosphere depends on the amount of radon progeny in the atmosphere of the mine. This in turn is dependant on the age of the ventilating atmosphere in the mine (Wymer, 2001). The acceptable limit for radiation exposure suggested by the International Commission on Radiological Protection (ICRP) is 1 mSv/year for the public, and 20 mSv/year for occupational

workers, including miners (International Commission on Radiobiological Protection, 1991). Exposure to the natural sources of radiation in mines cannot give rise to deterministic effects (Wymer, 2001). For occupational workers the 20 mSv/year may be averaged over 5 years, but may not be more than 50 mSv in any one year. For the public, in special circumstances, a higher level is allowed in a single year provided the average over 5 years does not exceed 1 mSv. It is the intention of these limits to prevent deterministic effects, and to limit the occurrence of stochastic effects to an acceptable limit. In animal studies following radon exposure, the major biological effect observed was life-span shortening, pulmonary emphysema, pulmonary fibrosis and respiratory carcinoma (Cross, 1985). Epidemiological studies in humans have not equivocally established the causal relationship between radon exposure and lung cancer.

Deterministic and stochastic effects

In acute exposures above a few Gray (Gy), radiation damage occurs principally because of cell death by apoptosis. This can give rise to organ and tissue damage and, in extreme cases, death (UNSCEAR, 1993). These effects, termed early or deterministic, occur principally above a threshold dose that must be exceeded before they are manifested as clinical damage, although damage to individual cells may occur at lower doses.

When energy imparted to the cell is below the deterministic threshold, the cell survives, although it may be altered in some way. This kind of effect is termed stochastic (meaning "of random or statistical nature"). Stochastic effects cause radiation-induced cancer to develop in a proportion of exposed persons (or hereditary disease in their descendants). Although the probability of both cancer and hereditary disease increases with radiation dose, it is generally considered that their severity does not (UNSCEAR, 1994).

The United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) supports a multistage model for the radiation-induced cancer (UNSCEAR, 1993). In this model, neoplastic initiation may be broadly defined as "…essentially irreversible changes to appropriate target somatic cells, driven principally by gene mutations that create the potential for neoplastic development.

Such tumour gene mutations can have profound effects on cellular behaviour and response, e.g. dysregulation of genes involved in biochemical signalling pathways associated with the control of cell proliferation and/or disruption of the natural processes of cellular communication, development, and differentiation…" (UNSCEAR, 2000).

Effects of ionizing radiation on the immune system

Investigations of the immune response to radiation have focused on the relative radiosensitivity of cells. It has been shown that the phagocytic function of macrophages is quite resistant to irradiation. Macrophages have been observed phagocytising debris of dead cells in irradiated lymphoid tissues (Anderson & Warner, 1976). Johnson has noted these findings in alveolar macrophages isolated from mice, after radon exposure (Johnson & Newton, 1994). Anderson found contradictory data existed concerning the ability of macrophages to catabolise, degrade and retain antigenic particles after irradiation. He suggests that the process may be antigen specific (Anderson & Warner, 1976). Ionizing radiation can mimic cytokine signals and lead to macrophage activation. Cells of the J774 macrophage cell line were found to be primed for antibody-dependent cell-mediated cytotoxicity following exposure to γ-radiation (Duerst & Werberig, 1991). Ibuki & Gotom, (1995), found that whole-body irradiation of mice with a low-dose of gamma-rays activated macrophages indirectly and consequently enhanced NO production from macrophages (Ibuki & Goto, 1995). In J774 and RAW264.7 murine macrophages, radiation alone (0.5-50 Gy) did not induce NO, but enhanced NO production in a dose-dependent manner when cells were exposed to IFN-γ or LPS 24 hours post-irradiation. The induction of NO is mediated by the induction of TNF-α (McKinney *et al.*, 1998). It is suggested that irradiation of guinea pigs decreases their ability to develop a delayed hypersensitivity reaction to purified protein derivative. This effect is only temporary, and can be fully restored (Volkman & Collins, 1971).

T-lymphocytes, unlike B-lymphocytes, appear to function normally even after high doses of radiation. Harrington found that natural killer cells and $CD4^+$ cells were more resistant than $CD8^+$ to whole body ionization. He emphasizes that it is bone marrow stem cell depletion and the activation of suppressor cells that lead to opportunistic infections in irradiated individuals (Harrington *et al.*, 1997). Low-dose

radiation (0.1 - 0.5 Gy) was shown to stimulate mitogen-induced proliferation and function of suppressor/cytotoxic T-lymphocytes (Gualde & Goodwin, 1984). Using a mouse model, Liu *et al.*, (2001), found that the stimulatory affect of low-dose radiation on the proliferative response of lymphocytes to Con A required the presence of APC's. B7-1/2 expression on APCs was upregulated after both low and high doses of radiation. Furthermore, there was up-regulation of CD28 expression on splenic and thymic lymphocytes after low-dose radiation, and suppression after high dose radiation. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) was expressed in the opposite manner. The authors conclude that the status of CD28/CTLA-4 expression on T lymphocytes in the presence of upregulated B7 expression on APC's determines the outcome of the immune changes in response to radiation. Up-regulation of CD28 after low-dose radiation results in immunoenhancement, and up-regulation of CTLA-4, associated with down-regulation of CD28 after high dose radiation, leads to immunosuppression (Liu *et al.*, 2001).

Little is known about cytokine/lymphokine expression by irradiated cells. TNF-α has found to be upregulated following radiation, as is the expression of IL-1 (Sherman, 1991). In mouse peritoneal macrophages *in vitro*, gene expression of IL-1β and IL-6 increased 1-2 hours after 2 Gy irradiation, but then decrease to below the basal level by four hours post-radiation (Hosoi *et al.*, 2001). TNF-α, IL-1 and IL-6 have been shown to contribute to natural resistance to lethal irradiation (Neta, 1992). It has been found that IL-10 increases following radiation exposure (Broski & Halloran, 1994). In a mouse model, Liu *et al.*, (2001), found that IL-12 secretion by macrophages was stimulated after both low and high doses of radiation, but IL-10 synthesis by splenocytes was suppressed by low-dose radiation and upregulated by high dose radiation.

2.3.3 Association between radiation and tuberculosis

There is no clear link between radiation exposure and tuberculosis. There are a number of reports that suggest an increase in tuberculosis prevalence after radiation exposure:

- males located between 0 and 1399 meters of the Hiroshima atomic hypocentre (Wanebo *et al.*, 1968),
- persons living in the Byelorussian region of the former Soviet Union, following the Chernobyl nuclear accident (Kalechitis & Al'khimovich, 1990),
- catholic nuns working as radiologists (Morin *et al.*, 2000).

The radiation dose received implies that tuberculosis may be an effect of the deterministic effect of the radiation on the immune system. There are a number of reports that document disseminated and localized tuberculosis in immunocompromised hosts after radiation exposure:

- Fulkerson describes a case of rapidly progressive pulmonary tuberculosis following irradiation of a lung carcinoma (Fulkerson, 1970),
- Crumpacker describes a patient with reticuloendotheliosis who develops a pulmonary infection with a photochromogenic mycobacteria after mediastinal irradiation (Crumpacker, 1977),
- Rumans describes a case of pulmonary infection with *M. avium-intracellulare* complex, following irradiation for breast cancer (Rumans, 1980).

It is Rumans (1980), opinion that irradiation of a tuberculous lesion will cause its reactivation. This claim seems to be supported by findings that irradiated mice are more susceptible to tuberculosis infection than non-irradiated control animals (Anderson & Warner, 1976).

2.4 MONOCYTIC CELL LINES

The use of primary cell for *in vitro* cultures is limited by the cell's life span, heterogeneity of cell types, and the small quantities of cells that can be harvested from samples or an embryo. A cell line is a homogenous population of cells that has been cloned from a single cell, and has acquired the capacity for an unrestricted number of cell divisions (Widnell & Pfenninger, 1990). Cell lines have been used to study leukaemia, as well as the role of specific cells in the human immune response (Hass *et al.*, 1990a).

2.4.1 U-937 leukaemic cell line

U-937 was derived from a previously healthy 37-year old male who presented with generalized lymphoma (Sundström & Nilsson, 1976). Pleural fluid from the patient was concentrated by centrifugation and the isolated cells were cultured. Cells were shown to differ from lymphoblastoid cell lines, and their histiocytic origins were demonstrated by their capacity to produce lysozyme and esterase. It was concluded that the cell line U-937 is promonocytic *i.e.* it is a precursor of the monocyte/macrophage lineage.

Living U-937 cells are variable in shape, with both round and polyglonal cells present. Fixed stained cells have a moderate amount of cytoplasm, which contains numerous eosinophilic granules and a few vacuoles. The nuclear shape is variable; generally, the chromatin is course, with one or two nucleoli. Under electron microscopy, cells are found to contain no fat vacuoles or viral particles. A few perichromation granules can be observed, as well as a well-developed Golgi apparatus and mitochondria.

U-937 grows as a single-cell suspension, with a doubling time of 95-100 hours. Both complement (C3b/CR1) and Fcγ-receptors are present, but only on approximately 30% of the cell surfaces. Surface Ig- cannot be detected, nor could any Ig- be demonstrated in the supernatants of cell cultures. Approximately 20% of the cells are phagocytic to test particles. Phagocytising cells contained 2-5 particles per cell (Sundström & Nilsson, 1976). U-937 expresses detectable levels of leukocyte-function-antigens (LFA-1) CD11a and CD18.

The differentiation of U-937 leukaemic cells into immature macrophage intermediates is described by Hass *et al.* (1989). Treatment of U-937 with 12-O-tetrade-canoylphorbol-13-acetate (TPA) causes adherence of the cells to the substratum and to each other. Four to 12 hours after TPA, treatment cells become rounded with cell-substratum adherence observed between 12-24 hours post-treatment. Cell-cell adherence is observed between 48-72 hours post-treatment. Cell-cell adherence is associated with the extension of pseudopodia, and results in colonies that are characteristic of U-937 differentiation. Associated with cell adherence, is the arrest of the growth cycle (i.e. cells enter the G0 phase). This, however, is not associated with

cell death (Hass *et al.*, 1990a). Hass *et al.* (1990b) showed that alterations in cell-surface carbohydrates are fundamental to the attachment of U-937 after TPA treatment. CD11b mediated adhesion in the course of differentiation is also crucial to attachment (Prudovsky *et al.*, 2002).

A number of morphological changes are observed after TPA treatment: the nucleus shows deep indentations and lobules, numerous ribosomes are present in the cytoplasm, and large membrane-bound vesicles and glycogen deposits are frequently observed. Morphological alterations in the cell surface are accompanied by a redistribution and/or reorganization of cytoskeletal proteins (Hass *et al*., 1989). Within 48 hours of TPA treatment, U-937 acquires functional qualities that are associated with monocytes and macrophages. As compared to the untreated cells, TPA treated U-937 show a marked increase in CD11a (LFA-1), the -subunit CD18, and particularly CD11b (CR3/MAC1) and CD11c (gp 150,95) antigen expression (Hass *et al*., 1989). There is a significant down regulation in transferrin receptor and Major Histocompatibility Complex (MHC) class I expression. MHC class II and CD 14 antigens are not markedly increased following TPA treatment (Hass *et al*., 1989). There is an increased expression of the macrophage colony stimulating factor (M-CSF) receptor, platelet-derived growth factor (PDGF-1 and PDGF-2) and the prostanoids PGE2, PGI2 and TxB2 (Hass *et al.*, 1990a). TPA-treated cells acquire the capacity to produce and release TNF-α and IL-1β but not IL-1α or IL-6 (Hass *et al*., 1991). The evidence suggests that TPA-treated cells are immature macrophage intermediates rather than terminally differentiated cells.

Hass *et al.*, (1990a), showed that differentiated U-937 maintained in long-term culture (more than 28 days), spontaneously retract their pseudopodia, detach from the substratum and begin to proliferate. Retrodifferentiation is associated with a reversal of all parameters which are altered during differentiation, and needs to be distinguished from dedifferentiation in which some parameters which accompanied the previous differentiation are not reverted (e.g. cessation of proliferation) (Hass *et al.*, 1990a). Retrodifferentiation is not unique to U-937, as it has been shown that differentiation is also transient in THP-1 and HL-60 cells (Hass, 1992).

Differentiation may also be induced by treating U-937 with phorbol 12-myristate 13-acetate (PMA), IFN-γ or 1,25-dihydroxyvitamin D_3 (VD_3) (Passmore *et al*., 2001). Passmore, (1999), compared the efficiency of differentiation between these substances and found that, although all arrested the cell cycle, differentiation with PMA in particular resulted in almost complete arrest (85-93%). PMA is a Protein kinase C (PKC) inhibitor that causes exit from the cell cycle, and G1 arrest (Cartee *et al.*, 2001). Analysis of the effects of PMA, IFN-γ and VD_3 induced differentiation on cell surface marker expression revealed a number of differences in the markers expressed following differentiation with the different substances. These structural changes affect the function of the cell lines, as U-937 were found to incorporate *M. tuberculosis* quite efficiently following PMA, but not IFN-γ or VD_3, differentiation (Passmore *et al.,* 2001). This may be due to CR3 expression, which is closely related to PMA activation (Gilbert *et al.*, 1985).

U-937 and radiation

The response of eukaryotic cells to ionizing radiation includes cell cycle arrest and activation of DNA repair. G1 and/or G2 arrest is associated with increased expression of p53 protein (Kastan *et al*., 1991). In U-937, p53 expression is associated with apoptosis and differentiation (Chylicki *et al*., 2000; Ehinger *et al*., 1998). Studies performed using irradiated U-937 cells have focused on the mechanisms by which ionizing radiation causes apoptosis.

A study to determine the effects of non-lethal low-dose irradiation and p53 induction on U-937 found that p53 was not induced by low-dose radiation (< 1 Gy), although the cell line was arrested in G2/M from higher doses (3 Gy) of γ-radiation (Hendrikse *et al.*, 2000). It was also noted that p21 levels in U-937 were unaltered by low-dose radiation. This is in contrast to the findings of Roig & Traugh (1999) that showed a 2-5 fold increase in activation of p21-activated kinase following ionizing doses ranging between 1 and 100 Gy.

Galan *et al.*, (2001), found that treatment of U-937 with the stress inducers, including X-rays (20 Gy), stimulated caspase-3 activity and caused death by apoptosis. The authors suggest that ionizing radiation causes intracellular oxidation (as measured by peroxide and/or anion superoxide accumulation), but that oxidation is not a general

requirement for apoptosis. The duration of the oxidant state seems to be critical in determining the mode of death (Galan *et al.*, 2001).

Datta *et al.*, (1997), found that ionizing radiation of U-937 was associated with internucleosomal DNA fragmentation and cleavage of Poly (ADP-ribose) polymerase (PARP) and PKCδ. The mechanism by which this occurred was via a p35-sensitive mechanism, and represented a distinct signalling cascade, different from the TNF-α pathway normally associated with radiation induced apoptosis (Datta *et al.*, 1997). Another pathway that is associated with apoptosis after radiation is described by Kharbanda *et al.*, (1994). It was shown that ionizing radiation of U-937 is associated with the induction of mitogen-activated protein kinase activity, and that this event is temporally related to activation of the 40S ribosomal protein S6 kinase, pp90rsk. The findings of the study suggest that activation of the mitogen-activated protein kinase/pp90rsk cascade is involved in the response of U-937 to ionizing radiation (Kharbanda *et al*., 1994).

U-937 and silica

Hetland *et al.*, (2000) describes the mechanism of silica uptake by U-937. The study showed that plasma-treated silica activates the alternate complement pathway, but not the classical pathway. Despite complement activation, only 1% of C3b/iC3b bound the silica particles in these experiments. Scanning electron microscopy demonstrated that more silica particles were phagocytosed by differentiated U-937 than undifferentiated cells. Flow cytometry phenotyping of U-937 receptors indicated that PMA differentiation induced CR4, but down-regulated FcγR1 and CR3 expression. IFN-γ differentiation induces expression of FcγR1, CR1, CR3 and CR4. Hetland *et al.*, (2000) concludes that plasma-treated silica bind immunoglobulin passively, and are internalised by IFN-γ differentiated U-937 via FcγR1. The authors hypothesize that uptake of opsonized silica by FcγR1, and not CR3 alone, may trigger the production of ROS in these cells.

Hass *et al*,. (1990b) examined the alterations in glycosylation and lectin pattern during differentiation, and showed that alterations in cell-surface carbohydrates are central to attachment of differentiating U-937 cells. Trabelsi *et al.*, (1997) examined the effect of mineral particles on protein glycosylation in differentiated U-937. Jacalin, a lectin

specific for O-glycosylated structures, was increased in particle-treated cells. *Sambucus nigra* agglutinin, *Maackia amurensis* agglutinin and *Ricinus communis* agglutinin revealed a complex pattern of alterations in glycoprotein glycosylation after silica treatments. No significant modifications were observed with Con A. The potential functional implications of these changes are still to be determined. Trabelsi *et al.*, (1998) also showed that shedding of CD44 from PMA differentiated U-937 was enhanced by mineral particles. Shedding was particle specific, but the ability of the particles to enhance CD44 shedding was not directly dependent on their cytotoxic potency.

Mycobacterial growth and U-937

CR3-mediated lectin-sugar recognition mechanisms have been shown to play a major role in the nonopsonic phagocytosis of several pathogens, including *M. tuberculosis* (Cywes *et al.*, 1996). Using Chinese hamster ovary cells stably transfected with CR3, Lé Cabec *et al.* (2000) showed that CR3 is able to mediate internalization of zymosan and pathogenic mycobacteria (*M. kansasii* and *M. avium*), but not that of non-pathogenic mycobacteria (*M. smegmatis* and *M. phlei*). Using 9-*cis* retinoic acid and VD_3 differentiated U-937; it was observed that zymosan ingestion through CR3 induced superoxide production, whereas phagocytosis of viable or heat-killed *M. kansasii* did not. Lack of superoxide anion production during phagocytosis of *M. kansasii* was not due to inhibition of Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) oxidase or superoxide anion scavenging. It would appear that nonopsonic phagocytosis of zymosan and *M. kansasii* by CR3 implicates different molecular mechanisms involving multiple and distinct epitopes of CD11b (Lé Cabec *et al.,* 2000).

Intracellular survival plays a central role in the pathogenesis of *M. tuberculosis*. Wei *et al.*, (2000) identified a gene that is makes it possible for *M. smegmatis* to survive within differentiated U-937 after phagocytosis. The gene was isolated from one clone (p69), and shown to enhance intracellular survival of *M. smegmatis* 2.4- to 5.3- fold, 24 and 48 hours after infection. The gene was named the enhanced intracellular survival (*eis*) gene. *Eis* was found in the genomic DNA of various *M. tuberculosis* strains and in *M. bovis* BCG but not in *M. smegmatis* or ten other non-pathogenic mycobacterial species suggesting a possible role for *eis*, and its protein product, in the intracellular survival of *M. tuberculosis* (Wei *et al.*, 2000). Further studies suggested that *eis* was associated

with the cell surface of mycobacteria. Forty percent of the sera from pulmonary tuberculosis patients tested for anti-*eis* antibody gave positive reactions in Western blot analysis (Dahl *et al.*, 2001).

Takii *et al.*, (2001) suggested an *in vitro* system, using differentiated U-937, to evaluate the pathogenesis of mycobacteria. U-937 and THP-1 cells were killed by virulent mycobacteria, after they were phagocytosed using this system. Twenty-four *M. tuberculosis* clinical isolates (some of which were drug resistant [n = 13]) and five *M. avium* strains were evaluated. All *M. tuberculosis* strains exhibited strong cytotoxic activity, but *M. avium* strains had only weak activity. There was no significant difference in cytotoxicity between drug susceptible and drug-resistant strains (Takii *et al*., 2001). Passmore *et al.*, (2001) evaluated U-937 cells as an *in vitro* model for HLA class I-restricted presentation of mycobacterial antigens, as HLA class I is constitutively expressed at high levels by U-937 cells in the absence of detectable HLA class II or CD1 molecules. Differentiated U-937 cells were capable of efficient phagocytosis of *M. tuberculosis* but did not generate a subsequent respiratory burst response, and were permissive for intracellular growth of both *M. bovis* BCG and *M. tuberculosis* H37Rv. CTL activity was restricted to live mycobacterial organisms and was shown to be mediated by *M. tuberculosis*-specific, HLA class I-matched, purified $CD8^+$ CTL lines and $CD8^+$ T-cell clones (Passmore *et al.*, 2001).

2.4.2 THP-1 leukaemic cell line

THP-1 was derived from the blood of a one-year-old boy with acute monocytic leukaemia (Tsuchiya *et al*., 1980). At the time of isolation and culture, 70% of the patient's leukocytes were monoblasts. Isolated cells were shown to differ from lymphoblastoid cell lines, and their histiocytic origins were demonstrated by their capacity to produce lysozyme and esterase. It was concluded that the cell line THP-1 is promonocytic *i.e.* it is a precursor of the monocyte/macrophage lineage.

THP-1 cells grow as a single cell suspension. In culture, stained smears show that THP-1 cells are either round or polygonal, with a diameter of 12-14 µm. The cells have a moderate amount of basophilic cytoplasm, which contain small azurophilic

granules, and a few vacuoles. The cytoplasm contains mitochondria, a rough endoplasmic reticulum and Golgi apparatus. The nucleus is folded, with a well-defined nucleolus. There are a number of cored vesicles. Investigations to determine the presence of surface receptors yield both complement (C3b/CR1) and Fcγ-receptors. No surface immunoglobulin can be detected. THP-1 cells are able to ingest one or more latex particles or sheep red blood cells. Phagocytosis increases after cells are sensitized. 12.7% of cells actively phagocytise one to six sensitized sheep red blood cells. Phagocytosis via Fc-receptors is completed in less than 30 minutes.

The differentiation of THP-1 leukaemic cells into activated macrophages is described by Tsuchiya *et al.*, (1982). TPA induces THP-1 cells to adhere to the substratum. Approximately 80% of the cells are adherent three hours after TPA treatment. A number of morphological changes are observed 24 hours after TPA treatment: cytoplasmic vacuoles are increased in number, the nuclei become reniform and distinct nucleoli can be observed. Cells on the substratum are irregular shaped and flattened. The nuclear pole may be slightly raised, with a complete loss of microvilli and flaps. Many vesicles, including the cored vesicles observed in the untreated cells, disappear upon TPA treatment (Tsuchiya *et al*., 1982).

Tsuchiya *et al.*, (1982) examined the effect of TPA treatment on the expression of surface receptors. Ninety seven percent of cells were IgG Fc receptor positive after TPA treatment. Little change in the expression of IgG Fc, C3b and C3d are noted. Compared to untreated cells, TPA treated cells show a dramatic increase in their ability to phagocytise yeasts and sensitized sheep red blood cells. Approximately 74% of TPA treated cells are able to phagocytise yeasts compared to 9.8% of the untreated cells.

Adherent TPA treated THP-1 cells are considered to be part of the mononuclear phagocytic series by virtue of their lysozyme activity, presence of IgG Fc receptors, increased phagocytosis and positive naphthyl butyrate esterase staining. TPA treatment differentiates THP-1 cells to form activated macrophages (Tsuchiya *et al*., 1982).

THP-1 and radiation

Studies utilising irradiated THP-1 cells have focused on the free radical production that is associated with radiation damage. Irradiation increases the generation of reactive oxygen intermediates, including hydrogen peroxide. Headlam & Davies, (2003), used THP-1 cells to investigate the intracellular reduction of peptides and protein hydroperoxides. Protein hydroperoxides are decomposed by transition metal ions, reducing agents, UV light and heat, with the formation of a range of reactive radicals that are capable of initiating cellular damage. In THP-1, the free radicals detected following peptide and hydroperoxide reduction, were similar to those detected from metal-ion catalysed reduction. The free radicals were generated externally to the cell, and it was found that intact cells were required for rapid hydroperoxide decomposition (Headlam & Davies, 2003).

Hachiya *et al.*, (2000) examined the potential protective effects of myeloperoxidase (MPO) on THP-1 and HL60 cells, following free radical production. MPO is a heme-containing glycoprotein located in the primary granules of polymorphonuclear leukocytes and monocytes. It catalyses the dismutation of hydrogen peroxide to water and hypochlorous acid, and plays an important role in the oxygen-dependent antimicrobial system. Hachiya *et al.*, (2000) found that THP-1 cells contained high concentrations of MPO transcripts, the expression of which was reduced following irradiation. There was a concomitant increase in TNF-α mRNA expression in a dose-dependent manner. Furthermore, the study reported increased transcription of several genes including GM-CSF and manganese superoxide dismutase (MnSOD) following irradiation of HL60 cells. MnSOD is a mitochondrial enzyme that scavenges superoxide anions. In a previous study, Hachiya *et al.*, (1997) found that irradiation increased TNF production in THP-1 cells, and TNF increased the levels of MnSOD transcripts. The authors conclude that increases in MnSOD mRNA caused by irradiation are regulated by both transcriptional and post-transcriptional mechanisms through a pathway involving PKC activation.

THP-1 and silica

Early studies on silica and THP-1 did not focus on the study of the pathogenesis of silicosis, but rather on utilising silica to produce IL-1. Krakauer & Oppenheim, (1983), conclude that THP-1 cells exposed to silica can serve as a valuable source of

relatively homogeneous human IL-1, which can be used for further purification and molecular characterization.

The first report that utilized THP-1 to study the pathogenesis of silicosis are described by Savisi, (1994). Savisi, (1994), examined the release of TNF from mononuclear phagocytes. Silica causes the release of TNF in THP-1 cells by up regulating the TNF gene. It was found that there was a dose-dependent increase in TNF mRNA three hours after silica exposure. THP-1 cells cultured for various periods in the presence of silica released TNF into the cell supernatants (Savici *et al*., 1994).

Chen *et al.*, (1996) found that silica is a potent inducer of nitric oxide production in THP-1 cells, suggesting that in the lung, silica has the ability to induce tissue damage by initiating an excessive production of nitric oxide from macrophages. It was found that silica elicited a marked stimulation of NO production in a time-dependent manner by THP-1 cells *in vitro* following PMA differentiation. Both NO synthase inhibitor, N-monomethyl-L-arginine, and xanthine oxidase inhibitor, allopurinol, were found to partially suppress silica-induced NO production in PMA-differentiated THP-1 cells. Northern blot analysis indicated that, after two hours of silica exposure, PMA-primed THP-1 cells began to express induced NO synthase (iNOS) mRNA, which reached peak expression after 8 hours.

Further studies utilising THP-1 to understand the pathogenesis of silicosis were performed by Mao, (2000). In a rat model, it was found that the supernatant of silica-stimulated THP-1 cell cultures could effectively enhance proliferation of fibroblasts and inhibit the migration of pulmonary alveolar epithelial cells (CCL-64). The supernatants from silica-stimulated pulmonary alveolar macrophages and THP-1 cell culture were also found to cause early silicotic nodule-like lesions in rats (Mao *et al.*, 2000).

THP-1 and mycobacterial growth

THP-1 is widely used for studies to identify mycobacterial virulence factors (Guerardel *et al*., 2003), to determine the mechanism of mycobacterial intracellular survival (Agranoff *et al*., 1999; Maiti *et al*., 2001), for drug susceptibility testing

(Arain *et al.*, 1996) and for identifying potential drug target sites (Harth & Horwitz, 1999).

Stokes & Doxsee, (1999), provide an important report that describes the interaction of *M. tuberculosis* with THP-1. In this study, the interaction of *M. tuberculosis* with THP-1 and human monocyte-derived macrophages is compared. It was found that *M. tuberculosis* grows progressively in THP-1 and human monocyte-derived macrophages. Serum-mediated binding is much greater than non-opsonic binding. Non-opsonic binding of *M. tuberculosis* to both cells types could be inhibited by antibodies recognizing CD11b and by mannan and glucan. Differentiated THP-1 cells bind IgG and complement-coated particles at levels similar to those of monocyte-derived macrophages. However, binding of zymosan by THP-1 cells is significantly lower than that seen for monocyte-derived macrophages (Stokes & Doxsee, 1999). Riendeau (2003) evaluated the potential for PMA-differentiated THP-1 cells to mimic this response of primary macrophages. *M. tuberculosis* infection primes human alveolar macrophages for TNF-α mediated apoptosis, and macrophage apoptosis is associated with killing internalized bacilli. Virulent mycobacterial strains elicit much less apoptosis than attenuated strains, implying that apoptosis is a defence against intracellular infection (Miyazaki *et al.*, 1999). Consistent with the behaviour of alveolar macrophages, *M. tuberculosis* H37Ra and *M. bovis* BCG strongly induce THP-1 apoptosis, a process that requires endogenous TNF. It was later found that *M. bovis* BCG induces TNF-α secretion in human monocytic THP-1 cells (Oliveira *et al.*, 2001). THP-1 apoptosis is associated with reduced viability of infecting *M. bovis* BCG. In contrast, virulent wild-type *M. tuberculosis* H37Rv and *M. bovis* do not increase THP-1 apoptosis over baseline. *M. bovis* BCG induces the early activation of caspase 10 and 9, followed by caspase 3. In contrast, wild-type *M. bovis* infection fails to activate any caspases in THP-1 cells. (Riendeau and Kornfeld, 2003). TK and PKC signals are activated during the interaction of human monocytes with both pathogenic and attenuated species of mycobacteria. (Lima *et al.*, 2001).

After phagocytosis, monocytes present the mycobacterial 65-kD heat shock protein (*hsp*) on their cell surface to αβ- and γδ- T lymphocytes. Cytotoxic $CD4^+$ cells then lyse the monocytes expressing the hsp as part of the antigen-specific T-cell-mediated immunity against mycobacteria. Friedland *et al.*, (1992) demonstrated that THP-1

cells are capable of cytokine production. Following extraction of bacterial lipopolysaccharide, purified recombinant mycobacterial *hsp* directly activates THP-1 to secrete TNF, IL-6 and IL-8 in a dose dependant manner (Friedland *et al*., 1993). *In vitro* labelling of mycolic acids during growth of *M. tuberculosis* H37Rv within THP-1 cells reveals a substantial increase in ketomycolate and alphamycolate synthesized by intracellularly grown mycobacteria. These results suggest a critical role of mycolate composition in proper cell wall function during the growth of *M. tuberculosis in vivo* (Yuan *et al.*, 1998). *M. tuberculosis* and *M. bovis* BCG encode Natural resistance associated protein gene (Nramp) homologue called Mramp. Nramp homologues transport divalent cations such as iron, magnesium, zinc and copper within the macrophage. *M. bovis* BCG cultured axenically and within THP-1 cells expresses mRNA encoding Mramp. Within phagosomes, Mramp and Nramp may compete for the same divalent cations, with implications for intracellular survival of mycobacteria (Agranoff *et al.* 1999). Further studies have identified an inhibitor of *M. tuberculosis* glutamine synthetase that selectively blocks the growth of pathogenic *M. tuberculosis* in axenic cultures and within THP-1 cells (Harth & Horwitz, 1999).

De Lerma (1999), investigated expression of HLA and CD1b molecules in THP-1. It was found that during the first two to three days, cell surface expression of HLA class II and CD1b is drastically down modulated, whereas HLA class I expression is up-regulated. In the following days, both HLA class II and CD1b expression first returns to normal, then increases, and finally returns to normal with kinetics similar to that observed for the steadily increased HLA class I. The initial down-modulation of HLA class II and CD1b cell surface antigens is dependent on phagocytosis of the mycobacteria. The authors suggest that the existence of a transient block of transport of mature HLA class II heterodimers to the cell surface in the first days after phagocytosis of mycobacteria may have both negative and positive consequences for the cell. One the one hand it may decrease early APC function, but on the other hand it may increase APC function later by favouring optimal loading of bacterial antigens in cellular compartments at high concentration of antigen-presenting molecules (De Lerma *et al.*, 1999).

Takii *et al.*, (2001) suggested an *in vitro* system, using differentiated THP-1, to evaluate the pathogenesis of mycobacteria. U-937 and THP-1 cells were killed by

virulent mycobacteria, after they were phagocytosed using this system. Twenty-four *M. tuberculosis* clinical isolates (some of which were drug resistant [n = 13]) and five *M. avium* strains were evaluated. All *M. tuberculosis* strains exhibited strong cytotoxic activity, but *M. avium* strains had only weak activity. There was no significant difference in cytotoxicity between drug susceptible and drug-resistant strains (Takii *et al.*, 2001).

One report, by Guerardel *et al.*, (2003), describes the interaction between *M. kansasii* and THP-1. The detailed structures of the of *M. kansasii* LAM (KanLAM), as well as one of its biosynthetic precursors, lipomannan (KanLM), were determined in a clinical strain isolated from a HIV-positive patient. The ability of these glycolipids to induce apoptosis of THP-1 cells was examined. In contrast to KanLAM, KanLM was a potent apoptosis-inducing factor in THP-1 cells. The authors suggest that LM may be a potential virulence factor in *M. kansasii* infections by inducing apoptosis of macrophages (Guerardel *et al.,* 2003).

2.5 TUBERCULOSIS RISK FACTORS ON SOUTH AFRICAN MINES

Occupational tuberculosis is defined as tuberculosis disease contracted in the course of specific employments that result in the contact with the tubercle bacilli as an inherent factor in such employment. Kleinschimidt and Churchyard (1997) identified a number of specific risk factors associated with tuberculosis in South African gold miners. In a retrospective cohort analysis, 5000 medical and occupation records of mineworkers were examined to determine the incidence ratios of different categories of worker. This study suggested a strong association between age and tuberculosis, even after adjustment for known confounders. There was an association between silicosis and age, which suggested that the high incidence of tuberculosis in older men might be due to undetected silicosis. The study also found a significant association between tuberculosis incidence and occupations associated with dust and/or silica. This finding confirms the findings of the Leon Commission (Leon *et al.*, 1995), in which it was found that there was an association between occupation and tuberculosis incidence.

Kleinschimidt and Churchyard (1997), report that since 1991 there has been a dramatic increase in the prevalence of HIV infection amongst mineworkers. In a case control study, Corbett reports on the strong association between tuberculosis and HIV infection in mineworkers in South Africa (Corbett *et al.*, 1999b). In this study, the HIV prevalence of miners with tuberculosis was 14.2%, compared to a prevalence of 5.6% in a control group that had no symptoms. Churchyard *et al.* (1999), suggests that HIV infection is the greatest risk factor for developing tuberculosis on the mines. In a retrospective cohort study of 305 HIV infected and 984 HIV uninfected miners, Mallory *et al.* (2000), reports that the high recurrence rate among HIV infected workers requires further investigation to distinguish between relapse and re-infection in this group.

The risk of HIV infected individual developing tuberculosis is further complicated by silicosis. In a retrospective cohort study, Corbett *et al.*, (2000) found that that the risks of silicosis and HIV infection combine multiplicatively so that HIV positive silicotics have considerably higher tuberculosis incidence than other HIV positive Africans. These findings are similar to those of Churchyard who found HIV infection and silicosis to be associated with an increased risk of death from opportunistic infections (Churchyard *et al.*, 1999).

2.5.1 NTM disease on South African mines

It is known that both *M. kansasii* and *M. scrofulaceum* are associated with pulmonary disease in dusty occupations in South Africa (Nel, 1977). Corbett, estimates the incidence of *M. kansasii* disease to be 66/100 000 person years (Corbett *et al.*, 1999c). Kleeberg (1981) was unable to isolate *M. kansasii* from rural populations, suggesting that its high prevalence of *M. kansasii* is an anomaly specific to the mines. The widespread occurrence of NTM's (specifically *M. kansasii*) in the mines is reported by both Corbett *et al.* (1998) and Cowie (1990). Cowie is unable to verify the role of the NTM's isolated in a drug resistance study (NTM's were isolated in 21% of miners, 67% of which were *M. kansasii*) as the single cultures submitted do not fulfil the diagnostic criteria of NTM disease. Corbett *et al*. (1998) reports that in miners isolates of *M. kansasii* and *M. scrofulaceum* are usually associated with positive

sputum smears, new radiographic changes and specific risk factors such as silicosis. In a retrospective case series, she investigated the clinical significance of NTM's in HIV negative miners (Corbett *et al.*, 1999d). She concludes that, as in other studies (O'Brien *et al.*, 1987), the isolation of either *M. kansasii* or *M. scrofulaceum* from sputum is likely to represent pulmonary NTM disease.

In a retrospective case-control investigation, Corbett *et al.* (1999a) examined the risk factors for developing non-tuberculous pulmonary mycobacterial disease and tuberculosis in gold miners. This study found that the historically high incidence of pulmonary disease from NTM's was largely attributable to chronic chest disease from silica (Odd's ratio 5.0 and 7.4), and previous tuberculosis (Odd's ratio 9.6). In cases of pulmonary tuberculosis, chronic chest disease from silica (Odd's ratio 4.9) and HIV infection (Odd's ratio 4.5) were the greatest risk factors. Unlike Kleinschimidt and Churchyard, (1997), this study did not find age to be an independent risk factor for tuberculosis. The reason for this may be due to the selection of the control group and study design, but it is emphasized that the determination of age-related changes in tuberculosis susceptibility is very difficult given the association between age and cumulative silica exposure. When found in association with HIV infection, it was noted that NTM disease occurs at an early stage of immunosuppression (median $CD4^+$ count 3.8×10^7), and has a good outcome, with cure rates greater than 80% (Corbett *et al.*, 1999b). Furthermore, there appears to be no significant difference in the bacteriological spectrum of NTM isolated from HIV positive and HIV negative miners. However, there were differences in the way disease manifested in these two groups. HIV positive miners were less likely to have new cavitations and less clear-cut upper field predominance on chest radiograph (Corbett *et al.*, 1999b).

CHAPTER 3: GENERAL PRINCIPLES AND METHODOLOGY

Considering the high prevalence of pulmonary disease associated with *M. tuberculosis* and *M. kansasii* in South African miners, it was hypothesized that silica and low-dose ionizing radiation exposure may both be involved in predisposing gold mine workers to pulmonary tuberculosis (or pulmonary disease due to *M. kansasii*) by their effects on impairing macrophage ability to resist mycobacteria.

The objective of this study was to establish if a synergistic relationship exists between the effects of silica and low-dose ionizing radiation in predisposing promonocytic cell lines U-937 and THP-1, to mycobacterial infection. The study:

- examined the cytotoxicity of silica or low-dose γ-radiation exposure or infection by *M. bovis* BCG and *M. kansasii* on U-937 and THP-1, and compared this to cytotoxicity of silica and/or low-dose γ-radiation exposure on U-937 and THP-1 cells subsequently infected with *M. bovis* BCG or *M. kansasii,*

- examined the mechanisms of death (apoptosis/necrosis) in U-937 or THP-1 after silica or low-dose γ-radiation exposure or infection by *M. bovis* BCG and *M. kansasii*, and compared this to the mechanisms of death in U-937 and THP-1 after silica and/or low-dose γ-radiation exposure in U-937 and THP-1 cells subsequently infected with *M. bovis* BCG or *M. kansasii,*

- examined the DNA damage caused by silica or low-dose γ-radiation exposure or infection by *M. bovis* BCG and *M. kansasii* on U-937 and THP-1, and compared this to DNA damage caused by silica and/or low-dose γ-radiation exposure on U-937 and THP-1 cells subsequently infected with *M. bovis* BCG or *M. kansasii,*

- examined the ability of U-937 and THP-1 cell lines to control the intracellular growth of *M. bovis* BCG and *M. kansasii* after exposure to silica and/or low-dose γ-radiation,

- examined the TNF-α production of U-937 and THP-1 following exposure to silica or low-dose γ-radiation exposure or infection by *M. bovis* BCG and *M. kansasii*, and compared this to the TNF-α production following exposure to silica and/or low-dose γ-radiation in U-937 and THP-1 cells subsequently infected with *M. bovis* BCG or *M. kansasii.*

3.1 U-937 AND THP-1 CELLS

Logistic restrictions and heterogeneity within, and between human mononuclear cells present a significant obstacle in the study of macrophage-mycobacterial interactions. U-937 and THP-1 are promonocytic cell lines that are precursors of the monocyte/macrophage lineage. The homogenous nature of these two cell lines makes them ideal to study the interaction between macrophages and mycobacteria (Passmore *et al.*, 2001). In this study, U-937 and THP-1 cell lines are chosen for the contrasting manner in which they control tuberculous infection. Passmore (2001) found that differentiated U-937 cells were capable of efficient phagocytosis of *M. tuberculosis*, and were permissive for intracellular growth of both *M. bovis* BCG and *M. tuberculosis* H37Rv. Stokes & Doxsee, (1999), found that attenuated strains such as H37Ra and *M. bovis* BCG induce apoptosis, and are associated with the reduced viability of the infecting organism in THP-1. Combined, the two cell lines represent possible scenarios by which the alveolar macrophage may respond to infection with mycobacteria, and represent different levels of maturation of the macrophage. THP-1 cells are considered more mature, behaving like macrophages in many ways, only differing significantly in their expression of lectin-like receptors (Stokes & Doxsee, 1999).

U-937 (ATCC CRL-1593.2) and THP-1 (ATCC TIB-202) were routinely maintained in suspension culture in RPMI-1640 containing 25 mM HEPES and L-glutamine (BioWitakker, Walkersville, ML, USA) and supplemented with 10% heat-inactivated fetal bovine serum (FCS) (Delta Bioproducts, Kempton Park, RSA). Cultures were incubated at 37˚C in 3-5% carbon dioxide (CO_2). Cultures were maintained by the addition of fresh medium or replacement of medium every three to four days. The cell density was maintained between 1×10^5 and 2×10^6 cells/ml. Frozen stocks were prepared in 80% FCS and 20% dimethyl sulphoxide (Sigma, St. Louis, MO, USA).

Cultures were washed in RPMI-1640 supplemented with 5% FCS before subculture. Cultures were induced to differentiate by exposing cells 5 ng/ml PMA (Sigma, St. Louis, MO, USA) in 96 or 24 well plates for 48 hours.

3.2 *M. BOVIS* BCG AND *M. KANSASII*

The two mycobacterial strains chosen for use in this study are *M. bovis* BCG and *M. kansasii*. *M. bovis* BCG belongs to the *M. tuberculosis* Complex, and is a good immunological surrogate for *M. tuberculosis* (van Reyn & Vuola, 2002). It should be noted though, *M. bovis BCG* is an avirulent mycobacterial strain, and is only a surrogate for *M. tuberculosis* infection. *M. kansasii* is the most common NTM isolated from miners with pulmonary disease other than tuberculosis (Corbett *et al.*, 1999d). *M. bovis* BCG (Copenhagen strain) and *M. kansasii* (ATCC 25294) were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% Oleic Albumin Dextrose Citrate (OADC) (Remel, Lenexa, KS, USA) at 37°C 5-10% CO_2. Mid-log phase cultures were supplemented with 20% glycerol, divided into aliquots, and stored at -70°C until needed. Viability of the frozen mycobacterial strains were determined by determining the Colony Forming Units (CFU) of 10-fold serial dilutions on Middlebrook 7H11 agar (Difco Laboratories, Detroit, MI, USA) supplemented with 10% OADC. CFU determination was determined after incubation of plates at 37°C 5-10% CO_2 for three weeks. Frozen aliquots were thawed and clumps were immediately dispersed by repeated passage through a 25-gauge tuberculin needle (Passmore *et al.*, 2001). Mycobacteria were opsonized in AB- human serum at 37˚C for 30 minutes. U-937 and THP-1 were infected by adding the mycobacteria to a multiplicity of 0.1:1 and 10:1 respectively.

3.3 U-937 AND THP-1 EXPOSURE TO SILICA, γ-RADIATION, *M. BOVIS* BCG AND *M. KANSASII*

U-937 and THP-1 cells in mid-logarithmic phase growth were concentrated by centrifugation. Cells were re-suspended in fresh RPMI-1640 supplemented with 10% FCS to give 1×10^5 cells/ml. Aliquoted cells were induced to differentiate by treatment with PMA (5 ng/ml) for 48 hours at 37˚C 3-5% CO_2. After differentiation,

the wells were examined microscopically for signs of differentiation. Two models for experimentation were used in this study (a diagrammatic representation of testing is shown in Appendix 1):

(i) U-937 and THP-1 cells were either exposed to silica [only] or γ-radiation [only] (these are referred to as exposed cells) or infected by *M. bovis* BCG or *M. kansasii* [only] (these are referred to as infected cells). Control cells were neither infected nor exposed.

(ii) To determine the effect of silica and radiation exposure in infected cells, U-937 and THP-1 cells were exposed to combinations of silica and γ-radiation, and then infected with *M. bovis* BCG or *M. kansasii*. Control cells were infected, but not exposed.

The choice of silica concentration for these studies is based on findings from similar *in vitro* silicosis models. Allison *et al.* (1966) describes in detail the cytotoxic effects of silica on human macrophages. In these studies, PBMC's were exposed to between 10 and 100 μg/ml silica. Hetland *et al.*, (2000) describes the mechanism of silica uptake by U-937. In this study, a single concentration of 100 μg/ml silica is used throughout the study. The exposure time for the former study was up to 30 hours, while for the latter it was 24 hours. A range of silica concentrations including, and exceeding that used in previous studies, was chosen (25 μg/ml, 100 μg/ml and 500 μg/ml). Initial exposure times of 24-48 hours were chosen, with observations being made 90 minutes, 24, 72 and 120 hours post-infection. Experiments that included infection by mycobacteria resulted in exposure times of 144 – 168 hours, significantly longer than in previous studies. Hetland *et al.*, (2000) conclude that silica is taken up via complement receptors making the opsonization of silica in these experiments essential. The experiments described in the present study are designed to mimic *in vivo* silicosis, radiation and mycobacterial exposure. However, silicosis is a chronic illness, the effects on the lung being the result of years of cumulative damage, and the exposure times used in this study are short and thus cannot accurately mimic chronic silicosis (although they exceed the exposure times used in previous studies).

In the current study, experiments requiring silica exposure, SiO_2 (Sigma, St. Louis, MO, USA) particles, 0.5-10 µm (80% between 1-5 µm), were opsonized in AB^- human serum at 37˚C for 30 minutes. SiO_2 was added to the wells of differentiated U-937 or THP-1 to give final concentrations of 25, 100 and 500 µl/ml. The cells were re-incubated for 48 hours at 37˚C 3-5% CO_2. The SiO_2 concentrations used in the study are high when considering the limited concentration of SiO_2 particles that can penetrate the lung defence. The silicosis model that is suggested represents the endpoint of exposure and/or infection. The radiation hazard associated with underground atmospheres in mines is highly variable. In South African gold mines, the concentration of uranium may be as much as twenty-five times higher than in the earth's crust (Wymer, 2001). In South Africa, only 2.6% of miners receive more than 20 mSv/year, the upper limit recommended by the ICRP (International Commission on Radiobiological Protection, 1991). The estimated α-particle dose of radon progeny to the peripheral lung is 9.8 ±1.2 mGy/WLM (Johnson & Newton, 1994), but this is not associated with decreased macrophage function (Johnson & Newton, 1994; Taya *et al.*, 1994). The choice of radiation dose for these studies is based on the lowest practical dose that could be achieved, and the highest non-lethal dose, as based on the relative sensitivity of undifferentiated cells (Hendrikse *et al.*, 2000). It is estimated that 25% of U-937 and THP-1 cells are apoptotic two hours after receiving 20 Gy γ-radiation (A. Hunter- personal communication). The radiation dose received by miners is an accumulation of α-, β- and γ- particles (Cothern, 1985). Work with α-, β-particles are impractical in such a study, so thus the type of radiation used is not truly representative of *in vivo* exposure. It is hypothesized cumulative radiation damage to alveolar macrophages may be the cause of failure to defend against mycobacterial infection. Cumulative radiation dose could not be reproduced, and neither was the adaptive response investigated. The radiation dose to the cell lines is high considering the low-dose radiation that most miners receive, however, both U-937 and THP-1 are cancerous cell lines, and are inherently more resistant to radiation than other human tissues. In this regard though, it should be noted that macrophages themselves are also less radiosensitive than other leukocytes (Mettler & Upton, 1995). In the current study, in experiments requiring γ-radiation exposure, U-937 and THP-1 were expose to 1 Gy, 3 Gy or 10 Gy ^{60}Co γ-rays, at a dose rate of 0.75 and 0.88 Gy/min.

3.4 STATISTICAL ANALYSIS

Statistical analysis was performed using the two-sample t-test of means (2-tailed), One Way-Analysis of variance (ANOVA), 95% confidence interval and linear regression using Analyse-it for Microsoft Excel, Leeds, UK (http://www.analyse-it.com/Analyse-it) or AcaStat™ software, Ashburn, USA (http://www.acastat.com). The homogeneity of variance was determined before performing t-test and ANOVA computations. In the case of unequal variances, alternative calculation methods, to adjust for heterogeneity of variance (Cochran Cox method), were employed. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant. All graphs were plotted using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The number of experiments (n) represents the number of independent experiments performed. The number of replicates in each independent experiment varies between experiments (2 to 5). Error bars on the graphs represent one standard deviation of the mean. Box-plots show the median, 25^{th} and 75^{th} percentile, whiskers are the maximum and minimum readings.

CHAPTER 4: CELLULAR MORPHOLOGY AND CYTOTOXICITY

4.1 INTRODUCTION

Cytotoxicity is defined as the quality or state of being cytotoxic, and refers specifically to agents that are directly toxic to cells *i.e.* preventing their reproduction or growth. Cytotoxic agents damage healthy, cells or tissues. *In vitro* studies found that silica is toxic for human macrophages (Allison, 1971; Allison *et al.*, 1966). In these studies, silica demonstrated two forms of cytotoxicity; a rapid cytotoxicity produced when relatively large amounts of silica is added to serum-free medium, and a delayed cytotoxicity that occurred in serum-containing medium after cells have digested the protein coat on the silica particle (deShazo, 1982). In U-937 cells exposed to silica it was found that plasma treated silica particles activated the alternative complement pathway, and that there was increased expression of CR4 after PMA treatment (Hetland *et al.*, 2000). THP-1 cells have a wide application in study of the pathogenesis of silicosis and production of silicosis-related cytokine and oxygen free radicals *in vitro* (Mao *et al*., 2000). However, experiments to date have not examined the cytotoxic effect of silica on the morphological and functional characteristics of U-937 and THP-1.

The exposure of cultured cells to sub-lethal doses of ionising radiation results in transient changes in their cell cycle. These changes include G1 and/or G2 arrest, as observed by increased expression of p53 protein (Kastan *et al.*, 1991). A study to determine the effects of non-lethal low dose irradiation and p53 induction on U-937 found that p53 was not induced by low dose radiation (< 1 Gy), although the cell line was arrested in G2/M from higher doses (Hendrikse *et al.*, 2000). Studies using irradiated THP-1 cells have focused on free radical production in these cells. No studies have been performed that examine the cytotoxic effect of radiation on U-937 and THP-1.

In macrophages, avirulent or attenuated bacilli (*M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. kansasii*) induce significantly more cytotoxicity than virulent strains (*M. tuberculosis* H37Rv, Erdman, *M. tuberculosis* clinical isolate BMC 96.1, and *M. bovis* wild type) (Keane *et al*., 2000). Consistent with the behaviour of alveolar macrophages,

attenuated *M. tuberculosis* H37Ra and *M. bovis* BCG strongly induce THP-1 apoptosis and are thus cytotoxic for THP-1. In contrast, U-937 are permissive for intracellular growth of both *M. bovis* BCG and *M. tuberculosis* H37Rv (Passmore *et al*., 2001). The cytotoxic effects of *M. kansasii* have not been studied in either U-937 or THP-1.

It was the objective of the experiments described in this chapter, to determine the extent of cytoxicity in exposed and/or infected U-937 and THP-1. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide (MTT) assay was first described by Mosmann (1983), and is based on the cellular conversion of a tetrazolium salt into a formazan product by viable cells that is detectable as an optical density (OD) using an Enzyme Linked Immunosorbant Assay (ELISA) spectrometer. The MTT assay has been used in proliferation, apoptosis and cytotoxicity assays (Nikš *et al.*, 1990) on a number of cell types, including U-937 (van de Loosdrecht *et al.*, 1991) and THP-1 (Gebre-Hiwot *et al.*, 1992).

4.2 MATERIALS AND METHODS

4.2.1 Cellular morphology

In 8 or 16-well Lab-Tek® Chamber Slides™ (Nunc Inc, IL, USA), 0.5 ml U-937 or THP-1 cells were aliquoted to wells in triplicate. Cells were exposed to SiO_2, or γ-radiation or infected with *M. bovis* BCG or *M. kansasii*. Alternatively, cells were exposed to varying combinations of SiO_2 and/or γ-radiation and then infected with *M. bovis* BCG or *M. kansasii*. Following 90 minutes of infection (Day 0), on Day 3 and again on Day 5 following infection, cells were examined for microscopic changes to their structure.

4.2.2 MTT assay

The MTT assay was standardized for use with U-937 and THP-1 cells. U-937 and THP-1 were maintained in suspension culture in RPMI-1640 supplemented with 10% FCS. Cultures were incubated at 37˚C in 3-5% CO_2. Cells were concentrated by centrifugation and then re-suspended to give a final concentration of 1×10^5 cells/ml.

Cells were diluted by 5-fold serial dilution in RPMI-1640 supplemented with 10% FCS to give a final concentration between 0 and 1×10^5 cells/ml. 0.1 ml of each dilution was aliquoted to three replicates in a 96-well microtitre plate. 5 mg/ml of the MTT dye solution was added to each well. The 96-well microtitre plates were incubated for four hours at 37˚C 3-5% CO_2. 0.1 ml of the MTT solubilisation/stop solution (isopropranol) was added to each well. The plates were re-incubated for one hour at 37˚C 3-5% CO_2. Cell density was determined on a spectrophotometer at an absorbance of 540 nm.

U-937 and THP-1 were concentrated as described in Chapter 3.3. Cells were aliquoted to wells of a 24-well plate (0.5 ml), or to wells of a 96-well plate (0.2 ml), and differentiated. Cells were exposed to SiO_2, or γ-radiation or infected with *M. bovis* BCG or *M. kansasii*. A control was not exposed or infected. Alternatively, cells were exposed to varying combinations of SiO_2 and/or γ-radiation and then infected with *M. bovis* BCG or *M. kansasii*. A control was infected, but not exposed. Following 90 minutes (Day 0), Day 3 and Day 5 post infection, adherent cells were dislodged by treatment with 0.05% Trypsin/0.02% EDTA. 0.05 ml Trypsin was added to each well and the plates were incubated for 10 minutes at 37˚C 3-5% CO_2. The cells were removed from the substratum by repeated mixes with a pipette. The MTT assay was performed as described above after 0.01 ml of the cells was transferred to a 96 well plate in duplicate (or triplicate).

4.3 RESULTS

4.3.1 Cellular morphology

In vitro U-937 grows as a single-cell suspension. Living U-937 cells are variable in shape, with both round and polyglonal cells present. Following treatment with PMA, cells attach to the substratum and to each other. The typical large clumps of differentiated U-937 are shown in Figure 4-1A.

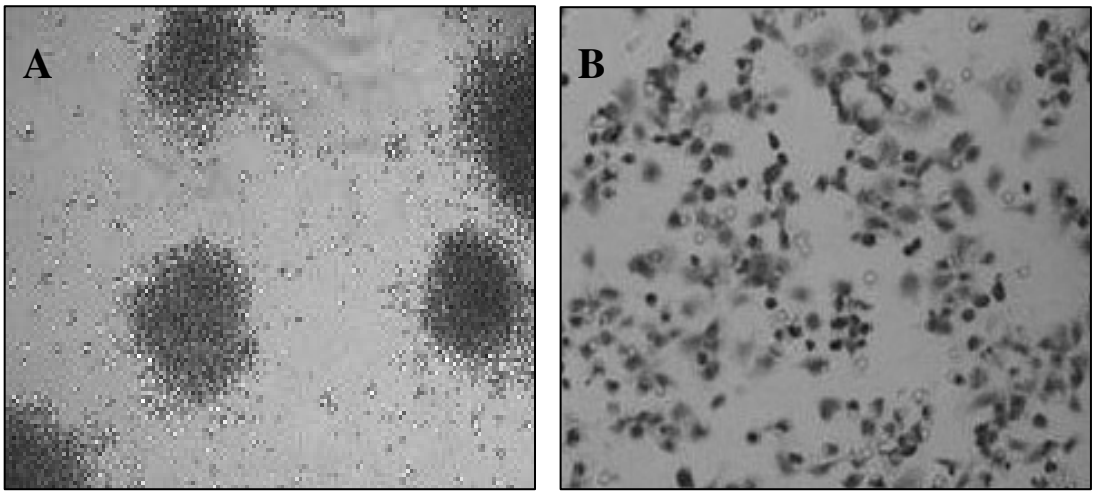

Figure 4-1 Microscopic appearance of differentiated U-937 (A), and differentiated THP-1 (B) (500x).

In vitro THP-1 cells grow as a single cell suspension. Living THP-1 cells are variable in shape, with both round and polyglonal cells present. Following treatment with PMA, THP-1 cells adhere to the substratum. Cells on the substratum are irregular shaped and flattened. The typical clump formation seen in U-937 is not present, although small groups of aggregated cells are common (Figure 4-1B).

In U-937 and THP-1, no notable differences were observed between the cell structures exposed to no radiation, and those that were exposed to between 1 and 10 Gy for either *M. bovis* BCG or *M. kansasii* infected cells. There were noticeable difference in the colour of cells exposed to SiO_2. In U-937 and THP-1 cells that were exposed to SiO_2 in doses between 25 and 500 µg/ml, there was a significant darkening of cells. The darkening was not concentration dependant, but it was noted that there was significant quantities of free SiO_2 on the substratum in wells containing 100 and 500 µg/ml SiO_2.

Distinct morphological changes were noted in early experiments conducted with U-937 (Figure 4-2). In all experimental conditions, but notably more significant in SiO_2 and irradiated cells, there was a rounding up of cells, followed by clump disruption, and eventual cell detachment from the substratum. These changes are consistent with retrodifferentiation as described (Hass, 1992), and occurred within five days post-differentiation. The changes occurred in both *M. bovis* BCG and *M. kansasii* infected cells, and are not thought to be related to the infecting strain. The reason for

"retrodifferentiation" phenomenon is not fully understood. It is suggested that the addition of SiO_2 and/or mycobacteria, and the removal of supernatant may reduce the critical concentration below that required to achieve differentiation in this cell type. The "retrodifferentiation" phenomenon was not observed in THP-1, despite retrodifferentiation being described in this cell type (Hass, 1992). The thresholds for differentiation for this cell line may be lower than U-937. The observation of retrodifferentiation necessitated a change in protocol, with the addition of PMA to all fresh medium added to differentiated cells.

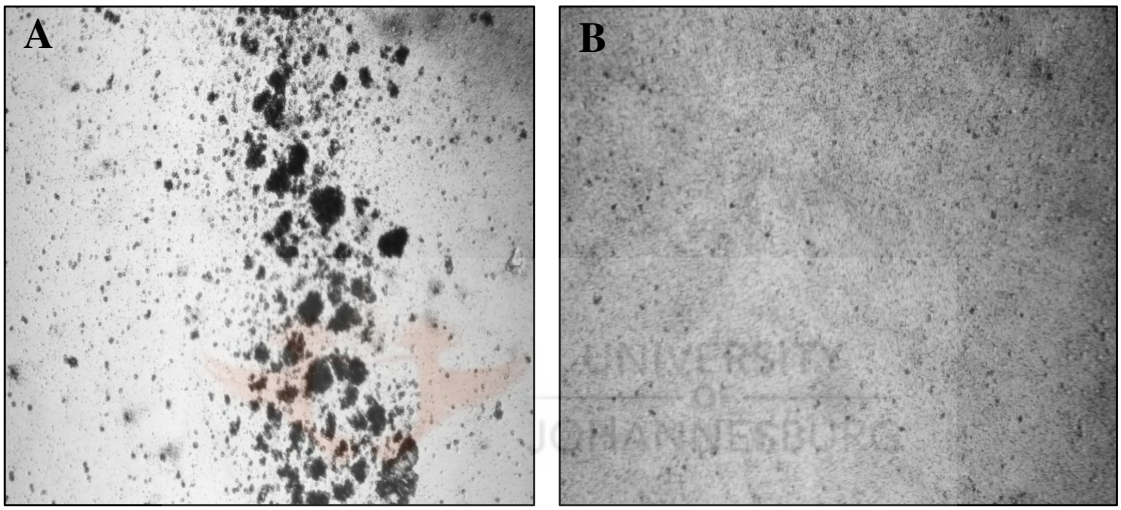

Figure 4-2 Differences in micro/macroscopic appearance of U-937 following the addition of SiO_2 (A) and the disruption of clump formation associated with retrodifferentiation (B) (500X).

4.3.2 MTT assay

The MTT assay was standardized for use with U-937 and THP-1 cells. Viable undifferentiated cells (as determined by Trypan blue staining) were diluted by 5-fold serial dilution in RPMI-1640 supplemented with 10% FCS to give a final concentration between 0 and 1×10^5 cells/ml. The MTT assay was performed. The relationship between optical density and cell concentration is shown in Figure 4-3**.** Linear regression lines for trend indicate for U-937 $r^2 = 0.9923$ and for THP-1 $r^2 = 0.9805$.

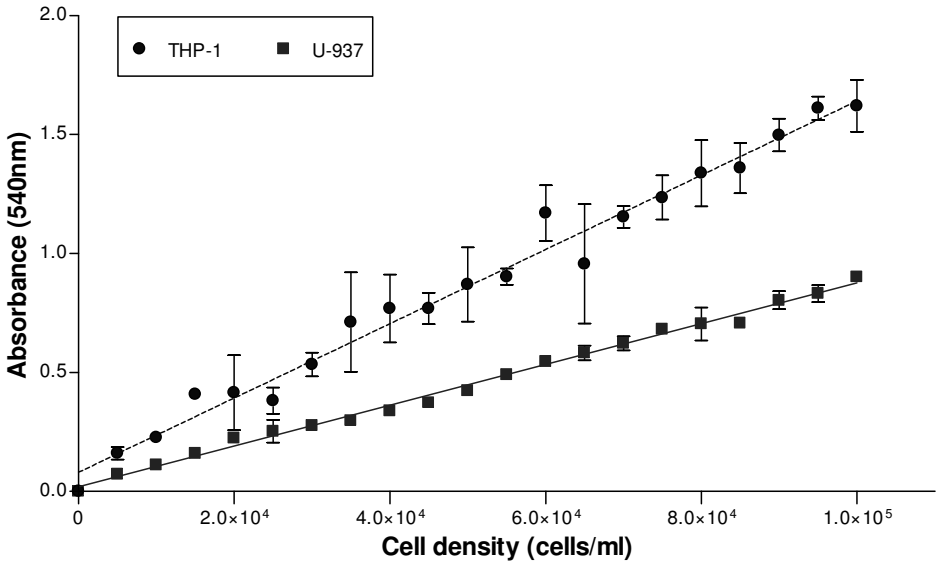

Figure 4-3 Standardization curve of U-937 and THP-1 cells using the MTT assay. Results show the linear relationship between the cell density (cells/ml) and the absorbance (OD) at 540 nm (n = 2).

This data substantiates the use of the MTT assay as a means to measure metabolic activity in U-937 and THP-1, with a concentration dependant increase in absorbance with increasing number of cells.

Differentiated U-937 were exposed to SiO_2 (25, 100 and 500 µg/ml), or γ-radiation (1, 3 and 10 Gy), or infected with *M. bovis* BCG or *M. kansasii*. A control was neither exposed nor infected. The cytotoxic effect was determined by measuring the metabolic activity of the cells by the MTT assay, 90 minutes (Day 0), 3 and 5 days post-infection and comparing these to a control (Figure 4-4). In control U-937 cells, the mean metabolic rate was 0.593 (OD 540 nm), which equates to 6.2×10^4 cells/ml after extrapolation with the standard curve (Figure 4-3). The number of cells plated was 5×10^4 cells/ml. A comparison between the mean metabolic rates of the controls and cells exposed to SiO_2 on Day 0 was made. Using the two sample t-test of means (2-tailed), it was determined that there was significantly more metabolic activity in cells exposed to 100 and 500 µg/ml SiO_2 ($p = 0.046$ and 0.001 respectively). As the number of cells initially plated was the same as for the control, this result would suggest either a stimulatory effect of SiO_2, or interference of SiO_2 with MTT read-out.

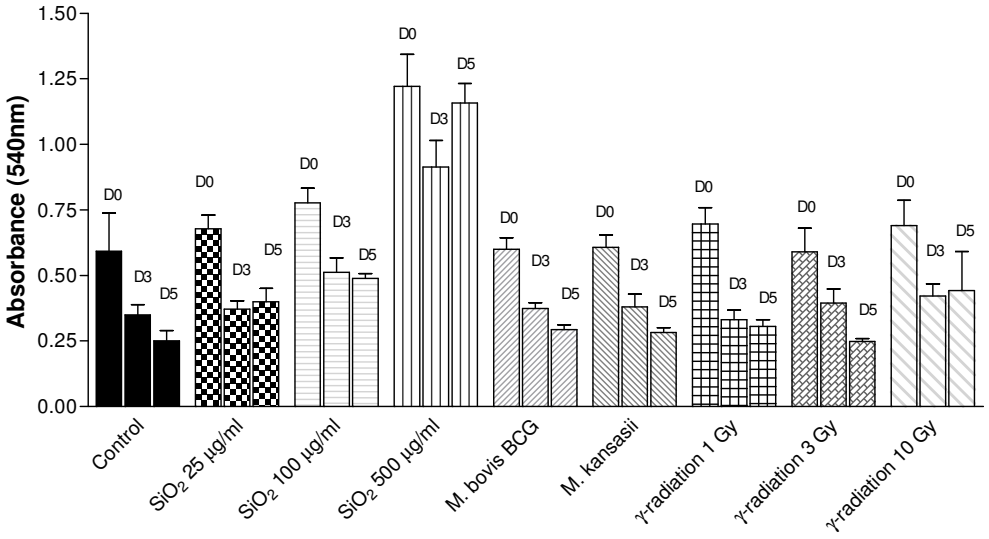

Figure 4-4 Viability of U-937 exposed to SiO_2 (25, 100 or 500 µg/ml), or γ-radiation (1, 3 or 10 Gy) or infected with *M. bovis* BCG or *M. kansasii*. The control was unexposed and uninfected. Cytotoxic effect was measured by the MTT assay, and was determined 90 minutes (D0), Day 3 (D3) and Day 5 (D5) post-infection (n = 2).

There was no significant difference between the control and the other parameters tested on Day 0. A comparison between the mean metabolic rates between Day 0 and Day 5 was made. There was a decrease in the mean metabolic activity in all parameters between Day 0 and Day 5, including the control ($p = 0.003$). A comparison between the difference between mean metabolic activity of the control cells [Day 0 – Day 5], and exposed or infected cells was made. The mean metabolic rate of the control cells was reduced by 57.5% over the five days. None of the other parameters tested reduced the metabolic rate more than the control. These results would suggest that neither SiO_2, nor γ-radiation, nor infection with either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on U-937.

Differentiated THP-1 cells were exposed to SiO_2 (25, 100 and 500 µg/ml), or γ-radiation (1, 3 and 10 Gy), or infected with *M. bovis* BCG or *M. kansasii*. A control was neither exposed nor infected. The cytotoxic effect was determined by measuring the metabolic activity of the cells by the MTT assay, 90 minutes (Day 0), 3 and 5 days post-infection and comparing these to a control (Figure 4-5). In control THP-1 cells, the mean metabolic rate of cells was 0.442 (OD 540 nm), which equates to 2.7×10^4 cells/ml after extrapolation with the standard curve (Figure 4-3). The number of cells plated was 5×10^4 cells/ml. A comparison between the mean metabolic rates of the

control and cells exposed to SiO_2, or γ-radiation or infected with either *M. bovis* BCG or *M. kansasii* on Day 0 was made. Using the two sample t-test of means (2-tailed), it was determined that there was significantly more metabolic activity in cells exposed to 100 and 500 µg/ml SiO_2 ($p = 0.005$ and 0.001 respectively). This result is consistent with the finding observed in U-937 cells exposed to SiO_2.

There was no significant difference between the control and the other parameters tested on Day 0. A comparison between the mean metabolic rates of THP-1 cells was made between Day 0 and Day 5, post-infection. There was a decrease in the mean metabolic activity in all parameters over the 5 days (exceptions were 25 µg/ml SiO_2 and 10 Gy γ-radiation). There was a significant decrease in the metabolic activity of control cells (*i.e* cells not exposed or infected) [$p = 0.003$], cells infected with *M. bovis* BCG ($p = 0.013$), and cells exposed to 1 Gy γ-radiation ($p = 0.023$). A comparison between the difference between mean metabolic activity of the control cells (not exposed or infected) [Day 0 – Day 5], and exposed or infected cells was made. The mean metabolic rate of the control cells was reduced by 28.2% over the five days. Infection by *M. bovis* BCG or *M. kansasii* resulted in 7.7% and 10.4% further reduction in the cells than the control respectively. These results would suggest that neither SiO_2, nor γ-radiation have a significant cytotoxic effect on THP-1. Infection by either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on THP-1, reducing the metabolic activity below that of the control.

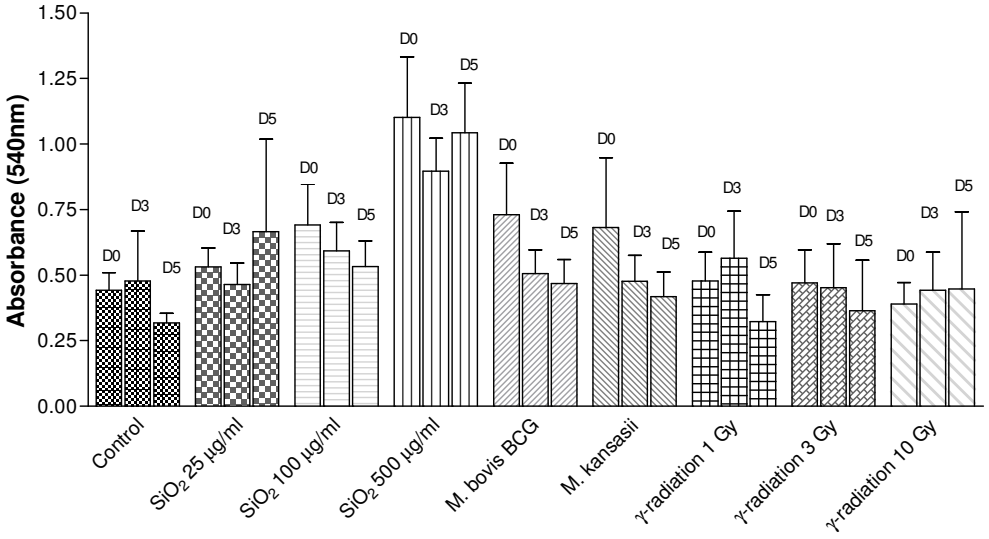

Figure 4-5 Viability of THP-1 exposed to SiO_2 (25, 100 or 500 µg/ml), or γ-radiation (1, 3 or 10 Gy) or infected with *M. bovis* BCG or *M. kansasii*. The control was unexposed and uninfected. Cytotoxic effect was measured by the MTT assay, and was determined 90 minutes (D0), Day 3 (D3) and Day 5 (D5) post-infection (n = 2).

To evaluate for any the synergistic effect of exposure to SiO_2 and γ-radiation on infected cells, differentiated U-937 were exposed to combinations of SiO_2 (25, 100 and 500 µg/ml) and γ-radiation (1, 3 and 10 Gy), and then infected with *M. bovis* BCG. Controls were either not exposed to SiO_2 or γ-radiation, or were exposed to combinations of SiO_2 and γ-radiation (Figure 4-6). In control U-937 cells, the mean metabolic rate of cells was 0.1838 (OD 540 nm), which equates to 1.5×10^4 cells/ml after extrapolation with the standard curve (Figure 4-3). The number of cells plated was 2×10^4 cells/ml. The control U-937 cells showed a decrease in the mean metabolic rate over the five days that the MTT was measured. Using the two sample t-test of means (2-tailed), it was determined that the decrease was not significant ($p = 0.736$). The decrease resulted in a loss of 10% of the cells (Table 4-1).

Table 4-1 **Difference in the number of U-937 cells (MTT assay) between Day 0 (D0) and Day 5 (D5) as determined by the two sample t-test of means (2-tailed) and expressed as a percentage. Cells were exposed to SiO_2 and/or γ-radiation and then infected with *M. bovis* BCG. A control was unexposed.**

U-937 M. bovis BCG	*D0 vs. D5 (p)*	*Difference (D0 – D5)*
Control	0.736	10%
0 Gy 25 µg/ml	< 0.01	81%*
0 Gy 100 µg/ml	< 0.01	261%*
0 Gy 500 µg/ml	0.503	77%*
1 Gy 0 µg/ml	0.048	63%
1 Gy 25 µg/ml	0.006	71%
1 Gy 100 µg/ml	0.036	58%
1 Gy 500 µg/ml	0.005	74%
3 Gy 0 µg/ml	0.316	34%
3 Gy 25 µg/ml	0.32	58%
3 Gy 100 µg/ml	0.358	46%*
3 Gy 500 µg/ml	0.001	88%
10 Gy 0 µg/ml	0.063	245%*
10 Gy 25 µg/ml	0.04	274%*
10 Gy 100 µg/ml	0.025	122%*
10 Gy 500 µg/ml	0.555	66%*

* Increase in metabolic activity between Day 0 and Day 5

Exposure to SiO_2 in infected cells resulted in an increase in metabolic activity between Day 0 and Day 5 (significant increases for 25 µg/ml and 100 µg/ml). Exposure to γ-radiation resulted in a decrease in metabolic activity for cells exposed to 1 Gy and 3 Gy ($p = 0.048$ and 0.316 respectively). There was an increase in metabolic activity for cells exposed to 10 Gy γ-radiation (significant increases for 10 Gy 25 µg/ml and 10 Gy 100 µg/ml). There were significant decreases in the mean metabolic rate of U-937 cells exposed to combinations of SiO_2 and γ-radiation when compared to a control that was unexposed (Table 4-1). These included 1 Gy 0 µg/ml, 1 Gy 25 µg/ml, 1 Gy 100 µg/ml, 1 Gy 500 µg/ml and 3 Gy 500 µg/ml. Decreases in mean metabolic rate ranged between 34% and 88%.

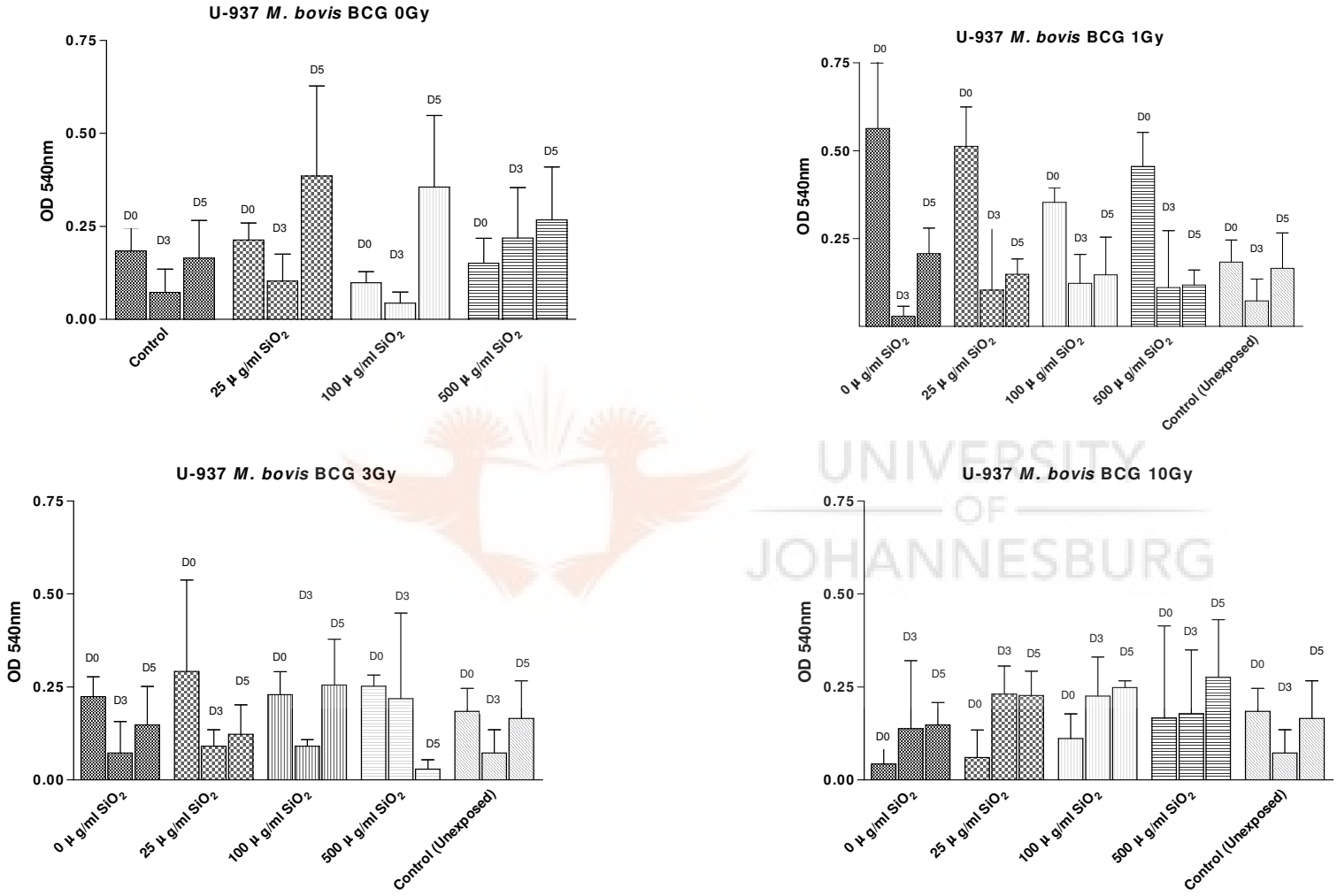

Figure 4-6 **Viability of U-937 infected with *M. bovis* BCG and exposed to SiO_2 (0, 25, 100 or 500 µg/ml) and/or γ-radiation (0, 1, 3 or 10 Gy). The control was unexposed. Cytotoxic effect was measured by the MTT assay, and was determined 90 minutes (D0), Day 3 (D3) and Day 5 (D5) post-infection (n = 2).**

To evaluate for any synergistic effect of exposure to SiO_2 and γ-radiation on infected cells, differentiated U-937 were exposed to combinations of SiO_2 (25, 100 and 500 µg/ml) and γ-radiation (1, 3 and 10 Gy), and then infected with *M. kansasii*. Controls were either not exposed to SiO_2 or γ-radiation, or were exposed to combinations of SiO_2 and γ-radiation (Figure 4-7). In U-937 control cells, the mean metabolic rate was 0.1938 (OD 540 nm), which equates to 1.8×10^4 cells/ml after extrapolation with the standard curve (Figure 4-3). The number of cells plated was 2×10^4 cells/ml. The control U-937 cells showed a decrease in the mean metabolic rate over the five days that the MTT was measured (Figure 4-7). Using the two sample t-test of means (2-tailed), it was determined that the decrease was not significant ($p = 0.054$). The decrease resulted in a loss of 58% of the cells (Table 4-2).

Table 4-2 Difference in the number of U-937 cells (MTT assay) between Day 0 (D0) and Day 5 (D5) as determined by the two sample t-test of means (2-tailed) and expressed as a percentage. Cells were exposed to SiO_2 and/or γ-radiation and then infected with *M. kansasii*. A control was unexposed.

U-937 M. kansasii	*D0 vs. D5 (p)*	*Difference (D0 – D5)*
Control	0.054	58%
0 Gy 25 µg/ml	0.853	5%
0 Gy 100 µg/ml	0.001	47%
0 Gy 500 µg/ml	0.100	53%
1 Gy 0 µg/ml	0.052	51%
1 Gy 25 µg/ml	0.010	75%
1 Gy 100 µg/ml	0.006	47%
1 Gy 500 µg/ml	0.001	48%
3 Gy 0 µg/ml	0.247	26%
3 Gy 25 µg/ml	0.348	30%
3 Gy 100 µg/ml	0.008	33%
3 Gy 500 µg/ml	0.323	32%
10 Gy 0 µg/ml	0.055	34%
10 Gy 25 µg/ml	0.711	11%*
10 Gy 100 µg/ml	0.125	10%*
10 Gy 500 µg/ml	0.697	18%

* Increase in metabolic activity between Day 0 and Day 5

Exposure to SiO_2 in infected cells resulted in a decrease in metabolic activity for cells exposed to 25, 100 and 500 µg/ml ($p = 0.853$, 0.001 and 0.1 respectively). Exposure to γ-radiation in infected cells resulted in a decrease in metabolic activity for cells exposed to 1 Gy, 3 Gy and 10 Gy ($p = 0.052$, 0.247 and 0.055 respectively).

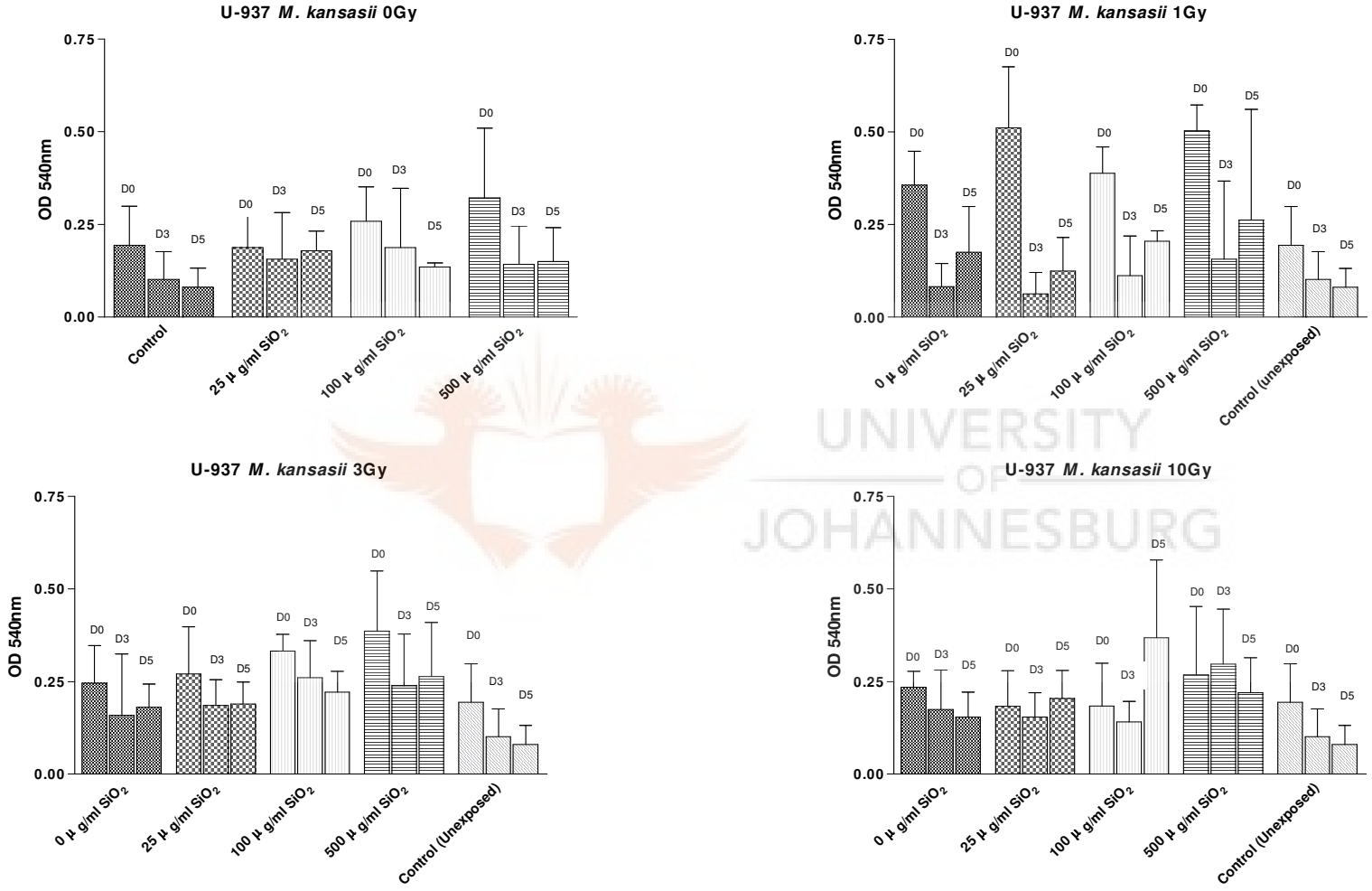

Figure 4-7 **Viability of U-937 infected with *M. kansasii* and exposed to SiO_2 (0, 25, 100 or 500 µg/ml) and/or γ-radiation (0, 1, 3 or 10 Gy). The control was unexposed. Cytotoxic effect was measured by the MTT assay, and was determined 90 minutes (D 0), Day 3 (D3) and Day 5 (D5) post-infection (n = 2).**

There were significant decreases in the mean metabolic rate of U-937 cells exposed to combinations of SiO_2 and γ-radiation when compared to a control that was unexposed (Table 4-2). These included cells exposed to 1 Gy 25 µg/ml, 1 Gy 100 µg/ml, 1 Gy 500 µg/ml and 3 Gy 100 µg/ml. Decreases in mean metabolic rate ranged between 18% and 75%. Increases in metabolic rate were not significant.

To evaluate for any synergistic effect of exposure to SiO_2 and γ-radiation on infected cells, differentiated THP-1 were exposed to combinations of SiO_2 (25, 100 and 500 µg/ml) and γ-radiation (1, 3 and 10 Gy), and then infected with *M. bovis* BCG. Controls were either not exposed to SiO_2 or γ-radiation, or were exposed to combinations of SiO_2 and γ-radiation (Figure 4-8). In control THP-1 cells, the mean metabolic rate was 0.0966 (OD 540 nm), which equates to 5×10^3 cells/ml after extrapolation with the standard curve (Figure 4-3). The number of cells plated was 2×10^4 cells/ml. The control THP-1 cells showed a decrease in the mean metabolic rate over the five days that the MTT was measured. Using the two-sample t-test of means (2-tailed), it was determined that the decrease was not significant (Table 4-3). The decrease resulted in a loss of 30% of the cells.

Table 4-3 Difference in the number of THP-1 cells (MTT assay) between Day 0 (D0) and Day 5 (D5) as determined by the two sample t-test of means (2-tailed) and expressed as a percentage. Cells were exposed to SiO_2 and/or γ-radiation and then infected with *M. bovis* BCG. A control was unexposed.

THP-1 M. bovis BCG	*D0 vs. D5 (p)*	*Difference (D0 – D5)*
Control	0.523	30%
0 Gy 25 µg/ml	0.983	1%*
0 Gy 100 µg/ml	< 0.01	89%
0 Gy 500 µg/ml	0.378	51%
1 Gy 0 µg/ml	0.067	59%
1 Gy 25 µg/ml	0.059	60%
1 Gy 100 µg/ml	0.026	65%
1 Gy 500 µg/ml	< 0.01	85%
3 Gy 0 µg/ml	0.047	50%
3 Gy 25 µg/ml	0.852	3%
3 Gy 100 µg/ml	0.442	21%
3 Gy 500 µg/ml	0.769	11%
10 Gy 0 µg/ml	0.612	22%*
10 Gy 25 µg/ml	0.96	2%
10 Gy 100 µg/ml	0.057	112%*
10 Gy 500 µg/ml	0.863	7%

* Increase in metabolic activity between Day 0 and Day 5

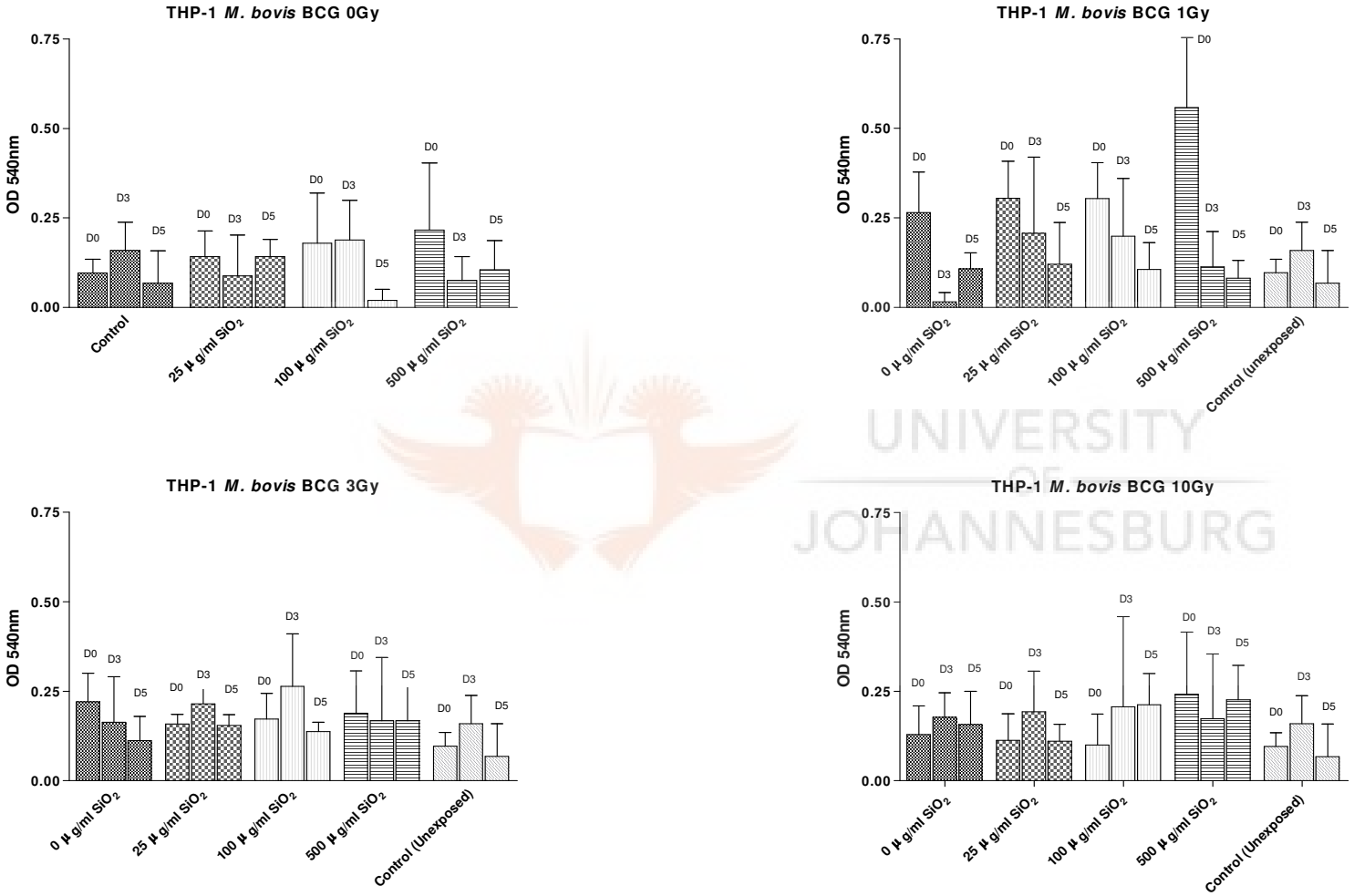

Figure 4-8 **Viability of THP-1 infected with *M. bovis* BCG and exposed to SiO_2 (0, 25, 100 or 500 µg/ml) and/or γ-radiation (0, 1, 3 or 10 Gy). The control was unexposed. Cytotoxic effect was measured by the MTT assay, and was determined 90 minutes (D0), Day 3 (D3) and Day 5 (D5) post-infection (n = 2).**

Exposure to SiO_2 in infected cells resulted in a decrease in metabolic activity for cells exposed to 100 and 500 µg/ml ($p < 0.001$ and 0.378 respectively). The metabolic rate for cells exposed to 25 µg/ml SiO_2 remained the same between Day 0 and Day 5. Exposure to γ-radiation in infected cells resulted in a decrease in metabolic activity for cells exposed to 1 Gy and 3 Gy ($p = 0.067$ and 0.047 respectively). Cells exposed to 10 Gy showed an increase in mean metabolic rate between Day 0 and Day 5. Increases in metabolic rate were not significant.

To evaluate for any synergistic effect of exposure to SiO_2 and γ-radiation on infected cells, differentiated THP-1 were exposed to combinations of SiO_2 (25, 100 and 500 µg/ml) and γ-radiation (1, 3 and 10 Gy), and then infected with *M. kansasii*. Controls were either not exposed to SiO_2 or γ-radiation, or were exposed to combinations of SiO_2 and γ-radiation (Figure 4-9). In control THP-1 cells, the mean metabolic rate was 0.1192 (OD 540 nm), which equates to 5×10^3 cells/ml after extrapolation with the standard curve (Figure 4-3). The number of cells plated was 2×10^4 cells/ml. The control THP-1 cells showed a decrease in the mean metabolic rate over the five days that the MTT was measured. Using the two-sample t-test of means (2-tailed), it was determined that the decrease was not significant (Table 4-4). The decrease resulted in a loss of 25% of the cells.

Exposure to SiO_2 resulted in a decrease in metabolic activity for cells exposed to 25, 100 and 500 µg/ml ($p = 0.703$, 0.351 and 0.085 respectively). Exposure to γ-radiation resulted in a decrease in metabolic activity for cells exposed to 1 Gy, 3 Gy and 10 Gy ($p = 0.001$, 0.272 and <0.001 respectively). The results from combinations of SiO_2 and γ-radiation exposure and *M. kansasii* infection are shown in Table 4-4.

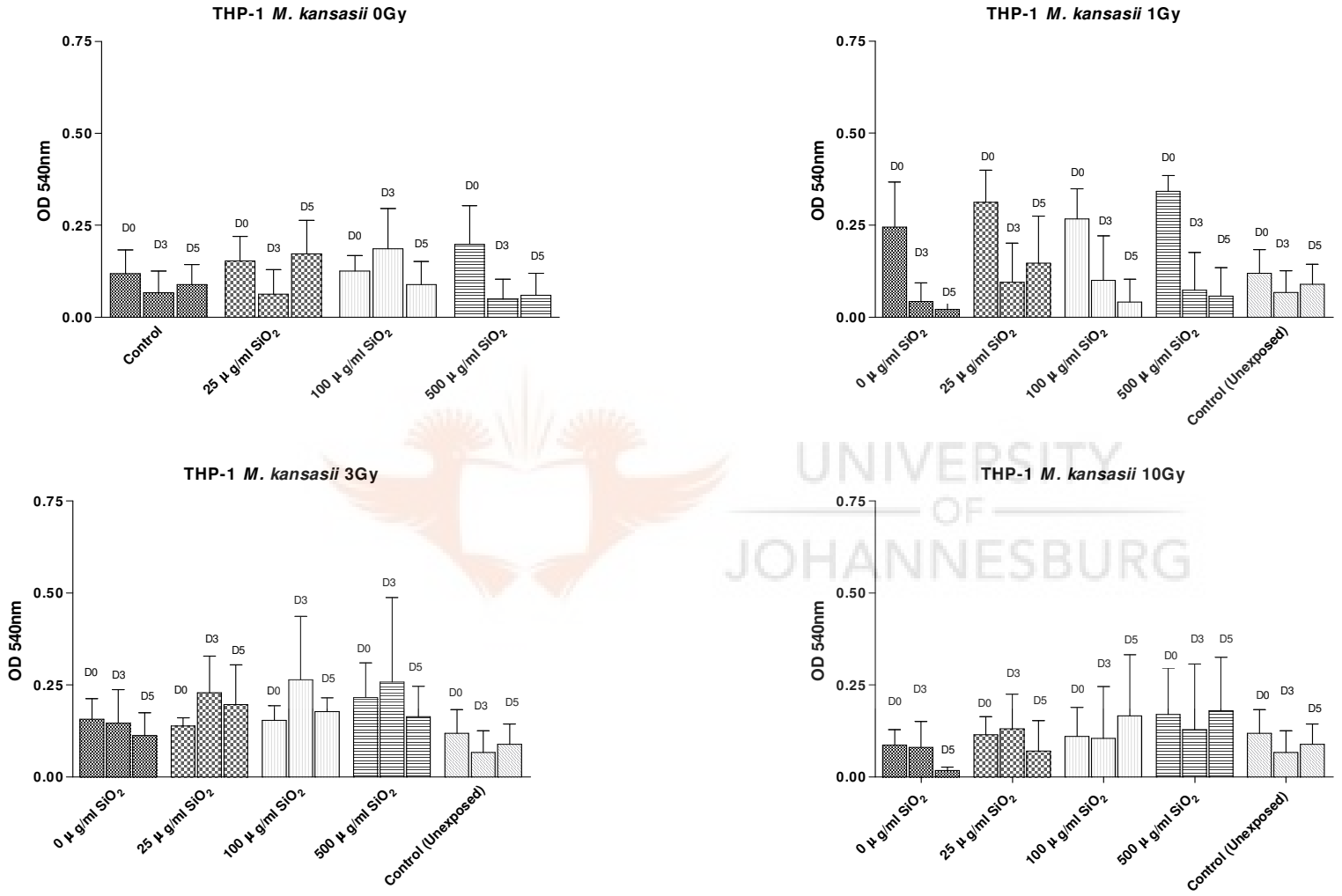

Figure 4-9 **Viability of THP-1 infected with *M. kansasii* and exposed to SiO_2 (0, 25, 100 or 500 µg/ml) and/or γ-radiation (0, 1, 3 or 10 Gy). The control was unexposed. Cytotoxic effect was measured by the MTT assay, and was determined 90 minutes (D0), Day 3 (D3) and Day 5 (D5) post-infection (n = 2).**

Table 4-4 **Difference in the number of THP-1 cells (MTT assay) between Day 0 (D0) and Day 5 (D5) as determined by the two sample t-test of means (2-tailed) and expressed as a percentage. Cells were exposed to SiO_2 and/or γ-radiation and then infected with *M. kansasii*. A control was unexposed.**

THP-1 M. kansasii	*D0 vs. D5 (p)*	*Difference (D0 – D5)*
Control	0.524	25%
0 Gy 25 µg/ml	0.703	13%
0 Gy 100 µg/ml	0.351	29%
0 Gy 500 µg/ml	0.085	70%
1 Gy 0 µg/ml	< 0.01	91%
1 Gy 25 µg/ml	0.067	53%
1 Gy 100 µg/ml	0.007	84%
1 Gy 500 µg/ml	< 0.01	83%
3 Gy 0 µg/ml	0.272	28%
3 Gy 25 µg/ml	< 0.01	42%*
3 Gy 100 µg/ml	0.342	16%
3 Gy 500 µg/ml	0.384	24%
10 Gy 0 µg/ml	< 0.01	79%
10 Gy 25 µg/ml	0.335	39%
10 Gy 100 µg/ml	0.515	51%
10 Gy 500 µg/ml	0.907	6%*

* Increase in metabolic activity between Day 0 and Day 5

4.4 DISCUSSION

In this study, the cytotoxic effect of SiO_2 on two heterogeneous promonocytic cell lines, U-937 and THP-1, was measured by determining their metabolic activity using the MTT assay and comparing these to controls that were uninfected and unexposed. In control U-937 cells, the mean metabolic rate equated to marginally more cells than were plated as suggested by the extrapolation with the standard curve. The difference was not significant ($p = 0.095$). In control U-937 cells there was a significant decrease (57.5%) in the metabolic activity between Day 0 and Day 5. Decreases in the mean metabolic activity of varying magnitude, were observed in controls and test parameters in both U-937 and THP-1. The decreases are in keeping with the slowing of metabolic activity associated with differentiation, and may also suggest cell death with prolonged differentiation (Hass, 1992).

A comparison between the mean metabolic rates of the control (*i.e* cells not exposed or infected) and cells exposed to SiO_2 showed a dose dependant increase in metabolic activity with increasing SiO_2 concentrations (significant for 100 µg/ml and 500 µg/ml). As the number of cells plated was identical to the control, and it is unlikely that SiO_2

has a protective effect on U-937, the results suggest interference of the SiO_2 with the MTT assay. In a methodological study on the use of MTT with U-937 van de Loosdrecht *et al.,* (1991), found that the background absorbance of the test varied considerably depending on the concentration of FCS used in the solvents and culture media. The higher the FCS concentration, the higher the test background. 10% FCS was used in all experiments conducted in the current study, and its potential effect would be cancelled by comparison with control. A study conducted by Hetland *et al.*, (2000) found that SiO_2 is taken up via complement receptors. Based on these findings the opsonization of SiO_2 in the current experiments was considered essential. In this protocol, the volume of AB^- serum increased with the increasing SiO_2 concentrations, suggesting that AB^- may interfere with the MTT assay in a similar manner to FCS. Since the effect of AB^- serum on the MTT assay can't be excluded, the results from the current study are inconclusive.

In acute γ-radiation exposures above a few Gy, radiation damage occurs principally because of cell death by apoptosis (UNSCEAR, 1993). In undifferentiated U-937, non-lethal low-dose irradiation (< 1 Gy) was found not to induce p53, although the cell line is arrested in G2/M from higher doses (3 Gy) of γ-radiation (Hendrikse *et al.*, 2000). In the current results, there was no significant difference between the metabolic rate of the control and the metabolic rate of cells exposed to γ-radiation. Similiarly, there was also no significant difference between the metabolic rate of the control and the metabolic rate of cells infected by *M. bovis* BCG and *M. kansasii*. It is concluded from this data that, since none of the other parameters tested reduced the metabolic rate more than the control, neither SiO_2, γ-radiation nor infection with either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on U-937.

In THP-1, the mean metabolic rate of the control cells (*i.e.* cells not exposed or infected) equated to fewer cells than were plated as suggested from extrapolation with the standard curve. The difference was significant ($p = 0.001$). The reason for the decrease is not known, but it is hypothesized that THP-1 may be more susceptible to Trypsin/EDTA than U-937, and a proportion of the cells may have died in the process of lifting the adherent cells before the metabolic rate was measured.

A comparison between the mean metabolic rates of the controls (*i.e* cells not exposed or infected) and cells exposed to SiO_2 showed a dose dependant increase in metabolic activity with increasing SiO_2 concentrations (significant for 100 µg/ml and 500 µg/ml). As the number of cells was identical to the control, the data suggests that the same mechanism that interferes with the MTT assay in U-937, interferes with the MTT assay in THP-1. Since the effect of AB^- serum on the MTT assay can't be excluded, the results from the current study are inconclusive.

In THP-1, the mean metabolic rate of the control cells was reduced by 28.2% between Day 0 and Day 5. Infection by *M. bovis* BCG or *M. kansasii* resulted in a decrease of 7.7% and 10.4% more than the control respectively. These results support the findings of Keane *et al.*, (1997) that found mycobacterial associated cytotoxicity in human macrophages. None of the other parameters tested reduced the metabolic rate more than the control. It is concluded that neither SiO_2 nor γ-radiation have a significant cytotoxic effect on THP-1. Infection by either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on THP-1, reducing the metabolic activity below that of the control.

It is the hypothesis of the current study, that silica and low-dose ionizing radiation exposure may both be involved in predisposing gold mine workers to pulmonary tuberculosis by their effects on the macrophage's ability to contain mycobacteria. Further experiments were performed to determine if there was a synergistic cytotoxic effect when combining SiO_2 and γ-radiation exposure in U-937 and then infecting with *M. bovis* BCG or *M. kansasii*. As was noted earlier, infection with *M. bovis* BCG or *M. kansasii* is not significantly cytotoxic for U-937. The mean metabolic activivity of cells used in these experiments was lower than the mean metabolic activivity observed in earlier experiments. This difference may be explained by an additional dilution of cells that was not present in the earlier experiments. This dilution also reduced the effect of the AB^- concentration in the test suspension, stengthening the hypothesis made in this chapter, that AB^- inteferes with the MTT assay.

The mean metabolic rate of the U-937 control cells (*i.e.* cells infected with *M. bovis* BCG, but not exposed to SiO_2 or γ-radiation) was decreased by 10% between Day 0 and

Day 5 (Table 4-1). Exposure to SiO_2 resulted in an increase in metabolic activity between Day 0 and Day 5 (significant for 25 µg/ml and 100 µg/ml). Exposure to γ-radiation (alone and with combinations of SiO_2) resulted in a decrease in metabolic activity which ranged between 34% and 88%. A comparison was made between the 95% confidence intervals of the control mean and each of the significant parameters (for both increases and decreases between Day 0 and Day 5). The confidence intervals were overlapping, suggesting that there is no synergistic cytotoxic effect on U-937 infected with *M. bovis* BCG and exposed to combinations of SiO_2 and/or γ-radiation. A similar comparison was made in U-937 infected with *M. kansasii* and exposed to SiO_2 and/or γ-radiation (Table 4-2). No evidence could be found to suggest a synergistic cytotoxic effect on U-937 infected with *M. kansasii* and exposed to combinations of SiO_2 and/or γ-radiation.

It was determined by experiments described in this chapter, that infection with *M. bovis* BCG or *M. kansasii* is significantly cytotoxic for THP-1. Further experiments were performed to determine if there was an additional synergistic cytotoxic effect when combining SiO_2 and γ-radiation exposure in THP-1 cells subsequently infected with *M. bovis* BCG or *M. kansasii*. The mean metabolic activivity of cells used in these experiments was lower than the mean metabolic activivity observed in earlier experiments. This difference may be explained by an additional dilution of cells that was not applicable in the earlier experiments, and is again evidence that a AB^- serum interferes with the MTT assay.

The mean metabolic rate of the THP-1 control cells (*i.e.* cells infected with *M. bovis* BCG, but not exposed to SiO_2 or γ-radiation) was decreased by 30% between Day 0 and Day 5 (Table 4-3). Exposure to SiO_2 resulted in a decrease in metabolic activity between Day 0 and Day 5. Exposure to γ-radiation (alone and with combinations of SiO_2) resulted in a decrease in metabolic activity which ranged between 2% and 89%. A comparison was made between the 95% confidence intervals of the control mean and each of the significant parameters (for both increases and decreases between Day 0 and Day 5). The confidence intervals were overlapping, suggesting that there is no synergistic cytotoxic effect on THP-1 infected with *M. bovis* BCG after exposure to combinations of SiO_2 and γ-radiation. A similar comparison was made in THP-1 infected with *M. kansasii* and exposed to SiO_2 and/or γ-radiation (Table 4-4).

Exposure to SiO_2 resulted in a decrease in metabolic activity between Day 0 and Day 5. Although the decrease in metabolic activity appears to be dose dependant, the effect was not statistically significant. Exposure to γ-radiation (alone and with combinations of SiO_2) resulted in a decrease in metabolic activity which ranged between 16% and 91%. A comparison was made between the 95% confidence intervals of the control mean and each of the significant parameters (for both increases and decreases between Day 0 and Day 5). The confidence intervals were overlapping, suggesting that there is no synergistic cytotoxic effect on THP-1 infected with *M. kansasii* after exposure to combinations of SiO_2 and γ-radiation.

In summary, the data described in this chapter suggests that γ-radiation (1 Gy, 3 Gy or 10 Gy) does not have a significant cytotoxic effect on U-937 or THP-1. Infection by either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on THP-1, but not U-937, reducing the metabolic activity below that of the control. The results from experiments to determine the cytotoxicity of SiO_2 were inconclusive. No evidence was found to support the hypothesis that combinations of SiO_2 and/or γ-radiation have a synergistic cytotoxic (or protective) effect on U-937 or THP-1 infected with *M. bovis* BCG or *M. kansasii*.

CHAPTER 5: CELL DEATH

5.1 INTRODUCTION

The cytotoxicity experiments described in Chapter 4, determined the extent of cell death following exposure to SiO_2, γ-radiation and infection with either *M. bovis* BCG or *M. kansasii.* While γ-radiation was not found to be cytotoxic for either cell line, infection with either *M. bovis* BCG or *M. kansasii* was found to be cytotoxic for THP-1, but not U-937. Results from cytotoxicity experiments on SiO_2 were inconclusive due to technical reasons. The mechanism by which cytotoxicity causes cell death is apoptosis or necrosis. Apoptosis is programmed cell death, and is characterized by cell dehydration, loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Necrosis is a passive, degenerative process that occurs in response to gross injury, and is characterized by swelling of the cell mitochondria, rupture of the cell membrane and release of the cytoplasmic contents into the external cellular environment (Darzynkiewicz *et al*., 1997).

It was the objective of the experiments performed in this chapter to determine the mechanism of cell death in exposed and infected U-937 and THP-1. This was determined by flow cytometry using Annexin/Propidium Iodide (PI) staining and Acridine Orange Ethidium Bromide staining of differentiated cells. A multifaceted approach was taken as it is widely believed that no single assay is sufficient to determine the mechanism of death. Acridine Orange/Ethidium Bromide staining was specifically chosen as it is useful in determining the mechanism of cell death in differentiated (adherent) cells. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for phospholipid phosphatidylserine, and binds to cells with exposed phospholipid phosphatidylserine (Koopman *et al.*, 1994). Annexin may be conjugated to fluorochromes such as PI and this way it is possible to measure viable, early apoptotic and late apoptotic/necrotic cells. Interpretation of Annexin- Fluorescein Isothiocyanate (FITC) PI flow cytometry is based on analysis of light scattering properties, and combinations of Annexin-FITC and PI results (Table 5-1).

Table 5-1 Summary of the interpretation of Annexin-FITC PI results for viable, early apoptotic, late apoptotic and necrotic cells.

	Annexin-FITC	***PI***
Viable	Negative	Negative
Early apoptotic	Positive	Negative
Late apoptotic	Positive	Positive
Necrotic	Negative	Positive

Acridine Orange and Ethidium Bromide are intercalating, nucleic acid specific, fluorochromes that emit a green and orange fluorescence, respectively, when they are bound to DNA (Thresher & Griffith, 1990). The Acridine Orange/Ethidium Bromide stain (Becton Dickinson, Towson, MD) is a simple fluorescent stain that has been used to determine the viability of many cell types (Nairn & Rolland, 1980; Taylor & Milthorpe, 1980). Acridine Orange can cross the plasma membrane of viable and early apoptotic cells, staining their nuclei bright green. Early apoptotic cells contain dense green areas of chromatin condensation (*i.e.* apoptotic nuclei). Late apoptotic cells have an orange nucleus with condensation of chromatin (*i.e.* non-viable cell with apoptotic nuclei) and necrotic cells display an orange nucleus with intact structure (*i.e.* non-viable cells with normal nuclei) (Figure 5-1).

Figure 5-1 Examples of viable (A), apoptotic (B) and nectrotic (C) cells stained by Acridine Orange Ethidium Bromide staining.

5.2 MATERIALS AND METHODS

5.2.1 Flow cytometry using Annexin Propidium Iodide staining

The Annexin Propidium Iodide assay (Sigma, St. Louis, MO, USA) was standardized for use with U-937 and THP-1. Mid-logarithmic phase undifferentiated U-937 and THP-1 cells were concentrated by centrifugation. Cells were re-suspended in 10% FCS RPMI-1640 to give 1×10^6 cells/ml. Differing concentrations of hydrogen peroxide (H_2O_2) was added to the cells to give final concentrations of 0.1, 0.5 and 10 mM H_2O_2. Control cells were not exposed to H_2O_2. Cells were incubated overnight, and then washed twice in phosphate buffered saline (PBS). The cells were re-suspended in binding buffer (100 mM Hepes/NaOH (pH 7.5) 1.40 M NaCl, 25 mM $CaCl_2$) and were transferred to a 12 x 75 mm 5 ml tubes (Falcon, Becton Dickinson, NJ, USA). Clumps of cells were dispersed by repeated passage through a 25-gauge tuberculin needle. Annexin FITC (0.005 µl) and PI (0.01 µl) was added. The cells were incubated for 10 minutes at room temperature in the dark, and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, NJ, USA) using CellQuest software (Becton Dickinson, NJ, USA). Excitation was at 488 nm, and the emission filters used were 515-545 BP (green; FITC) and 572-588 BP (orange; PE). 1×10^4 cells were counted per sample, and the results were graphed on histograms showing Annexin binding and PI uptake, and on dotplots showing Annexin vs. PI. For flow cytometric analysis, CellQuest software (Becton Dickinson, NJ, USA) was used.

U-937 and THP-1 were concentrated as described in Chapter 3.3. Cells were aliquoted to wells of a 24-well plate (0.5 ml) and differentiated. Cells were exposed to SiO_2, or γ-radiation or infected with *M. bovis* BCG or *M. kansasii*. A control was neither exposed nor infected. Following 90 minutes of infection, adherent cells were dislodged by treatment with 0.05 ml 0.05% Trypsin/0.02% EDTA. The plates were incubated for 10 minutes at 37˚C 3 - 5% CO_2. The cells were removed from the substratum by repeated mixes with a pipette. Cells were washed in 5% FCS in RPMI-1640, the supernatant was decanted and the cells were re-suspended in 10% FCS RPMI-1640 for overnight incubation. The following day, cells were washed twice in PBS, and re-suspended in binding buffer and processed as described above.

5.2.2 Acridine Orange Ethidium Bromide staining

Acridine Orange Ethidium Bromide staining was used to determine the extent of apoptosis/ necrosis in exposed and infected THP-1 and U-937 cells. U-937 and THP-1 were concentrated as described in Chapter 3.3. Cells were aliquoted to wells of a 24-well plate (0.5 ml), and differentiated. Cells were exposed to SiO_2, or γ-radiation or infected with *M. bovis* BCG or *M. kansasii*. Alternatively, cells were exposed to varying combinations of SiO_2 and/or γ-radiation and then infected with *M. bovis* BCG or *M. kansasii*. Ninety minutes (Day 0) and 24 hours (Day 1) post-infection cells were stained with Acridine Orange/Ethidium Bromide. The adherent cells were examined under a fluorescent microscope to determine percentage of viable, apoptotic and necrotic cells. Experiments with longer time points (up to Day 5) were also evaluated, but these were abandoned due to the high number of apoptotic or necrotic cells in the unexposed controls. To reduce subjectivity, early and late apoptotic cells were enumerated together.

5.3 RESULTS

5.3.1 Flow cytometry using Annexin Propidium Iodide staining

To evaluate the ability of Annexin PI staining to determine apoptosis and necrosis by flow cytometry, undifferentiated U-937 and THP-1 cells were exposed to differing concentrations of H_2O_2 and analyzed. Cells that were not exposed to H_2O_2 were used as a control. The results from the experiments are tabulated and are shown in Table 5-2.

Table 5-2 Standardization of the Sigma Annexin-FITC Apoptosis Detection kit showing the proportion of nectrotic, viable, late and early apoptotic THP-1 and U-937 in undifferentiated cell cultures exposed to 0.1 and 0.5 mM H_2O_2. A control was not exposed to H_2O_2 (n = 2).

	Necrotic	*Viable*	*Late apoptotic*	*Early apoptotic*	*Apoptotic Index*
THP-1 Control	0.3	88.92	7.67	3.11	10.78
THP-1 0.1 mM H_2O_2	0.22	84.75	11.54	3.49	15.03
THP-1 0.5 mM H_2O_2	0.33	29.61	66.74	3.32	70.06
U-937 Control	1.57	77.2	19.12	2.11	21.23
U-937 0.1 mM H_2O_2	3.5	74.74	15.21	6.55	21.76
U-937 0.5 mM H_2O_2	1.59	44.54	49.03	4.85	53.88

In control cell cultures of THP-1 and U-937, not exposed to H_2O_2, 88.92 and 77.2% of cells were viable respectively. The number of apoptotic cells (Apoptotic Index) was 10.78 and 21.23 for THP-1 and U-937 cells that were not exposed to H_2O_2. Addition of H_2O_2 reduced the number of viable cells, resulting in a dose dependant increase in the Apoptotic Index in both cell types. Thus, at a higher concentration of 0.5 mM H_2O_2, the Apoptotic Index was 70.06 and 53.88 for THP-1 and U-937 respectively. Good correlation was achieved between the Apoptotic Index and increasing H_2O_2 concentrations. From these results, it was concluded that the Annexin PI is able to distinguish between undifferentiated viable, apoptotic and necrotic U-937 and THP-1 cells.

Annexin PI staining was used to measure apoptosis and necrosis in differentiated U-937 and THP-1 cells by flow cytometry. Typical histograms and dotplots of Annexin binding (ordinate) vs. PI uptake (abscissa) of control U-937 and THP-1 cells are shown in Figure 5-2. The histogram [Figure 5-2A (i) and Figure 5-2B (i)] shows cells that stain positive for Annexin (upper panel) or PI (lower panel) for THP-1 and U-937 respectively. In Figure 5-2A (ii) and Figure 5-2B (ii) four populations of cells are defined by use of dual antibodies in the dot-plot. In the lower left quadrant, 83.66% and 55.88% of THP-1 and U-937 cells are viable (PI and Annexin negative) respectively. The lower right quadrant indicates 1.36% and 8.53% early apoptotic THP-1 and U-937 cells (Annexin +/PI-) respectively. Upper right quadrant identifies 13.34% and 33.10% late apoptotic THP-1 and U-937 cells (Annexin+/PI+) respectively. Upper left quadrant identifies 1.64% and 2.49% necrotic THP-1 and

U-937 cells (Annexin-/PI+) respectively. The results from the experiments with THP-1 are tabulated in Table 5-3.

Table 5-3 The proportion of necrotic, viable, late and early apoptotic (Apoptotic Index) THP-1 lifted from adherent cell cultures after exposure to SiO_2, or γ-radiation or infected with *M. bovis* BCG or *M. kansasii* (n = 2).

	Necrotic	*Viable*	*Late apoptotic*	*Early apoptotic*	*Apoptotic Index*
THP-1 Control	*3.08*	*57.74*	*35.64*	*3.53*	38.28
THP-1 25 µg/ml 0 Gy	*1.74*	*57.54*	*37.41*	*3.33*	37.62
THP-1 100 µg/ml 0 Gy	*1.50*	*52.66*	*43.31*	*2.54*	41.15
THP-1 500 µg/ml 0 Gy	*1.39*	*47.95*	*47.98*	*2.73*	46.06
THP-1 0 µg/ml 1 Gy	*8.01*	*55.36*	*35.29*	*1.35*	51.52
THP-1 0 µg/ml 3 Gy	*8.14*	*50.61*	*39.67*	*1.58*	54.53
THP-1 0 µg/ml 10 Gy	*5.91*	*58.33*	*34.71*	*1.09*	39.15
THP-1 *M. bovis* BCG	*3.48*	*33.10*	*61.24*	*2.17*	57.48
THP-1 *M. kansasii*	*18.03*	*48.54*	*31.50*	*1.94*	46.95

In this study, the extent of apoptosis and necrosis in U-937 following exposure to SiO_2, or γ-radiation or infection with *M. bovis* BCG or *M. kansasii*, could not reliably be determined. The failure to standardize the assay for use with differentiated U-937 was due to the unacceptable amount of auto-fluorescence on flow cytometry. Auto-fluorescence was generally viewed in the lower right quadrant of the PI-PE Annexin-FITC histogram (Figure 5.2Bii). Gating on the cells showed that they were uniformly distributed throughout the cell population. The results suggested that the auto-fluorescence was due to the uptake of dye by aggregated live cells. The aggregation of U-937 is a well-known feature of the cell type (Hass *et al.*, 1989), and despite numerous strategies (EDTA, Accutase™ [Innovative Cell Technologies Inc., San Diego, CA, USA] and scraping) the clumps could not be dissolved adequately. Difficulties with adherent cells and determination of apoptosis/necrosis by flow cytometry are quite common (Darzynkiewicz *et al*., 2001).

In THP-1 cells, there was a loss of cell viability when adherent cells were detached and analyzed. Approximately 58% of cells were viable in the control (unexposed and uninfected), with an Apoptotic Index of 38.28. Exposure of THP-1 cells to SiO_2

increased the Apoptotic Index in a dose dependant manner, with a related decrease in the number of viable cells (Apoptotic Index = 37.62, 41.15 and 46.06 for 25, 100 and 500 µg/ml SiO_2 respectively). The decrease in viability was not significant. There was also no significant difference in the number of viable cells in THP-1 exposed to γ-radiation as compared to the control. (Apoptotic Index = 51.52, 54.53 and 39.15 for 1, 3 and 10 Gy respectively). Infection by *M. bovis* BCG led to the most significant loss of viability, and increased Apoptotic Index (57.48) greatest of all the potentially cytotoxic agents tested ($p = 0.014$). The decrease in viability for THP-1 infected with *M. kansasii* was not significant ($p = 0.074$). There was an increase in cell death associated with necrosis in *M. kansasii* infected THP-1.

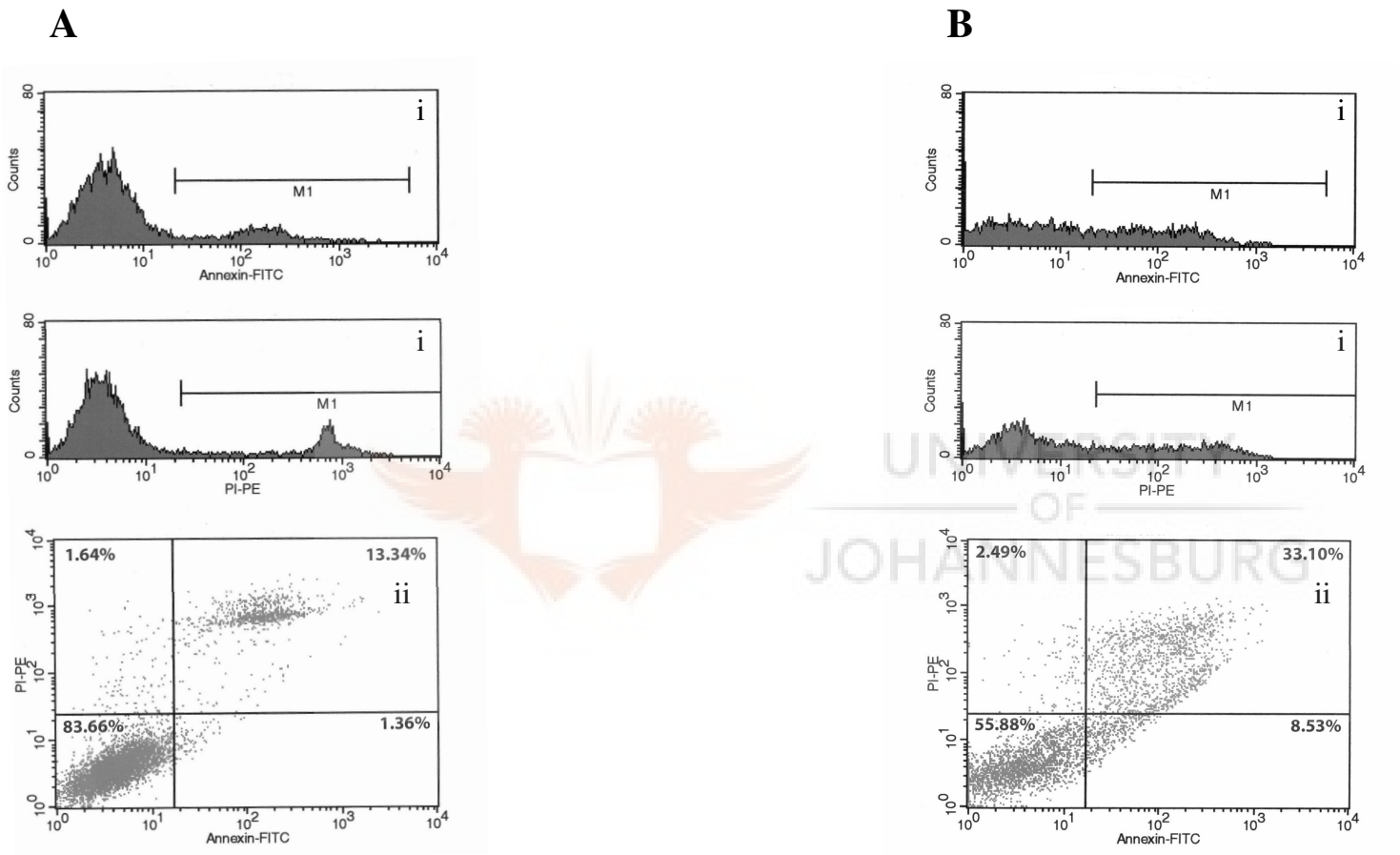

Figure 5-2 **Typical histograms (i) and dotplots (ii) of Annexin binding (ordinate) vs. PI uptake (abscissa) of control THP-1 (A) and U-937 (B) cells.**

5.3.2 Acridine Orange Ethidium Bromide staining

Given the multifaceted approach that was taken in this study to determine the mechanism of cell death, the failure to standardise the Annexin PI assay for use with U-937, and considering the potential impact on viability that lifting of adherent cells may have had on THP-1, Acridine Orange/Ethidium Bromide staining was used to determine the extent of apoptosis/necrosis in THP-1 and U-937 cells exposed to SiO_2 (25, 100 and 500 µg/ml) or γ-radiation (1, 3 and 10 Gy), or infected with *M. bovis* BCG or *M. kansasii*.

In control U-937 cells on Day 0, the mean percentage viable, apoptotic and necrotic cells was 93%, 4% and 3% respectively (Figure 5-3). The mean percentage viable and necrotic cells decreased to 89% and 2% after 24 hours post infection respectively. These differences were not statistically significant. To determine the effect of SiO_2 on apoptosis/necrosis in U-937, cells exposed to 25, 100 and 500 µg/ml SiO_2 were examined on Day 0 and Day 1. There was no significant difference in mean percentage viable, apoptotic and necrotic cells on Day 0 as compared to Day 1 ($p = 0.404$ to $p = 0.628$). A comparison was made between the mean percentage viable, apoptotic and necrotic cells of the control on Day 1, and 25, 100 and 500 µg/ml SiO_2 on Day 1. There was no significant difference between the control cells and those exposed to SiO_2. However, there was a decrease of between 6% and 12% in the number of viable cells in SiO_2 containing wells as compared to the control on Day 1. The decreases in the number of viable cells were not significant. Conversely, the mean percentage apoptotic cells in SiO_2 containing wells increased. The increase in apoptosis appeared to be dose related for 25, 100 and 500 µg/ml SiO_2 (9%, 12.5% and 19.5% respectively).

To determine the effect of infection with *M. bovis* BCG or *M. kansasii* on apoptosis and necrosis in U-937, exposed cells were examined on Day 0 and Day 1. There was no significant difference in mean percentage viable, apoptotic and necrotic cells on Day 0 as compared to Day 1 ($p = 0.293$ and $p = 0.284$ respectively).

To determine the effect of γ-radiation on apoptosis and necrosis in U-937, cells exposed to 1 Gy, 3 Gy or 10 Gy were examined on Day 0 and Day 1. There was no significant

difference in mean percentage viable, apoptotic and necrotic cells on Day 0 as compared to Day 1 for cells exposed to 3 Gy and 10 Gy γ-radiation. There was a significant difference for cells exposed to 1 Gy γ-radiation between Day 0 and Day 1 ($p < 0.001$), but when compared to the control this difference was not significant ($p = 0.25$). There was a decrease by 11% in cells exposed to 1 Gy γ-radiation as compared to the control on Day 1. The decrease was not significant. Conversely, the mean percentage apoptotic cells increased.

In control THP-1 cells on Day 0 the mean percentage viable, apoptotic and necrotic cells was 80%, 15% and 5% respectively (Figure 5-3). The mean percentage viable and necrotic cells Day 1 post infection was 78%, 18% and 4% respectively (not significant). To determine the effect of SiO_2 on apoptosis/necrosis in THP-1, cells exposed to 25, 100 and 500 µg/ml SiO_2 were examined on Day 0 and Day 1. There was no significant difference between the control cells and those exposed to 25 µg/ml and 100 µg/ml SiO_2. The difference between the control cells and those exposed to 500 µg/ml was significant ($p = 0.024$). There was a decrease of between 7% and 20% in the number of viable cells in SiO_2 containing wells as compared to the control on Day 1. Conversely, the mean percentage apoptotic cells in SiO_2 containing wells increased. The increase in apoptosis was highest in cells exposed to 500 µg/ml SiO_2 (19% compared to control), but the relationship between concentration and apoptosis did not appear to be dose-response related.

To determine the effect of infection with *M. bovis* BCG or *M. kansasii* on apoptosis and necrosis in THP-1, exposed cells were examined on Day 0 and Day 1 (Figure 5-3). There was a significant difference between the control cells and those infected by *M. bovis* BCG or *M. kansasii* ($p = 0.013$ and $p = 0.034$ respectively). The percentage viable cells in wells infected with *M. bovis* BCG and *M. kansasii* decreased by 28% and 26% respectively, compared to the control. Conversely, the mean percentage apoptotic cells increased.

To determine the effect of γ-radiation on apoptosis and necrosis in THP-1, cells exposed to γ-radiation were examined on Day 0 and Day 1. There was no significant difference in mean percentage viable, apoptotic and necrotic cells on Day 0 as compared to Day 1 for cells exposed to 1 Gy, 3 Gy or 10 Gy γ-radiation.

Acridine Orange/Ethidium Bromide staining was used to determine the extent of apoptosis/necrosis in THP-1 and U-937 cells exposed to SiO_2 (25, 100 and 500 µg/ml), and/or γ-radiation (1, 3 and 10 Gy), and then infected with *M. bovis* BCG or *M. kansasii*. In control U-937 cells infected with *M. bovis* BCG (not exposed), the mean percentage viable, apoptotic and necrotic cells was 85%, 8% and 7% respectively (Figure 5-4). There was no significant difference in the number of viable cells on Day 1 compared to the mean number of viable cells observed on Day 0. To determine the effect of SiO_2 and/or γ-radiation on apoptosis and necrosis; U-937 cells infected with *M. bovis* BCG were exposed to 25, 100 and 500 µg/ml SiO_2 and/or 1 Gy, 3 Gy and 10 Gy γ-radiation. There was no significant difference in mean percentage viable, apoptotic and necrotic cells as compared to the control for cells exposed to SiO_2, γ-radiation, and combinations of SiO_2 and γ-radiation.

In U-937 cells infected with *M. kansasii,* and exposed to neither SiO_2 nor γ-radiation (*i.e.* control), the mean percentage viable, apoptotic and necrotic cells was 80%, 10% and 10% respectively (Figure 5-4). There was no significant difference in the number of viable cells on Day 1 compared to the mean number of viable cells observed on Day 0. To determine the effect of SiO_2 and/or γ-radiation on apoptosis and necrosis; U-937 cells infected with *M. kansasii* were exposed to 25, 100 and 500 µg/ml SiO_2 and/or 1 Gy, 3 Gy and 10 Gy γ-radiation. There was no significant difference in mean percentage viable, apoptotic and necrotic cells as compared to the control for cells exposed to SiO_2, γ-radiation, and combinations of SiO_2 and γ-radiation.

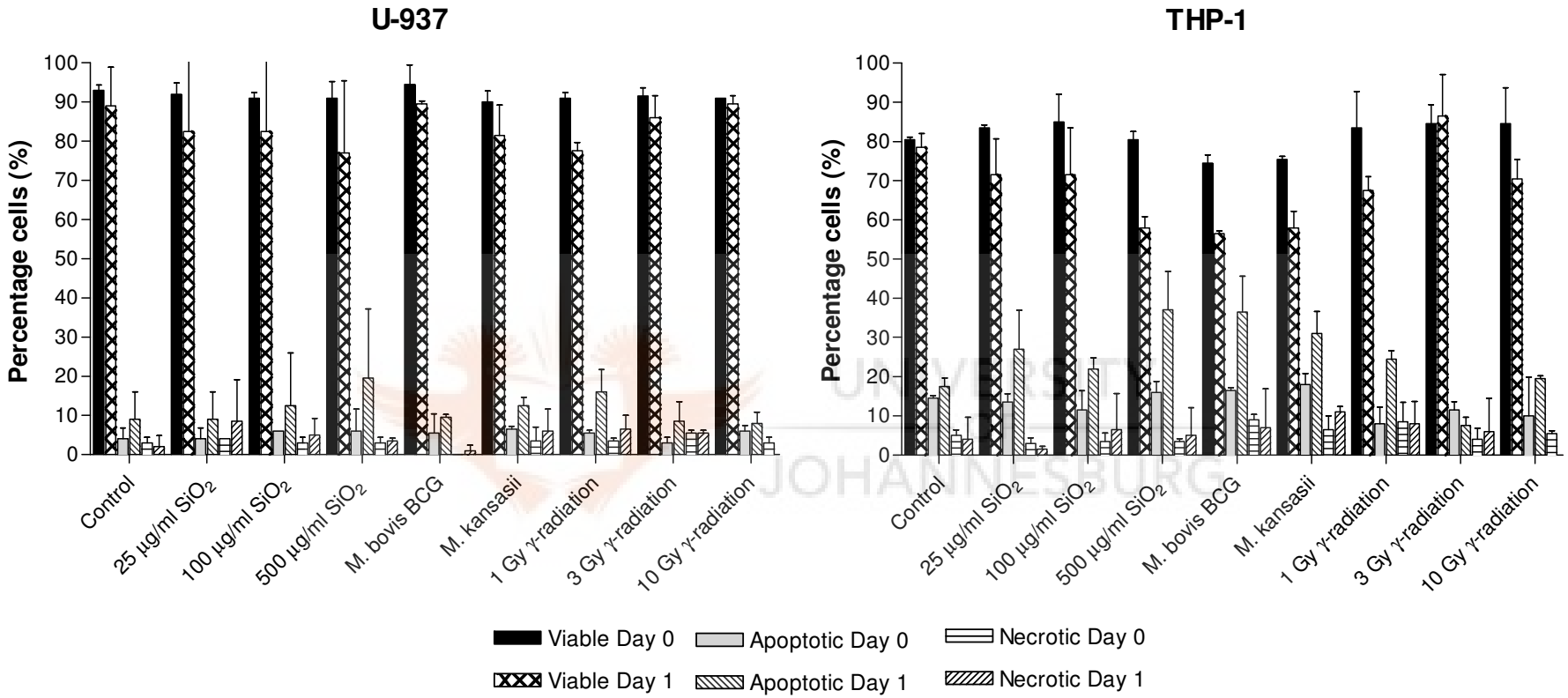

Figure 5-3 **Percentage viable, apoptotic and necrotic differentiated U-937 and THP-1 cells as determined by Acridine Orange Ethidium Bromide staining after exposure to SiO_2, or γ-radiation, or infection with *M. bovis* BCG or *M. kansasii*, 90 minutes (Day 0) and 24 hours (Day 1) post-infection. The controls were unexposed and uninfected (n = 2).**

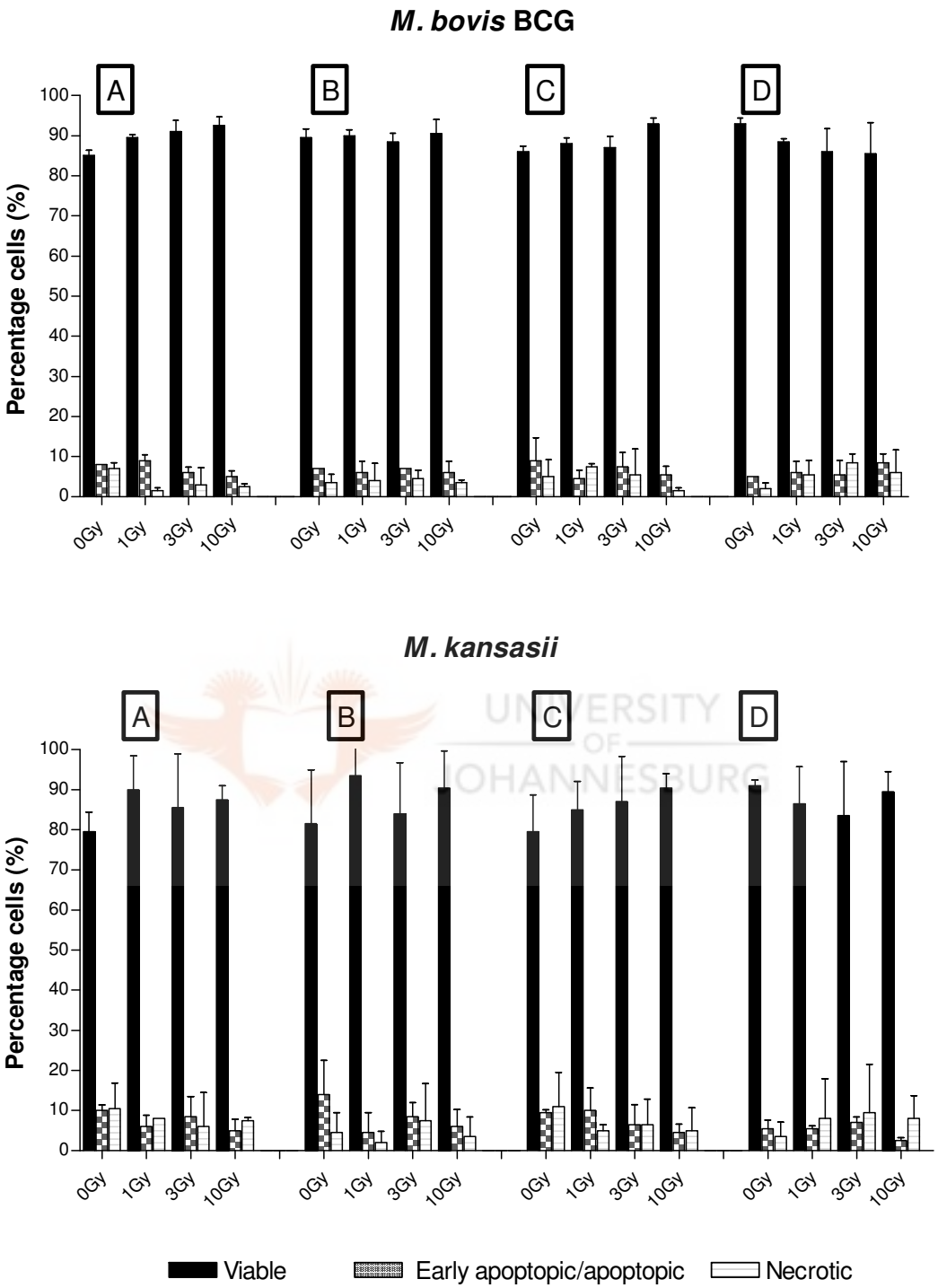

Figure 5-4 **Percentage viable, apoptotic and necrotic differentiated U-937 cells as determined by Acridine Orange Ethidium Bromide staining after exposure to SiO_2 (A= 0 µg/ml; B= 25 µg/ml; C= 100 µg/ml and D= 500 µg/ml) and/or γ-radiation (0, 1, 3 and 10 Gy), 24 hours post-infection with *M. bovis* BCG or *M. kansasii*. A control was not exposed (n = 2).**

In control THP-1 cells infected with *M. bovis* BCG, the mean percentage viable, apoptotic and necrotic cells was 73%, 19% and 8% respectively (Figure 5-5). There was no significant difference in the number of viable cells on Day 1 compared to the mean number of viable cells observed on Day 0. To determine the effect of SiO_2 and/or γ-radiation on apoptosis and necrosis; THP-1 cells infected with *M. bovis* BCG were exposed to 25, 100 and 500 µg/ml SiO_2 and/or 1 Gy, 3 Gy and 10 Gy γ-radiation. There was no significant difference in mean percentage viable, apoptotic and necrotic cells as compared to the control for cells exposed to SiO_2, γ-radiation, and combinations of SiO_2 and γ-radiation.

In control THP-1 cells infected with *M. kansasii* the mean percentage viable, apoptotic and necrotic cells was 74%, 19% and 8% respectively (Figure 5-5). There was no significant difference in the number of viable cells on Day 1 compared to the mean number of viable cells observed on Day 0. To determine the effect of SiO_2 and/or γ-radiation on apoptosis and necrosis; THP-1 cells infected with *M. bovis* BCG were exposed to 25, 100 and 500 µg/ml SiO_2 and/or 1 Gy, 3 Gy and 10 Gy γ-radiation. There was no significant difference in mean percentage viable, apoptotic and necrotic cells as compared to the control for cells exposed to SiO_2, γ-radiation, and combinations of SiO_2 and γ-radiation.

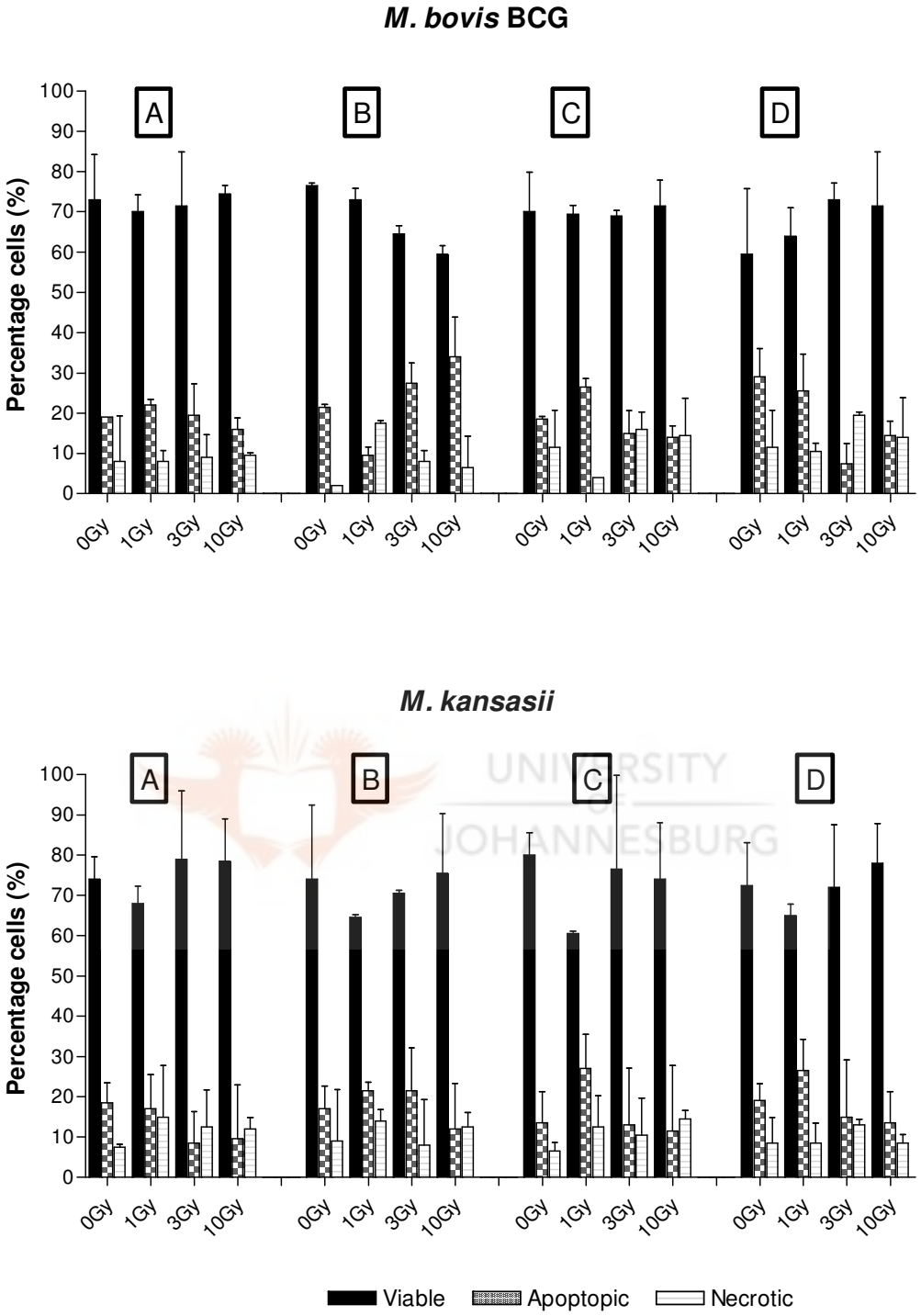

Figure 5-5 **Percentage viable, apoptotic and necrotic differentiated THP-1 cells as determined by Acridine Orange Ethidium Bromide staining after exposure to SiO_2 (A= 0 µg/ml; B= 25 µg/ml; C= 100 µg/ml and D= 500 µg/ml) and/or γ-radiation (0, 1, 3 and 10 Gy), 24 hours post-infection with *M. bovis* BCG or *M. kansasii*. A control was not exposed (n = 2).**

5.4 DISCUSSION

In this study, the extent of apoptosis and necrosis in U-937 following exposure to SiO_2, or γ-radiation or infection with *M. bovis* BCG or *M. kansasii*, could not reliably be determined by Annexin PI flow cytometry. Differentiated U-937 cells were stained by Ethidium Bromide/Acridine Orange and examined under a fluorescent microscope to determine percentage of viable, early apoptotic/apoptotic and necrotic cells. Exposure of U-937 to 25 µg/ml, 100 µg/ml and 500 µg/ml SiO_2 increased the percentage of apoptotic cells in a dose related manner, but this was not statistically significant. In the absence of reliable Annexin PI and MTT data, it is concluded that SiO_2 is not associated with significant cell death in U-937.

Exposure of THP-1 to 25 µg/ml, 100 µg/ml and 500 µg/ml SiO_2 increased the percentage of apoptotic cells. Analysis of data from the Annexin PI assay showed that in THP-1 cells, SiO_2 increased the Apoptotic Index in a dose dependant manner, with a related decrease in the number of viable cells, but that this was not statistically significant. Using Acridine Orange/Ethidium Bromide staining, it was determined that there was a statistically significant decrease in viable THP-1 cells that were exposed to 500 µg/ml SiO_2 only. Conclusions drawn from analysis of data from the MTT assay (Chapter 4) were inconclusive. The results from this chapter would suggest that SiO_2 is associated with cell death through apoptosis in THP-1, but that the amount of apoptosis is only slightly above background. In this study, SiO_2 was not associated with cell death through necrosis. SiO_2 has traditionally been associated with necrotic cell death (Carter & Driscoll, 2001; Porter *et al*., 2002) however, as shown by these results, there is increasing evidence to suggest that SiO_2 can initiate caspase activation and apoptosis.

Thibodeau *et al.* (2003) demonstrated that *in vitro*, using a mouse macrophage cell line (MH-S cells), α-quartz silica exposure (12.5 µg/cm^2 to 50 µg/cm^2) elicits activation of caspase 3 and caspase 9, whereas anatase titanium dioxide (non-fibrogenic particle) does not. Silica exposure induced apoptosis after six hours, as measured by the appearance of sub-diploid cell fragments on flow cytometry. Further work by this group found that following silica exposure, lysosomal injury preceded apoptosis. The apoptotic signalling pathway in this cell line includes cathepsin D and

acidic sphingomyelinase (Thibodeau *et al*., 2004). In a murine model, it has been shown that silica-induced apoptosis plays an inflammatory role in the lung parenchyma, and creates immunologic abnormalities in regional lymph nodes, with pathogenic implications for the host (Borges *et al*., 2002). This group have also found that silica induces Fas ligand expression in alveolar macrophages *in vitro* and *in vivo*, and this promotes FAS ligand–dependent macrophage apoptosis (Borges *et al*., 2001). Furthermore, elevated ROS levels may act as initiators, leading to caspase activation and PARP cleavage to execute the apoptotic process (Shen *et al*., 2001).

In neither U-937 nor THP-1, was there any significant increase in apoptosis or necrosis after exposure to γ-radiation. It is concluded that exposure to low-dose γ-radiation (< 10 Gy) is not associated with significant cell death in differentiated U-937 or THP-1. It should be noted however, higher doses of γ-radiation are associated with apoptosis, and apoptosis is a key defence mechanism against radiation. Datta *et al.,* (1997) demonstrated that exposure of undifferentiated U-937 cells to 20 Gy γ-radiation leads to apoptosis. Apoptosis removes severely DNA damaged cells, which, if they were to survive would increase the risk of tumours (Harms-Ringdahl *et al*., 1996).

In U-937, there was no significant difference in the proportion of viable, apoptotic and necrotic cells following infection with *M. bovis* BCG or *M. kansasii*. In THP-1, infection by *M. bovis* BCG and *M. kansasii* led to the significant loss of viability, and increased apoptosis as measured by Acridine Orange/Ethidium Bromide staining and Annexin PI (statistically significant for *M. bovis* BCG only in the latter). The increase in the number of necrotic cells following *M. kansasii* infection could not be confirmed by Acridine Orange/Ethidium Bromide staining. It was concluded from the MTT data presented in Chapter 4, that *M. bovis* BCG and *M. kansasii* are cytotoxic for THP-1, but not U-937 cells. The experiments presented in this chapter confirm the finding of no significant loss of viability in U-937, and conclude that the mechanism of cell death following infection in THP-1, is apoptosis.

In respect of *M. bovis* BCG, this finding is consistent with the finding of Riendieau & Kornfeld (2003). They examined the potential for PMA differentiated THP-1 cells to mimic this response of alveolar macrophages to mycobacterial infection. Consistent

with the behaviour of alveolar macrophages, attenuated *M. tuberculosis* H37Ra and *M. bovis* BCG strongly induce THP-1 apoptosis. THP-1 apoptosis is associated with reduced viability of infecting *M. bovis* BCG. In contrast, virulent wild-type *M. tuberculosis* H37Rv and *M. bovis* do not increase THP-1 apoptosis over baseline. *M. bovis* BCG induced early activation of caspase 10 and 9, followed by caspase 3. In contrast, wild-type *M. bovis* infection failed to activate any caspases in THP-1 cells.

As was noted by Riendieau & Kornfeld (2003), human macrophages respond to avirulent mycobacterial infection by undergoing apoptosis. Virulent mycobacteria override this reaction by inducing necrosis, which leads to uncontrolled *M. tuberculosis* replication. In *M. tuberculosis,* infected macrophages, high levels of mitochondrial membrane integrity, low levels of caspase activation, and diminished mitochondrial Cytochrome C release are hallmarks of apoptosis and effective antimycobacterial activity. The breakdown of mitochondrial membrane integrity and increased caspase activation are characteristic of necrosis and uncontrolled *M. tuberculosis* replication (Duan *et al*., 2002). Evasion of apoptosis by virulent *M. tuberculosis* is achieved by enhanced release of sTNFR2 by infected macrophages and subsequent formation of inactive TNF-alpha-TNFR2 complexes (Fratazzi *et al*., 1999).

Further studies have examined the extent of apoptosis of alveolar macrophages following infection by *M. tuberculosis* complex strains of differing virulence and by *M. kansasii* (Keane *et al*., 2000). Avirulent or attenuated bacilli (*M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. kansasii*) induced significantly more apoptosis than virulent strains (*M. tuberculosis* H37Rv, Erdman, *M. tuberculosis* clinical isolate BMC 96.1, and *M. bovis* wild type). The findings in respect of *M. kansasii*, are consistent with the conclusions drawn in this study *viz. M. kansasii* is cytotoxic for THP-1, and the mechanism by which this occurs is apoptosis.

No evidence to suggest a synergitic relationship between SiO_2 and/or γ-radiation on the induction of cell death could be found in either cell type. Furthermore, no evidence could be found to suggest a protective or stimulatory effect on U-937 or THP-1 after SiO_2 and/or γ-radiation exposure. This is in keeping with the conclusion drawn from cytotoxicity experiments performed in Chapter 4. As has been

demonstrated in these experiments, apoptosis occurs widely in THP-1 cells in response to exposure to SiO_2, or infection by *M. bovis* BCG or *M. kansasii*. In this study, the pathway to cell death has not been elucidated, but other authors have hypothesized that the way in which oxidants lead to apoptosis can differ from substance to substance (Kim *et al.*, 1999). Apoptosis induced via stimulation of several different cell surface receptors *e.g.* SR receptors; FAS (CD95) and TNF receptors would appear to be a most likely pathways (Cohen, 2000). Irradiation induced apoptosis is most likely through p53 (Hendrikse *et al.*, 2000). The relationship between TNF-α release and apoptosis will be explored further in Chapter 8.

CHAPTER 6: DNA DAMAGE

6.1 INTRODUCTION

In Chapter 5, it was concluded that in U-937 there is no significant increase in apoptosis or necrosis in cells exposed to SiO_2, or γ-radiation or infected by *M. bovis* BCG or *M. kansasii*, as compared to an unexposed uninfected control. In THP-1, there is no significant increase in apoptosis or necrosis in cells exposed to γ-radiation. However, there is a significant increase in apoptosis in cells exposed to SiO_2 (500 µg/ml only), and cells infected by *M. bovis* BCG or *M. kansasii* only, as compared to an unexposed uninfected control.

As was noted previously, apoptosis is a form of programmed cell death that is associated with chromatin condensation and nuclear fragmentation. The key morphological changes that are associated with apoptosis precede internucleosomal DNA cleavage (Cohen *et al*., 1992). Östling and Johanson (1984), were the first to develop a microgel electrophoresis technique for detecting DNA damage to the single cell. Using this technique, cells were embedded in agarose on a microscope slide, lysed, and then liberated DNA was electrophoresed. It was noted that cells with double-strand DNA breaks displayed increased migration toward the anode. The migrating DNA was quantified by staining the gel with Ethidium Bromide. Singh *et al.*, (1988) changed the neutral pH at which the assay ran, to alkaline thereby improving the early form of the Single Cell Gel Electrophoresis (SCGE) or Comet assay. With this change, it is able to detect single-strand and single-strand breaks associated with incomplete excision repair sites. The assay that is widely used today to identify the effects genotoxic agents is based on further changes introduced by Olive *et al.* (1990). The intensity of the tail reflects the number of DNA strand breaks present (Dušinská & Collins, 1996). The sensitivity of the Comet assay can be increased by using the bacterial repair enzyme, Endonuclease III. Endonuclease III recognises oxidized pyrimidines, cut them out, and nicks the DNA at the resulting apurinic/apyrimidinic site, thus promoting the comet formation (Collins, 2000).

The Comet assay is a simple method that measures the level of DNA damage at the level of single cells. The Comet assay is finding increasing popularity in investigating the level of DNA damage in terms of strand breaks and alkaline liable sites in bio-monitoring studies, and appears to be very informative in the monitoring of populations chronically exposed to genotoxic agents (Maluf *et al.*, 2001). It was the objective of the experiments described in this chapter to measure the level of DNA damage in U-937 and THP-1 cells exposed to SiO_2, or γ-radiation or infection by *M. bovis* BCG or *M. kansasii*. The Comet assay was also used to measure the level of DNA damage in U-937 and THP-1 exposed to SiO_2, and/or γ-radiation and then infected by *M. bovis* BCG or *M. kansasii*.

6.2 MATERIALS AND METHODS

U-937 and THP-1 were concentrated as described in Chapter 3.3. Cells were aliquoted to wells of a 24-well plate (0.5 ml), and differentiated. Cells were exposed to SiO_2, γ-radiation and/or infected with *M. bovis* BCG or *M. kansasii*. Following 90 minutes of infection (Day 0), adherent cells were dislodged by treatment with 0.05 ml 0.05% Trypsin/0.02% EDTA. The plates were incubated for 10 minutes at 37˚C 3-5% CO_2. The cells were removed from the substratum by repeated mixes with a pipette. The cells were washed in 1 ml 5% FCS RPMI, by centrifuging for two minutes at 2000 rpm (4˚C). The supernatant was decanted and the deposit was re-suspended in freezing solution consisting of 10% FCS, 10% dimethyl sulphoxide and 80% RPMI-1640.

The Comet assay was performed as described by Collins (2000). In brief, slides for the Comet assay were prepared by dipping conventional microscope slides in molten 1% agarose (Bio-rad Laboratories, Hercules, CA, USA) and allowing them to dry overnight. The frozen cells were thawed and concentrated by centrifugation at 2000 rpm for two minutes. The cells were lightly dispersed, and 0.14 ml 1% low melting point agarose (Life Technologies, Gaithersburg, MD, USA) was added. The cells and agarose were briefly mixed with a pipette, and two drops were placed either side of the gel. The drops were covered with 18×18 mm coverslips. The gels were placed in the fridge for five minutes, after which the coverslips were removed. The gels were

placed in lysis solution containing 1% Triton X-100 (Sigma, St. Louis, MO, USA), for one hour at 4˚C (Appendix 2 for the methods of preparation of the various buffers, solutions and stains).

After lysis, gels were washed in three changes of enzyme reaction buffer (five minutes per wash). Endonuclease III and enzyme reaction buffer were placed on the respective gels (0.05 ml respectively), and 18×18 mm coverslips were placed on top. The gels were incubated at 37˚C for 45 minutes. The coverslips were removed, and the gels were placed in electrophoresis solution for 40 minutes at 4˚C. The gels were electrophoresed in the electrophoresis solution for 30 minutes at 25 volts. The gels were neutralized by three washes in neutralizing buffer at 4˚C (5 minutes each). After the last wash, the gels were dipped in distilled water, and dried overnight. 4',6-Diamidine-2'phenylindole dihydrochloride (DAPI) (0.02 ml) was placed on each gel, a 18×18 mm coverslip was placed on top and the gels were read on a fluorescent microscope. One hundred random, consecutive cells per gel were analyzed. Cells were scored 0 - 4, based on the length of migration and/or the perceived relative proportion of the DNA in the tail. Quantification of the Arbitrary units was done using Comet Assay Evaluation software developed by R. Dušinský (1995). Examples of "comets" stained with DAPI, and visualized under a fluorescent microscope, are shown in Figure 6-1.

6.3 RESULTS

The numbers of Arbitrary units for U-937 and THP-1 that was exposed to SiO_2 (25, 100 and 500 g/ml), or γ-radiation (1, 3 or 10 Gy), or infected with *M. bovis* BCG or *M. kansasii* are shown in Figure 6-2. The mean number of Arbitrary units for the U-937 was low for all parameters tested, and was not to be influenced by exposure to SiO_2, γ-radiation or infection by *M. bovis* BCG or *M. kansasii*. In U-937, there was no significant difference in the Arbitrary units visualized between cells stained with no enzyme, and those stained with Endonuclease III.

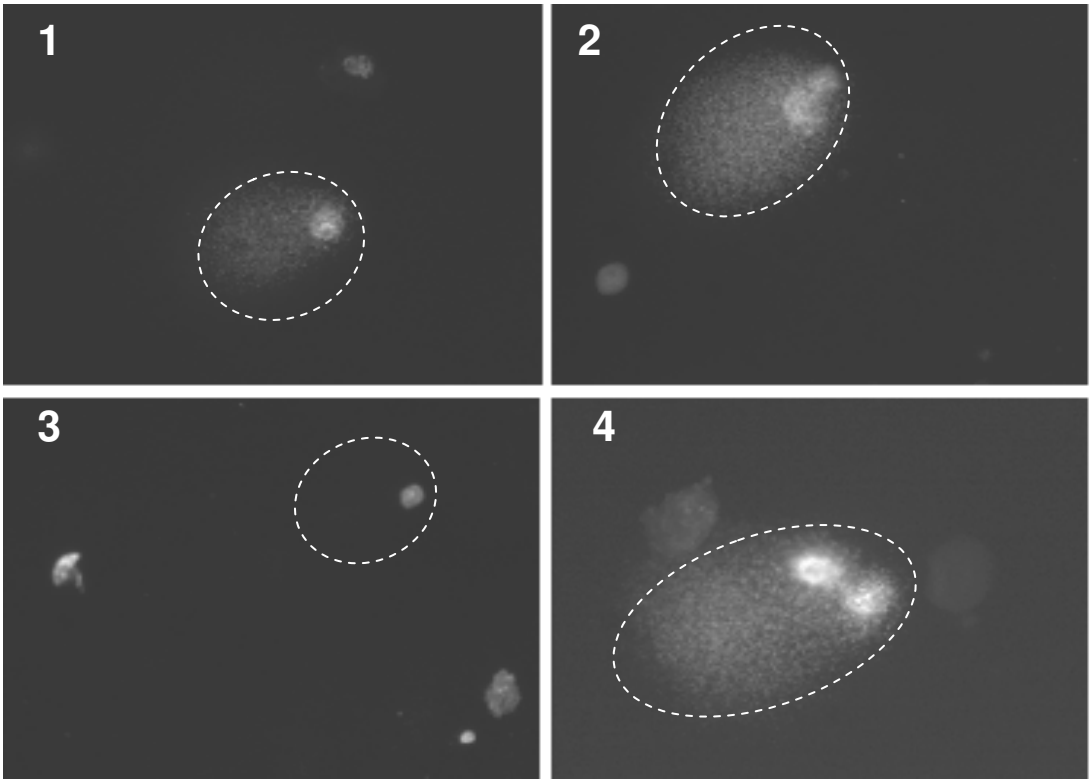

Figure 6-1 **THP-1 cells (1, 2 and 4) and U-937 cells (3) "comets" stained with DAPI, and visualized under a fluorescent microscope. The cell's periphery is marked by a dashed line.**

The mean number of Arbitrary units for the THP-1 control was significantly higher than for U-937 ($p = 0.03$). In THP-1, the number of Arbitrary units increased in a dose related manner for both SiO_2 ($p = 0.142$, 0.001 and < 0.001 for 25, 100 and 500 µg/ml respectively) and γ-radiation ($p = 0.781$, 0.005 and 0.001 for 1, 3 or 10 Gy respectively). The mean number of Arbitrary units for THP-1 infected by *M. bovis* BCG or *M. kansasii* was significantly higher than control ($p < 0.001$). In THP-1, there was no significant difference in the Arbitrary units visualized between cells stained with no enzyme, and those stained with Endonuclease III.

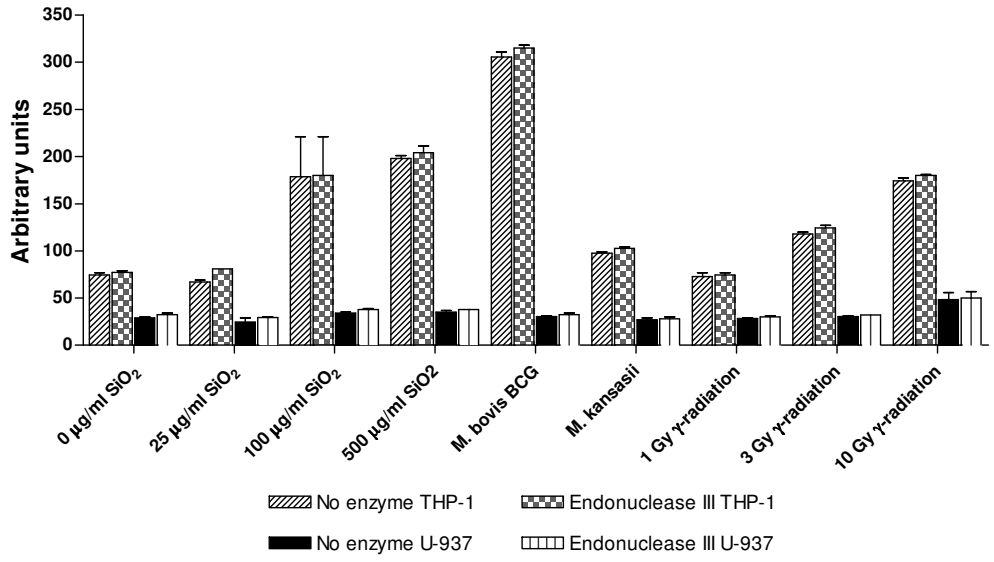

Figure 6-2 Arbitrary units of U-937 and THP-1 exposed to SiO_2 (25, 100 and 500 µg/ml),or γ-radiation (1, 3 and 10 Gy) or infected with *M. bovis* BCG (BCG) or *M. kansasii* 90 minutes (Day 0) post-infection. The controls were unexposed and uninfected (n = 2).

The Comet assay was used to determine the extent of DNA damage in U-937 cells exposed to SiO_2 and/or γ-radiation and then with *M. bovis* BCG or *M. kansasii* (Figure 6-3 and 6-4). The mean number of Arbitrary units for each parameter was low, and there was no significant difference between the number of Arbitrary units visualized between cells that were stained with no enzyme, and those that were stained with Endonuclease III. There was no statistically significant difference in the number of Arbitrary units between U-937 infected with *M. bovis* BCG, and U-937 infected with *M. kansasii*. The effect of SiO_2 on U-937 was determined by comparing the number of Arbritary units from the control (*i.e* no SiO_2 or γ-radiation exposure), to cells exposed to 25, 100 or 500 µg/ml SiO_2. There was no significant statistical difference in Arbitrary units between cells exposed to no SiO_2, and those that were exposed to 25, 100 and 500 µg/ml SiO_2 and then infected with either *M. kansasii* or *M. bovis* BCG. The effect of γ-radiation on U-937 was determined by comparing the number of Arbitrary units from the control (*i.e* no SiO_2 or γ-radiation exposure), to cells exposed to 1 Gy, 3 Gy or 10 Gy γ-radiation. There was no significant statistical difference in Arbitrary units between the control and 1 Gy or 3 Gy; however, there was a significant difference in cells exposed to 10 Gy γ-radiation ($p < 0.001$).

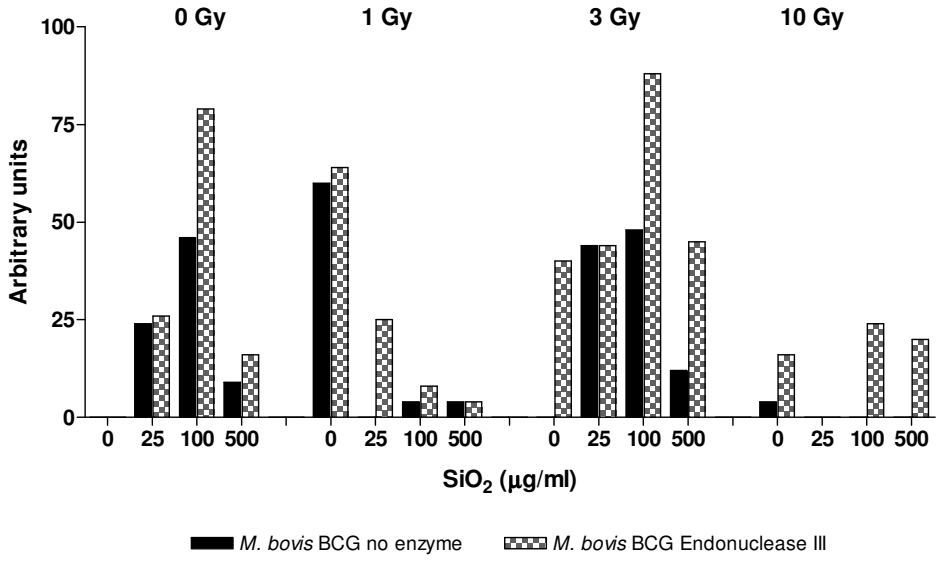

Figure 6-3 Arbitrary units of U-937 exposed to γ-radiation (0, 1, 3 and 10 Gy) and/or SiO_2 (0, 25, 100 and 500 µg/ml), and then infected with *M. bovis* BCG 90 minutes (Day 0) post-infection. A control was infected, but not exposed (n = 1).

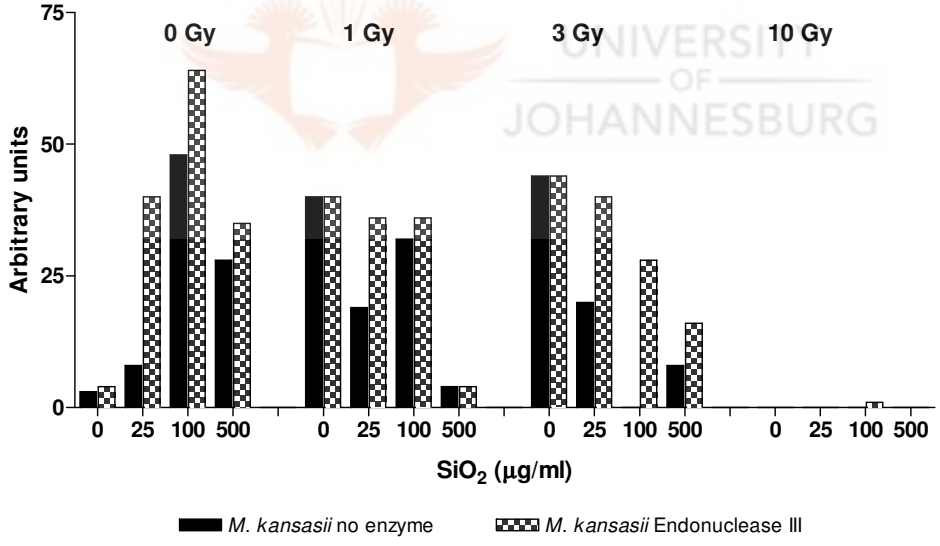

Figure 6-4 Arbitrary units of U-937 exposed to γ-radiation (1, 3 and 10 Gy) and/or SiO_2 (25, 100 and 500 µg/ml), and then infected with *M. kansasii* 90 minutes (Day 0) post-infection. A control was infected, but not exposed (n = 1).

The Comet assay was used to determine the extent of DNA damage in THP-1 cells exposed to SiO_2 and/or γ-radiation and then infected with *M. bovis* BCG or *M. kansasii* (Figure 6-5 and 6-6). The mean number of Arbitrary units for each parameter was high, and there was no significant difference between the number of Arbitrary

units visualized between cells that were stained with no enzyme, and those that were stained with Endonuclease III. There was no statistically significant difference in the number of Arbitrary units between THP-1 infected with *M. bovis* BCG and THP-1 infected with *M. kansasii*. The effect of SiO_2 on THP-1 was determined by comparing the number of Arbitrary units from the control (*i.e* no SiO_2 or γ-radiation exposure), to cells exposed to 25, 100 or 500 µg/ml SiO_2. There was no significant statistical difference in Arbitrary units between cells exposed to no SiO_2, and those that were exposed to 25, 100 and 500 µg/ml SiO_2 and co-infected with either *M. kansasii* or *M. bovis* BCG. There was no significant statistical difference in Arbitrary units between the control and cells exposed to 1 Gy, 3 Gy or 10 Gy γ-radiation and then infected with *M. bovis* BCG and *M. kansasii*.

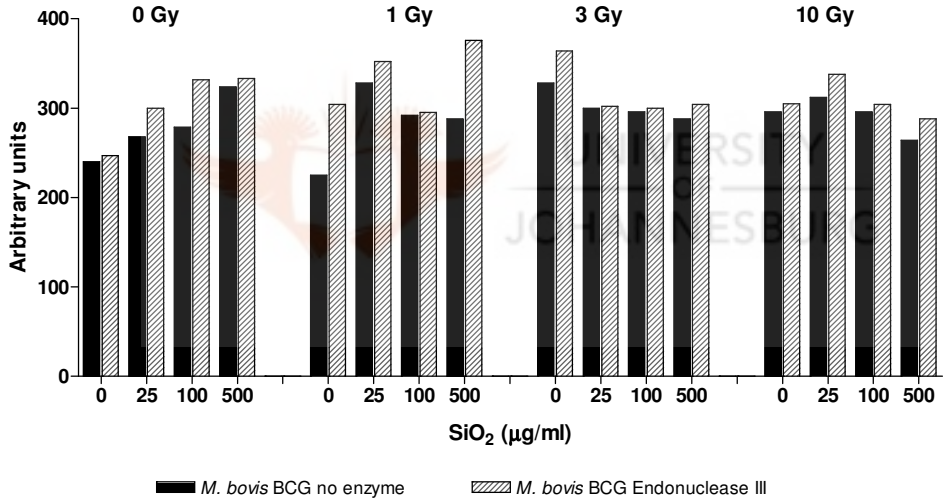

Figure 6-5 Arbitrary units of THP-1 exposed to γ-radiation (1, 3 and 10 Gy) and/or SiO_2 (25, 100 and 500 µg/ml), and then infected with *M. bovis* BCG 90 minutes (Day 0) post-infection. A control was infected, but not exposed (n = 1).

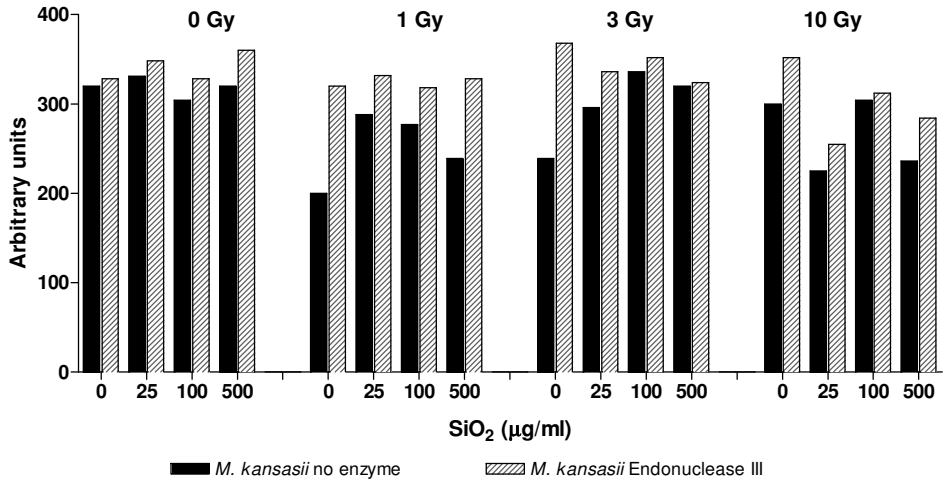

Figure 6-6 Arbitrary units of THP-1 exposed to γ-radiation (1, 3 and 10 Gy) and/or SiO_2 (25, 100 and 500 µg/ml), and then infected with *M. kansasii* 90 minutes (Day 0) post-infection. A control was infected, but not exposed (n = 1).

6.4 DISCUSSION

Using the Comet assay, Basaran *et al.* (2003) investigated the potential hazard associated with the occupational exposure to silica in the peripheral lymphocytes of 30 foundry and 22 pottery workers and compared these to 52 healthy controls. Occupational exposure of silica from foundry and pottery workplaces was associated with the increased DNA damage in the workers, but not the controls. Under the auspices of the International Agency for Research on Cancer (IARC), Kawami & Ebihara (2000) found similar abnormalities in patients with pneumoconiosis, including silicosis. There findings prompted them to lobby for the re-classification of crystalline silica as a carcinogenic substance from Group 2A to Group 1.

In the experiments described in this chapter, the Comet assay was used to measure the level of DNA damage in U-937 and THP-1 following exposure to SiO_2. The U-937 control cells (*i.e.* cells not exposed or infected) had significantly less Arbitrary units than the THP-1 control cells. This finding supports the hypothesis postulated in Chapter 4 that THP-1 is more susceptible to cellular damage by Trypsin/EDTA. Compared to the U-937 control, the number of Arbitrary units was not significantly increased after exposure to SiO_2, γ-radiation or infection by *M. bovis* BCG or *M. kansasii*.

In Chapter 5, no conclusion could be drawn regarding the toxic effect of SiO_2 on THP-1, although the data suggested that exposure to SiO_2 increases apoptosis in THP-1. Compared to the THP-1 control (*i.e.* cells not exposed or infected), the number of Arbitrary units was significantly increased after exposure to 100 μg/ml and 500 μg/ml SiO_2. It is concluded from these results that SiO_2 is genotoxic to THP-1 in a dose dependant manner. This finding is in keeping with the findings from other *in vitro* studies. Nagalakshmi *et al.* (1995) examined the genotoxic potential of SiO_2 using cultured Chinese hamster lung fibroblasts (V79) and human embryonic lung (Hel 299) cells. One-day-old cultures were treated with silica at concentrations between 40 and 320 $\mu g/cm^2$. The study found that the higher concentrations tested induced micronuclei formation in both cell types. Micronuclei were also induced by lower concentrations in V79 cells, suggesting that they are relatively more sensitive to silica than Hel 299 cells. In a similar study, Liu *et al.* (1996) challenged Chinese hamster lung fibroblasts (V79 cells) with respirable silica particles. Some of the silica dusts used were pre-treated with simulated pulmonary surfactant to model *in vivo* exposure conditions. The frequency of micro-nucleated cells in cultures treated with surfactant-coated silica was not significantly different from that of the non-treated control cultures. The study also showed that both crystalline and non-crystalline silica induced micronuclei in a dose-dependent manner.

In the experiments described in this chapter, compared to the THP-1 control (*i.e.* cells not exposed or infected), the number of Arbitrary units was significantly increased after exposure to γ-radiation. DNA damage was found in THP-1 exposed to 3 or 10Gy γ-radiation, but was not detected by cytotoxicity or flow cytometry/AOEB testing as described in Chapter 4 and 5. The finding as decribed in experiments in this chapter are in keeping with others that noted that high doses of γ-radiation are associated with apoptosis, and apoptosis is a key defence mechanism against radiation (Datta *et al.,* 1997).

There is substantial evidence that exposure to uranium and radon at the levels found in the mines increases the risk of lung cancer. In a study done using the fluorescent *in situ* hybridization (FISH) assay it was shown that long-term exposure to low-dose uranium increases the risk of biological radiation damage, which might lead to malignant disease. This was confirmed by tests done by Popp *et al.* (2000), on former East

German uranium miners who had increased frequencies of chromosomal aberrations in blood lymphocytes. Although mineworkers are exposed to a variety of airborne pollutants, their carcinogenic effect appears to be small as compared with radon exposure.

Houreld and Abrahamse (2004), examined blood collected from gold mineworkers to determine if they showed an increase in oxidative DNA damage. The Comet assay was performed on lymphocytes isolated on two occasions from 13 actively working gold mineworkers and the results were compared to those of case-matched controls. The mean values of strand breaks, oxidized pyrimidines and altered purines were significantly higher as compared to the control group on both occasions. There was no statistical difference between the means from the first and second round of analysis. It was concluded that occupational exposure to ionizing radiation elevates the levels of oxidative stress. In this study there appeared to be no accumulation of radiation induced DNA damage. It cannot be conclusively proven that radiation exposure can cause disease from infectious agents. Hooper (2000) makes some startling findings regarding the incidence of *Pneumocystis carinii* pneumonia (PCP) in children in Eastern Europe after World War II. One of the major silver mining regions in Europe was Jachymov in the northwest Czech Republic. The ore in this area is high in uranium and radium, and provided the source for the Pierre and Marie Curie experiments in the early 1900's, for the Third Reich in World War II, and the Soviet Union in the Cold War era. PCP is a highly opportunistic pathogen to which most humans are exposed early in life without any harmful consequence. An epidemic of PCP in children was recorded in Eastern Europe between 1945 and 1953. The epi-centre of the outbreak was located close to the mining town of Jachymov. Furthermore, the spread of the epidemic followed the rail routes that were used to transport 1.5 million tons of ore to the atomic and nuclear projects initiated in the Soviet Union.

In the experiments described in this chapter, compared to the THP-1 control (*i.e.* cells not exposed or infected), the number of Arbitrary units was significantly increased after exposure to *M. bovis* BCG and *M. kansasii*. It is concluded from these results that infection by *M. bovis* BCG and *M. kansasii* is genotoxic to THP-1. These findings are consistent with other findings in this study, that *M. bovis* BCG and *M. kansasii* are cytotoxic to THP-1 cells, that that the mechanism of death is apoptosis. Furthermore,

two reports document the presence of micronuclei in patients with tuberculosis. Masjedi *et al.* (2000) examined the peripheral lymphocytes of 36 patients diagnosed with pulmonary tuberculosis, and compared these to 36 healthy controls. The study found that the number of chromosomal aberrations and micronuclei were significantly higher ($p < 0.05$) in the tuberculosis patients than in the controls. Furthermore, the number of chromosomal aberrations and micronuclei increased significantly after anti-tuberculosis treatment. Similarly, a significant difference in oxidative DNA damage was noted between 28 patients with tuberculous pleurisy compared to healthy controls ($p < 0.01$) (Liu *et al.*, 2003).

The occurrence of DNA damage in THP-1 cells exposed to SiO_2 and γ-radiation was not detected by cytotoxicity or assays used to determine the mechanism of cell death. The contrast in results between this chapter, and results described in Chapter 4 (cytotoxicity) can attributed to the lack of sensitivity of the latter assay. As has been postulated in Chapter 4, cells with significant apoptotic damage may be metabolically active.

The occurrence of DNA damage in THP-1 gives insight into the establishment of apoptosis in this cell line by toxic agents. The pathways through which this occurs is most likely p53 (γ-radiation), TNF-α and/or FAS (SiO_2) (Kim *et al,.* 1999; Borges *et al*., 2001). The results described in this chapter would suggest that damage to the cell occurs soon after exposure to SiO_2 or γ-radiation (DNA damage occurs early in the progression of apoptosis). It is hypothesized that three mechanisms may play a part in the modulation of apoptosis in THP-1 cells exposed to SiO_2 or γ-radiation:

- The cell may progress rapidly to cell death via the apoptotic pathway:

If the cell progressed rapidly to death there would be a significant decrease in the number of cells exposed to SiO_2 or γ-radiation by five days post-exposure. In Chapter 4, it was concluded that there was no significant cytotoxic effect after SiO_2 or γ-radiation exposure five days post-exposure, and thus this mechanism is unlikely. Rapid progression to cell death is observed in THP-1 cells infected by *M. bovis* BCG and *M. kansasii*.

- The DNA damage is repaired, and the cell does not progress to cell death:

If the THP-1 cell repaired the DNA damage, apoptosis would be averted, and would not be detected experimentally. Repair of DNA damage occurs continuously in the cell, as does the removal of DNA damaged cells by apoptosis (Harms-Ringdahl *et al*., 1996). The reversal of early p53-inducted apoptosis has been described, and is a possible explanation for results in this study (Geske *et al.*, 2001). No evidence could be found to suggest reversal of apoptosis through TNF-α and/or FAS induction following extrinsic stimuli.

- The cell progress slowly to cell death via the apoptotic pathway:

After exposure to SiO_2 or γ-radiation, DNA damage occurs, but this does not progress to measurable apoptosis within 24 hours post-exposure. Furthermore, DNA damage does not progress to measurable cytotoxicity within five days post-exposure. The slow progress (relative to the experimental time-line) of apoptosis in THP-1 would account for the results described in this chapter, and those described in Chapter 4 and 5.

The results from experiments described in this chapter found no synergistic effect of SiO_2 and/or γ-radiation on DNA damage in U-937 and THP-1 cells exposed to *M. bovis* BCG or *M. kansasii*. Similiarly, no evidence of a protective effect of SiO_2 and/or γ-radiation exposure could be found. It was concluded that a combination of SiO_2 and/or γ-radiation was no more genotoxic to U-937 and THP-1 than infection with *M. bovis* BCG or *M. kansasii* alone.

The impact of SiO_2 and irradiation on the macrophage's ability to contain mycobacteria was explored next in a functional growth assay, and the results are presented in Chapter 7.

CHAPTER 7: MYCOBACTERIAL GROWTH ASSAY

7.1 INTRODUCTION

Alveolar macrophages are the initial cells that defend against infection with *M. tuberculosis* (Schluger, 2001). Evidence suggests that survival of the tubercle bacilli in the macrophage is crucial for the pathogenesis of tuberculosis (Hsu, 1971). Organisms which escape the initial intracellular destruction (by their own virulence, or inability to be killed by the host), will multiply and destroy the alveolar macrophage (Schluger & Rom, 1998). This will lead to active tuberculosis. Alveolar macrophages respond to mycobacterial infection by undergoing apoptosis. Virulent mycobacteria override this reaction by inducing necrosis, which leads to uncontrolled *M. tuberculosis* replication (Keane *et al*., 1997; Kornfeld *et al*., 1999).

In the current study, it was determined that in U-937 there is no significant increase in apoptosis or necrosis in cells exposed to SiO_2, or γ-radiation or infection by *M. bovis* BCG or *M. kansasii* (or combinations of the above). In THP-1, there is no significant increase in apoptosis in cells exposed to γ-radiation. However, there is a significant increase in apoptosis in THP-1 cells exposed to SiO_2 (500 µg/ml only), and cells infected by *M. bovis* BCG or *M. kansasii*. There was no significant increase in apoptosis or necrosis in cells exposed to combinations of SiO_2, and/or γ-radiation and then infected with *M. bovis* BCG or *M. kansasii*.

It was the objective of the experiments described in this chapter, to determine whether the apoptosis associated with *M. bovis* BCG or *M. kansasii* infection in U-937 and THP-1 can be correlated with intracellular growth. To evaluate if SiO_2 or γ-radiation potentiates mycobacterial growth, experiments were performed after exposure to SiO_2 or γ-radiation, or combinations of SiO_2 and γ-radiation.

7.2 MATERIALS AND METHODS

The triatiated uridine assay for enumeration of mycobacteria was performed as described by Rook and Rainbow (1981) and Fazal *et al.* (1992). In brief, frozen aliquots, of known concentration, of *M. bovis* BCG and *M. kansasii* were thawed. Clumps were dispersed by repeated passage through a 25-gauge tuberculin needle. In a 96-well plate, a serial dilution of the mycobacteria was performed in Middlebrook 7H9 broth supplemented with 10% OADC (10^0 to 10^{-7}). Radio-labelled 5,6-^{3}H uridine (0.05 ml) (Amersham, NJ, USA) was added to give a final concentration of 10 μCi/ml. The plate was sealed and incubated at 37˚C 3-5% CO_2 for 72 hours to allow the incorporation of the radiolabel into the proliferating mycobacteria. The mycobacterial cultures were harvested with distilled water onto glass fibre filter paper using an automated cell harvester (TitreTek, Fisher Scientific, IL, USA). The filtermats were dried, the wells removed to scintillation vessels (Zinsser Analytic, Frankfurt, De), and 9 ml of scintillation fluid (Quicksafe A; Zinsser Analytic, Frankfurt, De) was added. Counting was performed on a Tri-Carb 1900CA (Packard, Canberra Company, IL, USA) liquid scintillation counter with 60 second readings. λ correction counts per minute (CPM) was performed by subtracting absorbance of the background reading (scintillation fluid only) from CPM of the samples.

The effects on intracellular growth of mycobacteria after U-937 and THP-1 cells were exposed to SiO_2 and/or γ-radiation were determined. U-937 and THP-1 were concentrated as described in Chapter 3.3. Cells were aliquoted to wells of a 24-well plate (0.5 ml), or to wells of a 96-well plate (0.2 ml), and differentiated. Cells were exposed to SiO_2, or γ-radiation and then infected with *M. bovis* BCG or *M. kansasii*. Alternatively, cells were exposed to SiO_2 and γ-radiation, and then infected. The control was unexposed (*i.e.* was not exposed to SiO_2 or γ-radiation). Following 90 minutes of infection (Day 0), on Day 3 and again on Day 5 following infection, the supernatants were removed, and the adherent cells were solubilised with 0.25% SDS PBS. Mycobacterial growth was measured by 5,6-^{3}H uridine incorporation as described above.

7.3 RESULTS

Frozen aliquots, of known concentration, of *M. bovis* BCG and *M. kansasii* were thawed and diluted. 5,6-^{3}H uridine was added to the suspensions, and after incubation, it was harvested. The amount of triatiated uridine incorporated was determined on a liquid scintillation instrument. The linear relationship between *M. bovis* BCG and *M. kansasii* CFU and the counts per minute (CPM) is shown in Figure 7-1. Linear regression lines for trend indicate for *M. bovis* BCG $r^2 = 0.7144$ and for *M. kansasii* $r^2 = 0.73$. This data indicates significant correlation between the amount of uridine incorporation and viable organisms as determined by CFU. Therefore, 5,6-^{3}H uridine was used as a surrogate for CFU in these experiments.

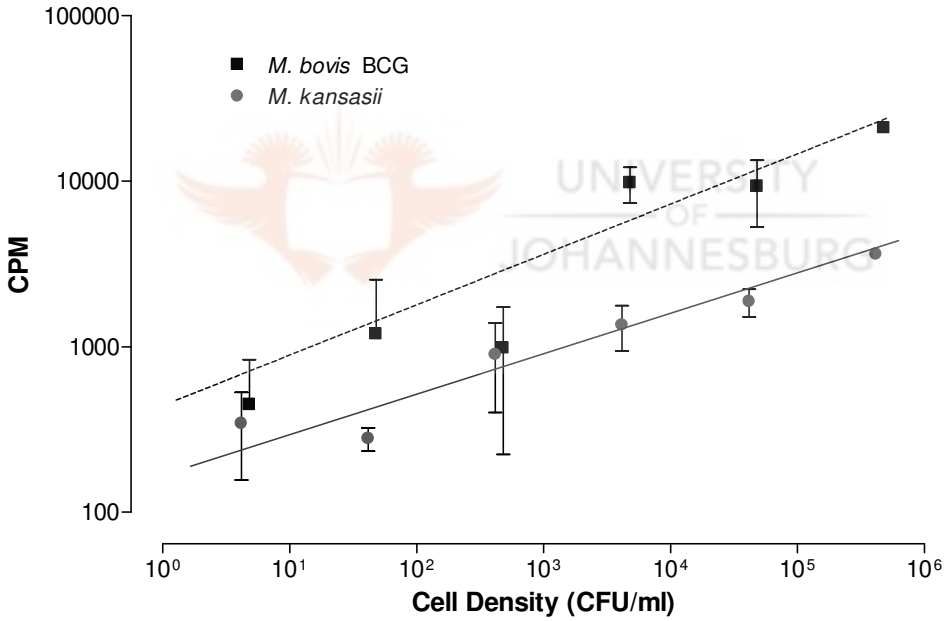

Figure 7-1 The linear relationship between the cell density of *M. bovis* BCG and *M. kansasii* (CFU/ml) and triatiated uridine counts per minute (CPM) (n = 2).

Differentiated U-937 cells were exposed to SiO_2 (0, 25, 100 and 500 µg/ml) and/or γ-radiation (0, 1, 3 and 10 Gy) and then infected with *M. bovis* BCG or *M. kansasii*. CPMs of the triatiated uridine were plotted against the time points (Figure 7-2 and 7-3). U-937 is permissive for the growth of *M. bovis* BCG *in vitro* (Figure 7-2). The student t-test was used to determine whether the differences in CPM between Day 0 and Day 5 were significant. There were significant increases in the CPMs in U-937

infected with *M. bovis* BCG, U-937 infected with *M. bovis* BCG and exposed to SiO_2 (25, 100 and 500 µg/ml), γ-radiation (1, 3 and 10 Gy) and combinations of SiO_2 and γ-radiation ($p < 0.01$). To determine whether exposure to SiO_2 and/or γ-radiation affected U-937 permissiveness for the growth of *M. bovis* BCG, the two-sample t-test of means was used to compare the CPMs of the control and exposed cells on Day 5. Despite higher CPMs in exposed cells, there was no statistical difference between the control and exposed cells.

U-937 is permissive for the growth of *M. kansasii in vitro* (Figure 7-3). The two-sample t-test of means (2-tailed) was used to determine whether the differences in CPM between Day 0 and Day 5 were significant. In all cases, there was an increase in CPMs between Day 0 and Day 5, although these increases were not always statistically significant (Table 7-1). There were significant increases in the CPM in U-937 infected with *M. kansasii*, U-937 infected with *M. kansasii* and exposed to 500 µg/ml SiO_2 ($p < 0.01$), 3 Gy and 10 Gy γ-radiation ($p < 0.01$) and combinations of SiO_2 and γ-radiation.

To determine whether exposure to SiO_2 and/or γ-radiation affected U-937 permissiveness for the growth of *M. kansasii*, the two-sample t-test of means was used to compare the CPMs of the control (not exposed to SiO_2 or γ-radiation) and exposed cells on Day 5 (Table 7-1). The mean CPMs for the control was higher than the means of the exposed cells. There were significant decreases in CPMs ($p < 0.001$) from U-937 infected with *M. kansasii* BCG and exposed to 500 µg/ml SiO_2 and 1 Gy γ-radiation as compared to the control). There were significant decreases in CPMs ($p < 0.001$) from U-937 infected with *M. kansasii* BCG and exposed to combinations of SiO_2 and γ-radiation as compared to the control.

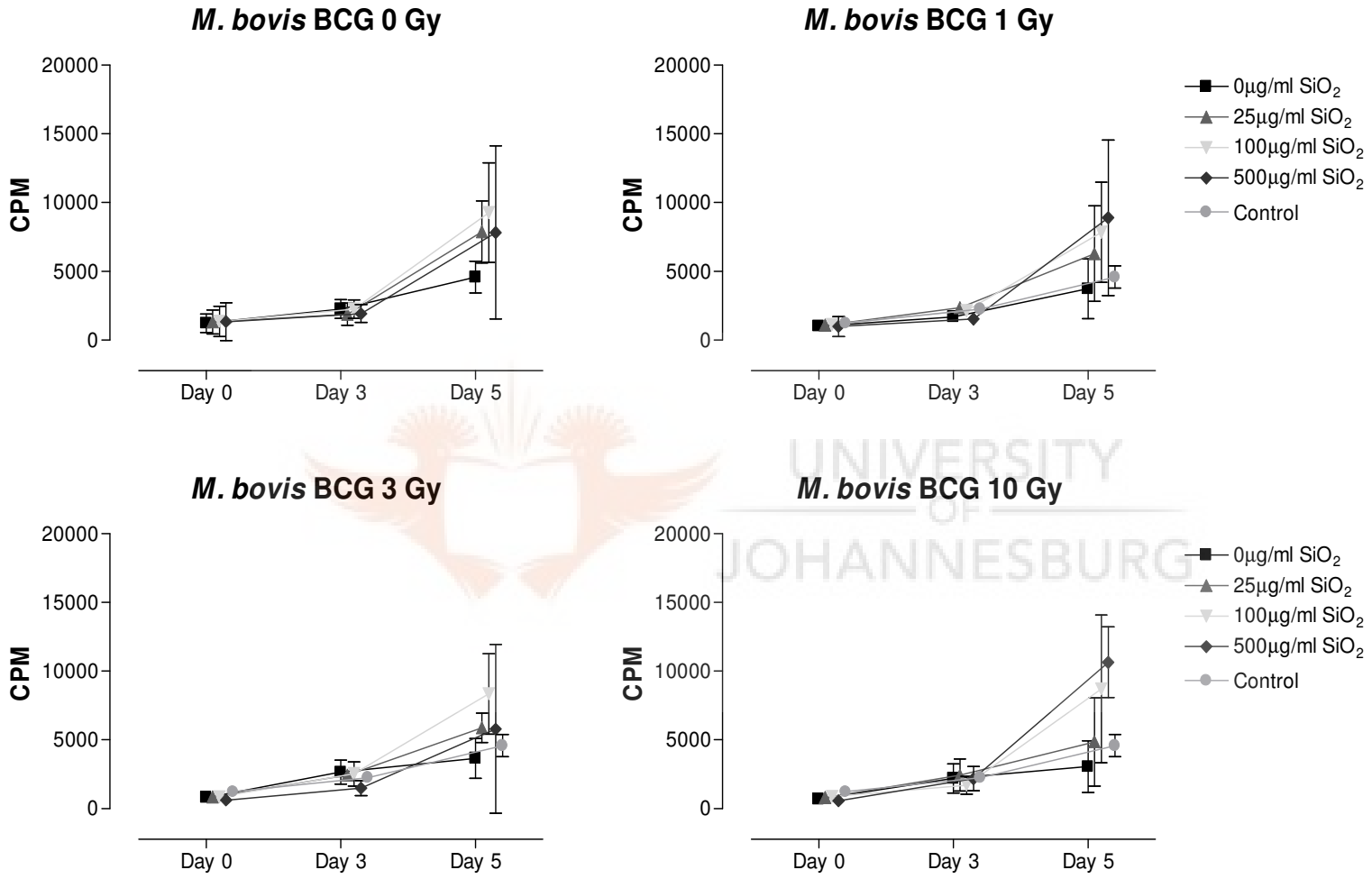

Figure 7-2 Growth of *M. bovis* BCG in U-937 infected cells exposed to SiO_2 (25, 100 and 500 µg/ml) and γ-radiation (1, 3 or 10 Gy). Two controls are shown, both of which have been infected- an unexposed control (0 µg/ml), and a control exposed to γ-radiation only (n = 2).

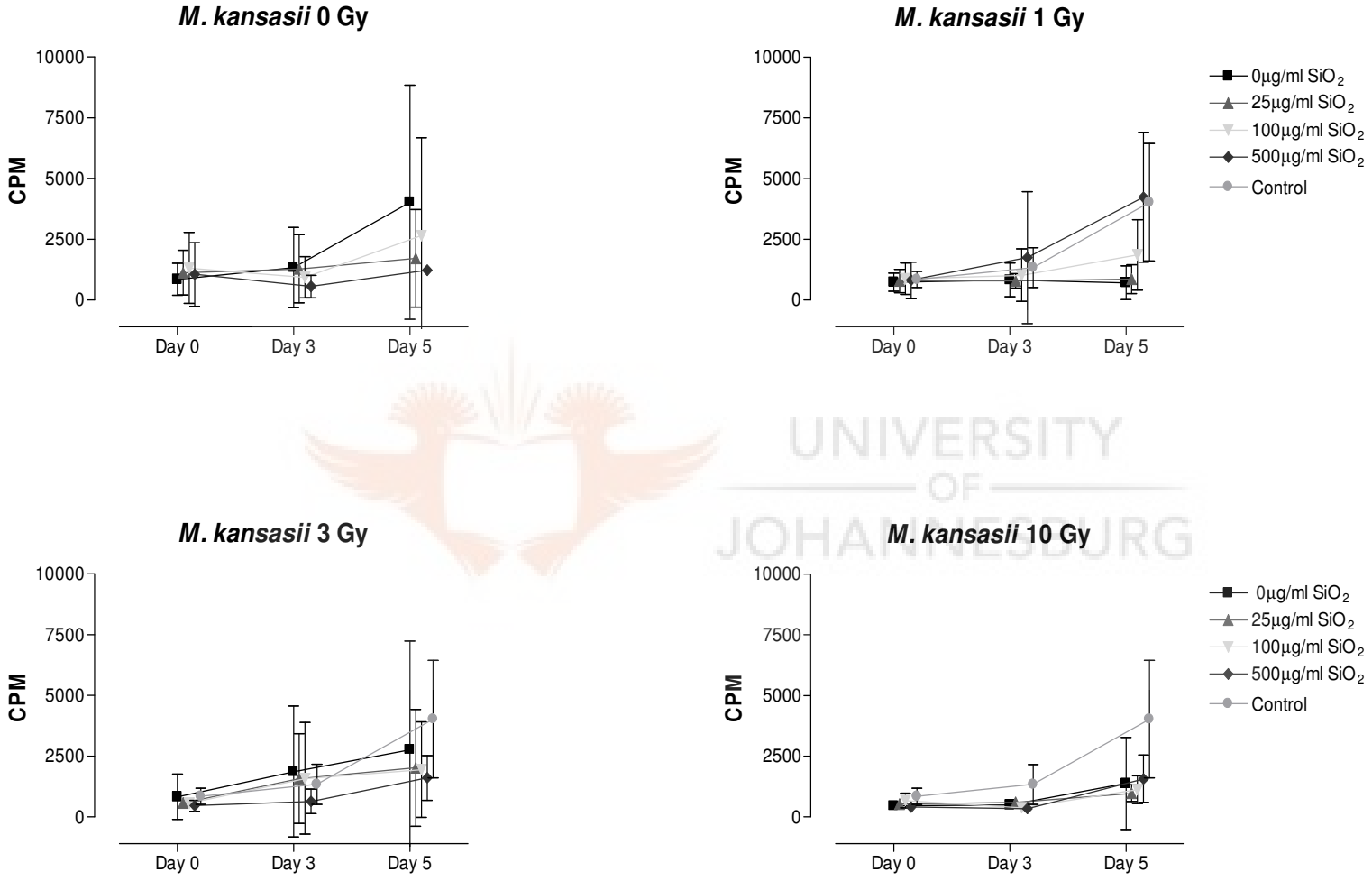

Figure 7-3 **Growth of *M. kansasii* in U-937 infected cells exposed to SiO_2 (25, 100 and 500 µg/ml) and γ-radiation. Two controls are shown, both of which have been infected- an unexposed control (0 µg/ml), and a control exposed to γ-radiation only (n = 2).**

Table 7-1 **Statistical significance as determined by the two-sample t-test of means (2-tailed) between Day 0 (D0) and Day 5 (D5) for U-937 infected with *M. kansasii* and exposed to SiO_2, and/or γ- radiation or combinations of SiO_2 and γ- radiation. Also shown is the statistical significance as determined by the two-sample t-test of means (2-tailed) between the unexposed control and exposed tests on D5.**

	D0 vs. D5 (p)	*Control vs. (p)*
Control	< 0.01	-
0 Gy 25 µg/ml	0.612	0.41
0 Gy 100 µg/ml	0.557	0.677
0 Gy 500 µg/ml	< 0.01	< 0.01
1 Gy 0 µg/ml	0.949	< 0.01
1 Gy 25 µg/ml	0.832	< 0.01
1 Gy 100 µg/ml	0.257	< 0.01
1 Gy 500 µg/ml	< 0.01	0.941
3 Gy 0 µg/ml	< 0.01	0.715
3 Gy 25 µg/ml	< 0.01	0.485
3 Gy 100 µg/ml	< 0.01	0.454
3 Gy 500 µg/ml	< 0.01	< 0.01
10 Gy 0 µg/ml	< 0.01	0.345
10 Gy 25 µg/ml	< 0.01	< 0.01
10 Gy 100 µg/ml	0.219	< 0.01
10 Gy 500 µg/ml	< 0.01	< 0.01

Differentiated THP-1 cells were exposed to SiO_2 (0, 25, 100 and 500 µg/ml) and γ-radiation (0, 1, 3 and 10 Gy). Cells were infected with *M. bovis* BCG or *M. kansasii* and concentrations of these organisms were measured 90 minutes, 3 and 5 days post infection. The CPMs of the tritiated uridine were plotted against the time points (Figure 7-4 and 7-5). THP-1 controls the growth of *M. bovis* BCG *in vitro* (Figure 7-4). The two-sample t-test of means was used to determine whether the differences in CPM between Day 0 and Day 5 were significant. There was a significant increase in the CPMs in THP-1 infected with *M. bovis* BCG ($p = 0.036$) however; the 95% confidence intervals of the means between Day 0 and Day 5 were overlapping. THP-1 is permissive for the growth of *M. bovis* BCG *in vitro* in cells exposed to SiO_2 (25 µg/ml, 100 µg/ml) and/or γ-radiation (1, 3 and 10 Gy). To determine whether exposure to SiO_2 and/or γ-radiation affected THP-1 ability to control the growth of *M. bovis* BCG, the two-sample t-test of means was used to compare the CPMs of the control (not exposed to SiO_2 or γ-radiation) and exposed cells on Day 5. The mean CPMs for the control was lower than the means of the exposed cells ($p < 0.01$), except in cells exposed to a combination of 500 µg/ml SiO_2 and 10 Gy γ-radiation ($p = 0.716$).

THP-1 controls the growth of *M. kansasii in vitro* (Figure 7.5). The two-sample t-test of means was used to determine whether the differences in CPM between Day 0 and Day 5 were significant. There were significant decreases in the CPMs in THP-1 infected with *M. kansasii* and THP-1 infected with *M. kansasii* and exposed to 25, 100 and 500 µg/ml SiO_2 ($p < 0.001$). The decreases in cells exposed to γ-radiation (1, 3 and 10 Gy) were not significant. Combinations of SiO_2 (all concentrations) and 3 Gy γ-radiation were statistically significant ($p < 0.01$). To determine whether exposure to SiO_2 and/or γ-radiation affected THP-1 ability to control the growth of *M. kansasii*, the two-sample t-test of means was used to compare the CPMs of the control (not exposed to SiO_2 or γ-radiation) and exposed cells on Day 5 (Table 7-2). The mean CPMs for the control was lower than the means of the exposed cells, except in cells exposed to 3 Gy γ-radiation and combinations of 3 Gy γ-radiation and SiO_2 in which they were higher.

Table 7-2 **Statistical significance as determined by the two-sample t-test of means (2-tailed) between Day 0 (D0) and Day 5 (D5) for THP-1 infected with *M. kansasii* and exposed to SiO_2, and/or γ- radiation or combinations of SiO_2 and γ- radiation. Also shown is the statistical significance as determined by the two-sample t-test of means (2-tailed) between the unexposed control and exposed tests on D5.**

	D0 vs. D5 (p)	*Control vs. (p)*
Control	< 0.01	-
0 Gy 25 µg/ml	< 0.01	0.968
0 Gy 100 µg/ml	< 0.01	<0.01
0 Gy 500 µg/ml	< 0.01	0.695
1 Gy 0 µg/ml	0.669	< 0.01
1 Gy 25 µg/ml	0.879	< 0.01
1 Gy 100 µg/ml	0.913	< 0.01
1 Gy 500 µg/ml	0.976	< 0.01
3 Gy 0 µg/ml	< 0.01	< 0.01
3 Gy 25 µg/ml	< 0.01	< 0.01
3 Gy 100 µg/ml	< 0.01	< 0.01
3 Gy 500 µg/ml	< 0.01	< 0.01
10 Gy 0 µg/ml	0.95	0.602
10 Gy 25 µg/ml	0.898	0.37
10 Gy 100 µg/ml	0.688	0.652
10 Gy 500 µg/ml	0.644	< 0.01

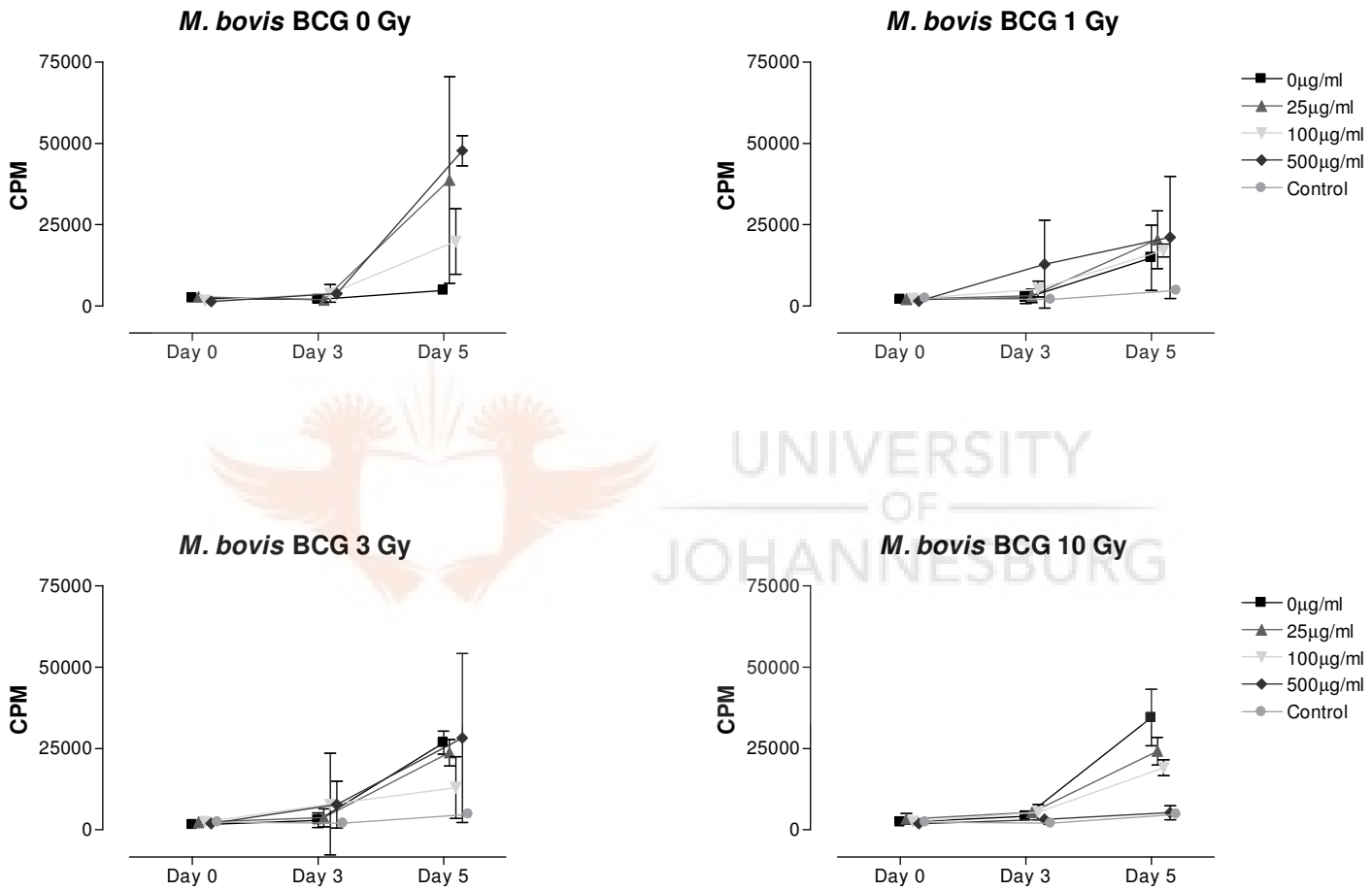

Figure 7-4 **Growth of *M. bovis* BCG in THP-1 infected cells exposed to SiO_2 (25, 100 and 500 µg/ml) and γ-radiation. Two controls are shown, both of which have been infected- an unexposed control (0 µg/ml), and a control exposed to γ-radiation only (n = 3).**

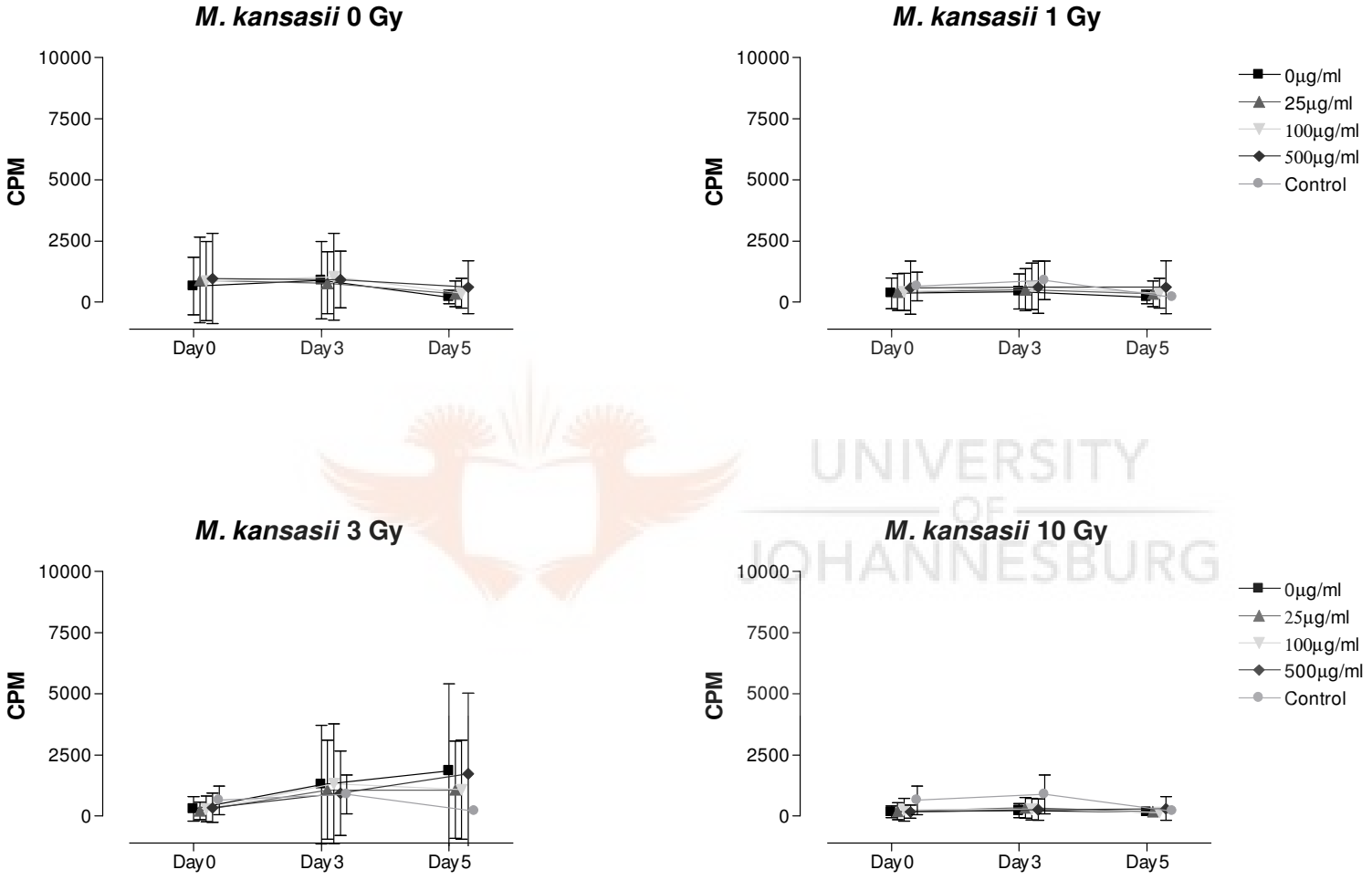

Figure 7-5 **Growth of *M. kansasii* in THP-1 infected cells exposed to SiO_2 (25, 100 and 500 µg/ml) and γ-radiation. Two controls are shown, both of which have been infected- an unexposed control (0 µg/ml), and a control exposed to γ-radiation only (n = 2).**

7.4 DISCUSSION

In vitro studies have examined the extent of apoptosis of alveolar macrophages following infection by *M. tuberculosis* complex strains of differing virulence and by *M. kansasii* (Keane *et al*., 2000). Avirulent or attenuated bacilli (*M. tuberculosis* H37*Ra*, *Mycobacterium bovis* BCG, and *M. kansasii*) induced significantly more apoptosis than virulent strains (*M. tuberculosis* H37*Rv*, Erdman, *M. tuberculosis* clinical isolate BMC 96.1, and *M. bovis* wild type). Since apoptosis has been associated with containment of growth of mycobacteria (Riendieau and Kornfeld 2003, Passmore *et al.*, 2001), it was of interest to evaluate this in U937and THP-1 cell lines exposed to SiO_2 and/or radiation and then infected with mycobacteria.

In control U-937 cells infected with *M. bovis* BCG (*i.e.* not exposed to SiO_2 or radiation), there was a significant increase in CPM between Day 0 and Day 5 ($p = 0.009$). This result suggests that U-937 is permissive for the growth of *M. bovis* BCG *in vitro*. This finding supports the conclusions of Chapter 4, 5 and 6, which found no cytotoxic/apoptotic/ DNA damaging effect on U-937 following infection by that *M. bovis* BCG. This result is consistent with the findings of Passmore *et al.*, (2001) who found that differentiated U-937 cells were capable of efficient phagocytosis of *M. tuberculosis* but did not generate a subsequent respiratory burst response, and are hence permissive for intracellular growth of *M. bovis* BCG.

U-937 was permissive for the growth of *M. bovis* BCG in SiO_2 concentrations of 25, 100 and 500 µg/ml SiO_2 ($p < 0.01$), as well as in cells exposed to 1 Gy, 3 Gy and 10 Gy γ-radiation. The permissive growth of *M. bovis* BCG in U-937 exposed to SiO_2 and/or γ-radiation has not been previously demonstrated, and is in keeping with the lack of apoptosis as determined by experiments described in Chapter 4, 5 and 6. It was the hypothesis of this study that SiO_2 and γ-radiation exposure may both be involved in predisposing mine workers to pulmonary tuberculosis by their effects on the macrophage's ability to contain mycobacterial proliferation. Despite higher CPMs in U-937 exposed to SiO_2 and γ-radiation, there was no statistical difference between the control and exposed cells, and it is concluded that SiO_2 and/or γ-radiation do not potentiate the growth of *M. bovis* BCG in U-937.

In control U-937 cells infected with *M. kansasii* (*i.e.* not exposed to SiO_2 or radiation), there was a significant increase in CPM between Day 0 and Day 5 ($p < 0.01$). This result suggests that U-937 is permissive for the growth of *M. kansasii in vitro.* This finding supports the conclusions of Chapter 4, 5 and 6, which found no cytotoxic/apoptotic/ DNA damaging effect on U-937 following infection by that *M. kansasii*. The permissive growth of *M. kansasii* in U-937 has not been previously demonstrated. Furthermore, in comparison to the unexposed control, it was noted that U-937 was permissive for the growth of *M. kansasii* in SiO_2 or after γ-radiation. Not all the increases were significant (Table 4-7). As was noted in Chapter 5, exposure of U-937 to 25 µg/ml, 100 µg/ml and 500 µg/ml SiO_2 increased the percentage of apoptotic cells in a dose related manner, but this was not statistically significant. In the absence of reliable Annexin and MTT data, it was concluded that SiO_2 is not associated with significant cell death in U-937. It was also concluded that γ-radiation is not associated with significant cell death in U-937. The permissiveness of U-937 for the growth of *M. kansasii* is in keeping with these findings. It is the hypothesis of this study that SiO_2 and γ-radiation exposure may both be involved in predisposing mine workers to pulmonary disease due to NTM's by their effects on the macrophage's ability to contain mycobacterial proliferation. CPMs in exposed cells were consistently lower than the control, but the differences were not always significant, and it was concluded that SiO_2 and/or γ-radiation do not potentiate the growth of *M. kansasii* in U-937 cells.

No studies to date have examined the effect of γ-radiation on mycobacterial potentiation. Inability to document potentiation of growth of *M. bovis* BCG or *M. kansasii* in U-937 cells using the uridine assay may represent maximal metabolic activity of the mycobacteria, which are not able to be additionally stimulated by SiO_2 or γ-radiation exposure. In contrast, Allison & D'Arcy Hart (1968) and Policard *et al.* (1967), demonstrated that infections with *M. tuberculosis* and *M. kansasii* in experimental animals are aggravated by injection or inhalation of SiO_2. It is hypothesized that discrepancies in the effect of SiO_2 between the latter two studies and the data reported here may be due to the differences in control of mycobacterial proliferation demonstrated by animal and *in vitro* models, the lack of maturity of the U937 cell line, and/or differences in mycobacterial growth obtained by CFU as compared to uridine assay.

In THP-1 control cells (*i.e.* not exposed SiO_2 or radiation) infected with *M. bovis* BCG, there was a negible increase in CPM between Day 0 and Day 5 (1.7 higher on Day 5 than on Day 0). The negligible increase may be due to technical difficulties associated with obtaining consistent suspensions of mycobacteria *in vitro* (due to clumping), and lead to the conclusion that THP-1 controls the growth of *M. bovis* BCG *in vitro*. This is consistent with the findings in Chapter 4, 5 and 6 that concluded that *M. bovis* BCG is associated with cytotoxicity, death by apoptosis and DNA damage in THP-1. The finding is also consistent with the findings of Riendieau & Kornfeld (2003), who examined the potential for PMA differentiated THP-1 cells to mimic this response of alveolar macrophages to mycobacterial infection. Consistent with the behavior of alveolar macrophages, attenuated *M. tuberculosis* H37*Ra* and *M. bovis* BCG strongly induced THP-1 apoptosis, and was associated with reduced viability of *M. bovis* BCG.

In comparison to an unexposed control, THP-1 was permissive for the growth of *M. bovis* BCG in SiO_2, γ-radiation and combinations of SiO_2 and γ-radiation. This potentiation of growth of *M. bovis* BCG in THP-1 cannot be explained by changes in apoptosis, since the findings of Chapter 4, 5 and 6, indicate that exposure to SiO_2 and/or γ-radiation resulted in the same amount of cytotoxic/apoptotic/DNA damaging effects as did infection by *M. bovis* BCG alone. There is no evidence to suggest a protective effect that would result in more viable cells able to support the intracellular growth of *M. bovis* BCG. Nonetheless, the findings of the current study are in keeping with those of Allison & D'Arcy Hart (1968), who recognized that infections with *M. tuberculosis* in experimental animals are aggravated by injection or inhalation of SiO_2. Allison & D'Arcy Hart offer two possible explanations for their findings, *viz.* after ingestion of silica the metabolism of macrophage is altered to favour the proliferation of mycobacteria, or there is a direct effect of silica (or silicic acid) on the mycobacteria which causes its more rapid multiplication. Impairment of monocyte function by SiO_2 cannot entirely be excluded, taking into account the impact of SiO_2 on apoptosis (Chapter 5), genotoxicity (Chapter 6), and potentiation of *M. bovis* BCG growth data (this chapter), and considering that the influence on metabolic activity of the cell lines (Chapter 4) was attributed to interference by AB- serum with the MTT assay. Since the uridine growth assay primarily measures microbial metabolic activity, SiO_2 may also augment metabolic activity rather than mycobacterial growth (as measured by CFU). While these measurements usually correlate as shown in

Figure 7.1, dissociation of these parameters could theoretically occur, resulting in increased metabolic activity without concommitant growth. Alternatively, Allison & D'Arcy Hart's hypothesis that there is a direct effect of silica on stimulating the growth of the mycobacteria could account for the SiO_2 data from this study and could also explain the findings that suggest potentiation of growth of *M. bovis* BCG by γ-radiation. Such potentiation of growth of mycobacteria *in vitro* has not previously been described, but of interest is that irradiated mice are more susceptible to tuberculosis infection than non-irradiated control animals (Anderson & Warner, 1976). Malatsi (2004) could find no evidence to suggest that irradiation reactivates dormant mycobacteria. An alternative hypothesis explaining the potentiation of growth by irradiation is that low dose ionizing radiation has an immunoenhancing effect on the body or cell (Luckey, 1999). This would result in either more viable cells, or alternatively the cells that are viable are better able to support the intracellular growth of *M. bovis* BCG. Against this possibility accounting for the data reported here are the results from Chapter 4, 5 and 6 which found no evidence of an increase in the number of viable THP-1 cells after exposure to γ-radiation. In fact, evidence from the Comet assay (Chapter 6) would suggest a decrease in the number of viable cells, and a corresponding increase in DNA damaged cells after exposure to γ-radiation (alone).

In THP-1 control cells (*i.e.* not exposed SiO_2 or radiation) infected with *M. kansasii*, there was a significant decrease in CPM between Day 0 and Day 5 ($p < 0.001$). It is concluded that THP-1 controls the growth of *M. kansasii in vitro*. The ability of THP-1 to control the growth of *M. kansasii in vitro* has not been previously demonstrated, and is in keeping with the findings in Chapter 5 that suggested that *M. kansasii* induces apoptosis in THP-1. The finding is also in keeping with that of Guerardel *et al.*, (2003). This group demonstrated that the *M. kansasii* biosynthetic precursor lipomannan (KanLM), is a potent apoptosis-inducing factor in THP-1 cells. Although the authors provide no details of the growth of *M. kansasii* in THP-1, the apoptosis induction would provide a mechanism whereby THP-1 controls the growth of *M. kansasii*

Furthermore, additional apoptosis caused by exposure to SiO_2 and/or γ-radiation may contribute to the control of *M. kansasii* in THP-1 cells exposed to 3Gy γ-radiation

(alone and with SiO_2), as this potentiated the growth of *M. kansasii*, although there were wide confidence intervals around the mean and this result needs to be treated with caution and requires confirmation. However, Policard *et al.* (1967), recognized that infections with *M. kansasii* in macrophages of guinea pigs are aggravated by the addition of pulmonary dust, and differences in the magnitude of this effect could be due to the difference between *in vitro* and *ex vivo* models.

In conclusion, the potentiation of *M. bovis* BCG growth following SiO_2 and/or radiation may support the hypothesis of this study that SiO_2 and γ-radiation exposure may both be involved in predisposing mine workers to pulmonary tuberculosis by their effects on the macrophage's ability to contain mycobacterial proliferation. However, *M. kansasii* growth was controlled by THP-1 following SiO_2 and γ-radiation exposure. The different manner in which THP-1 controlled the metabolic activity (and by extrapolation proliferation) of the mycobacterial strains occurred despite similar findings of cytotoxicity, apoptosis and genotoxicity between the strains. Differences in virulence factors and the manner in which *M. bovis* BCG and *M. kansasii* establish infection are likely explanations.

CHAPTER 8: IMMUNOLOGICAL RESPONSE

8.1 INTRODUCTION

Cytokines are small soluble molecules secreted by one cell that can alter the behaviour or properties of the cell itself, or of another cell. TNF-α is a pro-inflammatory cytokine that is a 185 amino acid glycoprotein peptide hormone, cleaved from a 212 amino acid-long pro-peptide on the surface of macrophages. TNF-α release is stimulated by several mediators, such as IL-1 and bacterial endotoxin (Carswell *et al*., 1975). U-937 releases increasing levels of TNF-α into the culture medium during differentiation. TNF-α concentrations peak 48 hours post-differentiation, while in undifferentiated cells they remain at the detection limit (Hass *et al*., 1991). Hass *et al.* (1991), describes the TNF-α concentration in supernatants of 5×10^5 cells/ml examined by RIA. It was found that TNF-α concentrations remain at peak concentration (9 ng/ml) for up to six days post-differentiation before declining over two days to pre-differentiation levels. Similarly, THP-1 releases increasing levels of TNF-α into the culture medium during differentiation. Oliveira *et al*. (2001), describe a dose dependant increase TNF-α secretion in response to PMA 48 hours after differentiation.

TNF-α has a wide spectrum of biological activities and generally acts together with IL-1 and IL-6. Tumor necrosis factor is produced mainly by activated macrophages, and is the major extrinsic mediator of apoptosis. In the current study, it has been determined that in U-937 there is no significant increase in apoptosis or DNA damage in cells exposed to SiO_2, or γ-radiation or infection by *M. bovis* BCG or *M. kansasii* (or combinations of the above). In THP-1, there is no significant increase in apoptosis in cells exposed to γ-radiation, but there was an increase in DNA damage. There is a significant increase in apoptosis and DNA damage in THP-1 cells exposed to SiO_2.

TNF-α activities also include the augmentation of neutrophil and eosinophil functional activity, the stimulation of fibroblast growth (Dubois *et al*., 1989), granuloma formation (Amiri *et al*., 1992) and intracellular killing (Liew *et al*., 1990). In the current study, it was demonstrated that apoptosis occurs widely in THP-1 in response to *M. bovis* BCG

or *M. kansasii* infection. Consistent with this finding, it was demonstrated that U-937 is permissive for the growth of *M. bovis* BCG and *M. kansasii in vitro*, and THP-1 controls the growth of *M. bovis* BCG and *M. kansasii in vitro*.

It was the objective of these experiments to quantify the TNF-α release by U-937 and THP-1 after exposure to SiO_2, or γ-radiation or infection by *M. bovis* BCG or *M. kansasii* and after exposure to SiO_2, and/or γ-radiation followed by infection with *M. bovis* BCG or *M. kansasii*.

8.2 MATERIALS AND METHODS

Becton Dickinson Opt ELISA Human TNF-α cytokine assay (Becton Dickinson, Towson, MD) was standardized for use with U-937 and THP-1 cells. ELISA plates were prepared as instructed in the package insert. 96-well plates (Falcon, Becton Dickinson, NJ, USA) were coated with 0.1 ml capture antibody per well diluted (1:250) in coating buffer (0.1 M Sodium Carbonate [pH9.5], 8.4 g $NaHCO_3$, 3.56 g Na_2CO_3; made up to 1 L; pH 9.5), and stored overnight at 4˚C. The wells were aspirated and washed with wash buffer (PBS with 0.05% Tween-20) three times before blocking with 0.2 ml assay diluent per well for one hour at room temperature. After incubation, the wells were re-washed in wash buffer three times.

Standards were prepared as instructed in the pack insert. The stock standard was reconstituted with 1 ml of deionized water (the reconstituted stock can be aliquoted and stored at -70˚C). Serial dilutions were performed to yield standard TNF-α concentrations between 500 pg/ml and 7.8 pg/ml.

Each standard dilution was aliquoted to a well of the ELISA plate in triplicate (0.1 ml). The plate was incubated for two hours at room temperature. The plate was washed with wash buffer five times. Working detector (0.1 ml) (detection antibody and avidin-HRP reagent) was added to each well. The plate was sealed and incubated for one hour at room temperature. The plate was washed with wash buffer seven times. Before incubating the plate for 30 minutes at room temperature (in the dark), 0.1 ml substrate solution (TMB, Becton Dickinson, Towson, MD) was added to each

well. Finally, 0.05 ml stop solution (2 N H_2SO_4) was added to each well, and the absorbance was measured. The λ correction was performed by subtracting absorbance at 540 nm from absorbance at 450 nm. A combined standard curve is shown in Figure 8-1. Linear regression lines for trend indicate $r^2 = 0.9294$ to 0.9521. Good correlation between the OD 540 and TNF-α concentration was achieved. The standard curves were used for interpolation of the TNF-α concentration.

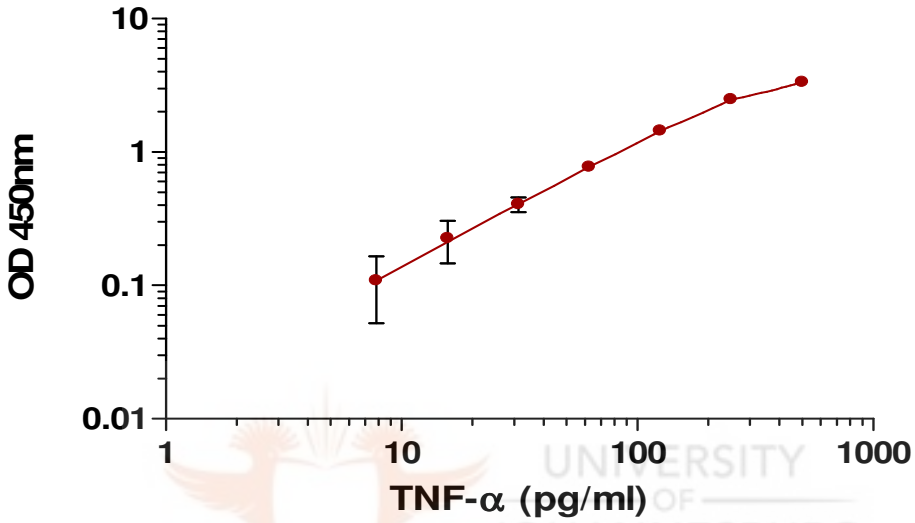

Figure 8-1 **Standard curve for the Becton Dickinson Opt ELISA Human TNF-α cytokine assay. The graph shows the linear relationship between absorbance (OD) at 450 nm (λ corrected 570 nm) and TNF-α concentration (pg/ml) (n = 5).**

The effects on intracellular growth of mycobacteria after U-937 and THP-1 cells were exposed to SiO_2 and/or γ-radiation was determined. U-937 and THP-1 were concentrated as described in Chapter 3.3. Cells were aliquoted to wells of a 24-well plate (0.5 ml), or to wells of a 96-well plate (0.2 ml), and differentiated. Cells were exposed to SiO_2, γ-radiation and/or infected with *M. bovis* BCG or *M. kansasii*. Following 90 minutes of infection, the supernatants were collected. The supernatants were frozen at -20˚C until the day of testing. The Becton Dickinson Opt ELISA Human TNF-α cytokine assay was performed as described. The TNF-α concentration was determined by extrapolation from the standard curve.

8.3 RESULTS

In U-937, the mean TNF-α production by differentiated control cells (*i.e* not exposed or infected) was 134 pg/ml (Figure 8-2). There was a dose dependant decrease in TNF-α production with increasing SiO_2 concentrations (110, 55 and 37 pg/ml for 25, 100 and 500 µg/ml respectively). There was a significant difference in the TNF-α concentration produced by the control compared to cells exposed to 100 µg/ml and 500 µg/ml SiO_2 ($p = 0.001$ and 0.001 respectively). The mean TNF-α production by U-937 for cells infected by *M. bovis* BCG and *M. kansasii* only was 108 pg/ml ($p = 0.04$) and 128 pg/ml ($p = 0.835$) respectively. The mean TNF-α production by U-937 for cells exposed to γ-radiation was higher than the control ($p = 0.004$ to $p = 0.011$), and increased marginally with increasing exposure (184 pg/ml, 188 pg/ml and 192 pg/ml for 1 Gy, 3 Gy and 10 Gy respectively). There was no significant difference in TNF-α production between the different γ-radiation exposures.

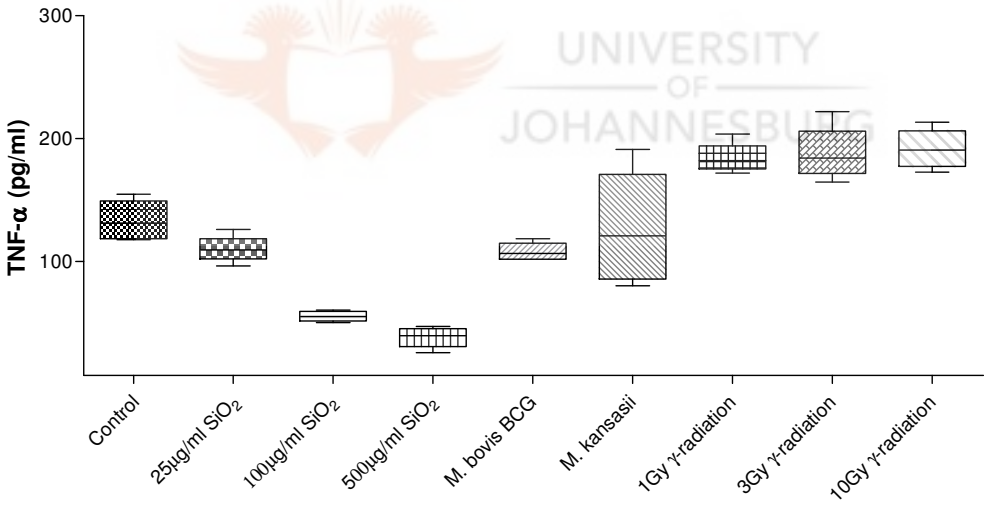

Figure 8-2 TNF-α production in U-937 cells exposed to SiO_2 (25, 100 and 500 µg/ml), or γ-radiation (1, 3 and 10 Gy) or infected with *M. bovis* BCG or *M. kansasii*. The control was uninfected and unexposed (n =2).

In THP-1, the mean TNF-α concentration produced by differentiated control cells (*i.e.* not exposed or infected) was 22.5 pg/ml, and was significantly lower than the control U-937 cells ($p < 0.001$) (Figure 8-3). There was no significant difference in TNF-α concentration between the control and cells exposed to SiO_2 (25 µg/ml, 100 µg/ml and

500 µg/ml), infected by *M. bovis* BCG and *M. kansasii* or exposed to γ-radiation (1 Gy, 3 Gy and 10 Gy).

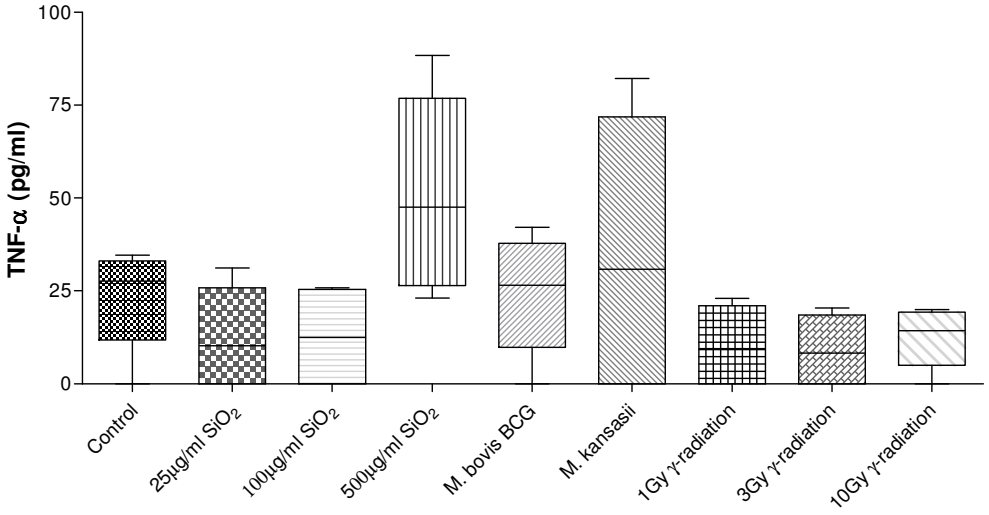

Figure 8-3 TNF-α production in THP-1 cells exposed to SiO_2 (25, 100 and 500 µg/ml), or γ-radiation (1, 3 and 10 Gy) or infected with *M. bovis* BCG or *M. kansasii*. The control was uninfected and unexposed (n = 2)

TNF-α concentration produced by U-937 cells exposed to SiO_2 (25, 100 and 500 µg/ml), and/or γ-radiation and then infected with *M. bovis* BCG are shown Figure 8-4. A control was infected, but not exposed. Due to the similarity of results between 1Gy, 3Gy and 10Gy, only data from the 10Gy analysis are shown. In the control U-937 cells, the mean TNF-α concentration was 262 pg/ml. There was a dose related decrease in TNF-α concentration with increasing SiO_2 concentrations. These were significantly lower than the control ($p = 0.003$, 0.001 and 0.001 for 25 µg/ml, 100 µg/ml and 500 µg/ml SiO_2 respectively). There was no significant difference between the control and cells exposed to 10 Gy γ-radiation. Decreasing TNF-α concentrations were also seen in cells exposed to both SiO_2 and γ-radiation (Table 8-1).

Table 8-1 **TNF-α concentrations (pg/ml) of U-937 and THP-1 infected by *M. bovis* BCG and *M. kansasii* and exposed to SiO_2 (25, 100 and 500 µg/ml) and 10 Gy γ-radiation, as well as significance value (p) compared to the control as determined by the two-sample t-test of means (2-tailed).**

U-937 M. bovis BCG	*TNF-α concentration(pg/ml)*	*Control vs. (p)*
10 Gy 25 µg/ml	127	0.035
10 Gy 100 µg/ml	34	0.001
10 Gy 500 µg/ml	14	0,001
U-937 M. kansasii		
10 Gy 25 µg/ml	79.3	0.382
10 Gy 100 µg/ml	26.2	0.09
10 Gy 500 µg/ml	8.2	0.007
THP-1 M. kansasii		
10 Gy 25 µg/ml	47	0.002
10 Gy 100 µg/ml	68.7	0.001
10 Gy 500 µg/ml	90.1	0.001

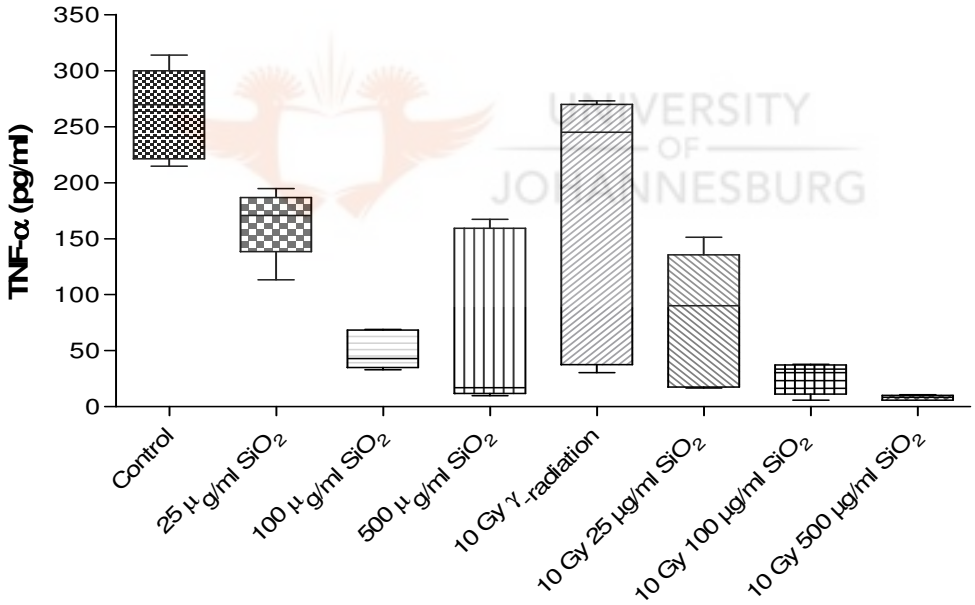

Figure 8-4 **TNF-α production of U-937 cells infected with *M. bovis* (BCG) exposed to SiO_2 (25, 100 or 500 µg/ml) and/or 10 Gy γ-radiation. The control was infected, but not exposed (n = 2).**

TNF-α concentration produced by U-937 cells exposed to SiO_2 (25, 100 and 500 µg/ml), and/or γ-radiation and then infected with *M. kansasii* are shown Figure 8-5. A control was infected, but not exposed. Due to the similarity of results between 1Gy, 3Gy and 10Gy, only data from the 10Gy analysis are shown. In U-937 control cells

(*i.e.* infected but not exposed), the mean TNF-α concentration was 52 pg/ml. There was no significant difference in TNF-α concentration between the control and cells exposed to 25 µg/ml and 500 µg/ml SiO_2. There was a significant difference in TNF-α concentration between the control and cells exposed to 100 µg/ml SiO_2 ($p = 0.019$). There was no significant difference between the control and cells exposed to 10 Gy γ-radiation. Decreasing TNF-α concentrations were also seen in cells exposed to both SiO_2 and γ-radiation (Table 8-1).

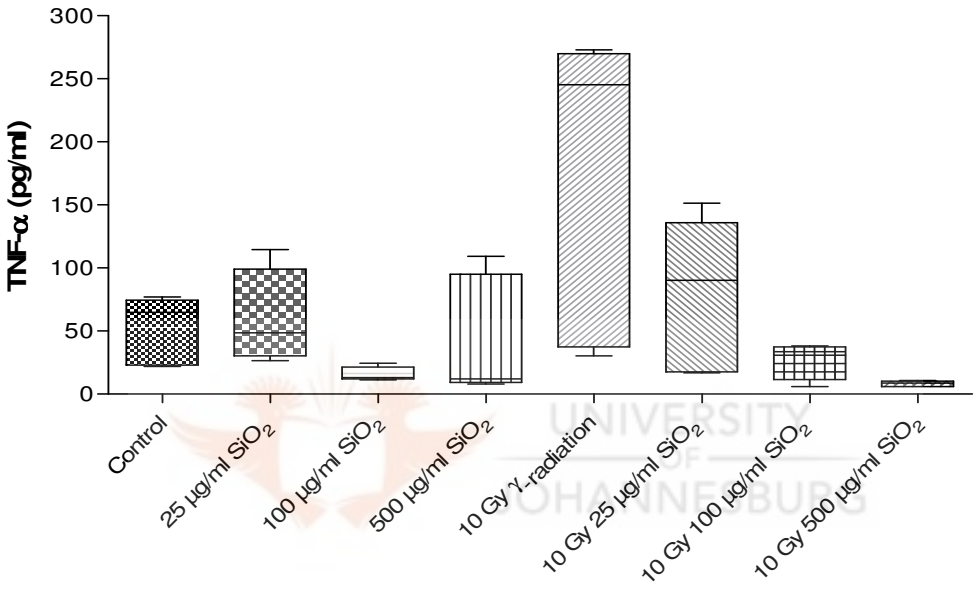

Figure 8-5 TNF-α production of U-937 cells infected with *M. kansasii* exposed to SiO_2 (25, 100 or 500 µg/ml) and/or 10 Gy γ-radiation. The control was infected, but not exposed (n = 2).

TNF-α concentration produced by THP-1 cells exposed to SiO_2 (25, 100 and 500 µg/ml), and/or γ-radiation and then infected with *M. bovis* BCG are shown Figure 8-6. Due to the similarity of results between 1Gy, 3Gy and 10Gy, only data from the 10Gy analysis are shown. In control THP-1 cells (infected with *M. bovis* BCG and not exposed to SiO_2 or γ-radiation), the mean TNF-α concentration was 36.2 pg/ml. There was no significant difference in TNF-α concentration between the control and cells exposed to 25 µg/ml, 100 µg/ml and 500 µg/ml SiO_2, although the trend was for increasing TNF-α concentration with increasing SiO_2 concentrations. There was no significant difference between the control and cells exposed to 10 Gy γ-radiation. There was a dose related increase in TNF-α concentration with increasing SiO_2 concentrations. The TNF-α concentration in THP-1 cells exposed to 10 Gy 100 µg/ml

were significantly higher than the control (p = 0.048), but not for cells exposed to 10 Gy 25 µg/ml and 10 Gy 500 µg/ml.

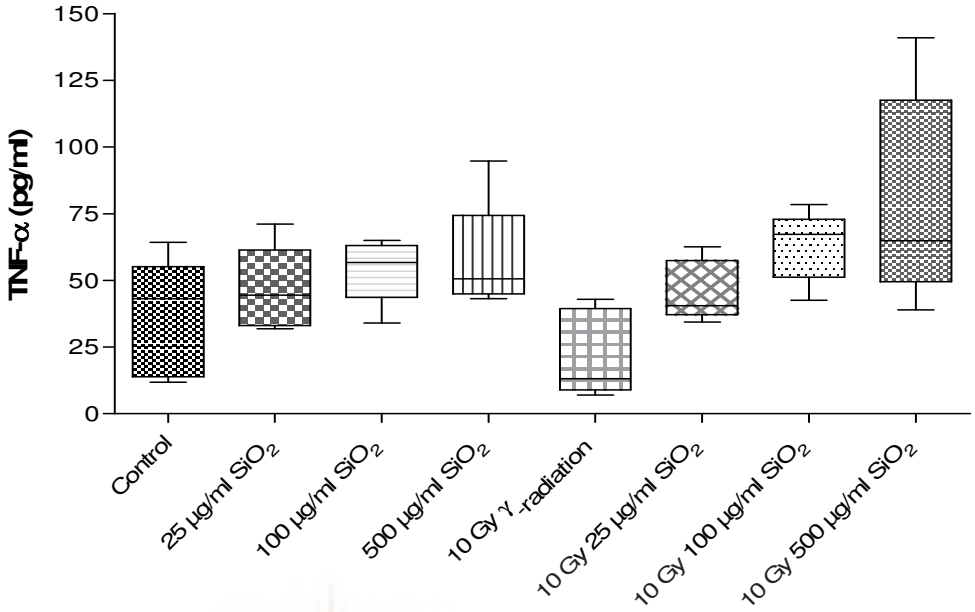

Figure 8-6 TNF-α production of THP-1 cells infected with *M. bovis* BCG and exposed to SiO_2 (25, 100 or 500 µg/ml) or γ-radiation (10 Gy). The control was infected, but not exposed (n = 2).

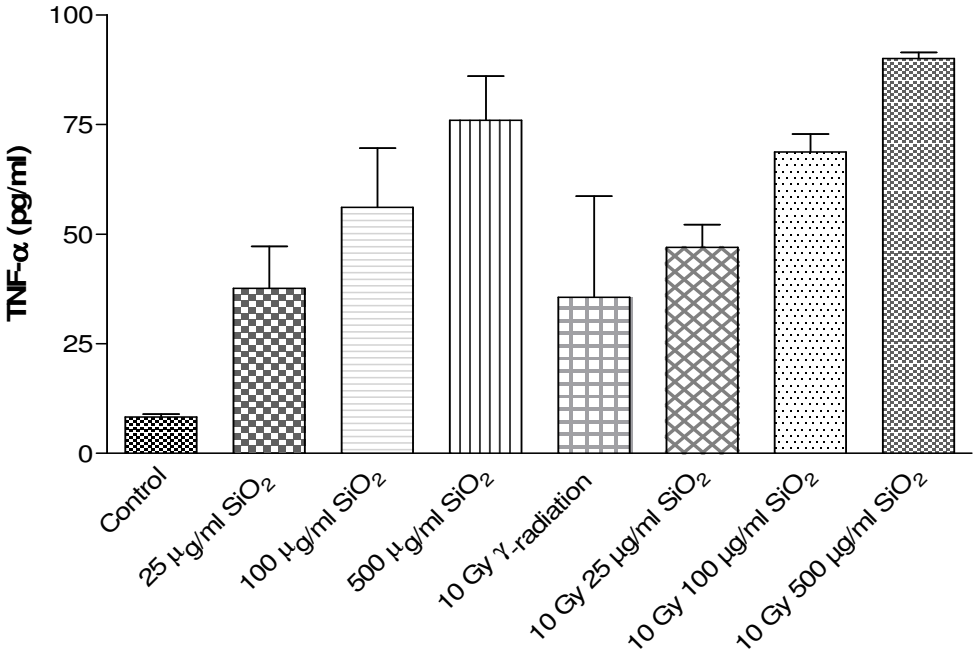

Figure 8-7 TNF-α production of THP-1 cells infected with *M. kansasii* and exposed to SiO_2 (25, 100 or 500 µg/ml) or γ-radiation (10 Gy). The control was infected, but not exposed (n = 2).

TNF-α concentration produced by THP-1 cells exposed to SiO_2 (25, 100 and 500 µg/ml), and/or γ-radiation and then infected with *M. kansasii* are shown Figure 8-7. Due to the similarity of results between 1Gy, 3Gy and 10Gy, only data from the 10Gy analysis are shown. In THP-1 infected with *M. kansasii* and not exposed to SiO_2 or γ-radiation (*i.e.* control), the mean TNF-α concentration was 8.3 pg/ml. There was a dose related increase in TNF-α concentration with increasing SiO_2 concentrations. These were significantly higher than the control ($p = 0.037$, $p = 0.024$ and $p = 0.003$ for 25 µg/ml, 100 µg/ml and 500 µg/ml SiO_2 respectively). There was no significant difference between the control and cells exposed to 10 Gy γ-radiation. Increasing TNF-α concentrations were also seen in cells exposed to both SiO_2 and γ-radiation (Table 8-1).

8.4 DISCUSSION

In the experiments described in this chapter, SiO_2 was found to have divergent effects on TNF-α release in U-937 and THP-1 cell lines. There was a dose dependant decrease in TNF-α concentration with increasing SiO_2 concentrations in U-937 cells. Although there is some evidence from this study that SiO_2 is associated with cytotoxicity, apoptosis or genotoxicity, this could not be conclusively proven. In the absence of evidence to suggest a decrease in the number of cells after SiO_2 exposure, it is suggested that SiO_2 may inhibit gene events that regulate TNF-α release. No reports could be found describing TNF-α concentrations in differentiated U-937 cells.

In contrast to the above findings, there was an increase in TNF-α concentration with increasing SiO_2 concentration in THP-1, despite evidence to suggest SiO_2 associated apoptosis and genotoxicity. The findings of the experiments described in this chapter are keeping with the findings of Savici, (1994). Using THP-1 cells as a model, he demonstrated that silica causes the release of TNF in THP-1 cells by up-regulating the TNF gene. THP-1 cells cultured for various periods in the presence of silica released TNF into the cell supernatants (Savici *et al.*, 1994). This trend is also observed in human macrophages, which produce increased amounts of TNF-α and TGF-β in response to silica (Driscoll & Maurer, 1991). Furthermore, Arcangeli *et al.* (2001) showed that both TNF-α and TGF-β are able to stimulate the proliferation of human

lung fibroblasts in culture. Lung fibrosis is an important feature of chronic and advanced silicosis. It is speculated that the increase in TNF-α release may be associated with neutrophil and eosinophil modulation and granuloma formation in the silicotic lung.

In the experiments described in this chapter, when compared to the unexposed control, there was increase in TNF-α concentration with exposure to γ-radiation in U-937. The finding in the current study is in keeping with those of Sherman (1991) who found TNF-α was up-regulated following irradiation. While it is thought that TNF-α provides protection against radiation damage (Urbaschek *et al*., 1987), the exact mechanism by which this occurs has not be elucidated. It has been reported that p53 is not up-regulated by low dose irradiation in undifferentiated U-937 (Hendrikse *et al*,. 2000). In THP-1, there was no significant difference in TNF-α concentration between the unexposed control, and cells exposed to γ-radiation. In the current study, there is some evidence to suggest that irradiation causes genotoxicity in THP-1 (Chapter 6), but there was no evidence to suggest a decrease in the number of viable THP-1 cells following irradiation (Chapter 4 and 5). These results would appear to contradict those obtained by Hachiya *et al.*, (1997). He found that irradiation increased TNF-α production, but his experiments were performed in undifferentiated THP-1 cells. The results from the current study would not suggest the implication of the TNF-receptor in the modulation of THP-1 response to irradiation, while leaving open the role of p53 which was not addressed.

The interaction of *M. tuberculosis* and *M. kansasii* with human macrophages at the level of macrophage cytokine expression was investigated by Beltan *et al.* (2000). The results from this study indicate that the levels of TNF-α, Granulocyte monocyte colony stimulating factor (GM-CSF), IL-6 and IL-1 expression differ only slightly between these two species. A comparison between viable and heat-killed bacilli as initiators of cytokine expression indicate that viable *M. kansasii* elicits a stronger response than heat-killed bacilli. Heat-killed *M. tuberculosis* however, elicits strong responses of TNF-α and IL-6. It is thought that this latter observation indicates that once killed, *M. tuberculosis* loses some of the heat-unstable virulence components and is unable to inhibit phagosome-lysosome fusion (Hart *et al.*, 1987). A comparison of TNF-α and

IL-6 expression in human monocytes infected with either *M. avium* or *M. kansasii* showed that *M. kansasii* elicited the production of significantly lower amounts of TNF-α and IL-6 than *M. avium*. The authors hypothesize that the extent of cytokine production might be relevant for the clinical manifestation of mycobacterial disease (Stauffer *et al.*, 1994). In the experiments described in this chapter, there was no significant difference in TNF-α concentration between U-937 infected with *M. bovis* BCG or *M. kansasii* and the uninfected control. It is concluded from this data that TNF-α production in U-937 is neither stimulated nor suppressed following infection with *M. bovis* BCG and *M. kansasii*. These findings are in keeping with conclusions drawn from Chapter 4, 5 and 6 which found no cytotoxic, apoptotic or genotoxic effect following infection by *M. bovis* BCG or *M. kansasii.* No reports could be found describing TNF-α concentrations in differentiated infected U-937 cells. In the experiments described in this chapter, there was also no significant difference in TNF-α concentration between THP-1 infected with *M. bovis* BCG or *M. kansasii* and the uninfected control. It was previously found that bacterial lipopolysaccharide purified recombinant mycobacterial heat shock protein directly activates THP-1 to secrete TNF-α, IL-6 and IL-8 in a dose dependant manner (Friedland *et al.*, 1993). Similarly, *M. bovis* BCG was found to induce TNF-α secretion in THP-1 (Oliveira *et al*., 2001). The results from the experiments described in this study, would suggest that TNF-α release by THP-1 is up-regulated sufficiently to compensate for the loss of cells due to *M. bovis* BCG or *M. kansasii* infection. It is thus concluded that the findings of the current study are in keeping with those of Oliveira *et al.* (2001) and Friedland *et al.* (1993), and are also consistent with the similar findings in alveolar macrophages (Valone *et al*., 1988). No reports could be found that described TNF-α release after *M. kansasii* infection in THP-1. The increase in TNF-α release in THP-1 following infection with *M. bovis* BCG and *M. kansasii* suggests that cytokine production is relevant for the clinical manifestation of mycobacterial disease since TNF- is associated with granuloma formation. Furthermore, the significance of GM-CSF and TNF-α expression in *M. kansasii* infection is highlighted by a case of persistent disseminated *M. kansasii* infection in a 27-year-old HIV negative woman. It was found that the patient's peripheral blood monocytes were abnormally permissive to the intracellular growth of *M. kansasii*. Extensive investigation yielded a normal number of interferon receptors, but reduced numbers of receptors to GM-CSF and TNF-α (Bermudez *et al*., 1994).

In experiments in which U-937 cells were exposed to SiO_2 and/or γ-radiation and then infected with *M. bovis* BCG or *M. kansasii*, the results were consistent with those seen with cells were exposed alone. The results suggest that in U-937, combinations of SiO_2, γ-radiation and infection with *M. bovis* BCG or *M. kansasii* does not stimulate or suppress the secretion of TNF-α more than SiO_2, γ-radiation or infection with *M. bovis* BCG or *M. kansasii* alone. These results are in keeping with the findings of a lack of cytotoxicity, apoptosis or genotoxicity in U-937 cells exposed and then infected.

Similiarly, in experiments in which THP-1 cells were exposed to SiO_2 and/or γ-radiation and then infected with *M. bovis* BCG or *M. kansasii*, the results were consistent with those seen with cells that were exposed alone. The results suggest that in THP-1, combinations of SiO_2, γ-radiation and infection with *M. bovis* BCG or *M. kansasii* does not stimulate or suppress the secretion of TNF-α more than SiO_2, γ-radiation or infection with *M. bovis* BCG or *M. kansasii* alone. As described earlier, this occurs despite cytotoxicity, apoptosis or genotoxicity caused by infection in THP-1. Since neither U-937 or THP-1 were associated with significant alterations in TNF-α release in experiments designed to to test synergy between SiO_2 and irradiation, there is no evidence to suggest that impaired granuloma formation could take place in miners exposed to SiO_2 and γ-radiation.

CHAPTER 9: CONCLUSIONS

The objective of this study was to establish if a synergistic relationship exists between the effects of silicosis and low-dose γ-radiation in predisposing promonocytic cells, U-937 and THP-1, to mycobacterial infection.

- In Chapter 4, the study examined the cytotoxicity of SiO_2, γ-radiation and the cytotoxicity following infection with *M. bovis* BCG and *M. kansasii* on U-937 and THP-1. It was established that neither γ-radiation nor infection with either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on U-937. It was also established that γ-radiation does not have a significant cytotoxic effect on THP-1. Results from cytotoxicity studies on U-937 and THP-1 were inconclusive due to technical reasons. Infection by either *M. bovis* BCG or *M. kansasii* has a significant cytotoxic effect on THP-1, reducing the metabolic activity below that of the control. The significance of this finding is the possibility that THP-1 cells act like alveolar macrophages in their response to mycobacterial infection. No synergism between SiO_2 and γ-radiation that resulted in an increase in cytotoxic effect could be determined in either cell type.

- In Chapter 5, the study examined the mechanisms of cell death of U-937 and THP-1 following SiO_2, γ-radiation exposure, and following infection with *M. bovis* BCG and *M. kansasii*. It was established that in U-937 there is no significant increase in apoptosis or necrosis in cells exposed to SiO_2, γ-radiation or infected by *M. bovis* BCG or *M. kansasii*. In THP-1, there is no significant increase in apoptosis or necrosis in cells exposed to γ-radiation. However, there is a significant increase in apoptosis in cells exposed to SiO_2 (500 μg/ml only), and cells infected by *M. bovis* BCG or *M. kansasii*, which was in keeping with the findings from other studies. No synergism between SiO_2 and γ-radiation that resulted in an increase in apoptotic effect could be determined in either cell type.

- In Chapter 6, the study examined the DNA damage caused by SiO_2, γ-radiation and DNA damage following infection with *M. bovis* BCG and *M. kansasii*. It was established that neither SiO_2, γ-radiation nor infection by *M. bovis* BCG nor *M. kansasii* was genotoxic to U-937. In THP-1, it was found that exposure to γ-radiation, exposure to SiO_2 and infection with either *M. bovis* BCG or *M. kansasii* were genotoxic to THP-1. The study suggested that the progression to cell death in THP-1 exposed to toxic agents was slow, in comparison to the progression in infected cells. No synergism between SiO_2 and γ-radiation that resulted in an increase in DNA damage could be determined in either cell type.

- In Chapter 7, the study examined the ability of U-937 and THP-1 cell lines to control the intracellular growth of *M. bovis* BCG and *M. kansasii* after exposure to SiO_2 and/or γ-radiation. It was found that U-937 was permissive for the growth of *M. bovis* BCG and *M. kansasii in vitro*. U-937 was also permissive for the growth of *M. bovis* BCG and *M. kansasii* in cells exposed to SiO_2 and/or γ-radiation. Potentiation of growth of *M. bovis* BCG or *M. kansasii* due to SiO_2 and/or γ-radiation in U-937 could not be demonstrated. In contrast, THP-1 controlled the growth of *M. bovis* BCG and *M. kansasii in vitro*. The results of this study were in keeping with those from other published studies. The growth of *M. bovis* BCG was potentiated by SiO_2 and/or γ-radiation, despite apoptosis. This may support the hypothesis of this study that SiO_2 and γ-radiation may both be involved in potentiating the growth of *M. bovis* BCG *in vitro*. The growth of *M. kansasii* was not potentiated by SiO_2 and γ-radiation exposure.

- In Chapter 8, the study examined the TNF-α production caused by SiO_2 and/or low-dose γ-radiation in U-937 and THP-1 alone, and following infection with *M. bovis* BCG and *M. kansasii*. It can be concluded that TNF-α release in U-937 was suppressed by exposure to SiO_2, and stimulated in THP-1. TNF-α release was increased in response to γ-radiation exposure in U-937, but remained unaltered in THP-1. There was no significant differences in TNF-α release in infected U-937 or THP-1, but due to the difference in number of viable cells, it was concluded that infection by M. bovis BCG or *M. kansasii*

stimulated TNF-α release. In THP-1 and U-937, combinations of SiO_2, γ-radiation and infection with *M. bovis* BCG or *M. kansasii* did not stimulate or suppress the secretion of TNF-α more than SiO_2, γ-radiation or infection with *M. bovis* BCG or *M. kansasii* alone.

It was the primary objective of this study, to establish if a synergistic relationship exists between the effects of SiO_2 and low-dose γ-radiation in predisposing promonocytic cells, U-937 and THP-1, to mycobacterial infection. In U-937, no synergistic relationship could be determined between the effects of SiO_2 and/or γ-radiation and the potentiation of mycobacterial growth by U-937. When the results from Chapter 4, 5 and 6 were considered together it was concluded that U-937 is an immature precursor to the alveolar macrophage, and that it is inert in its response to exposure to toxic agents and infection by mycobacteria. Using U-937 as a model of monocyte function, there is insufficient evidence to sustain the hypothesis that SiO_2 and radiation exposure may both be involved in predisposing gold mine workers to pulmonary tuberculosis by their effects on the macrophage's ability to contain mycobacterial proliferation.

In THP-1, *M. bovis* BCG, *M. kansasii*, SiO_2 and γ-radiation were associated with apoptosis or DNA damage. THP-1 cells are considered to be more mature than U-937, and responded to toxic agents and mycobacterial infection by undergoing apoptosis. No synergistic effect of SiO_2 and γ-radiation could be determined in THP-1 cells infected with *M. kansasii*. However, the growth of *M. bovis* BCG was potentiated by SiO_2 and/or γ-radiation, despite apoptosis, which may support the hypothesis that SiO_2 and radiation exposure are both involved in predisposing gold mine workers to pulmonary tuberculosis by their effects on the macrophage's ability to contain mycobacterial infection. It is hypothesized that SiO_2 has a direct effect on stimulating mycobacteria proliferation and/or the THP-1 cell's protective state is inhibited by γ-radiation.

9.1 FURTHER STUDIES

In this study it was suggested that THP-1 cells progress to cell death slowly. This hypothesis could be further investigated in further studies by measurement of caspase 9 activity, and the elucidation of the pathway through p53. The relation of the TNF-receptor to apoptosis could measured through quantification of caspase 3 or 8, and the effect of anti-TNF antibodies. Further investigation of the pathway through FAS is also required, particularly in cells exposed to γ-radiation. The relevance of the potentiation of *M. bovis* BCG following SiO_2 and γ-radiation requires further investigation. While the advantage of using homogenous cell lines is in the elimination of differences between individuals, such differences in genetic regulation of innate monocyte function may well be relevant to susceptibility to tuberculosis. This would best be evaluated in peripheral blood monocytes from normal donors in comparison to miners, and in cells isolated from bronchial alveolar lavage, and/or in an appropriate animal model. The findings in this study are also limited by the fact that experiments measured only acute, short term effects. Studies in miners would have the advantage of measuring the cumulative impact of chronic exposure to silica and radiation, such studies could also measure TNF-α release, in the presence and absence of mycobacterial infection, including virulent *M. tuberculosis*.

Although this study only indentified tentative evidence to suggest a synergistic effect between silica and low-dose radiation exposure and mycobacterial proliferation, it would be prudent for mine safety committees to take cognisance of the dangers of silica and radiation, and try where possible, to minimise mineworkers' exposures to these toxic agents.

CHAPTER 10: REFERENCES

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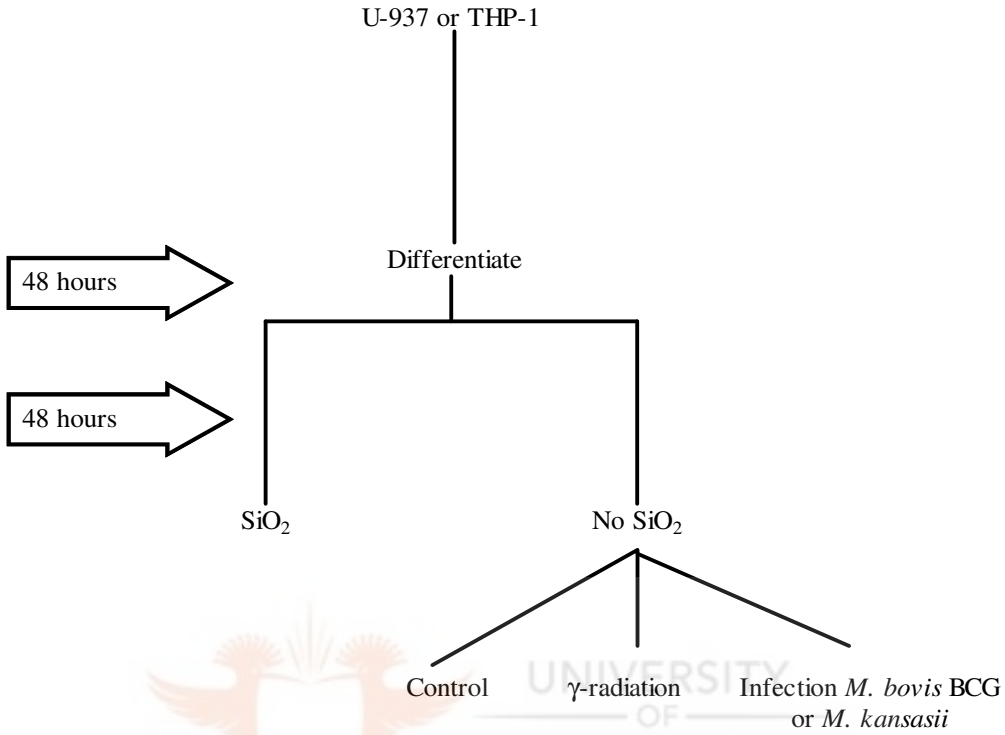
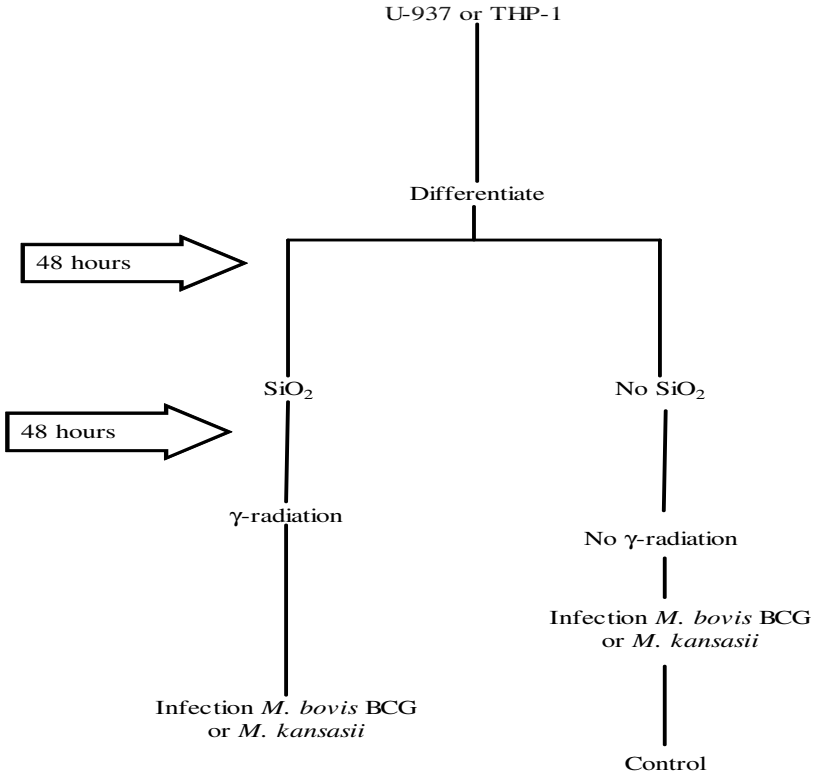
APPENDIX 1: DIAGRAMMATIC REPRESENTATION OF EXPERIMENTAL DESIGN

Experimental design 1:



Experimental design 2:



APPENDIX 2: REAGENT PREPARATION FOR SINGLE CELL GEL ELECTROPHORESIS (COMET ASSAY)

Lysis solution

2.5 M NaCl
0.1 M EDTA
10 Mm Tris
Make up to 1 L in distilled water. Set to pH 10 with either solid NaOH, or preferably, 10 M NaOH solution (add approximately 35 ml NaOH immediately to dissolve the EDTA, and then dropwise to pH 10). Add 1 ml Triton X-100 per 100 ml immediately just before use.

Enzyme reaction buffer for Endonuclease III

40 mM HEPES
0.1 M KCl
0.5 mM EDTA
0.2 mg/ml BSA
Make up to 5 L in distilled water. Adjust to pH 8.0 with concentrated KOH.

Endonuclease III

Prepare a working solution by diluting the stock 1: 1000 with enzyme reaction buffer.

Electrophoresis solution

0.3 M NaOH
1 mM EDTA
Make up to 10 L in distilled water.

Neutralising buffer

0.4 M Tris
Make up to 1 L in distilled water. Adjust to pH 7.5 with concentrated HCl.

DAPI

DAPI was reconstituted in 10 ml distilled water to yield a 1 mg stock solution. The stock solution was diluted in distilled water to yield a working solution of 1 µg/ml.