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A Human Macrophage Model for Selective Evaluation of $CD8^+$ and $\gamma\delta^+$ Cytotoxic T cell Function in Tuberculosis

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Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Medicine
UNIVERSITY OF CAPE TOWN

June, 1999

ACKNOWLEDGEMENTS

I wish to express my appreciation to the following:

- Associate Prof. Stan Ress, supervisor, for providing me the opportunity to be part of his research group, for his constant enthusiasm, and for his passionate appreciation and extensive understanding of all aspects of immunology.
- Dr Pauline Lukey, supervisor, for her expert academic and technical guidance, for teaching me the basics of immunology and much more, for being supportive when it counted most, and for persevering with me despite being so far away.
- Dr Mustapha Keraan, co-supervisor, for being available for advice and guidance.
- Richard Glashoff for being supportive throughout my PhD, for providing very useful advice when it was needed most, for the hundreds of tea-time discussions, and for providing liters of your own blood for this study.
- Colleagues in the Clinical Immunology Laboratory, including Marcia Watkins for constant entertainment and endless helpful advice, Barbara Mohr and Meryl Abrahams for their assistance at times with this study, Edgar Carelse and Francie Cleaphas.
- Prof. Gerry Blekkenhorst, Alistair, Andre, and Fatima of the Department of Radiobiology, Groote Schuur Hospital, for assistance with irradiating cells.
- Dr Mohammed Jaffer of the Electron Microscopy Unit, University of Cape Town for expert advice and assistance with the TEM aspects of the project.
- Dereck Taljaard and Lorraine Hope of the Department of Tissue Immunology, University of Cape Town, for providing information about potential donors and for tissue typing numerous patients throughout the study.
- Dr Marx of the Department of Human Genetics, University of Stellenbosch, for karyotyping of U937 cells.
- To the many colleagues, patients and blood donors who kindly participated in this study.
- My parents for their constant love and support, without which I would never have achieved as much over the last twenty-seven years.
- Darren, a constant source of support, love and exceptionally good advice, for his motivation, for his clarity of thought when I doubted myself most, for taking the time to understand the intricacies of my project, and for painstakingly reading the final thesis.
- GlaxoWellcome *Action TB* and the Foundation for Research and Development are gratefully acknowledged for their financial support.

ABSTRACT

The human macrophage cell line U937 was investigated as an *in vitro* model for human macrophage function in mycobacterial infections. This involved evaluating the ability of differentiated U937 cells to phagocytose *Mycobacterium tuberculosis*, control intracellular mycobacterial growth, and present mycobacterial antigens to human HLA class I-matched cytotoxic T lymphocytes (CTLs).

Differentiation of U937 cells using IFN-γ, 1,25-$(OH)_2$ vitamin D_3, or PMA significantly enhanced their ability to phagocytose *M. tuberculosis* but failed to induce a subsequent respiratory burst response. Following infection, U937 cells were found to be permissive to the intracellular growth of both the virulent H37Rv strain of *M. tuberculosis* and the attenuated vaccine strain of *M. bovis* BCG. U937 cells have been shown to constitutively express high levels of cell surface HLA class I while expressing undetectable levels of HLA class II both at the mRNA level and at the cell surface. HLA class II expression was neither up-regulated following infection with *M. tuberculosis* nor inducible using IFN-γ, 1,25-$(OH)_2$ vitamin D_3, PMA, GM-CSF or a combination of these agents. In contrast, chronic infection of U937 cells with virulent H37Rv *M. tuberculosis* (but not with BCG) resulted in the cell surface expression of HLA class I being significantly up-regulated. Taken together, these characteristics made U937 cells a very attractive model for further investigations into their ability to present mycobacterial antigens to human HLA class I-restricted CTLs.

Differentiation of U937 cells was found to completely abrogate their sensitivity to non-antigen specific cytolysis mediated by NK or LAK cells. Following infection with *M. tuberculosis*, U937 target cells were lysed by *M. tuberculosis*-primed CTLs from HLA class I-matched donors in an antigen-specific manner and with a similar efficiency to autologous macrophage targets. This cytolytic activity was restricted to live organisms since only U937 cells infected with virulent H37Rv *M. tuberculosis* and BCG but not those pulsed with soluble PPD were lysed by the HLA class I-matched effector cells. On the other hand, *M. tuberculosis*-stimulated but HLA-mismatched CTLs failed to lyse infected U937 cells in an antigen-specific manner. T cell subset fractionation of the HLA class I-matched *M. tuberculosis*-primed CTL

population and limiting dilution cloning demonstrated that the cytolytic activity was mediated by $CD8^+$ cytolytic T cells and confirmed that $CD4^+$ T cells showed no significant ability to lyse infected U937 target cells. Furthermore, this study found that *M. tuberculosis*-infected U937 target cells were lysed by $CD8^+$ CTLs more rapidly and strongly than similarly infected autologous macrophage targets demonstrating the sensitivity of this *in vitro* model as an indicator for $CD8^+$ cytolytic function in mycobacterial infections.

M. tuberculosis-infected U937 cells were found to be highly sensitive to mycobacterial antigen-specific cytolysis mediated by $\gamma\delta^+$ CTL. Mycobacterial antigen-specific $\gamma\delta^+$ CTLs consistently showed stronger cytolytic activity against infected U937 target cells than $CD8^+$ CTL but were not restricted to classical HLA class I or class II molecules. A panel of cytolytic human *M. tuberculosis*-reactive $\gamma\delta^+$ CTL clones was established to investigate more thoroughly the role of $\gamma\delta^+$ CTL lytic activity in human mycobacterial infections. This study examined the mechanism of cellular cytotoxicity used by these mycobacterial-specific $\gamma\delta^+$ CTL clones against infected U937 targets and further investigated the effect of $\gamma\delta^+$ T cell-mediated cytolysis on intracellular mycobacterial survival. Cytolysis mediated by the $\gamma\delta^+$ T cell clones was found to be dependent on cell-to-cell contact. Furthermore, the ability of the $\gamma\delta^+$ CTL clones to lyse infected targets was found to be strongly Ca^{2+}-dependent, sensitive to cyclosporine A (a specific inhibitor of granule exocytosis), and completely abrogated following Sr^{2+}-induced de-granulation of the $\gamma\delta^+$ T cell effectors, indicating that cytoxicity was mediated predominantly by the granule exocytosis/perforin pathway. Despite being strongly cytolytic against infected U937 cells, however, the $\gamma\delta^+$ CTL clones did not have any impact on the survival of intracellular *M. tuberculosis*.

The major conclusions of this study are that U937 cells not only provide a useful *in vitro* human macrophage model allowing for selective evaluation of HLA class I-restricted $CD8^+$ CTL function in mycobacterial infections but also provided a highly sensitive indicator for $\gamma\delta^+$ CTL cytolytic activity.

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ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
Ab	Antibody
AB	AB^+ human serum
AFB	Acid fast bacilli
AICD	Activation induced cell death
APC	Antigen presenting cell
BCG	Bacillus Calmette Guerin
BFA	Brefaldin A
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming units
CLIP	Class II associated invariant chain peptide
CMI	Cell mediated immunity
cpm	Counts per minute
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
ER	Endoplasmic reticulum
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) *N, N, N', N'*-tetra-acetic acid
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluoroscein isothiocyanate
GMCSF	Granulocyte-macrophage colony-stimulating factor
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-ethane-sulphonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hsp	Heat shock protein
HSV	Herpes simplex virus
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon-γ
IL	Interleukin
IPP	Isopentenyl pyrophosphate
LAK	Lymphokine-activated killer
LAM	Lipoarabinomannan
LAMP	Lysosomal membrane glycoproteins
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MBP	Mannose binding protein
MCF	Mean channel fluorescence
MDM	Monocyte-derived macrophage
MHC	Major histocompatibility complex
MR	Mannose receptor
MW	Molecular weight
NBT	Nitroblue Tetrazolium
NK	Natural killer
OADC	Oleic acid-albumin-dextran-catalase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEG	Polyethylene glycol
PHA	Phytohaemaglutanin
PMA	Phorbol myristate acetate
PPD	Purified protein derivative of *M. tuberculosis*
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RPMI	Roswell Park Memorial Institute media
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SI	Stimulation index
SK-SD	Streptokinase-streptodornase
SpA	Surfactant protein A
SRBC	Sheep red blood cell
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TEM	Transmission electron microscopy
TNF	Tumour necrosis factor
TT	Tetanus toxoid
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume
ZN	Zhiel Nielsson

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Tuberculosis is the leading infectious cause of death world wide, being responsible for 3 million deaths annually. Among those aged over 5 years, tuberculosis kills more people than AIDS, malaria, diarrhoea, leprosy and all other tropical diseases combined (WHO, 1994). The tragedy of this situation is that treating tuberculosis is one of the most effective and inexpensive of all health interventions (WHO, 1994). It has been estimated that one-third (approximately 1.7 billion) of the world's population are infected with *Mycobacterium tuberculosis* with 60 million people developing clinical symptoms (WHO, 1997) and about 7.3 million new cases arising every year (Raviglione *et al.*, 1997). The tuberculosis morbidity and mortality figures in South Africa are presently regarded as one of the highest in the world and the WHO has categorized South Africa as one of 16 countries hampering global efforts to control tuberculosis (WHO, 1998). In 1998, the South African National Department of Health reported that South Africa has 108 382 confirmed cases of tuberculosis (251 cases per 100 000 population). Compared to the 21 337 cases reported in the USA during the same year (Centers for Disease Control and Prevention, United States, 1997), these figures are alarming. The Western Cape region of South Africa has a rate of pulmonary tuberculosis that is 170% higher than the national figure (South African National Department of Health, 1991) with prevalence rates being as high as 1 505 per 100 000 population (Mizrahi, 1998). Worsening the crisis is the emergence of multi-drug resistant (MDR) strains of *M. tuberculosis* that are resistant to the major, and sometimes all, conventional antibiotics used to treat the disease.

It is widely accepted that the clinical manifestations and outcome of tuberculosis in infected individuals is determined not only by the innate virulence of the tubercle bacillus but also by the host's immune response. It has been estimated that the overwhelming majority (90-95%) of individuals infected with *M. tuberculosis* never progress towards clinical disease (Bloom and Murray, 1992). Some will develop active disease later, however, in the event of some form of immune impairment such as that caused by HIV infection, malnutrition, or advanced malignancy. Furthermore, active tuberculosis in the pre-antibiotic era was not always fatal and a substantial proportion of patients eventually recovered without specific therapy (Weisner,

1922; Mitchell, 1955; Stephens, 1941). Even today, a small subset of patients with MDR-tuberculosis will recover (Turett *et al.*, 1995; Park *et al.*, 1996).

Despite the long history of tuberculosis and the existence of various effective treatments, there are still significant areas within the basic immunology of this infection that need to be elucidated. With renewed interest in this disease, information on the precise mechanisms of immune protection against tuberculosis is emerging.

1.2 CELL-MEDIATED IMMUNITY IN TUBERCULOSIS

Infection with *M. tuberculosis* triggers a broad spectrum of immune responses with efficient cell-mediated immunity (CMI) being pivotal to effective recovery from tuberculosis. Individuals with defective CMI (such as those infected with HIV or chronic renal failure) are significantly more susceptible to infection with *M. tuberculosis*, whereas individuals with defective humoral immunity (such as those with sickle cell disease or multiple myeloma) show no increased predisposition to tuberculosis (Barnes *et al.*, 1994). Macrophages and T cells in particular have been found to be key elements in the CMI response to tuberculosis (Orme and Collins, 1984). Macrophages play a dual role in tuberculosis, however, since they promote protection against the disease but also provide the preferred biotype of *M. tuberculosis* (Kaufmann and Andersen, 1998). In the same way, T cells are critical for protective immunity but also contribute to the pathology of the disease. A co-ordinated interaction between T cells and macrophages is essential for optimum protection against tuberculosis and this is best achieved in the granulomatous lesion (Chan and Kaufmann, 1994). Within a granuloma, the intricate interplay between T cells, cytokines and macrophages succeed in containing pathogenic mycobacteria (Kaufmann and Andersen, 1998).

Tuberculosis is typically a disease of the lung, being the major port of entry, the site of mycobacterial persistence, and the location of disease outbreak and manifestation (Kaufmann and Andersen, 1998). The disease is transmitted from one individual to another by coughing, sneezing and during speech and the principle risk factor for becoming infected with *M. tuberculosis* is through inhalation of infectious particles (Bloom and Murray, 1992). A sneeze

may contain over a million particles with diameters of less that 100 μm that may carry between 3 to 10 tubercle bacilli. Of the inhaled bacilli, it has been estimated that only 6% will reach the alveoli and produce tubercles while the majority of larger particles will settle in the upper respiratory mucosa and be expelled by the ciliated respiratory escalator. It has been estimated that the only particles able to remain suspended in the air-stream long enough to reach the alveolar spaces will contain no more than 3 bacilli (Riley *et al.*, 1995; Wells, 1955). Furthermore, the number that must be inhaled to establish infection is probably between 10 and 50 infectious units (Lurie, 1964; Riley *et al.*, 1962). Once *M. tuberculosis* reaches the alveolus, alveolar macrophages phagocytose the bacillus and often destroy it. Destruction depends on both the inherent microbicidal activity of the macrophage as well as the virulence of the invading organism (Lurie, 1964; Dannenberg & Tomashefski, 1988; Dannenberg, 1991).

If the original alveolar macrophage does not inactivate or destroy the inhaled organisms at this point, the bacilli multiply until that macrophage bursts. The released bacterial load is then ingested by other alveolar macrophages and/or by non-activated monocytes/macrophages that have emigrated from the blood stream resulting in the formation of a primary granuloma. Both types of macrophages are attracted to the site by released bacilli, cellular debris, and a variety of chemotactic factors released by the host. Macrophages from the circulation eventually become completely responsible for the fate of the early lesion. In the early lesions, the alveolar macrophages rarely participate because they remain peripheral, far from the centrally located bacilli. The newly recruited macrophages from the bloodstream phagocytose the released bacilli. Because macrophages have not yet been activated during this early stage of infection, the disease is poorly controlled with the bacilli multiplying exponentially despite the increasing number of macrophages being recruited and accumulating in the lesion.

As the primary granuloma develops and matures, logarithmic mycobacterial multiplication becomes static and the lesions undergo caseous necrosis in their centres. The bacillus can survive in this solid caseous material but cannot multiply because of the anoxic conditions, reduced pH, and the presence of inhibitory fatty acids (Poole and Florey, 1970; Hemsworth and Kochan, 1978). The host thus locally destroys its own tissues in order to control the uninhibited multiplication of bacilli that would otherwise be fatal (Canetti, 1955; Poole and Florey, 1970;

Dannenberg, 1991). Only after such control has been established can the activated macrophages that accumulate around the caseous focus prevent the disease spreading. Both the macrophage turnover rate and the accumulation of activated macrophages have peaked when T cell-mediated responses first develop. Within this now productive granuloma, the T cells come into close contact with infected macrophages at various differentiation and activation states. $CD4^+$ T cells are the predominant T cell subset within the granuloma. They function to induce mycobacteriostatic activity in macrophages at the centre of the granuloma by releasing interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and 1,25-dihydroxyvitamin D_3 (Denis, 1991; Rook *et al.*, 1987). These productive granulomas are also surrounded by mantle of $CD8^+$ cells. Cytolysis of poorly bactericidal or incapacitated macrophages by $CD8^+$ and $CD4^+$ cytotoxic T lymphocytes (CTLs) may directly impact on mycobacterial survival (De Libero *et al.*, 1988) but will also facilitate release and subsequent uptake of multiplying bacilli by more efficiently activated macrophages. The presence of $\gamma\delta^+$ T cells within the developing granuloma is clearly demonstrable but their role is poorly understood (Inoue *et al.*, 1991; Modlin *et al.*, 1989). In the majority of healthy individuals, the productive granuloma will successfully contain *M. tuberculosis* at this stage within distinct foci and prevent dissemination and progression towards active disease (Kaufmann and Young, 1992).

1.2.1 THE ROLE OF MACROPHAGES IN TUBERCULOSIS

Because the alveolar macrophage is one of the first lines of defence encountered by *M. tuberculosis* once it reaches the lower respiratory tract, it is likely that this interaction plays a key role in determining the outcome of infection. Alveolar macrophages and blood-derived monocytes/macrophages participate in defence against mycobacterial infection by fulfilling four important functions: (i) they are capable of phagocytosing *M. tuberculosis* efficiently; (ii) they are capable of producing proteolytic enzymes and other metabolites following phagocytosis that exhibit mycobactericidal effects; (iii) they process and present antigens derived from *M. tuberculosis* to T cells; and (iv) macrophages produce a characteristic pattern of cytokines in response to *M. tuberculosis* infection that have the potential to exert potent immunoregulatory effects and mediate many of the clinical manifestations of tuberculosis (Valone *et al.*, 1988; Toossi *et al.*, 1991; Barnes *et al.*, 1992; Zhang *et al.*, 1993; Barnes & Modlin, 1994).

Binding and phagocytosis of *M. tuberculosis* by monocytes and macrophages

Mycobacterial uptake by macrophages is generally accepted to be a macrophage-determined event that is initiated by direct bacterial binding to specific macrophage receptors (reviewed recently by Ernst, 1998). The most widely studied of these are the complement receptors (CR1, CR3 and CR4; Schlesinger *et al.*, 1990; Schlesinger and Horwitz, 1991), mannose receptors (Schlesinger, 1993), surfactant receptors (Downing *et al.*, 1995), scavenger receptors (Zimmerli *et al.*, 1996), and CD14 (Pugin *et al.*, 1994). Fc receptors (FcR) and the β-glycan receptor do not seem to be of major importance in mediating binding of *M. tuberculosis* (Schlesinger *et al.*, 1990; Czop *et al.*, 1991).

Complement receptors

Like many other bacteria, *M. tuberculosis* can activate the alternative pathway of complement, resulting in opsonization with C3b and C3bi (Schlesinger *et al.*, 1990). Bacilli that are sufficiently coated with these serum-derived ligands bind to CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and are subsequently phagocytosed in membrane-bound phagosomes (Schlesinger, 1993; Schlesinger *et al.*, 1990). Unlike other bacteria (such as *Leishmania mexicana*, *Staphylococcus aureus*, and *Listeria monocytogenes*), however, virulent mycobacteria have evolved additional mechanisms for acquiring opsonic C3. Pathogenic mycobacteria uniquely recruit the complement fragment C2a to form a C3 convertase and generate opsonically active C3b in the absence of early activation components of the alternative or classical pathways (Schorey *et al.*, 1997). In addition, two recent studies provided evidence for association of mannose-binding lectin (MBL) with the surface of mycobacteria (Polotsky *et al.*, 1997; Hoppe *et al.*, 1997). MBL and its associated serine proteases can activate both the classical and the alternative complement pathways (Matsushita & Fujita, 1996).

M. tuberculosis can also bind to CR3 at two distinct sites on the receptor. Opsonized *M. tuberculosis* binds CR3 at its C3bi binding domain, and nonopsonized *M. tuberculosis* uses its endogenous capsular polysaccharide to interact with the β-glucan binding site near the C

terminus of CD11b (Cywes *et al.*, 1996, 1997). *M. tuberculosis* can therefore exploit complement receptors through multiple mechanisms to bind to and enter macrophages. The mechanism and consequences that predominate *in vivo* may be determined by features of the individual bacterial strain (complement dependent or independent), the environment of the macrophage (such as the availability of complement proteins), and the state of differentiation or activation of the macrophage (Ernst, 1998). Although little is known about the trafficking of phagosomes that contain bacteria ingested by macrophages through complement receptors, it has been suggested that complement receptor-mediated entry of *M. tuberculosis* may provide these pathogens safe passage into the macrophage by allowing them to circumvent the toxic release of reactive oxygen intermediates during the respiratory burst response (Wright and Silverstein, 1983).

Mannose receptors

Mannose receptors (MR) have been implicated in mediating uptake of virulent stains of *M. tuberculosis* (H37Rv and Erdmann) but not the avirulent strains (H37Ra) (Schlesinger, 1993). A well-characterised mycobacterial-ligand for MR is lipoarabinomannan (LAM), which is abundant, peripherally exposed and contains terminal mannose residues that interact with MR (Schlesinger *et al.*, 1994, 1996). LAM of both Erdman and H37Rv *M. tuberculosis* contain core mannose units linked to long arabinose branches and Erdman differs from H37Ra LAM in that it contains mannose oligosaccharide "caps" on the arabinose saccharides at the terminal portions of the molecule. It is therefore possible that the involvement of MR in phagocytosis of virulent *M. tuberculosis* strains relates to the presence of mannose "caps" that serve as ligands for this receptor (Chatterjee *et al.*, 1992).

Surfactant protein A

In addition to CR and MR, accumulating evidence exists for an important role for surfactant protein receptors in mediating bacterial binding. Sp-A is a member of the collectin family of proteins, which also includes mannose binding protein (MBP) and complement component C1q. Sp-A enhances macrophage binding and uptake of *M. tuberculosis*, although the mechanism has

not been fully elucidated (Downing *et al.*, 1995). Purified Sp-A binds directly to *M. tuberculosis* H37Ra, and binding to the bacteria is dependent on calcium and on glycosylation of Sp-A (Pasula *et al.*, 1997). There is evidence to suggest, however, that Sp-A can exert its function on *M. tuberculosis* phagocytosis indirectly. Attachment of macrophages to Sp-A-coated surfaces enhances phagocytosis by FcRs, CRs and probably by MRs (Tenner *et al.*, 1989; Gaynor *et al.*, 1995). Because this mechanism extends to such structurally diverse receptors, it is improbable that Sp-A interacts physically with each of these receptors and it is more likely that this effect is exerted by Sp-A at a step in phagocytosis that is common to or downstream of these various receptors (Ernst, 1998).

CD14

The macrophage receptor CD14 has also been implicated in mycobacterial binding. It is best known as the high-affinity receptor for lipopolysaccharide (LPS) of gram-negative bacteria. In mycobacterial infections, however, CD14 has been shown to bind LAM of *M. tuberculosis.* Furthermore, this binding has been shown to induce interleukin-8 (IL-8) secretion by macrophages (Pugin *et al.*, 1994).

Scavenger receptors

Macrophage scavenger receptors bind polyanionic particles, including LPS of gram-negative bacteria and lipoteichoic acid of gram-positive bacteria (Krieger *et al.*, 1993; Dunne *et al.*, 1994). Class A scavenger receptors have recently been identified as important in the binding of *M. tuberculosis* to human monocyte-derived macrophages (Zimmerli *et al.*, 1996). Whether scavenger receptors directly activate the macrophage cytoskeleton to internalise mycobacteria, or whether these receptors act only to bind bacteria with phagocytosis being mediated by other receptors remains to be seen (Ernst, 1998).

It is likely that a particulate target such as *M. tuberculosis*, that displays numerous and diverse ligands on its surface, engages multiple receptor types simultaneously. Thus, *in vivo*, *M. tuberculosis* is probably not internalised by macrophages using a single receptor-mediated

pathway. The degree of differentiation or the state of activation of the macrophage may also bias the receptors used *by M. tuberculosis* to gain entry. Distinct types of receptors may also co-operate to optimise binding and internalisation of a target particle. Co-operation between CR1 and CR3 has been demonstrated in the binding of complement-opsonised particles (Sutterwala *et al.*, 1996), and co-operation between phosphatidylinositol glycan-linked FcγRs and CR3 markedly enhances phagocytosis of IgG-opsonized targets (Krauss *et al.*, 1994). Such co-operation may account for phagocytosis of particles bound to receptors that lack transmembrane and cytoplasmic domains, such as CD14 (Ernst, 1998).

Receptor choice and intracellular fate

Certain intracellular pathogens exploit specific macrophage receptors to ensure their own survival. *Leishmania major*, for instance, activates the alternative complement pathway to deposit C3b on its surface (Mosser and Edelson, 1987). When C3b-opsonized *L. major* metacyclic promastigotes bind to CR1, they survive and replicate successfully within the macrophage. When non-infective *L. major* promastigotes enter macrophages through the lectin-like domain of CR3, however, they are killed (Da Silva *et al.*, 1989). Similarly, while *Salmonella typhi* entering macrophages through CR3 become enclosed in a vacuole that eventually fuses with lysosomes, entry via CR1 allows *S. typhi* to survive in a phagosome which does not acquire lysosomal markers (Ishibashi and Arai, 1990). These observations suggest that successful pathogens may ensure their survival within macrophages by gaining entry using a receptor-mediated pathway that is not coupled to the activation of macrophage antimicrobial mechanisms. There have only been a limited number of studies investigating whether *M. tuberculosis* uses such a mechanism to facilitate its own intracellular survival. By using monoclonal antibodies or competitive ligands to block CR1, CR3, CR4, MR, and class A scavenger receptors during initial entry of *M. tuberculosis* into human macrophages, no apparent difference in the extent of survival or rate of intracellular growth of the virulent Erdman strain was observed (Zimmerli *et al.*, 1996). Earlier studies have shown, however, that entry of *M. tuberculosis* via the CR pathway may provide the bacterium safe passage into the macrophage by allowing it to avoid the toxic consequences of a respiratory burst (Wilson *et al.*, 1980; Wright and Silverstein, 1983; Yamamoto and Johnston, 1984).

Activation of macrophage anti-mycobacterial mechanisms

Once *M. tuberculosis* has gained entry into the macrophage and become engulfed within phagosomes, they are confronted with a variety of macrophage mycobactericidal and/or mycobacteriostatic mechanisms aimed at limiting their intracellular growth. The ability of infected macrophages to produce reactive oxygen intermediates (ROI) by oxidative metabolism and reactive nitrogen intermediates (RNI) by the L-arginine-dependant cytotoxic pathway are the two best-characterized mechanisms.

Reactive oxygen intermediates

Early reports indicated that ROI (such as superoxide anions and hydrogen peroxide molecules) generated during oxidative metabolism are important components of host defence against a wide variety of microorganisms. It has been demonstrated that (i) low virulence of *M. tuberculosis* in guinea pigs is correlated with the susceptibility of this bacterium to peroxide (Mitchison *et al.*, 1963); (ii) peroxide sensitive mutants of *M. tuberculosis* fare far less well in the lungs of guinea pigs than peroxide resistant strains (Jacket *et al.*, 1981); and (iii) addition of catalase abolishes killing of *M. microti* by murine macrophages activated with a lymphocyte culture supernatant (Walker and Lowrie, 1981). Circumstantial evidence in humans comes from the clinical observation that patients whose macrophages cannot mount an oxidative burst because of chronic granulomatous disease are more at risk of developing complications due to mycobacterial infection (Sbarra and Karnovsky, 1959; Iyer *et al.*, 1961; Klebanoff, 1980). Macrophages from these patients can, however, be activated to mount an enhanced respiratory burst that does not concomitantly enhance their antimycobacterial activity (Rook *et al.*, 1986).

More recent investigations have shown, however, that ROI have only a limited role to play in host defense against *M. tuberculosis*. Flesch and Kaufmann (1988), for example, infected murine bone marrow-derived macrophages with *M. bovis* BCG and determined the ability of these macrophages to inhibit mycobacterial growth in the presence and absence of scavengers of toxic oxygen species. In cell-free conditions, hydrogen peroxide, but not superoxide anions or

hydroxyl radicals, inhibited the growth of mycobacteria. They found, however, that growth inhibition of BCG was not reversed to any significant extent when superoxide dismutase or catalase was added to macrophages infected with BCG and stimulated with IFN-γ.

While the ability of ROI to restrict intracellular mycobacterial growth is unclear, the ability of various mycobacterial species to actively scavenge these toxic oxygen species has been clearly demonstrated. Several reports have implicated a variety of mycobacterial components or products, including LAM, sulfatides, and phenolicglycolipid I (PGL-I). LAM is a major cell wall-associated, phosphotidylinositol-anchored complex lipopolysaccharide produced by *M. tuberculosis* in large amounts (Hunter *et al.*, 1986; Hunter and Brennan, 1991). Two mechanisms have been proposed by which LAM may be capable of inactivating the toxic ROI-mediated onslaught. Firstly, LAM has been shown to be an effective ROI scavenger; and secondly, LAM can down regulate the oxidative burst by inhibiting the activity of protein kinase C (an enzyme which plays an important role in activation of the oxidative burst in phagocytic cells; Chan *et al.*, 1991). Both PGL-I and sulfatides also demonstrate the *in vitro* capacity to down regulate ROI production in macrophages and PGL-I has been found to directly scavenge oxygen radicals in a cell-free system (Chan *et al.*, 1989). While the sulfatides are widely expressed among the different strains of *M. tuberculosis* (Middlebrook *et al.*, 1959; Goren *et al.*, 1974, 1976), the expression of PGL-I is more restricted (Brennan *et al.*, 1990). In addition to these mycobacterial components, the most abundant proteins secreted by *M. tuberculosis* in short-term culture is superoxide dismutase and catalase (Andersen *et al.*, 1991).

Reactive nitrogen intermediates

Chan *et al.* (1992) demonstrated that murine macrophages stimulated with either IFN-γ, LPS or TNF-α are capable of inhibiting the growth of *M. tuberculosis* using a mechanism that is independent of the respiratory burst and ROI generation. They found that the antimycobacterial activity of these macrophages seemed to correlate with induction of L-arginine-dependent production of toxic nitrogen species, including nitric oxide (NO), nitrogen dioxide (NO_2), and nitric acid (HNO_3). A number of studies have demonstrated that RNI contributes to the activity of murine macrophages against *M. tuberculosis* (Denis, 1991; Chan *et al.*, 1992), *M. bovis* BCG (Flesch and Kaufmann, 1991), *M. leprae* (Adams *et al.*, 1991) and *M. avium* (Doi *et al.*, 1993). More recently, knockout mice containing an interferon regulatory factor (IRF-1) gene deletion, and whose macrophages are subsequently incapable of releasing NO, were shown to rapidly succumb to BCG infection (Kamijo *et al.*, 1994). MacMicking and colleagues (1997) recently confirmed that mice lacking the ability to produce inducible NO synthase (iNOS -/- knockout mice) showed more rapid *M. tuberculosis* replication and had a greater bacterial load than their wild-type littermates. Some evidence for up regulation of iNOS in alveolar macrophages from human patients with tuberculosis has also been presented recently (Nicholson *et al.*, 1996).

Apoptosis

Another potential mechanism involved in macrophage defense against *M. tuberculosis* is apoptosis or programmed cell death. Placido *et al.* (1997) reported that virulent *M. tuberculosis* H37Rv induces apoptosis in a dose-dependent manner in BAL cells recovered from tuberculosis patients. Klinger *et al.* (1997) have demonstrated that apoptosis associated with tuberculosis is mediated through a down-regulation of bcl-2, an inhibitor of programmed cell death. Furthermore, Molloy *et al.* (1994) have shown that apoptosis of macrophages is associated with reduced viability of intracellular mycobacteria.

1.2.2 MYCOBACTERIAL LOCALIZATION AND ANTIGEN PRESENTATION

Macrophages have evolved two clearly defined pathways for the processing of exogenous and endogenous antigens (Fig. 1.1). The first being the HLA class I or endogenous antigen processing pathway (Fig. 1.1a) wherein peptides derived from endogenous proteins are presented to $CD8^+$ T cells by HLA class I molecules. The second being the HLA class II or exogenous processing pathway (Fig. 1.1b) wherein peptides derived from extracellular sources are presented to $CD4^+$ T cells in association with HLA class II molecules (Germain, 1994). In general, *M. tuberculosis* follows the HLA class II processing pathway which predominantly elicits a $CD4^+$ T cell response (Kaufmann and Flesch, 1986; Ottenhoff *et al.*, 1988; Barnes *et al.*, 1989; reviewed by Kaufmann and Andersen, 1998).

More recent studies have demonstrated several additional antigen processing pathways operative within professional phagocytes. Alternative HLA class I pathways have been described that facilitate the HLA class I presentation of exogenously derived antigens to $CD8^+$ CTLs (Pfeifer *et al.*, 1993; Kovacsovics-Bankowski *et al.*, 1993; Harding and Song, 1994; Kovacsovics-Bankowski and Rock, 1995). Abundant evidence for $CD8^+$ CTL activity in both murine and human tuberculosis provides evidence for the existence of such a pathway in mycobacterial infections (Bonato *et al.*, 1998; Silva *et al.*, 1996; De Libero *et al.*, 1988; Turner & Dockrell, 1996; Tan *et al.*, 1997; Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998). The processing pathway used by the non-classical, highly conserved CD1 molecules to present non-proteinaceous mycobacterial antigens to $CD8^+$ and $CD4^-CD8^-$ $\alpha\beta$ T cells represents another alternative to the classical HLA class I and II processing pathways (Jullien *et al.*, 1996). Finally, presentation of antigens to $\gamma\delta^+$ T cells does not require HLA class I or class II processing and neither is $\gamma\delta^+$ T cell recognition restricted by either HLA class I, class II or CD1 (Morita *et al.*, 1996).

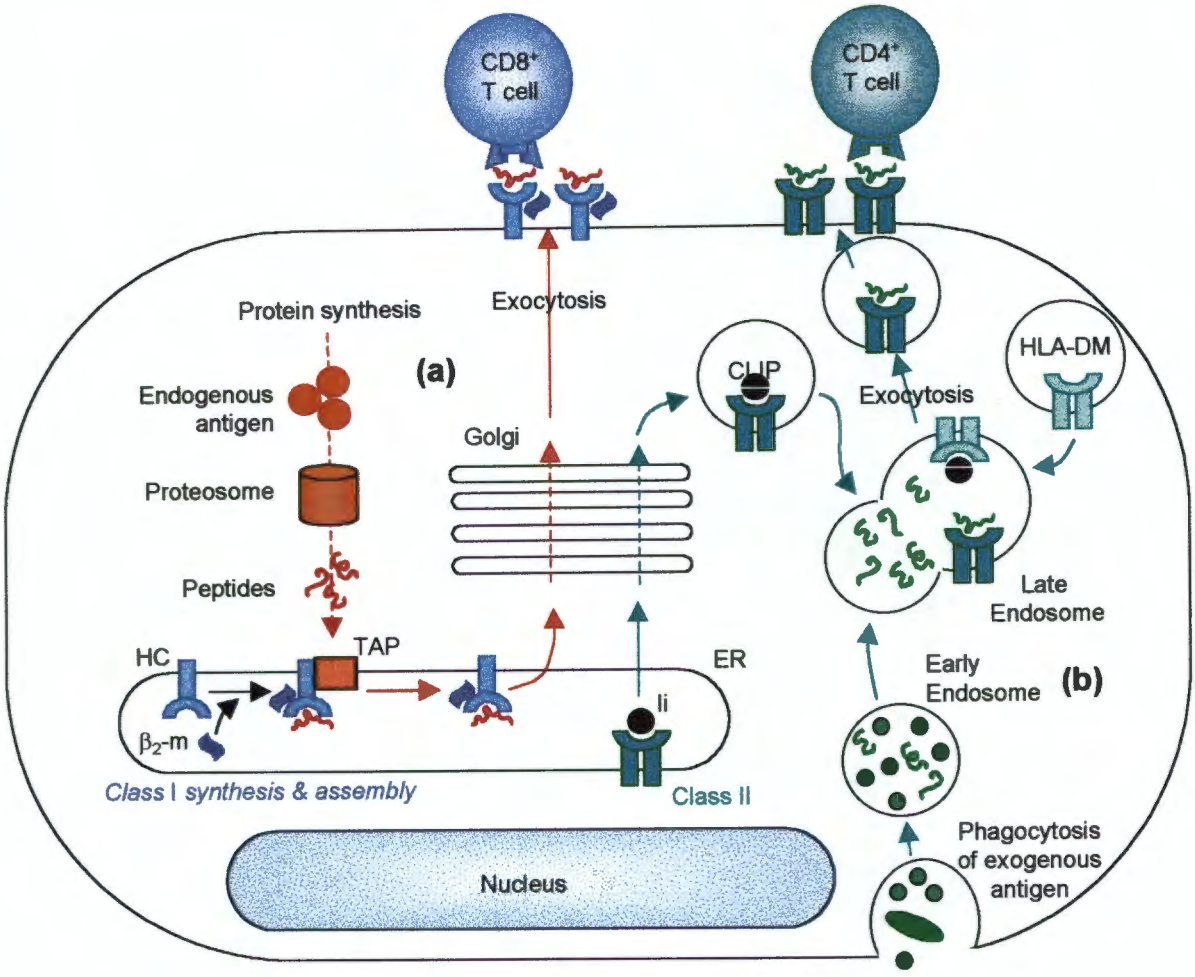


FIGURE 1.1 The classical HLA class I and class II antigen processing pathways. (a) HLA class I molecules acquire antigenic peptides derived from antigens present in the cytoplasm. In the endoplasmic reticulum (ER), newly synthesized HLA class I heavy chains bind β_2-microglobulin. The assembled class I heterodimer then associates with the transporter associated with antigen processing (TAP) and awaits a peptide. Peptides, generated from proteins in the cytosol by the proteasome, are transported into the ER by TAP. After binding peptide, class I molecules dissociate from TAP and are transported by the default exocytic pathway to the plasma membrane. Peptides are presented to $CD8^+$ CTL's bearing the appropriate T cell receptor. (b) HLA class II molecules, in contrast, acquire antigenic peptides derived from antigens that are internalized in the endocytic pathway. Newly synthesized HLA class II heterodimers assemble in the ER with invarient chains (Ii). At the trans-Golgi network, these complexes are targeted to HLA class II compartments in the endocytic pathway due to targeting signals within the Ii cytoplasmic tail. There, the HLA class II associated Ii is degraded leaving CLIP (class II associated invariant chain peptide) associated with the HLA class II peptide binding groove. CLIP can then be exchanged for antigenic peptides, a process catalyzed by HLA-DM molecules. Peptide loaded HLA class II complexes are then transported to the plasma membrane for presentation to $CD4^+$ T cells. In most cells, proteins in the extracellular fluids do not intersect the HLA class I processing pathway but follow the classical HLA class II antigen processing pathway (adapted from Engelhard, 1994; Pieters *et al.*, 1997; and Rock, 1996)

The HLA class II processing pathway and $CD4^+$ T cells

Internalized exogenous antigen initially enters early endosomes, then late endosomes, and finally lysosomes (Neefjes & Ploegh, 1992). A gradient of pH is observed between early endosomes (pH 6.0-6.5), late endosomes (pH 5.5), and lysosomes (pH 4.5-5.0) because of the action of ATP-dependent proton pumps in the endosomal membrane which pump H^+ from the cytosol into these compartments. It has been suggested that the pH gradient results in the activation of different proteases in these cellular compartments (Forquet *et al.*, 1993). Proteolytic degradation of exogenous antigens begins once they enter endosomes and continues to the lysosomes (Guagliardi *et al.*, 1990). The exact endocytic compartment in which HLA class II molecules bind to antigenic peptides still remains unclear, however. After peptide binding to MHC class II, the assembled complexes move towards the plasma membrane of the APC where they can be recognised by $CD4^+$ T cells. Studies have shown that $CD4^+$ T cells can become activated by as few as 100 to 200 peptide-HLA class II complexes on the APC surface (Demotz *et al.*, 1990; Harding & Unanue, 1990).

Mycobacterial intracellular trafficking and inhibition of phagosome-lysosome fusion

Once *M. tuberculosis* has become engulfed by the macrophage, the intracellular bacteria reside in membrane-bound phagosomes. Even if the organism is capable of surviving the onslaught of both ROI and RNI, it is confronted with the usual fate of phagocytosed material which are earmarked for the classical exogenous processing pathway and eventually undergo degradation in lysosomes, highly acidic compartments filled with hydrolases and other noxious products. It is thus not surprising that facultative intracellular pathogens, including *M. tuberculosis*, have evolved a variety of strategies to avoid this pathway of destruction. Intracellular pathogens follow one of three general pathways within the host cell leading either to escape into the cytoplasm (*Trypanosoma cruzi* and *Listeria monocytogenes*); residence in a phagolysosome (*Leishmania donovani* and *Coxiella burnetti*); or residence in a phagosome that does not fuse with lysosomes. Although it is generally accepted that *M. tuberculosis* resembles several other intracellular pathogens (including *Legionella pneumophila*, *Toxoplasma gondii* and *Chlamydia psittaci*) in following the latter pathway and residing in non-fused phagosomes, there have also

been isolated reports suggesting that mycobacteria exhibit haemolytic activity (King *et al.*, 1993) and demonstrate restricted ability to escape into the cytoplasm (McDonough *et al.*, 1993; Myrvik *et al.*, 1984).

The lack of mixing between *M. tuberculosis* phagosomes and lysosomal compartments in macrophages was first thoroughly documented in the classical studies by Armstrong and Hart (1971). While early studies described the ability of sulphatides produced by virulent *M. tuberculosis* to block the maturation of phagosomes to lysosomes, this observation turned out to be an artifact associated with entrapment of markers in polyanionic colloids (Goren *et al.*, 1976; 1987). Instead, attention has been turned to the inhibition of phagosomal-lysosomal fusion by mycobacterial products, such as ammonia which is found to accumulate in *M. tuberculosis* culture filtrates at concentrations of up to 20 mM (Gordon *et al.*, 1980). Later studies demonstrated that live *M tuberculosis* reside within non-acidified vacuoles (pH 6.0-6.5; Crowle *et al.*, 1991). Strugill-Koszycki and colleagues (1994) have subsequently demonstrated the selective exclusion of vescicular proton-ATPase on mycobacterial phagosomes. The proton translocating ATPase is usually present on the majority of vesicles of endocytic and exocytic pathways that become acidified to varing degrees, but appears to be absent on mycobacterial phagosomes. The precise trafficking events underlying this exclusion are not yet known, and it is not clear whether additional factors may play a role in modulating the pH of the phagosome. It is possible that the Na^+/K^+-ATPase persists on the *M. tuberculosis* phagosome, thereby counteracting the action of the proton pump (Clemens, 1996).

Although little is known about the precise trafficking events followed by mycobacterial vesicles, clues have been provided by investigations into the distribution of several markers on mycobacterial phagosomes (reviewed recently by Deretic *et al.*, 1997; and Clemens, 1996). The distribution of markers such as HLA molecules, transferrin receptors, late endosomal and lysosomal markers and rab proteins on mycobacterial phagosomes are best understood relative to the intracellular fates of either inert particles (such as latex beads) or *L. pneumophila* (Fig. 1.2).

HLA molecules

The *M. tuberculosis* phagosomal membrane shows relatively slow clearance of HLA class I molecules. Phagosomes containing latex beads or *L. pneumophila*, by comparison, show comparatively rapid HLA class I clearance with levels being barely detectable a day after phagocytosis (Clemens & Horwitz, 1992, 1993). Clemens and Horwitz (1995) demonstrated that HLA class II molecules also persisted on the mycobacterial phagosomal membrane. Whereas HLA class I molecules disappeared from the *M. tuberculosis* phagosome later than 2 days after phagocytosis, HLA class II molecules were still detectable on the phagosome 5 days post-phagocytosis. These HLA class II molecules may initially be acquired from both the plasma membrane internalised during phagocytosis and the interaction of the *M. tuberculosis* phagosome with HLA class II-containing endocytic vesicles at a later stage. By comparison, phagosomes containing mature *L. pneumophila* or latex beads have negligible staining for HLA class II (Clemens, 1996).

Tranferrin receptor

Whereas mature phagosomes containing latex beads or *L. pneumophila* do not stain for transferrin receptor, this receptor is clearly detectable on *M. tuberculosis* phagosomal membrane (Clemens & Horwitz, 1995; Strugill-Koszycki *et al.*, 1996). This suggests that, although mycobacterial phagosomes demonstrate limited fusogenicity with lysosomes, they do interact with other components of the intracellular trafficking pathway.

The presence of LAM in vesicles outside mycobacterial phagosomes may be another indication that *M. tuberculosis* phagosomes interact with the endocytic pathway (Xu *et al.*, 1994). This could, however, also be due to uptake of LAM from culture media or to degradation of dead mycobacteria. Russell and colleagues (1994, 1996) have also presented evidence to suggest that certain markers such as plasma membrane glycosphingolipids are able to traffic into and out of the mycobacterial phagosome.

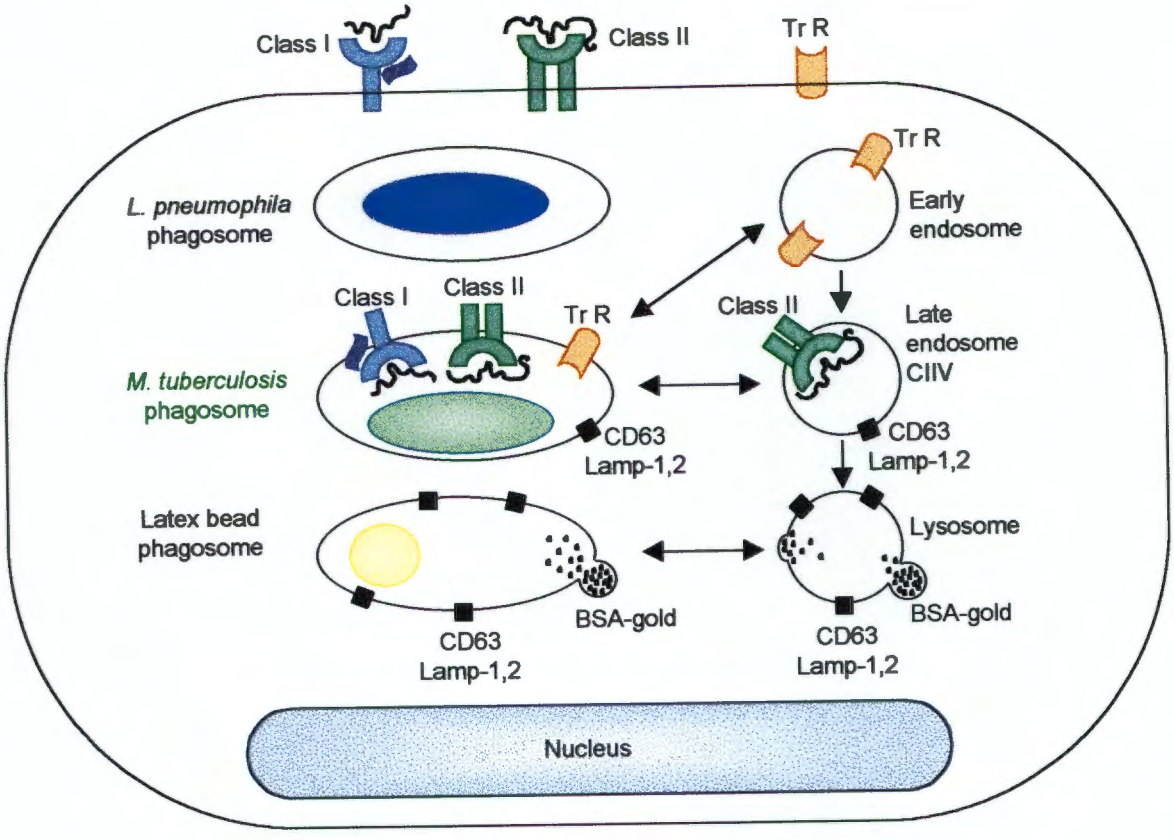


FIGURE 1.2. Postulated relationship of the endosomal-lysosomal pathway to phagosomes containing *Mycobacterium tuberculosis*, *Legionella pneumophila* or latex beads. *L. pneumophila* phagosomes interact minimally with the endosomal-lysosomal pathway and do not acquire endosomal or lysosomal markers. *M. tuberculosis* phagosomes show a delayed clearance of HLA class I molecules and interact with endosomes, acquiring various endosomal markers including HLA class II, transferrin receptor molecules and modest amounts of lysosomal membrane glycoproteins (CD63 and LAMP-1). However, the *M. tuberculosis* phagosome does not fuse with lysosomes and does not acquire the abundant amounts of lysosomal membrane glycoproteins present on lysosomes. Latex-bead-containing phagosomes, in contrast, fuse with lysosomes and acquire abundant amounts of lysosomal proteins (modified from Clemens, 1996).

Late endosomal and lysosomal markers

Both Clemens (1996) and Xu *et al.* (1994) have reported the presence of lysosomal membrane glycoproteins (LAMP) and cathepsin D in *M. tuberculosis*-containing phagosomes. While Xu *et al.* (1994) reported intense staining, however, Clemens & Horwitz (1995) demonstrated reduced levels of LAMP and cathepsin D compared with the levels seen on latex bead phagosomes in the same cells. Based on their study, Clemens (1996) has suggested that the limited acquisition of

LAMP and cathepsin D is consistent with both the restricted fusogenicity of the mycobacterial phagosome and the retarded maturation of the phagosome along the endosomal-lysosomal pathway. Alternatively, these results may reflect altered trafficking events within the endosomal pathway upstream of lysosomes. This is alluded to by the recent report by Rohrer and colleagues (1996) which showed that a significant portion of newly synthesized LAMP molecules are sorted in the trans-Golgi network for delivery to early/sorting endosomes and the plasma membrane, with subsequent trafficking to late endosomes and lysosomes. In keeping with this model, it has been suggested that mycobacterial phagosomes acquire LAMP and cathepsin D prior to the scheduled addition of proton-ATPase to the vesicles (Deretic *et al.*, 1997). In contrast to phagosomes containing *M. tuberculosis* or latex beads and consistent with their ability to persist outside the endosomal-lysosomal pathway, *L. pneumophila*-containing phagosomes do not acquire staining for LAMP or cathepsin D (Clemens & Horwitz, 1995).

Rab proteins

Further clues to intracellular mycobacterial trafficking have been provided by a recent study of the distribution of small GTP binding proteins on the membranes of mycobacterial phagosomes (Via *et al.*, 1997). Members of the rab subfamily of small GTP-binding proteins control vesicular trafficking in various compartments of eukaryotic cells (Zerial and Stenmark, 1993). Along the subdivisions of the endocytic pathway, rab4, rab5, rab7, and rab9 play distinct roles in regulating membrane fusion events (Zerial, 1995). For example, rab5 isoforms are associated with the plasma membrane and early endosomes and represent a rate-limiting factor in the process of membrane docking and fusion within the early endocytic pathway. Whereas rab4 is primarily involved in recycling steps (van der Slujis *et al.*, 1992), rab7 appears to regulate transport from early to the late endosomes (Feng *et al.*, 1995). The recycling of mannose-6-phosphate receptors from late endosomes back to the trans-Golgi network is stimulated by rab9 (Riederer *et al.*, 1994).

Compared to latex bead phagosomes which at first acquire and then gradually lose rab5, Via *et al.* (1997) demonstrated that rab5 continues to accumulate on the membrane of mycobacterial phagosomes. Furthermore, the latex bead phagosomes later acquire rab7, reflecting phagosome

maturation towards late endosomes and lysosomes. By contrast, mycobacterial phagosomes do not acquire any appreciable amounts of rab7. It seems likely therefore that rab5 accumulation is possibly at the expense of rab7, defining the checkpoint that has been compromised in the process of mycobacterial phagosome maturation (Via *et al.*, 1997).

The mechanism that allows for mycobacterial control of phagosome maturation and inhibition of phagosome-lysosome fusion is probably multi-factorial. It has been proposed that these mechanisms may include the involvement of major mycobacterial cell wall constituents, bio-active molecules secreted from viable intracellular mycobacteria, and the receptor pathway selected during phagocytosis (Clemens, 1996; Schlesinger, 1996). Regardless of the virulence determinants it uses to survive and multiply within the phagosome of host macrophages, *M. tuberculosis* have demonstrated an ability to modify both the composition of their phagosomes and the interaction of these phagosomes with other host-cell organelles that presumably results in a more hospitable phagosomal compartments. By residing in a phagosome that retains some, albeit restricted, interaction with endosomes, *M. tuberculosis* is also able to obtain both nutrients needed for intracellular growth and membrane components for its expanding phagosome (Deretic *et al.*, 1997).

Mycobacterial modulation of antigen presentation

Because macrophages form part of a complex network by communicating with and alerting other components of the CMI response, particularly T cells, the ability of *M. tuberculosis* to modify its intracellular phagosome and endosomal trafficking pathway is likely to have important implications with regard to mycobacterial antigen presentation. Pancholi *et al.* (1993) provided evidence for this by demonstrating that macrophages 'chronically' infected with BCG stimulated T cell proliferation poorly compared to cells infected for shorter periods of time, suggesting a defect in antigen presentation. Monocytes infected with *M. tuberculosis* and concomitantly pulsed with tetanus toxoid (TT) have been found to present TT poorly and to inefficiently prime TT-specific T cell proliferation due to both reduced expression of HLA class II antigens and reduced accessory cell function (Gercken *et al.*, 1994). A 25-kDa *M. tuberculosis* glycolipoprotein component has been reported to counteract the enhanced IFN-γ or LPS-induced

expression of HLA class II molecules on monocytes (Wadee *et al.*, 1995). More recently, Hmama *et al.* (1998) demonstrated that diminished cell surface HLA class II expression in cells infected with *M. tuberculosis* was due to intracellular sequestration of immature HLA class II molecules. *M. tuberculosis*-infected macrophages from susceptible mice were found to have reduced expression of the co-stimulatory molecule B7 and increased expression of the adhesion molecule ICAM-1. This was shown to result in T cell unresponsiveness, which appeared linked to prostaglandin synthesis in response to infection with live bacteria (Saha *et al.*, 1994). Similarly, incubation of human macrophage cell lines with *M. tuberculosis* or LAM results in increased expression of ICAM-1, which was partially due to secretion of TNF-α (Lopez-Ramirez *et al.*, 1994). Despite these mechanisms aimed at avoiding normal antigen presentation, a wide spectrum of T cell responses are elicited by *M. tuberculosis*-infected macrophages.

The dominance of $CD4^+$ T cells in protective immunity to both murine and human tuberculosis has been clearly demonstrated (Kaufmann and Flesch, 1986; Ottenhoff *et al.*, 1988; Barnes *et al.*, 1989; reviewed by Kaufmann and Andersen, 1998). Mice depleted of $CD4^+$ T cells prior to infection with avirulent BCG or virulent *M. tuberculosis* are unable to control mycobacterial growth (Muller *et al.*, 1987; Pedrazzini *et al.*, 1987) and adoptive transfer of $CD4^+$ T cells from sensitised animals confers protection against tuberculosis (Orme, 1987, 1988). Ladel and collaborators (1995) demonstrated that disruption of the HLA class II genes or of the gene for the β chain of the $\alpha\beta^+$ TCR, resulting in a deficiency of $CD4^+$ T cells, rendered mice more susceptible to mycobacterial infection. In humans, $CD4^+$ T cells are selectively expanded at the site of disease in patients with tuberculous pleuritis (Barnes *et al.*, 1989), and depletion of $CD4^+$ cells by HIV infection markedly increases susceptibility to primary and reactivation tuberculosis (Barnes *et al.*, 1991). Furthermore, in HIV-infected tuberculosis patients, clinical indicators of severe disease, such as extrapulmonary involvement, mycobacteremia and positive acid-fast smears, become progressively more common as the $CD4^+$ cell count declines (Jones *et al.*, 1993).

The two major functions mediated by $CD4^+$ T cells in mycobacterial infections are their ability to produce protective cytokines in response to infection and their ability to directly lyse infected target cells in an HLA class II-restricted manner. Studies in mice have shown that protective, mycobacterial-reactive $CD4^+$ T cells predominantly produce Th_1-type cytokines, IFN-γ and IL-2

(Huygen *et al.*, 1992; Kaufmann & Anderson, 1998). Studies of human tuberculosis, however, have yielded conflicting results. Some authors found that the majority of mycobacterial-specific $CD4^+$ T cell clones are Th_1-like, producing high concentrations of IFN-γ, but low concentrations of IL-4 and IL-5 (Del Prete *et al.*, 1991; Haanen *et al.*, 1991). In contrast, others have reported that human *M. tuberculosis*-specific $CD4^+$ cells secrete a broad spectrum of cytokines, including IFN-γ, IL-2, IL-4, IL-5 and IL-10 (Boom *et al.*, 1991; Barnes *et al.*, 1993a, 1993b). At the site of mycobacterial disease, however, mRNA for the Th_1 cytokines IFN-γ and IL-2 is greater in the tuberculous pleural fluid than in the peripheral blood, and concentrations of IFN-γ are 15-fold higher than the serum concentrations (Barnes *et al.*, 1993b). Furthermore, expression of mRNA for the Th_2 cytokine IL-4 was found to be lower in the pleural fluid than in the peripheral blood.

An alternative mechanism by which $CD4^+$ T cells may contribute to immune defense in mycobacterial infections is through direct cytolysis of macrophages and other phagocytic cells infected with *M. tuberculosis*. Human *M. tuberculosis*-specific CTLs cultured *in vitro* were found to be predominantly $CD4^+$ (Ottenhoff and Mutis, 1990; Lorgat *et al.*, 1992), and *M. tuberculosis*-specific cytolytic activity of $CD4^+$ cells at the site of disease was found to be greatly enhanced compared to that of peripheral blood mononuclear cells (Lorgat *et al.*, 1992).

The HLA class I processing pathway and $CD8^+$ T cells

The classical HLA class I processing pathway (Fig. 1.1) is operative in almost all cells and allows the immune system to monitor tissues for the presence of cytoplasmically-situated viral and bacterial infections, and tumours. Protein antigens in the cytosol are degraded primarily by proteosomes (large multicatalytic proteolytic particles), transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) where they become associated with newly synthesized HLA class I heavy chain/β_2-microglobulin (β_2m) heterodimers (reviewed in Heemels & Ploegh, 1995; York & Rock, 1996). The peptide/β_2m/class I heavy chain complexes are then routed through the Golgi complex and carried to the cell surface where they may be presented to $CD8^+$ CTLs.

In most cells, exogenous antigens cannot be presented by HLA class I because the antigens are unable to gain access to the cytosolic compartment (Moore *et al.*, 1988). And because HLA class I generally present peptides to CTLs, whose effector function is best restricted to actively infected cells, it makes biological sense that these HLA molecules should be limited in their ability to acquire peptides derived from proteins in the extracellular milieu (exogenous antigens). This would help prevent healthy cells from becoming sensitised for CTL lysis by foreign, non-infectious proteins released from neighbouring infected or transformed cells (Reis e Sousa and Germain, 1995).

Because *M. tuberculosis* is generally assumed to remain within the exogenous antigen processing pathway, within membrane-bound vacuoles, access of mycobacterial antigens to the classical HLA class I processing pathway seems improbable. Despite this, several studies have emerged clearly demonstrating the protective role of $CD8^+$ CTLs in mycobacterial infections. Adoptive transfer and cell depletion studies *in vivo* have demonstrated that $CD8^+$ T cells are involved in controlling *M. tuberculosis* infections (Orme & Collins, 1984; Orme, 1987; Muller *et al.*, 1987; Silva *et al.*, 1994) and are required for immunologic memory (Hubbard *et al.*, 1991). Mice with a disruption in the gene for CD8 were shown to be highly susceptible to infection with *M. tuberculosis* (D'Sousa *et al.*, 1998). Mycobacterium-specific $CD8^+$ T cell lines and clones have been isolated from both *M. tuberculosis* and BCG-immune mice and have been shown to directly lyse *M. tuberculosis*-infected cells in an antigen-specific manner and restrict the growth of intracellular *M. tuberculosis* (Bonato *et al.*, 1998; Silva *et al.*, 1996; De Libero *et al.*, 1988). Furthermore, mice with a targeted disruption in the β_2-microglobulin gene, lacking functional $CD8^+$ T cells, are significantly more susceptible to *M. tuberculosis* but not BCG infection than their wild type litter mates (Flynn *et al.*, 1992; Ladel *et al.*, 1995). These β_2-microglobulin-knockout mice were found to be able to form normal granuloma's but showed a ten-fold higher bacillary load in infected tissue when compared with control mice. This evidence is less compelling in light of the recent finding that β_2-microglobulin also associates with the non-classical CD1 molecule, which is able to present antigen to both $CD8^+$ CTLs (Stenger *et al.*, 1997) and $CD4^-CD8^-$ $\alpha\beta^+$ T cells (Beckman *et al.*, 1994).

Until recently, mycobacteria-responsive $CD8^+$ T cells had only rarely been isolated from patients with tuberculosis (Rees *et al.*, 1988) and little success had been achieved in determining the role of HLA class I-restricted $CD8^+$ T cells in human immunity to tuberculosis. Several studies have since emerged, however, demonstrating classical HLA class I-restricted mycobacterial antigen-responsive $CD8^+$ T cells which acquire activation markers following stimulation, are capable of mycobacterial-specific cytolytic activity and of IFN-γ production (Lewinsohn *et al.*, 1998; Turner & Dockrell, 1996; Tan *et al.*, 1997; Lalvani *et al.*, 1998; Mohagheghapour *et al.*, 1998). Although both murine and human $CD8^+$ T cells are capable of producing significant levels of IFN-γ in response to *M. tuberculosis* infection (De Libero *et al.*, 1988; Bonato *et al.*, 1998; Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998), evidence suggests that their strong cytolytic activity is more important for protective immunity in mycobacterial infections (Bonato *et al.*, 1998; Stenger *et al.*, 1997).

Although isolated reports have suggested that intracellular *M. tuberculosis* is able to escape into the cytoplasm (Leake *et al.*, 1984; McDonough *et al.*, 1993), more recent studies have clearly shown that *M. tuberculosis* resides exclusively within membrane-bound phagosomes (Xu *et al.*, 1994; Clemens & Horwitz, 1995). The mechanism by which *M. tuberculosis* antigens might gain access to the HLA class I presentation pathway is not well characterized. Mazzaccaro *et al.* (1996) has demonstrated that infection with *M. tuberculosis* is able to facilitate exchange of exogenous antigenic material between phagosomes and the cytoplasm. In a more recent study, Hess *et al.* (1998) made use of recombinant BCG expressing a biologically active listeriolysin fusion protein of *Listeria monocytogenes*. They found that while this did not enable BCG egression into the cytoplasm of infected cells, it did facilitate improved HLA class I presentation of co-phagocytosed soluble protein compared with wild type BCG.

The ability of exogenous antigens, such as *M. tuberculosis* and its secreted products, to gain access to the HLA class I processing pathway has become an area of great interest with a number of recent reports describing alternative pathways for HLA class I processing (Pfeifer *et al.*, 1993; Kovacsovics-Bankowski *et al.*, 1993; Harding and Song, 1994; Kovacsovics-Bankowski and Rock, 1995). A significant body of evidence now exists to indicate that HLA class I processing

can occur independently of microbial egression into the cytoplasm (Pfeifer *et al.*, 1993; Kovacsovics-Bankowski *et al.*, 1993).

Phagosomal "leakage" or indigestion model

The mechanism by which exogenous antigens intersect the HLA class I processing pathway and become associated with HLA class I molecules is one of the most important issues that remains to be resolved. Some antigens have been found to escape from phagosomes into the cytosolic compartment, where they presumably enter the endogenous processing pathway as judged by the inhibitory effects of proteosomal inhibitors and brefaldin A (BFA blocks HLA class I trafficking through the Golgi; Takahashi *et al.*, 1990; van Binnendijk *et al.*, 1992; Aggarwal *et al.*, 1990; Aldovini and Young, 1991; Stover *et al.*, 1991, 1993; Schafer *et al.*, 1992; Rock, 1996). Little is presently known about this "leakage" from phagosome to cytosol; whether it is an energy dependent process regulated by specific transporters, whether it depends on unspecified damage to the vesicular membrane, or whether it represents an important *in vivo* event is unknown. The phagosome to cytosol transfer process has been demonstrated with (i) internalised toxins, which lack the capacity to penetrate membranes but still can enter the cytosol to act on ribosomes (Reis e Sousa and Germain, 1995; Norbury *et al.*, 1995; Kovacsovics-Bankowski and Rock, 1995), (ii) fluoresceinated dextran and ISCOM-associated protein antigen, and (iii) in different intracellular microbial systems (Norbury *et al.*, 1995; Andrews, 1994; Morein *et al.*, 1994). Germain *et al.* (1996) and Jondal *et al.* (1996) proposed that HLA class I-presentation of some modified exogenous antigens may simply reflect a stochastic loss of phagosome integrity and leakage of antigen into the cytoplasm rather than the action of a novel intracellular transport system (Fig 1.3). Consistent with this, Orme (1993) suggested that high mycobacterial load in chronically infected macrophages may result in disruption of the phagosomal membrane, facilitating the escape of mycobacterial proteins into the cytoplasm.

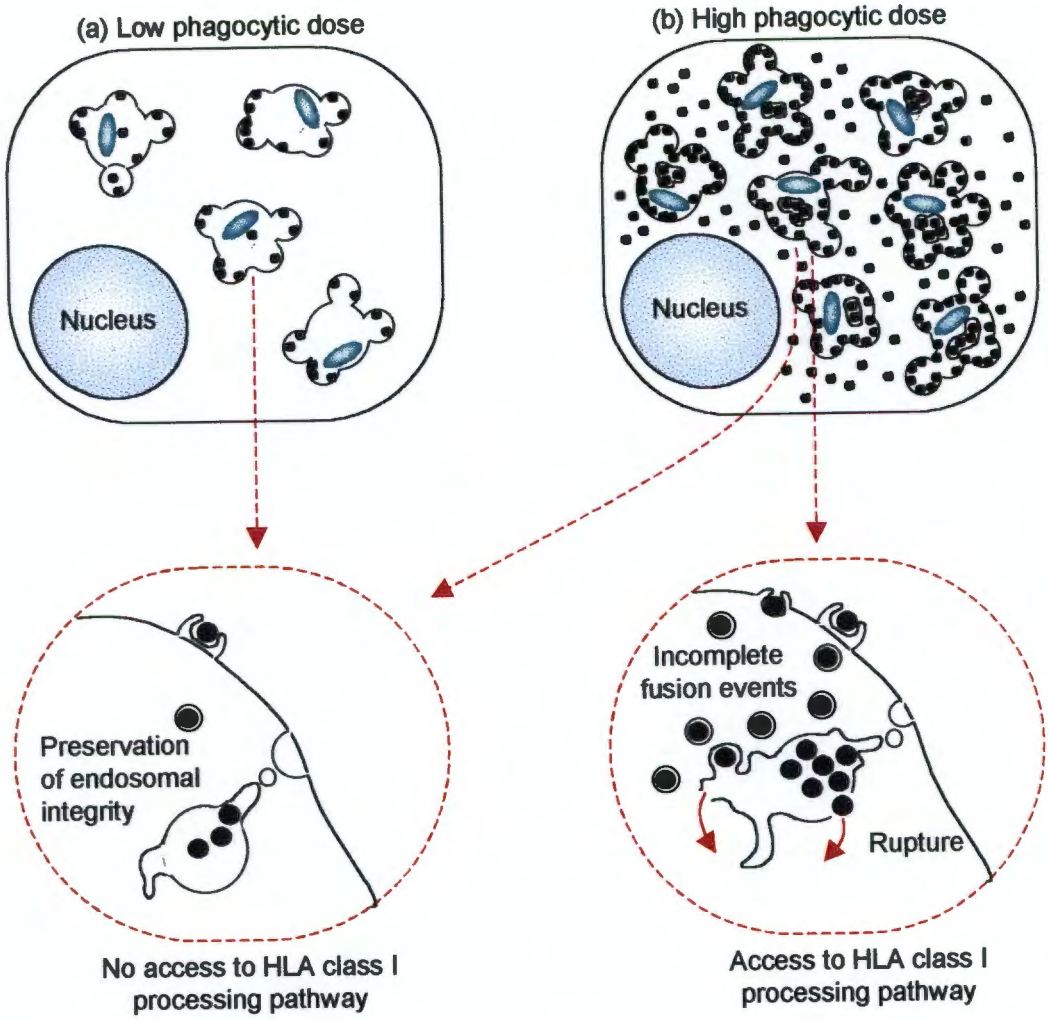


FIGURE 1.3 Access of exogenous antigen to the HLA class I pathway in a fraction of phagocytic cells. Cells given a low phagocytic 'dose' (upper left panel) internalise particles while preserving the integrity of the endocytic/phagocytic compartments (expanded view in lower left panel). In a small fraction of cells given a high phagocytic 'dose' (upper right panel), overloading of forming phagosomes leads to a leak of antigen into the cytosol because of incomplete membrane fusion events or rupture of the vesicle membrane (expanded view in lower right panel). Some leaks would permit escape of soluble material either cleaved from the particle by endosomal proteases or co-internalised during the phagocytic event. In some cases, entire particles may escape. Once in the cytosol, the antigen is available to the classical HLA class I processing and loading pathway, as with conventional endogenous antigens (reproduced from Germain *et al.*, 1996).

Transiting HLA class I loading model

'Transiting HLA class I loading' has also been proposed as a possible mechanism whereby newly formed HLA class I molecules exchange the peptides they capture in the ER for exogenous antigens in vesicular compartments whilst on their way from the ER to the cell surface (Fig. 1.4b). It is controversial whether 'empty' HLA class I molecules can leave the ER and be loaded with peptides in a vacuolar compartment. Despite the fact that the peptide presentation capacity of such chaperoned HLA class I molecules remains to be determined, the invariant chain used for trafficking of HLA class II molecules has been shown to direct a subset of HLA class I molecules directly to the endocytic compartment (Sugita and Brenner, 1995).

Endosomal compartment or recycling HLA class I loading

An alternative to transiting HLA class I loading or antigen translocation into the cytoplasm would be HLA class I loading within the endosomal compartment (Fig. 1.4c). HLA class I molecules within the endosomal compartment could be derived by recycling from the cell surface, from the formation of membrane vesicles containing correctly positioned HLA class I molecules, or by direct transport from the ER (Jondal *et al.*, 1996). Recycling of HLA class I molecules between the cell membrane and an endosomal compartment has been demonstrated in both T cells and macrophages and has been shown to exceed the *de novo* synthesis pathway in terms of membrane expression (Yewdell and Bennick, 1992; Reid and Watts, 1990). Phagocytic vesicles, derived from the plasma membrane, might contain peptide-receptive HLA class I molecules, or newly synthesised class I molecules might be directly transported to these with the help of chaperone proteins (De Bruijn *et al.*, 1995). Eventually, these class I molecules might be transferred to an endocytic compartment by vesicular transport. Two recent studies have described a thermostable population of 'empty' HLA class I molecules expressed on the surface that binds neither antigenic peptides, nor exogenous β_2-microglobulin (Smith *et al.*, 1993; Schirmbeck and Reimann, 1996). These presentation-incompetent HLA class I-isoforms recycle through endosomal compartments where they seem to be assembled into presentation-competent complexes (Schirmbeck and Reimann, 1996). This class of 'empty', recycling HLA class I molecules may be involved in endolysosomal loading of peptides from endocytosed exogenous antigens. This pathway also points to the possibility that following presentation of a 'first

generation' of peptides (generated by endogenous processing of endogenous antigens), a 'second generation' of antigenic peptides (generated by exogenous processing of exogenous antigens) can be loaded onto recycling HLA class I molecules. The precedent for this pathway comes from a recent study which demonstrated that recycling HLA class II molecules are able to capture a new set of antigenic peptides in an HLA-DM- and invariant chain-independent pathway following the initial HLA-DM- and invariant chain-dependent loading of nascent HLA class II (Lindner and Unanue, 1996).

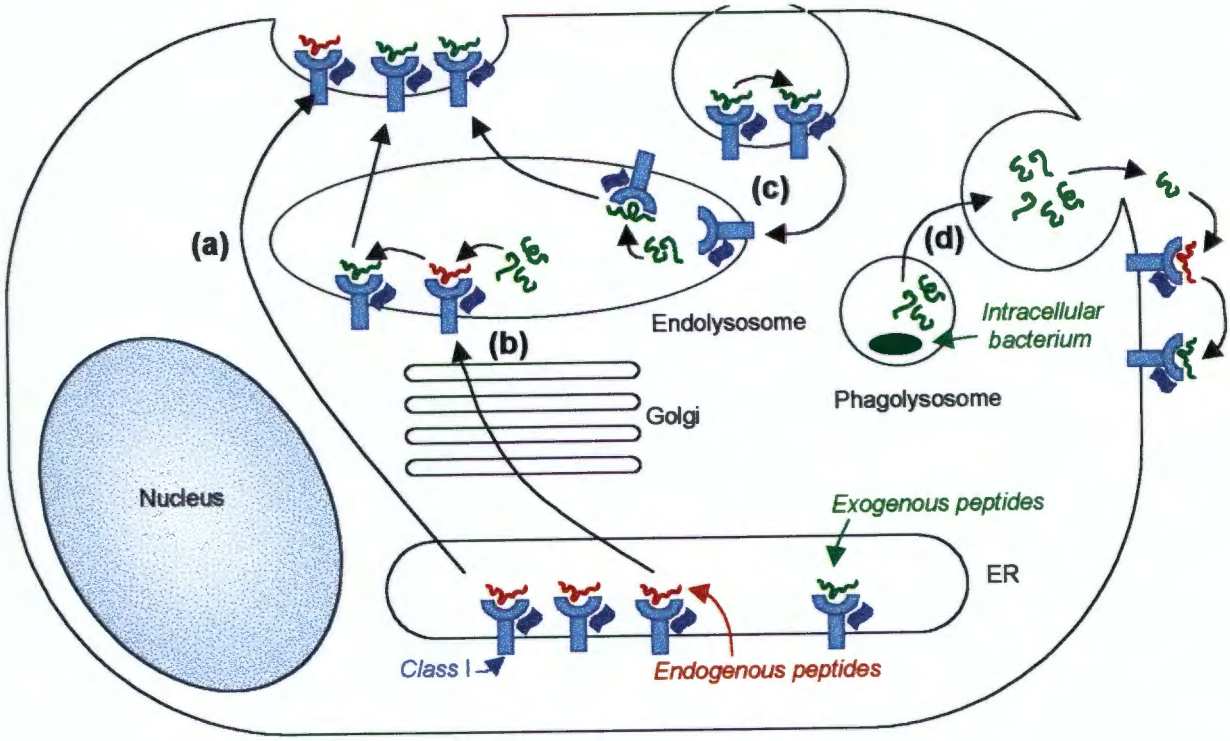


FIGURE 1.4 Peptide loading onto HLA class I molecules. After processing of microbial antigens, the resultant peptides are loaded onto HLA class I molecules for presentation to other cells of the immune system. Exogenous peptide loading has been suggested to occur by three alternative pathways: (a and b) during intracellular HLA class I transport; (c) during HLA class I recycling; and (d) during peptide exocytosis at the cell surface. (a) Endogenously derived peptides are loaded onto nascent HLA class I molecules in the ER lumen and are transported to the cell surface via the Golgi and endolysosomal compartments. (b) These peptides may then be exchanged for exogenously derived peptides during transit of the HLA class I molecules through endolysosomal vesicles. (c) Loading or exchange of peptides may also take place during recycling of surface-derived HLA class I molecules though endolysosomal vesicles. (d) Regurgitated peptides, derived from exogenous antigen processed in phagolysosomes, can bind or replace peptides of HLA class I molecules on the cell surface (from Reimann and Kaufmann, 1997).

Surface HLA class I loading or the 'regurgitation' model

Surface HLA class I loading may also be involved in presentation of exogenous antigens to HLA class I molecules. This could occur when secreted antigens from partially digested exogenous antigens in phagosomes can bind membrane HLA class I by 'regurgitation' (Fig. 1.4d; Harding, 1996). These peptides would unfortunately also sensitise 'bystander' cells in the immediate vicinity (Pfeifer *et al.*, 1993a; Rock, 1996). While some studies have demonstrated this pathway for the HLA class I presentation of exogenous ovalbumin (either present as a bacterial fusion protein or associated with beads), it has not been observable in others (Pfeifer *et al.*, 1993b; Reis e Sousa and Germain, 1995; Rock, 1996). 'Empty' HLA class I molecules (apparently not associated with β_2-microglobulin or peptides) are found on the surface of both murine and human cells (Ljunggren *et al.*, 1995; Smith *et al.*, 1993; Schirmbeck and Reimann, 1996).

Heat shock protein-mediated HLA class I loading

Stress proteins may play an important role in facilitating the processing of exogenous proteins and in peptide loading onto HLA class I molecules (Nieland *et al.*, 1996; Schirmbeck and Reimann, 1994; Suto and Srivastava, 1995; Roman and Moreno, 1996). Sequence homologies and secondary structure predictions of the peptide-binding domains of HLA class I and heat shock protein 70 (hsp 70) polypeptides suggest a phylogenetic relationship between both classes of molecules. Polypeptides non-covalently bound to molecules of the hsp70 and hsp90 families have been shown to efficiently prime CTL responses *in vivo* against microbial antigens, minor histocompatibility antigens, and tumour-associated antigens (Reimann and Kaufmann, 1997). Immunodominant viral peptides endogenously bound to stress protein gp96/grp94 have recently been isolated (Nieland *et al.*, 1996). How, and in which subcellular compartment, endogenous or exogenous hsp-bound peptides are 'loaded' onto HLA class I molecules remains unknown. A TAP-independent, hsp73-dependent processing pathway for the HLA class I-restricted presentation of endogenous peptides from truncated viral proteins has been identified (Schirmbeck and Reimann, 1994). A different pathway seems to operate for hsp96. In this system, peptides from endocytosed hsp96-peptide complexes seem to be loaded to nascent HLA

class I molecules in the ER of a subset of macrophages using a BFA-sensitive, chloroquine-resistant pathway (chloroquine inhibits endosomal acidification; Suto and Srivastava, 1995).

Dendritic cell and apoptotic body facilitated 'cross-presentation'

Accumulating evidence has identified dendritic cells as potent mediators of 'cross-presentation'. In a recent study, Albert *et al.* (1998a) have shown that immature dendritic cells are able to efficiently phagocytose apoptotic cells and cross-present antigens from these bodies to induce HLA class I-restricted CTLs. Previously, the ability of antigens to become highly concentrated within apoptotic bodies has been described (Casciola-Rosen *et al.*, 1994). Further investigations revealed that dendritic cells engulf apoptotic cells by receptor-mediated phagocytosis, involving CD36 and the integrin receptor $\alpha_v\beta_5$ (Albert *et al.*, 1998b). Following uptake, apoptotic cells were shown to enter the classical endocytic pathway. The site where apoptotic cell-derived antigens intersect the HLA class I processing pathway and are loaded onto HLA class I molecules is yet to be defined. The ability of apoptotic cells to deliver antigens to the HLA class I pathway has also been described in a recent report by Kurts *et al.* (1998). These authors found that CTL-mediated tissue destruction and apoptosis facilitated cross-presentation of exogenous antigens to $CD8^+$ CTLs. In tuberculosis, it is likely that target cell apoptosis may be a major determinate in HLA class I-presentation of mycobacterial antigens. *M. tuberculosis*-infected targets cells undergoing apoptosis would provide a ready source of antigens for cross-presentation to $CD8^+$ CTLs.

In summary, this review has presented new evidence from different *in vivo* and *in vitro* systems has indicated that priming HLA class I-restricted CTL responses by exogenous antigens is much more regular and efficient than previously thought. *In vitro* studies investigating the cell biology of the antigen processing pathways followed by endogenous and exogenous antigens for HLA class I-restricted epitope presentation have revealed a substantial number of alternative pathways, the characterization of which are still unclear (Reimann and Kaufmann, 1997).

The CD1 processing pathway

Unlike the classical HLA molecules, which present peptide fragments of antigens to most T cells, CD1 proteins are not polymorphic (Van Agthoven and Terhorst, 1982) but comprise different isotypes (CD1a, b, c, d, and e) that are conserved in several mammalian species (Calabi *et al.*, 1989). These glycoproteins have a typical HLA class I molecular structure, however. They are heterodimers consisting of an approximately 45kDa glycosylated heavy chain interacting non-covalently with the β_2-microglobulin light chain (Porcelli, 1995). Based on their lack of polymorphism, it was predicted that CD1 may have specialized in capturing and presenting antigens with limited structural variability (Beckman *et al.*, 1994). This prediction proved accurate since recent studies have demonstrated that CD1 molecules, unlike classical HLA molecules, present mycobacterial lipids (LAM and mycolic acid) rather than peptide antigens to T cells (Beckman *et al.*, 1994).

The CD1 presentation pathway appears to be unique, although it shares some features with the classical HLA pathways. The earliest functional studies of CD1-restricted mycobacterial antigen presentation suggested a need for antigen processing prior to T cell recognition (Procelli *et al.*, 1992). T cells do not respond to free mycobacterial lipids in the absence of APCs, and CD1-bearing APCs must be exposed to antigen for a discrete period of time (usually more than 30 minutes) before they can activate T cells. T cell recognition of all lipid antigens was abrogated by gluteraldehyde fixation of the surface of APCs before but not after exposure to antigen. In addition, lipid antigen recognition is also blocked by agents that inhibit acidification of endosomes by different mechanisms such as chloroquine, monensin and concanamycin A (Porcelli *et al.*, 1992; Sieling *et al.*, 1995). There is substantial co-localization of CD1b and markers of late endosomes and lysosomes (mannose-6-phosphate and LAMP-1, respectively). Furthermore, CD1b containing vesicles also contain HLA class II and HLA-DM (Sugita *et al.*, 1996). These characteristics demonstrate that these late endosomal compartments overlap extensively or completely with HLA class II compartments in which peptide loading onto HLA class II molecules is believed to occur.

Two candidate pathways for CD1b entry into the endosomal network have been considered and partially examined. CD1b may be directly trafficked to endosomes, in a manner similar to HLA class II proteins, which are diverted from the default secretory pathway, directly to endosomes because of targeting signals present in the associated invariant chain. Alternatively, CD1b may first be trafficked to the surface and then recycled via clathrin-mediated endocytosis back to the endosomal pathway. Recent studies have demonstrated that CD1b recycling is the major pathway accounting for localization of CD1b to the endocytic system (Jullien *et al.*, 1996).

Because the initial discovery of CD1 reactivity was made using $CD4^-CD8^-$ αβ or "double-negative" (DN) T cells (Porcelli *et al.*, 1989, 1992; Beckman *et al.*, 1994), initial work on CD1-restricted T cells focused on this relatively small T cell population. However, it is becoming increasingly evident that antigen presentation by CD1 is not exclusively restricted to this T cell subset with CD1-restricted $CD8^+$ αβ T cells also being implicated (Stenger *et al.*, 1997). CD1-restricted T cells may provide protection in mycobacterial infections by either secreting Th_1-type cytokines or by direct cytolysis of infected CD1-expressing phagocytes (Rook & Hernandez-Pando, 1996). Recently, Stenger *et al.* (1997) clearly demonstrated this by showing that $CD8^+$ CD1b-restricted cytolytic activity against *M. tuberculosis*-infected target cells was associated with a reduction in intracellular mycobacterial survival.

Direct presentation of mycobacterial antigens to $\gamma\delta^+$ T cells

$\gamma\delta^+$ T cells recognize an entirely different class of antigens through an extracellular presentation pathway that does not require antigen uptake, antigen processing or known antigen-presenting elements (Morita *et al.*, 1995). There are many indications that antigen recognition by $\gamma\delta^+$ T cells might be very different from antigen recognition by αβ T cells. Although human αβ and γδ T cells have antigen receptors which share a similar structure (both have a variable heterodimer that associated with the invariant CD3 signaling complex), αβ T cells have more than 50 Vα and 46 Vβ gene segments that can pair to make several thousand receptor combinations while γδ T cells have only 6 Vγ and 3-10 Vδ gene segments which could only yield between 18 and 60 different receptor combinations (Porcelli *et al.*, 1991). The actual peripheral blood repertoire of γδ T cells, however, is dominated by 2 main subsets, Vγ9/Vδ2 and Vγ(2,3,4)/Vδ1 (Parker *et al.*,

1990). Of these, the Vγ9/Vδ2 TCR subset comprises the major group making up approximately 75% of all γδ T cells. Furthermore, the *M. tuberculosis*-reactive γδ T cells are predominantly Vγ9/Vδ2 (De Libero *et al.*, 1991; Kabelitz *et al.*, 1991; Panchamoorthy *et al.*, 1991; Tanaka *et al.*, 1994).

Early studies described a rapid amplification of Vγ9/Vδ2 T cells following stimulation of PBL with *M. tuberculosis* extracts (Kabelitz *et al.*, 1990) and identified the stimulatory antigens as low molecular weight, protease-resistant compounds (Pfeffer *et al.*, 1990, 1992). The stimulatory antigens were subsequently found to be non-peptidic phosphorylated moeities (Constant *et al.*, 1994; Schoel *et al.*, 1994; Tanaka *et al.*, 1994; 1995; Burk *et al.*, 1995). Some of the more well characterized phosphorylated metabolites recognized by γδ T cells include TubAg1-4 moeities (Constant *et al.*, 1994), monoethylphosphate (Tanaka *et al.*, 1994), phosphoglycolic acid (Tanaka *et al.*, 1994), isopentenyl pyrophosphate (IPP; Tanaka *et al.*, 1995), dimethyl pyrophosphate (Tanaka *et al.*, 1995), farnesyl pyrophosphate, geranyl pyrophosphate (Tanaka *et al.*, 1995), and diphosphoglyceric acid (Burk *et al.*, 1995). IPP is not restricted to prokaryotes but is also widely produced by eukaryotic cells. It is an essential precursor for a variety of biological molecules such as cholesterol and its derivatives, vitamins, dolicol phosphates and ubiquinones and is required for the membrane anchoring of a number of important signal transduction molecules such as ras (Tanaka *et al.*, 1995; Morita *et al.*, 1996). Similar phosphorylated compounds can also be produced by stressed cells and may explain the ability of several different transformed cell lines (Penninger *et al.*, 1995) or cells infected with different pathogens to activate the same γδ T cell clone (Bukowski *et al.*, 1994; Wallace *et al.*, 1995; Ponniah *et al.*, 1996;).

Recognition by γδ T cells was found to be TCR-dependent since the *in vitro* activation of Vγ9/Vδ2 T cells by phosphoantigens was blocked by TCR-specific monoclonal antibodies (Munk *et al.*, 1990; Constant *et al.*, 1994; Tanaka *et al.*, 1994). More direct evidence has been provided by a recent study which showed that phosphoantigen recognition could be conferred when a TCR-deficient Jurkat T cell line was transfected with cDNAs encoding Vγ9/Vδ2 chains (Bukowski *et al.*, 1995).

The presentation of phosphoantigens does not require the presence of professional antigen-presenting cells (Morita *et al.*, 1995; Vila *et al.*, 1995). The majority of Vγ9/Vδ2 T cell clones have been found to respond to these antigens in the absence of additional APCs. Recognition was found to be rapid, occurring as soon as 2-3 minutes after the addition of phosphorylated antigens (Morita *et al.*, 1995). Furthermore, both non-fixed and paraformaldehyde-fixed APC were shown to present prenyl pyrophosphate antigens to APC-dependent Vγ9/Vδ2 T cell clones (Fisch *et al.*, 1990; Morita *et al.*, 1995). Together this data suggests that antigen uptake and processing are not requirements for γδ T cell antigen recognition. Presentation of prenyl pyrophosphate is dependent, however, on cell-cell contact (Morita *et al.*, 1995; Lang *et al.*, 1995). Even though the presentation of prenyl pyrophosphate antigens was found to require cell-cell contact, none of the known antigen-presenting molecules were necessary for their recognition (Fisch *et al.*, 1990; Holoshitz *et al.*, 1993; Morita *et al.*, 1995). Monoclonal antibodies against HLA class I, class II and CD1 molecules did not block the recognition of prenyl pyrophosphate antigens by γδ T cells (Morita *et al.*, 1995). In addition, mutant APCs lacking classical HLA class I, β_2-microglobulin or HLA class II molecules were as efficient as wild type APCs at presenting mycobacterial phosphoantigens to an APC-dependent γδ T cell clone (Morita *et al.*, 1995). It is not clear if a novel antigen-presenting element exists for the prenyl pyrophosphate antigens. Unlike most peptide antigens, culturing APCs with prenyl pyrophosphate antigens does not render the 'pulsed' APCs stimulatory for Vγ9/Vδ2 bearing T cells (Lang *et al.*,1995; Morita *et al.*, 1995) suggesting that the antigen does not form a stable association with the APC element on the cell surface.

A model for the extracellular presentation of prenyl pyrophosphate antigens has been proposed by Morita *et al.* (1996). Mycobacteria secrete IPP and related prenyl pyrophosphates extracellularly where they can associate with the surface of an APC, perhaps by binding to a novel antigen-presenting element. Vγ9/Vδ2 T cells can recognize these compounds in a TCR-dependent fashion. The T cells then become activated to secrete cytokines, proliferate, and kill neighboring cells. This process does not require that the presenting cell be a professional APC and does not require antigen uptake or processing.

The ability of γδ T cells to release cytokines following activation is rapid and can precede αβ T cell activation by several hours to several days (Ferrick *et al.*, 1995). This observation has led to the hypothesis that the initial pattern of cytokine production by γδ T cells may influence the ensuing αβ T cell response. Several studies have demonstrated that differentiation of $CD4^+$ αβ T cells into either Th_1 or Th_2 cells is influenced by the cytokine milieu in which the initial antigen priming of these cells occurs (Rocken *et al.*, 1992; Hsieh *et al.*, 1992; Seder *et al.*, 1992). Furthermore, Ferrick *et al.* (1995) have recently demonstrated that γδ T cells are able to discriminate between *L. monocytogenes* or *Nippostrongylus brasiliensis* early in infection by producing cytokines associated with the appropriate T-helper response. A similar mechanism may allow γδ T cells to influence cytokine production by NK cells (Ladel *et al.*, 1996) and Ig production by B cells (Hacker *et al.*, 1995; McMenamin *et al.*, 1995). The central role of γδ T cells in regulating both the innate and acquired immune response has been summarized in Fig. 1.5.

At the same time, an increasingly noted characteristic of TCR $\delta^{-/-}$ knockout mice is one of dysregulated, hyperactive immune function towards both foreign and self-antigens (Roberts *et al.*, 1996; Peng *et al.*, 1996; Szczepanik *et al.*, 1996). These studies have suggested that γδ cells ordinarily down-regulate αβ T cells of either Th_1 (Roberts *et al.*, 1996; Peng *et al.*, 1996; Szczepanik *et al.*, 1996; Mukasa *et al.*, 1995) or Th_2 (McMenamin *et al.*, 1994) function. In mycobacterial infections, γδ T cells have been shown to regulate local cellular trafficking in granuloma formation by both promoting the influx of protective lymphocytes and monocytes and limiting the access of inflammatory neutrophils that do not contribute to protection but may cause tissue damage (D'Souza *et al.*, 1997). It is conceivable that γδ regulation is mediated by their ability to produce both Th_1 and Th_2 cytokines (D'Souza *et al.*, 1997; Wen *et al.*, 1998).

With regard to their cytolytic ability, Vγ9/Vδ2 T cells demonstrate an inherent ability to lyse *M. tuberculosis*-infected target cells (Kabelitz *et al.*, 1990). This cytolytic activity is not restricted *to M. tuberculosis* infected cells, however, as the same γδ T cells are broadly cytotoxic towards various transformed cell lines, Daudi in particular (Haas *et al.*, 1993), as well as cells infected with a range of viruses (Bukowski *et al.*, 1994; Wallace *et al.*, 1995).

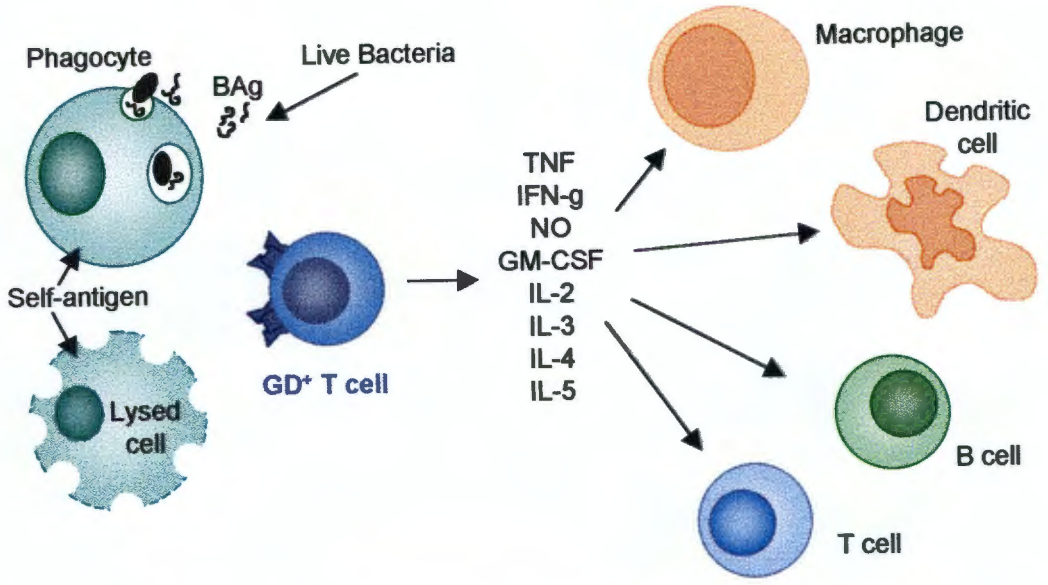


FIGURE 1.5 γδ T cells recognize bacterial and self-ligands on different antigen present cells (APCs). Bacterial ligand (BAg) is released by extracellular live bacteria or after their internalization within phagosomes. This cellular localization allows the surface display of the BAg in cells harboring live bacteria and, subsequently, the stimulation of γδ T cells. Self-generated metabolic ligands in intact cells do not have access to the cell surface, and therefore do not stimulate γδ T cells. However, following cell lysis (for example, due to persistent bacterial or viral infection or tissue necrosis), self-ligands are exposed on the surface and can stimulate γδ T cells. Activated γδ T cells secrete lymphokines capable of activating various types of cells and can induce functional maturation of professional APCs through soluble factors, thus facilitating the recruitment of antigen-specific αβ T cells. γδ T cells can also activate macrophages thus promoting uptake and destruction of invading bacteria. Furthermore, some γδ T cells are strongly cytolytic and can lyse infected phagocytes. Cytolysis may lead to the release of intracellular pathogens, which can then be internalized and destroyed by recently activated macrophages (adapted from De Libero, 1997).

1.3 CONCLUSION

In conclusion, it is obvious from this review of the literature that the human host response to tuberculosis is a complex reaction to infection with a vigorous pathogen that involves many different components. Unfortunately, research into the initial macrophage-mycobacterial interaction, the intracellular lifestyle of *M. tuberculosis*, the role of the many different T cell subsets and the mechanism by which mycobacteria are eliminated are only beginning to yield useful insights into this complex disease. A better understanding of the intricate cross talk between T cells, macrophages and infected host cells will no doubt directly promote the development of improved control measures.

1.4 PROJECT AIMS AND BACKGROUND

Although it is well recognized that macrophages are key components of a protective immune response to *M. tuberculosis*, the heterogeneity within and between even steady state human macrophages has presented a significant obstacle to the study of macrophage-mycobacterial interaction *in vitro*. In fact, O'Brien *et al.* (1994) recently observed that our present understanding of the interaction between macrophages and mycobacterial species is based on macrophages collected from different anatomical sites with differing maturational statuses, and from different animal species and the results presented seem to reflect this variability.

This situation has been partially remedied by the estalishment of several human leukemic cell lines, such as THP-1, Monomac-6, U937, HL-60 and KG-1, which are blocked at different steps along the monocyte to macrophage differentiation pathway (Sundstrom and Nilsson, 1976; Tsuchiya *et al.*, 1980; Collins, 1987; Ziegler-Heitbrook *et al.*, 1988). These cell lines have allowed the investigation of relatively homogenous cell populations during defined stages of maturation and differentiation. When I took up this challenge in 1994, one of the most well characterized and widely used of these human monocytic cell lines U937 had not been investigated in the context of mycobacterial infection.

The first major aim of this study was, therefore, to investigate the feasibility of using U937 as a representative *in vitro* model for human macrophage function in *M. tuberculosis* infections (Chapter 2) and to compare it to another commonly used monocytic cell line THP-1. It is appropriate, at this point, to briefly overview some of the salient characteristics of this cell line and it's potential for use as a model for human macrophage function.

Sundstrom and Nilsson (1976) first established the promonocytic U937 cell line from the pleural fluid of a patient with diffuse histiocytic lymphoma. These cells are not considered to be mature monocytes but are thought to be precursors of the monocyte/macrophage lineage. Accordingly, they possess many macrophage-like characteristics including (i) positive staining for non-specific esterases (Nilsson *et al.*, 1981), (ii) the ability to produce lysozyme (Ralph *et al.*, 1976),

(iii) the expression of Fc and complement receptors (Guyre *et al.*, 1983), and (iv) the ability to phagocytose exogenous antigens (Sundstrom and Nilsson, 1976). This cell line does, however, fail to exhibit some phenotypic and functional properties of mature macrophages. They lack monocyte-specific differentiation markers such as CD14 and HLA class II expression (Hass *et al.*, 1989), fail to generate leukotrienes (Kohler *et al.*, 1989; Kohler *et al.*, 1990), and do not produce detectable levels of IL-6 (Hass *et al.*, 1991a).

U937 cells can, however, be induced towards a more mature macrophage phenotype by the addition of various agents. These include 1,25-dihydroxyvitamin D_3 (Dodd *et al.*, 1983; Olsson *et al.*, 1983; Trinchieri *et al.*, 1987), retinoic acid (RA; Olsson and Breitman, 1982), phorbol esters such as phorbol miristate acetate (PMA; Hass *et al.*, 1989; Lotem and Sachs, 1979; Rovera and O'Brian, 1979), granulocyte/macrophage-colony stimulating factor (GM-CSF), dimethyl sulphoxide (DMSO; Nakamura et al, 1990), cyclic adenosine monophosphate (Olsson and Breitman, 1982), LPS (Ikewaki *et al.*, 1993), IFN-γ, and TNF-α (Harris *et al.*, 1985; Testa *et al.*, 1988). In the presence of these agents, U937 cells develop morphologic, cytochemical, phenotypical and functional characteristics that are consistent with cellular maturation. Following differentiation, these cells showed increased expression of CD11b (Grattage *et al.*, 1992), CD16, CD23 (Ikewaki *et al.*, 1993), GM-CSF receptor (Zuckerman *et al.*, 1988) and tissue factor VIIa (Rana *et al.*, 1988). In addition, they were shown to produce various cytokines [IL-1 (Knudsen *et al.*, 1986) and TNFα (Cannistra *et al.*, 1987)] as well as growth factors [macrophage-colony stimulating factor (M-CSF; Horiguchi *et al.*, 1989), platelet-derived growth factors (PDGF-1, PDGF-2; Sariban *et al.*, 1988), and prostanoids (PGE_2, PGS_2, PGI_2, TxB_2; Kohler *et al.*, 1989; Wiederhold *et al.*, 1988)]. Differentiation of U937 cells is often associated with inhibition of autonomous proliferative ability (Harris *et al.*, 1985; Palacios *et al.*, 1982) and induction of cellular adhesion (Hass *et al.*, 1989; Ikewaki *et al.*, 1993). The ability to further modulate and differentiate the phenotypic and functional properties of this cell line using various agents such as PMA, IFN-γ or vitamin D_3 may allow for more defined investigation of mycobacterial-macrophage interactions in tuberculosis.

The U937 cell line has also been widely used as a model in which to study the function of human macrophages in host defense against a wide range of other intracellular pathogens. These

include *Leishmania* spp. (Arena *et al.*, 1997), *Toxoplasma* spp. (Wing *et al.*, 1981; Sarciron *et al.*, 1997), *Legionella* spp. (Abu Kwaik *et al.*, 1998); *Salmonella* spp. (Brett *et al.*, 1993; Cheteau *et al.*, 1997; Scorneaux *et al.*, 1996), *Brucella* spp. (Caron *et al.*, 1996), and *Listeria* spp. (Caron *et al.*, 1994a, 1994b, 1994c, 1994d; Hauck *et al.*, 1997). They have been shown to be capable of both pinocytic and phagocytic uptake of various human pathogens, mounting a respiratory burst following infection, releasing specific cytokines (TNF-α, IL-8), and supporting the growth of virulent organisms while efficiently eliminating or restricting the growth of non-pathogenic counterparts (Abshire & Neidhardt, 1993; Bianchi *et al.*, 1997; Caron *et al.*, 1994a, 1994b, 1994c, 1994d; Husmann & Johnson, 1992; Iwamoto *et al.*, 1997; King *et al.*, 1991; Pearlman *et al.*, 1988)

Fewer reports have investigated the ability of this monocytic cell line to process and present exogenous or endogenous antigens. While U937 cells were found to express cell surface HLA class I (Sundstrom and Nilsson, 1976), they neither constitutively nor inducibly expressed HLA class II (Sundstrom and Nilsson, 1976; Peterlin *et al.* 1984). U937 cells have also been shown to possess an efficient HLA class I processing pathway that not only facilitates the normal presentation of endogenous self and viral antigens but also the presentation of exogenously derived soluble antigens in the context of HLA class I (Harris *et al.*, 1993; 1995).

Because U937 cells do not constitutively nor inducibly express HLA class II despite expressing high levels of cell surface HLA class I (Sundstrom and Nilsson, 1976; Peterlin *et al.* 1984), they may provide a unique system in which to evaluate the ability of human macrophages to present mycobacterial antigens to non-HLA class II-restricted T cells. This investigation formed the basis for the second major focus of this study (Chapter 3).

Although a number of reports have implicated cytolytic $CD8^+$ T cells in defense against murine mycobacterial infections (Orme & Collins, 1984; Orme, 1987; Muller *et al.*, 1987; Silva *et al.*, 1994; D'Sousa *et al.*, 1998), their role in human tuberculosis has proven much more difficult to demonstrate. At the conception of this study in 1994, few reports had been published demonstrating *M. tuberculosis*-reactive cytolytic $CD8^+$ T cells in human mycobacterial infections (Rees *et al.*, 1988). The aim of this study was therefore to investigate the role of $CD8^+$

T cells and HLA class I presentation in mycobacterial infections using the HLA class I^+ but HLA class II^- human macrophage cell line U937 (Chapter 3).

With the emergence of more sophisticated immunological techniques, and a corresponding renewed interest in the role of $CD8^+$ CTLs in tuberculosis, a number of studies have been published very recently demonstrating the existence of classical HLA class I-restricted *M. tuberculosis*-responsive human $CD8^+$ T cells (Turner and Dockrell, 1996; Tan *et al.*, 1997; Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998; Mohagneghpour *et al.*, 1998; Canaday *et al.*, 1999). Because of the inherent difficulties associated with demonstrating the cytolytic function of CTLs with such low precursor frequencies, many of these studies have restricted their focus to the ability of $CD8^+$ T cells to produce cytokines or to proliferate in response to stimulation with defined mycobacterial peptides (Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998; Canaday *et al.*, 1999). Those studies that have evaluated the cytolytic activity of $CD8^+$ T cells against *M. tuberculosis*-infected target cells have had to use very defined priming conditions to facilitate the generation of $CD8^+$ CTLs. Tan *et al.* (1997), for example, reported that mycobacterial-specific $CD8^+$ CTL activity was only demonstrable following *in vitro* co-culture with specific growth factors, while Mohagheghpour *et al.* (1998) made use of mycobacterial peptide pulsed dendritic cells to efficiently prime $CD8^+$ CTL effector cells. The results presented in this study (Chapter 3) describe a relatively simple, robust, and easily adaptable *in vitro* model using U937 cells to efficiently present mycobacterial antigens to human HLA class I-restricted $CD8^+$ CTLs.

An important finding which emerged during the course of this study was that U937 cells provided not only a useful *in vitro* human macrophage model for selective evaluation of HLA class I-restricted $CD8^+$ CTL function in mycobacterial infections but were also shown to be a sensitive indicator for $\gamma\delta^+$ CTL activity (Chapter 3 and 4). Although it is well recognized that $\gamma\delta^+$ T cells recognize an entirely different class of mycobacterial antigens through an extra-cellular presentation pathway that does not require antigen uptake, antigen processing or known antigen-presenting elements (Morita *et al.*, 1995), their role in protective immunity in human tuberculosis remains contentious. The establishment of strongly cytolytic human *M. tuberculosis*-specific $\gamma\delta^+$ CTL clones (Chapter 3) afforded a unique opportunity to investigate

more thoroughly the role of $\gamma\delta^+$ CTL lytic activity in human mycobacterial infections. This investigation formed the basis of the third and final focus of the present study (Chapter 4).

CHAPTER 2

THE HUMAN MONOCYTIC CELL LINE U937 AS AN *IN VITRO* MODEL FOR MACROPHAGE FUNCTION IN MYCOBACTERIAL INFECTIONS

2.1 INTRODUCTION

Macrophages are central to an efficient protective immune response following infection with *M. tuberculosis* and have evolved diverse anti-microbial mechanisms to deal with this intracellular pathogen (Chan and Kaufmann, 1994). Their four most important functions in mycobacterial infections are: (i) their ability to phagocytose *M. tuberculosis*; (ii) their ability to produce proteolytic enzymes and other metabolites following phagocytosis that exhibit mycobactericidal effects; (iii) their ability to process and present antigens derived from *M. tuberculosis* to T cells; and (iv) their ability to produce cytokines in response to *M. tuberculosis* infection that have the potential to exert potent immuno-regulatory effects (Valone *et al.*, 1988; Toossi *et al.*, 1991; Barnes *et al.*, 1992; Zhang *et al.*, 1993; Barnes & Modlin, 1994).

Unfortunately, the logistic restrictions and heterogeneity within and between even steady-state human mononuclear phagocytes at all levels of analysis (Forster and Landy, 1981; Fine, 1995) has presented a significant obstacle for the study of macrophage-mycobacterial interactions. Although being less variable, murine macrophages have been shown to differ quite significantly from human macrophages in both their production of cytokines and reactive nitrogen intermediates. While murine macrophages can easily be induced by cytokines to form nitrogen radicals in response to mycobacterial infection, reactive nitrogen intermediate production in human monocytes is more difficult to demonstrate (Albina *et al.*, 1995; Nicholson *et al.*, 1996; Jagannath *et al.*, 1998). In many respects, therefore, the murine model of tuberculosis does not closely mimic the human disease.

The establishment of several human leukemic cell lines, such as U937, THP-1, Monomac-6, HL-60 and KG-1, which are blocked at different steps along the monocyte to macrophage differentiation pathway (Sundstrom and Nilsson, 1976; Tsuchiya *et al.*, 1980; Collins, 1987; Ziegler-Heitbrook *et al.*, 1988), have allowed the investigation of relatively homogenous cell populations during defined stages of maturation and differentiation. One of the most well characterized and widely used of these human monocytic cell lines is U937.

The U937 cell line has been used extensively as a model in which to study the function of human macrophages in defense against a wide range of intracellular pathogens. These include *Leishmania* spp. (Arena *et al.*, 1997), *Toxoplasma* spp. (Wing *et al.*, 1981; Sarciron *et al.*,

1997), *Legionella* spp. (Abu Kwaik *et al.*, 1998), *Salmonella* spp. (Brett *et al.*, 1993; Cheteau *et al.*, 1997), *Brucella* spp. (Caron *et al.*, 1996), and *Listeria* spp. (Caron *et al.*, 1994a, 1994b, 1994c, 1994d; Hauck *et al.*, 1997). Despite being so widely used and well characterized, this study represents the first to investigate the usefulness of U937, compared with another commonly used human monocytic cell line THP-1, as a model for macrophage function in mycobacterial infections.

The aim of the research presented in this chapter was firstly to investigate the constitutive and inducible phenotypic and functional characteristics U937 cells and to compare these with another well established human monocytic cell line THP-1; and secondly, to investigate the usefulness of U937 as a representative human macrophage model in which to study mycobacterial infection. These investigations will form the basis from which more applied studies into the ability of U937 cells to present mycobacterial antigens to non-HLA class II-restricted T cells will be developed (Chapter 3). The results presented in this chapter confirm that U937 cells were inducible towards a more mature macrophage phenotype by the addition of PMA, IFN-γ, or 1,25-$(OH)_2$-vitamin D_3. The efficiency of monocytic differentiation was demonstrated by the acquisition of certain cell surface receptors (CD14, CD11b, and CD16) but not HLA class II, cessation of autonomous proliferation, growth arrest in the G_0 phase of the cell cycle and cellular adherence. Although U937 cells were found to be capable of phagocytosing *M. tuberculosis* following differentiation, their phagocytic ability was reduced compared with THP-1 cells. Both U937 and THP-1 cells, however, showed similar ability to mount a respiratory burst response following phagocytosis of *M. tuberculosis*. U937 cells were found to be more permissive to the intracellular growth of both virulent *M. tuberculosis* and avirulent BCG than THP-1 cells. Finally, chronic infection of U937 cells with virulent *M. tuberculosis* did not influence cell surface expression of HLA class II but did significantly up-regulate the cell surface expression of HLA class I compared with uninfected cells. Evidence presented in this chapter demonstrates that, in many important respects, U937 cells represent a useful model for investigating defined interactions between human macrophages and *M. tuberculosis*.

2.2 MATERIALS AND METHODS

2.2.1 Cell lines

U937 and THP-1 cells were maintained routinely in suspension culture in RPMI-1640 (Flow Laboratories, Irvine, Scotland) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa), 2 mM L-glutamine, and 10 mM HEPES at 37°C in a humidified atmosphere of 5% CO_2. THP-1 culture medium was additionally supplemented with 10^{-5} M 2-mercaptoethanol. Passage was performed every 2-3 days for U937 and every 4 days for THP-1. Cells used in experiments were in the logarithmic growth phase and viability exceeded 95% as measured by trypan blue exclusion. Mycoplasma infection was excluded by regular monitoring using the Bisbenzanide fluorochrome stain (Hoescht No. 33258, Germany).

2.2.2 Induction of Differentiation

Differentiation was induced by treating U937 or THP-1 cells (5 x 10^5 cells.ml^{-1} in 6- or 24-well plates) with PMA (5 ng.ml^{-1}; Sigma, St. Louis, MO; Hewison *et al.*, 1992), 1,25-$(OH)_2$-vitamin D_3 (10^{-7}-10^{-9}M; Roche Products Ltd, U.K.; Zuckerman *et al.*, 1988), GM-CSF (5 ng.ml^{-1}; Sandoz; Kelsey *et al.*, 1992), or recombinant human IFN-γ (100-200 U.ml^{-1} rhIFN-γ; Cetus; Roberts *et al.*, 1991) for at least 48 hours at 37°C, 5% CO_2. Morphological changes, adherence, autonomous proliferative ability, changes in cell cycling, and cell surface antigen expression following the various treatments were evaluated as indicators of terminal differentiation.

Autonomous proliferative ability

U937 and THP-1 cells (2 x 10^4 cells.well^{-1}) were seeded into 96-well round-bottomed sterile tissue culture plates (Greiner, Nurtingen, Germany) in 6-well replicates. Cells were either untreated or induced to differentiate by the addition of PMA, rhIFN-γ, or 1,25-$(OH)_2$-vitamin D_3 as described above. [^{3}H]-Thymidine (Amersham International, England; specific activity 185 MB) was added (2 μCi/well) at 0, 24, 48, and 72 hours for a further 16 hours of incubation at 37°C, 5% CO_2. The cells were harvested using an automated cell

harvester (Titertek^R 630; Flow Laboratories) and the radioactivity was measured using a liquid scintillation counter (Tricarb 4640, Packard, Meriden, CT). To normalize the results for each cell line following the respective treatments, the results have been expressed as a 'relative proliferative index' and calculated as follows: [^{3}H-thymidine incorporation (cpm) for differentiated cells] ÷ [^{3}H-thymidine incorporation (cpm) for untreated cells].

DNA and cell cycle analysis

U937 or THP-1 cells were untreated or induced to differentiate with PMA, rhIFN-γ, or 1,25-$(OH)_2$-vitamin D_3 as described above. Following 48 hours of differentiation, the cell concentration was adjusted to 2 x 10^6 cells.ml^{-1} in 10% FCS RPMI-1640 and at least 2 x 10^5 cells (in 100 µl) were used per analysis. Cells were permeablised, stained with propidium iodide and fixed using DNA Prep Kit (Coulter). An Epics Profile II flow cytometer (Coulter) was used to perform cell cycle analysis and histograms were gated on the U937 or THP-1 population by forward scatter (FS) versus log side scatter (LSS). Single parameter histograms of fluorescence 3 (propidium iodide), showing G_0/G_1, S and G_2/M, were analyzed using Cytologics Software (Coulter). The method of rectangular integration was used to analyze DNA histograms (Baisch *et al.*, 1975). Cells in G_0 phase of the cell cycle have diploid amount of DNA (2N). Cells in G_2/M phase have tetraploid amounts of DNA (4N). Cells in the S phase of the cell cycle have an intermediate amount of DNA (2N< S phase <4N). Chromosomal analysis was performed by Dr. M.P. Marx in the Department of Human Genetics, University of Stellenbosch.

Cell surface marker expression

U937 and THP-1 cells, either untreated or induced to differentiate with PMA, rhIFN-γ, or 1,25-$(OH)_2$-vitamin D_3 for 48 hours, were assessed for various macrophage cell surface antigens. An Epics Profile II flow cytometer (Coulter) was used to perform immuno-phenotyping and the monoclonal antibodies (Coulter, Hialeah, FL; Becktin-Dickinson, San Jose, CA) used were directed against HLA class I (W6/32), HLA class II (I3), CD14 (MY4), CD14 (Mo2), CD11b (Mo1), FcR (CD16), CD1a, CD1b, and CD1c. All the monoclonal antibodies (with the exception of W6/32, CD1a, CD1b, and CD1c) were directly conjugated with FITC (fluoroscein isothiocyanate) or RD1 (phycoerythrin; PE). Indirect labeling was

performed using mouse anti-human W6/32, CD1a, CD1b, CD1c and goat anti-mouse FITC. Antibodies were used at the concentrations suggested by the manufacturer. Histograms were gated on U937 or THP-1 populations by forward scatter (FS) versus log side scatter (LSS). Single parameter histograms of fluorescence 1 (FITC) and fluorescence 2 (RD1) were analyzed using Cytologics (Coulter) and WinMDI Software (Trotter, 1996). Corresponding isotypic control monoclonal antibodies were used in all cases to set cursors to allow 2% false positives.

2.2.3 Tissue-typing

HLA-typing of U937 and THP-1 cells was determined according to phenotype using the microdroplet lymphocyte cytotoxicity test described by Bodmer *et al.* (1977).

2.2.4 Isolation of human PBMC and monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMC) were obtained from healthy adult volunteers by centrifugation of heparinized venous blood over Ficoll-Hypaque density gradients (Sigma) as previously described (Boyum, 1968). Monocyte-derived macrophages were prepared by culturing PBMC (1×10^6 cells.ml^{-1} in 10% pooled AB$^+$ human serum RPMI-1640) in 24-well plates (1 ml.well^{-1}) for 4 to 6 days. Non-adherent cells were removed and adherent monocyte-derived macrophages were washed 3 times with warmed 10% AB RPMI-1640.

2.2.5 Mycobacterial growth conditions

M. tuberculosis H37Rv (ATCC) and *M. bovis* BCG (Trudeau strain) were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% OADC (Oleic Acid-Albumin-Dextran-Catalase growth supplement; State Vaccine, Cape Town, South Africa) and 0.02% Tween-80 (Merck, Darmstadt, Germany) at 37°C, 5% CO_2. Mid-log phase cultures were supplemented with 15% sterile glycerol, divided into aliquots, snap frozen in liquid nitrogen and immediately stored at -70°C until needed. Viability of the frozen mycobacterial stocks was monitored over time by determining colony forming units (CFU) of serial 10-fold dilutions of the bacterial preparations on Middlebrook 7H10 agar (Difco) supplemented with 10% OADC. Frozen aliquots were thawed immediately before

use in an assay and clumps were disrupted by repeated passage through a 25-gauge tuberculin needle. Where indicated, mycobacterial preparations were coated with serum opsonins by incubating thawed aliquots with an equal volume of fresh human serum for 30 minutes at 37°C.

For experiments using flow cytometric assessment of mycobacterial phagocytosis, *M. tuberculosis* was labeled with fluoroscein isothiocyanate (FITC) isomer I according to the method described by Drevets and Campbell (1991). Briefly, mycobacterial broth culture (6 x 10^7 CFU.ml^{-1}) was incubated with 0.1mg.ml^{-1} FITC isomer I (Sigma) in 0.1 M $NaHCO_3$, pH 9.0 for 1 hour at 25°C. Bacteria were washed three times with PBS by centrifugation at 11000 g for 5 min and reconstituted in original volume in 10% OADC Middlebrooks 7H10 broth. An Epics Profile II flow cytometer (Coulter) was used to assess the efficiency of FITC-labeling and histograms were gated on the mycobacterial population by forward scatter (FS) versus log side scatter (LSS). Viability of the FITC-labeled *M. tuberculosis* preparations was measured by determining CFU's as described above.

2.2.6 Phagocytosis of *M. tuberculosis*

U937 and THP-1 cells were untreated or induced to differentiate by the addition of PMA, rhIFN-γ, or 1,25-$(OH)_2$-vitamin D_3. Following 48 hours of differentiation, the non-adherent rhIFN-γ- or 1,25-$(OH)_2$-vitamin D_3-treated cells were re-adjusted to 0.5 x 10^6.ml^{-1}. Initial experiments using PMA-differentiated cells confirmed that their cell concentration did not significantly increase following treatment and, as a result, no concentration re-adjustment was necessary in subsequent experiments. U937 or THP-1 cells were infected with *M. tuberculosis* H37Rv (in the absence or presence of serum opsonins) and phagocytic ability was determined using three independent techniques: (i) mycobacterial binding assay using Ziehl-Neelson (ZN) staining for acid fast bacilli; (ii) transmission electron microscopy (TEM) of *M. tuberculosis*-infected cells; and (iii) flow cytometric evaluation phagocytosis using FITC-labeled *M. tuberculosis*.

Monitoring M. tuberculosis infection using Ziehl-Neelson staining

Untreated or differentiated U937 or THP-1 cells (treated for 48 hours with PMA, rhIFN-γ or 1,25-$(OH)_2$-vitamin D_3) were infected with *M. tuberculosis* H37Rv (10 CFU.cell^{-1}) for 90 minutes. Infected cells were washed 3 times in 10% FCS PBS to remove non-ingested bacilli and re-suspended in 10% FCS RPMI-1640 (2×10^6 cells.ml^{-1}). Suspension cells were coated onto duplicate glass slides and allowed to air-dry. Following heat-fixation, efficiency of infection was visualized using ZN staining for acid fast bacilli as described by Bishop and Neumann (1970). Fixed slides were stained for 10 minutes with carbol fuchsin (10g fuchsin; 50g phenol dissolved in 100 ml 95 % ethanol; diluted to 1l in distilled water). During this period, the carbol fuchsin flooded slide was gently heated twice. After 10 minutes, excessive stain was removed by rinsing under running water. The slides were de-stained by flooding repeatedly with acid alcohol (3% HCl diluted in 85% ethanol) and rinsed in between each de-colourizing step with water. Finally, the cells were counter-stained using 0.5% aqueous malachite green for 1 minute. The number of cells containing acid-fast *M. tuberculosis* was evaluated using oil immersion light microscopy and infected cells were expressed as a percentage of the total number of cells counted. At least 200 cells per slide were assessed for each treatment.

Transmission electron microscopy of infected U937 cells

U937 cells were differentiated with PMA for 48 hours and then infected with *M. tuberculosis* H37Rv (50 CFU.cell^{-1}) for 90 minutes at 37°C, 5% CO_2. The cells were EDTA-detached, washed 3 times in 10% FCS PBS, and fixed with 1% paraformaldehyde PBS for 1 hour at room temperature. Cells were washed three times with 0.1 M sodium cacodylate buffer containing 0.1 M sucrose (pH 7.2) and then fixed again with 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose (pH 7.2) overnight at 4°C. The cells were post-fixed with 1% osmium tetroxide, dehydrated in graded ethanol solutions, and embedded in Spurs resin. Thin sections were stained with uranyl acetate and lead citrate before examination with a Jeol 200 CX Electron microscope. A minimal estimate of the efficiency of infection with *M. tuberculosis* was determined by assessing at least 100 independent cells. This method of quantifying infection was considered to give a minimal estimate because infection was evaluated from only a single section through infected cells.

Flow cytometric evaluation of phagocytosis

Phagocytosis of FITC-conjugated *M. tuberculosis* by U937 cells, human monocytes and macrophages was assessed using the commercially available "Phagotest" kit (Orpegen, Heidelberg, Germany). FITC conjugated-*E. coli* (1×10^9.ml^{-1}), provided with the kit, was used as the positive control while FITC conjugated-*M. tuberculosis* (5×10^8 CFU.ml^{-1}) were prepared as described above. Fresh monocytes were derived from heparinized whole blood (100 µl per test) as described by the manufacturers. Monocyte-derived macrophages were prepared as described above. U937 cells were either undifferentiated or treated with PMA, rhIFN-γ, or 1,25-$(OH)_2$-vitamin D_3 for 48 hours as described above.

Untreated and differentiated U937 cells, fresh human monocytes, and monocyte-derived macrophages were infected with either *E. coli* (1000 bacteria.cell^{-1}) or *M. tuberculosis* (100 bacteria.cell^{-1}) for 90 minutes in the presence of serum opsonins at 37°C, 5% CO_2. Adherent macrophages and PMA-differentiated U937 cells were EDTA-detached following the 90 minutes of infection, and washed once with washing solution provided by the manufacturers. All the samples were placed into an ice water bath at the end of the 90-minute infection period to stop phagocytosis. Quenching solution (100 µl.sample^{-1}; provided by the manufacturer) was added to each sample to quench any extracellular fluorescence due to associated but not incorporated bacteria. Each sample was washed 3 times with washing solution to remove remaining non-ingested bacteria. Any contaminating red blood cells from the fresh monocyte population were lysed using the lysing solution provided with the kit and all the samples were fixed with 1% buffered paraformaldehyde PBS for 1 hour at room temperature. Samples were washed once with washing solution, stained with DNA staining solution (100 µl.sample^{-1}; provided by the manufacturers) and incubated for a further 10 minutes at 0°C in the dark before evaluating the results. Phagocytic efficiency was quantified by using an Epics Profile II flow cytometer (Coulter) and histograms were gated on U937 cells, monocytes or macrophages by forward scatter (FS) versus fluorescence 3 (propidium iodide; DNA stain provided by the manufacturers'). Single parameter histograms of fluorescence 1 (FITC) showing the percentage of the gated population having incorporated FITC-labeled *E. coli* or *M. tuberculosis* were used to determine phagocytic efficiency.

Uninfected monocytes, macrophages or U937 cells, prepared in parallel, were used in all cases to set cursors to allow 2% false positives.

2.2.7 Respiratory burst activity

Respiratory burst activity was measured using both nitroblue tetrazolium (NBT) reduction and the commercially available flow cytometric "Burst-Test" kit (Orpegen, Heidelberg, Germany).

NBT reduction

Measurement of respiratory burst activity using NBT reduction was carried out using the method described by Roberts *et al.* (1991) with the following modifications. U937 and THP-1 cells were untreated or induced to differentiate by the addition of PMA, rhIFN-γ, or 1,25-$(OH)_2$-vitamin D_3. Following 48 hours of differentiation, the cells were re-suspended in 10% FCS RPMI-1640 containing 0.05% NBT (1×10^6 cells.ml^{-1}). In the presence of NBT, U937 and THP-1 cells were stimulated with PMA (10 μg.ml^{-1}; Spittler *et al.*, 1997); infected with *M. tuberculosis* (100 bacteria.cell^{-1}) or left untreated for 1 hour at 37°C. Each independent experiment was carried out in duplicate and NBT reduction was quantified by counting at least 100 cells from each sample.

Flow cytometric evaluation of respiratory burst

Respiratory burst activity following infection of human monocytes, macrophages and U937 cells with either opsonized *M. tuberculosis* or *E. coli* was assessed using the commercially available "Burst-Test" kit (Orpegen, Heidelberg, Germany). The principle of the test is the oxidative conversion of non-fluorogenic dihydrorhodamin-123 to green fluorescent rhodamine-123 by activated cells exhibiting respiratory burst activity (Rothe *et al.*, 1988). Pre-opsonised *E. coli* (1×10^9 CFU.ml^{-1}) was provided by the manufacturers as a positive control and used according to their direction, while *M. tuberculosis* (5×10^8 CFU.ml^{-1}) was coated with fresh serum opsonins as previously described. Fresh monocytes were derived from heparinized whole blood (100 μl per test) as described by the manufacturers.

Monocyte-derived macrophages and differentiated U937 cells were prepared as described above.

Untreated and differentiated U937 cells, fresh human monocytes, and monocyte-derived macrophages were infected with pre-cooled, pre-opsonized *E. coli* (1000 bacteria.cell^{-1}), *M. tuberculosis* (100 bacteria.cell^{-1}) or with an equal volume of washing solution (negative control suggested by the manufacturer) for 10 minutes at 37°C, 5% CO_2. After 10 minutes, 20 µl freshly prepared dihydrorhodamin-123 substrate solution (provided by the manufacturer) was added to each sample and the cells were incubated for a further 10 minutes at 37°C. To terminate the reaction, 1% paraformaldehyde in PBS was added to each sample for 1 hour at room temperature. Adherent macrophages and PMA-differentiated U937 cells were subsequently EDTA-detached. Samples were washed once with washing solution, stained with DNA staining solution (100 µl.sample^{-1}; provided by the manufacturers) and incubated for a further 15 minutes on ice, in the dark before evaluating the results. Respiratory burst activity was quantified by using an Epics Profile II flow cytometer (Coulter) and histograms were gated on U937 cells, monocytes or macrophages by forward scatter (FS) versus fluorescence 3 (propidium iodide; DNA stain). Single parameter histograms of fluorescence 1 (rhodamine-123) showing the percentage of the gated population exhibiting oxidative conversion of dihydrorhodamine-123 to rhodamine-123 were used to determine respiratory burst potential of the different populations of cells. Uninfected U937 cells, monocytes and macrophages, prepared in parallel, were used in all cases to set cursors to allow 2% false positives.

2.2.8 Measurement of intracellular mycobacterial growth

U937 and THP-1 cells were untreated or induced to differentiate by the addition of PMA, rhIFN-γ, or 1,25-$(OH)_2$-vitamin D_3. Following 48 hours of differentiation, the cell concentration was re-adjusted to 0.5 x 10^6 cells.ml^{-1} in fresh medium containing PMA, rhIFN-γ or 1,25-$(OH)_2$-vitamin D_3. The cells were infected with pre-opsonized *M. tuberculosis* H37Rv or BCG (0.1 CFU.cell^{-1}). Following 90 minutes of infection (day 0), at day 3 and again at day 7 following infection, supernatants were removed and collected and the cells solubilized with 0.25% SDS PBS. The cell lysate and corresponding supernatant were pooled to compensate for mycobacteria released into the surrounding medium from

damaged cells during the course of the infection. Because U937 have demonstrated the ability to retro-differentiate (Hass *et al.*, 1991), each well was supplemented on day 3 with fresh medium containing PMA, rhIFN-γ or 1,25-$(OH)_2$-vitamin D_3. Mycobacterial growth was measured by [^{3}H]-Uridine (Amersham International; specific activity 185 MB; 1 μCi.well^{-1}) incorporation. Pooled cell lysate and supernatant (100 µl.well^{-1}) were incubated together with [^{3}H]-Uridine in 6-well replicates for 10 days at 37°C, 5% CO_2. On day 10, the cells were fixed by the addition of an equal volume of 1% paraformaldehyde for 1 hour, harvested using an automated cell harvester (Titertek^R 630), and the radioactivity (cpm) was measured using a liquid scintillation counter (Tricarb 4640).

2.2.9 Statistical analysis

Statistical analyses were performed using the Wilcoxon ranks test for paired non-parametric data and the Mann-Whitney *U*-test for unpaired non-parametric data (Kaplan, 1987).

2.3 RESULTS

2.3.1 Characterization and differentiation of U937 and THP-1 cells

The present study was designed to investigate and compare the phenotypic and functional characteristics displayed by the monocytic cell lines U937 and THP-1 both before and following differentiation. Both cell lines showed strong and diffuse cytoplasmic staining for non-specific esterase activity consistent with their monocytic cell ancestry. U937 and THP-1 cells were induced to differentiate by the addition of various agents such as PMA, rhIFN-γ, or 1,25-$(OH)_2$-vitamin D_3; and the efficiency of differentiation was compared by assessing (i) autonomous proliferative ability, (ii) cell cycle distribution, (iii) adherence, (iv) cell surface marker expression, (v) phagocytic ability, and (vi) respiratory burst activity following infection.

Autonomous proliferative ability and cell cycle distribution

An important indicator of monocyte/macrophage terminal differentiation is their arrest in the G_0/G_1 phase of the cell cycle and their reduced proliferative potential (Abrink *et al.*, 1994).

Although untreated U937 and THP-1 cells showed good ability to proliferate autonomously, differentiation with PMA, rhIFN-γ or 1,25-$(OH)_2$-vitamin D_3 differentially arrested this proliferative ability (Fig. 2.1). Differentiation with PMA, in particular, resulted in almost complete arrest of proliferation (85-93% reduction) and this effect was detectable as early as 24 hours post-treatment. Interestingly, this PMA-induced growth arrest followed an initial burst in [^{3}H]-thymidine uptake (0-16 hours) in U937 but not THP-1 cells. IFN-γ-induced differentiation of U937 and THP-1 cells resulted in a more gradual loss of proliferative ability with its effect reaching a peak of 65-87% inhibition after 72 hours of treatment. 1,25-$(OH)_2$-vitamin D_3-treatment (10^{-7} to 10^{-9} M), in contrast, resulted in only slight inhibition of cellular proliferation of U937 cells after 72 hours (48% inhibition) but had no effect on THP-1 proliferative ability.

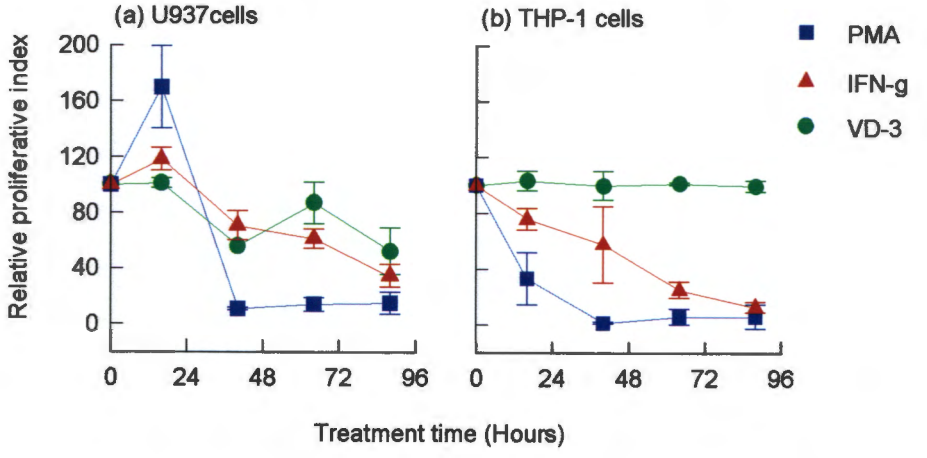


FIGURE 2.1 **The effect of differentiation on the autonomous proliferative ability of U937 and THP-1 cells. (a) U937 and (b) THP-1 cells were induced to differentiate by the addition of (■) PMA (5ng.ml^{-1}), (▲) rhIFNγ (200 iu.ml^{-1}) or (●) 1,25-$(OH)_2$-vitamin D_3 (10^{-7} M). Relative proliferative index was calculated as follows: [(^{3}H)-thymidine incorporation (cpm) for differentiated cells] ÷ [(^{3}H)-thymidine incorporation (cpm) for untreated cells] and expressed as a percentage. Each data point represents the mean percentage (error bars represent SD) of at least 3 independent experiments.**

Analysis of the effects of PMA-, rhIFN-γ-, and 1,25-$(OH)_2$-vitamin D_3-induced differentiation (48-72 hours) on the cell cycle distribution of U937 and THP-1 cells (Fig. 2.2) revealed only slight differences between these cell lines. Untreated, both cell lines showed a high proportion of the population cycling through S- and G_2/M-phases of the cell cycle (40-55%) consistent with their ability to proliferate autonomously. PMA-treatment of both cell lines resulted in a striking accumulation of cells in G_0 with 93.1% of U937 and 81.6% of

THP-1 cells having accumulated in this phase. rhIFN-γ or 1,25-$(OH)_2$-vitamin D_3-treatment, by comparison, showed only a slight accumulation of cells in the G_0-phase for U937 and no significant accumulation for THP-1 cells. Differential accumulation of cells in the G_0 phase of the cell cycle following treatment with PMA is consistent with the observed inhibition in the autonomous proliferation of both cell lines.

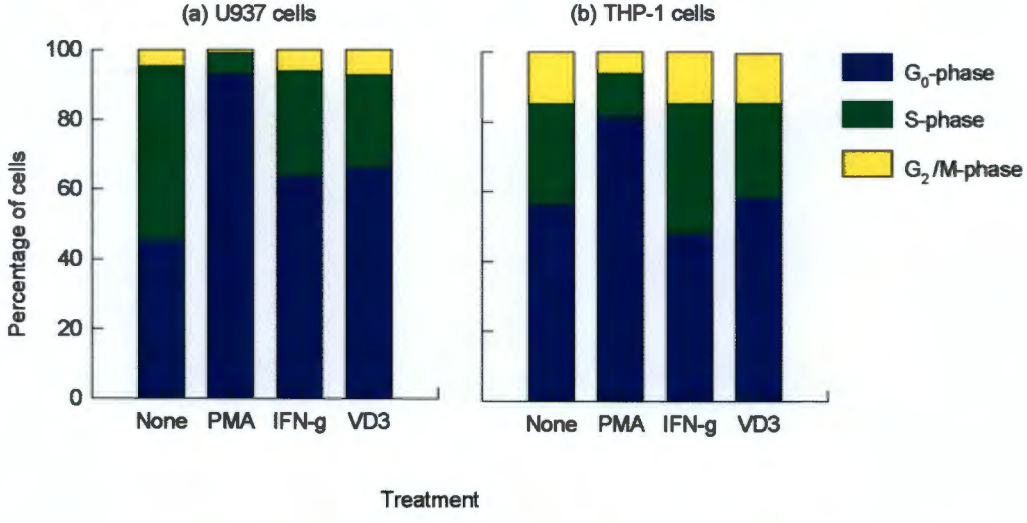


FIGURE 2.2 Cell cycle distribution of U937 and THP-1 cells following differentiation. (a) U937 and (b) THP-1 cells were either untreated or induced to differentiate by the addition of PMA (5 $ng.ml^{-1}$), rhIFNγ (200 $iu.ml^{-1}$) or 1,25-$(OH)_2$-vitamin D_3 (10^{-7} M) for 48-72 hours at 37°C. The blue bars represent cells in G_0 phase, the green bars represent cells in S phase, and the yellow bars represent cells in G_2/M phase of the cell cycle. Each bar represents the mean percentage of at least 3 independent experiments.

Comparison of the DNA content of human mononuclear cells, U937 and THP-1 cells showed that while both THP-1 cells and human mononuclear cells had diploid chromosome number (2N), U937 cells were clearly aneuploid. Chromosomal analysis confirmed that U937 had a mean of 57 chromosomes per cell (and a range of 50-63) with many of the chromosomes exhibiting extensive structural abnormalities.

Kinetics of Adherence

While THP-1 and U937 remained in suspension following 1,25-$(OH)_2$-vitamin D_3-treatment, differentiation of the U937 with PMA and THP-1 with PMA or rhIFN-γ resulted in the cells becoming adherent within 24-48 hours. Whereas THP-1 cells formed relatively uniform macrophage-like mono-layers following PMA- or rhIFN-γ-differentiation with a significant

proportion of the cells becoming elongated and spindle shaped, PMA-treated U937 cells formed irregular and 'patchy' mono-layers.

Cell surface marker expression

Surface expression of important macrophage markers, such as CD14, CD16 (Fcγ receptor), CD11b (CR3), and HLA class I and class II, were monitored following PMA-, rhIFN-γ- and 1,25-$(OH)_2$-vitamin D_3-treatment (Fig. 2.3 and Table 2.1).

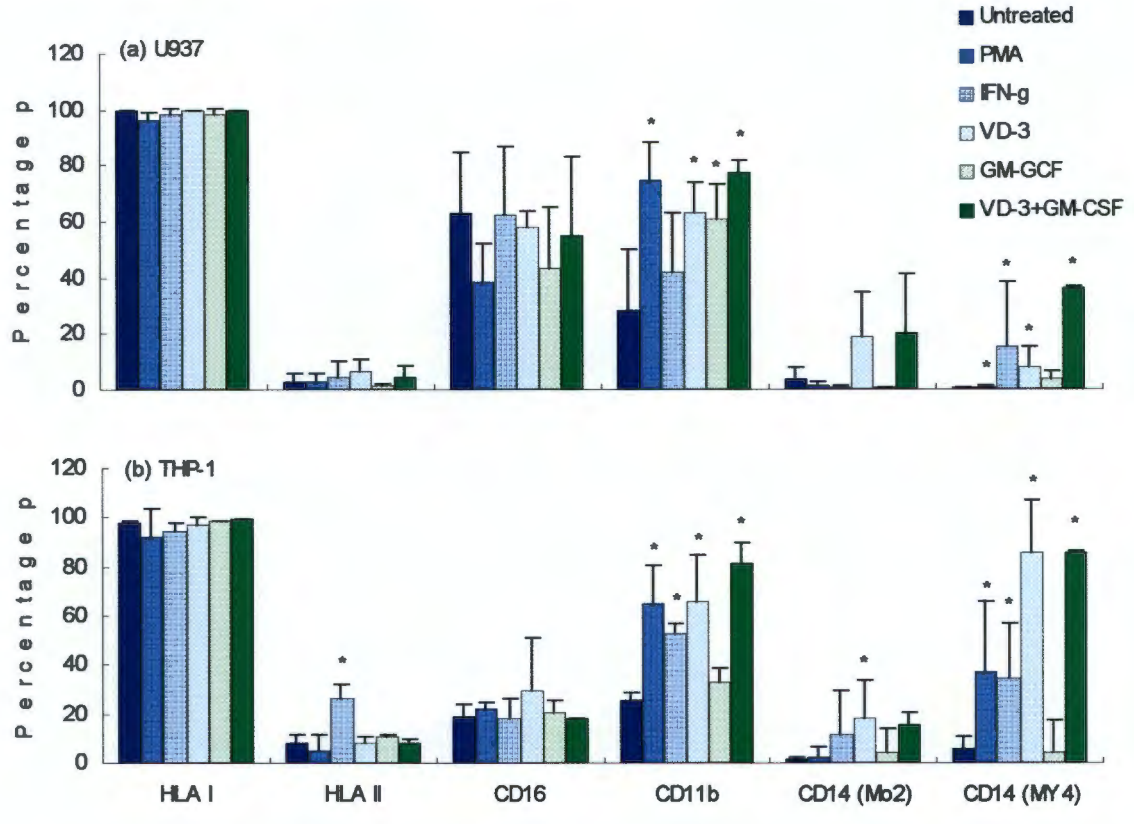


FIGURE 2.3 Effect of differentiation on U937 and THP-1 cell surface marker expression. The distribution of certain monocyte-specific cell surface markers were assessed on (a) U937 or (b) THP-1 cells which were either untreated (dark blue) or induced to differentiate by the addition of PMA (medium blue, 5 $ng.ml^{-1}$), rhIFN-γ (light blue, 200 $iu.ml^{-1}$), 1,25-$(OH)_2$-vitamin D_3 (pale blue, 10^{-7} M), GM-CSF (light green, 5 $ng.ml^{-1}$) or a combination of 1,25-$(OH)_2$-vitamin D_3 (10^{-7} M) and GM-CSF (5 $ng.ml^{-1}$, medium green). Each bar represents the mean of at least 5 independent experiments (error bars represent SD). (*) Represents significantly increased cell surface expression ($p<0.05$) on differentiated cells compared with untreated cells.

Table 2.1 *Mean channel fluorescence of U937 and THP-1 cells expressing macrophage-specific markers*

Cell type	Treatment	Cell surface antigen					
		HLA class I	HLA class II	CD16	CD11b	CD14 (Mo2)[a]	CD14 (MY4)[a]
Macrophage	None	nd[b]	12.1 (± 7.1)[c]	2.5 (± 0.4)	4.8 (± 1.6)	16.6 (± 12.4)	2.6 (± 0.1)
U937	None	31.7 (± 0.8)	13.8 (± 14.1)	10.9 (± 11.9)	22.4 (± 6.9)	10.0 (± 9.4)	8.9 (± 6.4)
	PMA	31.9 (± 17.4)	16.7 (± 15.9)	2.9 (± 0.5)	4.0 (± 1.6)	12.3 (± 7.6)	13.1 (± 10.0)
	IFN-γ	71.1 (± 0.9)	81.5 (± 57.3)	12.4 (± 13.8)	17.5 (± 16.6)	11.8 (± 10.3)	15.0 (± 12.6)
	VD3	56.5 (± 2.2)	25.6 (± 5.1)	9.5 (± 11.2)	30.6 (± 25.9)	13.4 (± 14.7)	11.6 (± 10.9)
	GM-CSF	64.0 (± 55.9)	16.3 (± 11.4)	15.7 (± 9.2)	37.4 (± 32.6)	26.9 (± 15.3)	19.5 (± 12.4)
	VD3/GM-CSF	44.1 (± 14.4)	21.8 (± 8.5)	26.6 (± 21.9)	44.5 (± 41.7)	37.9 (± 28.1)	19.7 (± 18.3)
THP-1	None	113.5 (± 0.0)	3.7 (± 1.0)	11.9 (± 7.9)	7.3 (± 0.5)	1.5 (± 0.4)	0.7 (± 0.2)
	PMA	193.7 (± 87.3)	2.6 (± 0.8)	14.6 (± 10.0)	26.3 (± 16.7)[d]	5.1 (± 4.9)[d]	11.3 (± 18.3)[d]
	IFN-γ	236.3 (± 59.4)	29.9 (± 17.9)	13.1 (± 5.4)	16.9 (± 8.6)[d]	6.7 (± 8.6)[d]	26.7 (± 48.2)[d]
	VD3	145.9 (± 32.2)	4.3 (± 1.8)	9.6 (± 4.5)	34.1 (± 25.7)[d]	8.7 (± 7.3)[d]	42.9 (± 44.8)[d]
	GM-CSF	73.4 (± 99.5)	2.5 (± 2.2)	60.5 (± 73.6)	8.0 (± 1.0)	5.1 (± 3.8)	1.5 (± 0.1)
	VD3/GM-CSF	128.0 (± 34.4)	3.3 (± 0.7)	7.9 (± 0.3)	23.4 (± 12.8)	4.6 (± 2.8)	19.8 (± 13.4)[d]

[a]CD14 Mo2 and CD14 MY4 are monoclonal antibodies directed against different epitopes on CD14. [b]nd (not determined).

[c]Mean of at least 5 independent experiments (± SD).

[d]Differentiated cells showed significant up-regulation ($p > 0.05$) in the indicated cell surface marker as compared to the untreated cells.

Both U937 and THP-1 cells were found to constitutively express high levels of cell surface HLA class I. Levels of HLA class I expression were further induced [as determined by increased mean channel fluorescence (MCF), Table 2.1] on U937 cells following differentiation with rhIFN-γ, 1,25-$(OH)_2$-vitamin D_3 and GM-CSF and on THP-1 cells following differentiation with PMA, rhIFN-γ and 1,25-$(OH)_2$-vitamin D_3. Tissue typing showed that U937 cells express cell surface HLA-A3, -B18, -B51, and -Cw1 while THP-1 express surface HLA-A2, -A9, and -B5.

U937 and THP-1 cell lines do not constitutively express detectable levels of HLA class II. Despite showing a relative increase in MCF following differentiation with rhIFN-γ and 1,25-$(OH)_2$-vitamin D_3, the absolute percentage of U937 cells staining positive for cell surface HLA class II was not concurrently up-regulated. Several earlier studies have characterized U937 as a HLA class II-deficient monocytic cell line and the inability of U937 cells to express HLA class II was found to reside at the level of gene transcription (Sundstrom and Nilsson, 1976; Peterlin *et al.*, 1984). DNA typing of U937 cells confirmed that they do possess the relevant genes for HLA class II (DR1, DQ2) despite their not being detectable at the cell surface. In contrast, THP-1 cells could be induced to express HLA class II following differentiation with rhIFN-(but not PMA, 1,25-$(OH)_2$-vitamin D_3 or GM-CSF. Both the percentage of cells expressing HLA class II (Fig. 2.3b) and the relative intensity of cell surface staining on individual cells (determined by increased MCF) was significantly increased following rhIFN-(-treatment ($p<0.05$).

U937 cells do not express detectable levels of the CD1a (2.3 ± 0.6%) (mean ± SD), CD1b (1.9 ± 0.6%), or CD1c (2.3 ± 0.4%). The expression levels of these highly conserved antigen presenting structures did not increase following PMA-, rhIFNγ- and 1,25-$(OH)_2$-vitamin D_3-treatments nor with a combination of GM-CSF and IL-4. This is consistent with the previously published findings of Kasinrerk *et al.* (1993) who demonstrated that U937 cells (unlike human monocytes and macrophages) could not be induced to express CD1.

Both cell lines were found to constitutively express very low levels of cell surface CD14. This important monocyte/macrophage marker was differentially inducible in both U937 and THP-1, however, with the percentage of cells expressing CD14 (as detected by Mo2 antibody staining) being up-regulated following differentiation with 1,25-$(OH)_2$-vitamin D_3 but not

PMA, rhIFN-γ, or GM-CSF. U937 and THP-1 cells showed similar levels of CD14 (Mo2) expression following 1,25-$(OH)_2$-vitamin D_3-differentiation. Induction of CD14 (as detected by MY4 antibody staining) on THP-1 (and U937 to a lesser extent) was greater when compared with CD14 (Mo2). PMA, rhIFN(and 1,25-$(OH)_2$-vitamin D_3 treatment of both cell lines resulted in a significant increase ($p<0.05$) in the percentage of cells staining positive for CD14 (MY4) and the relative intensity of cell surface staining for this marker.

Whereas 63.1 (± 21.8) % of untreated U937 cells express CD16 (Fcγ receptor), only 18.5 (± 5.1) % of THP-1 cells express CD16 constitutively. Neither U937 nor THP-1, however, showed significant changes in CD16 expression following exposure to PMA, rhIFNγ, 1,25-$(OH)_2$-vitamin D_3 or GM-CSF. Both cell lines express comparable levels of CD11b (complement receptor 3); and both the percentage of cells staining positive as well as the relative intensity of expression in both lines were similarly up-regulated following exposure to PMA, rhIFNγ, and 1,25-$(OH)_2$-vitamin D_3.

In summary, some important constitutive and inducible phenotypic differences have emerged when comparing U937 cells with THP-1. Although both cell lines express similar levels of cell surface CD11b (complement receptor 3), U937 but not THP-1 express detectable levels of CD16 (Fcγ receptor) while THP-1 to a greater extent than U937 cells can be induced to express CD14. Most importantly and unlike THP-1 cells, U937 cells do not constitutively express HLA class II nor can they be induced to do so despite expressing high levels of cell surface HLA class I.

2.3.2 Phagocytosis of *M. tuberculosis*

The ability of U937 cells to bind and subsequently phagocytose *M. tuberculosis* was investigated using three independent techniques: (i) mycobacterial binding assay using ZN staining; (ii) TEM of *M. tuberculosis*-infected cells; and (iii) flow cytometric evaluation of phagocytosis using FITC-labeled *M. tuberculosis*.

M. tuberculosis binding assay using Ziehl-Neelson staining

The ability of U937 and THP-1 cells (untreated, PMA-, IFN-γ- or vitamin D_3-treated) to bind *M. tuberculosis* (in the presence or absence of fresh human serum) was compared using ZN-staining and light microscopy (Fig. 2.4). THP-1 cells showed a greater ability to bind *M. tuberculosis* than U937 cells. Whereas untreated U937 cells showed poor ability to bind *M. tuberculosis* (2.0 ± 1.4 %; mean ± SD), 50.7 ± 19.0 % (mean ± SD) of undifferentiated THP-1 cells had associated mycobacteria. Both cell lines showed strong dependence on serum opsonins for mycobacterial binding as indicated by the 2- to 4-fold increase in associated bacteria in the presence of fresh human serum. Low levels of bacterial association were also found in the absence of serum components suggesting that while bacterial entry into both U937 and THP-1 cells is predominantly opsonic, a non-opsonic pathway is possibly also operative. For U937 specifically, differentiation with PMA-, rhIFN-γ-, or 1,25-$(OH)_2$-vitamin D_3 had a significant effect on their ability to incorporate *M. tuberculosis*. rhIFN-γ- and 1,25-$(OH)_2$-vitamin D_3-treatment of THP-1 cells, by comparison, did not result in a significant increase in their ability to phagocytose mycobacteria compared to the untreated population. For both cell lines, PMA-treatment showed the most striking results with 57% of the U937 cells and 97% of THP-1 cells binding one or more opsonised bacilli.

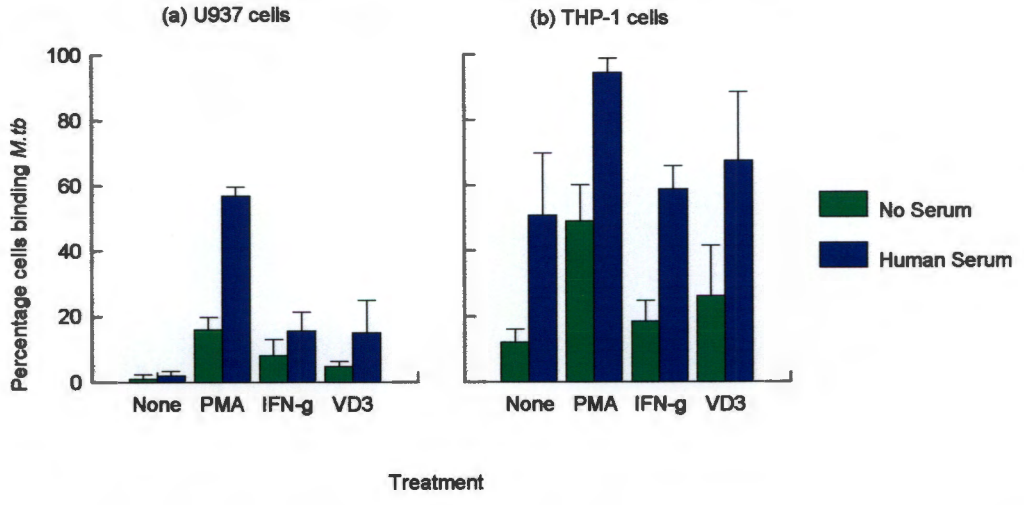


FIGURE 2.4. The ability of (a) U937 and (b) THP-1 cells, differentiated with PMA (5 $ng.ml^{-1}$), rhIFN-γ (200 $iu.ml^{-1}$) or 1,25-$(OH)_2$-vitamin D_3 (10^{-7} M), to bind *M. tuberculosis* was compared using ZN staining. U937 or THP-1 cells (0.5×10^6 $cells.ml^{-1}$) were infected with *M. tuberculosis* (10 $CFU.cell^{-1}$) in the absence (green bars) or presence (blue bars) of human serum for 90 minutes at 37°C. The bars represent the percentage of U937 or THP-1 cells with at least one associated mycobacterium. Each data point represents the mean (± SD) of at least 3 independent experiments, each experiment was carried out in duplicate and at least 200 cells from each sample was assessed.

Transmission electron microscopy

Transmission electron microscopy (TEM) of PMA-differentiated U937 cells infected with *M. tuberculosis* showed that intracellular mycobacteria were always found within membrane bound vacuoles which were usually tightly apposed (Fig. 2.5 a-c) although larger phagosomes containing *M. tuberculosis* were also evident (Fig. 2.5 d-e). The majority of infected U937 cells (35%) contained less than 5 bacilli per cell each contained within single vacuoles. Although infrequent, larger vacuoles containing clumps of up to 20 bacteria were also evident (Fig. 2.5 f). Quantitative analysis of the infected cells by TEM showed that 53% of PMA-treated U937 cells contained one or more bacilli. This level of infection correlates well with the results presented in Fig. 2.4 where 57% of PMA-differentiated U937 had associated mycobacteria.

Flow cytometric evaluation of phagocytosis

The ability of U937 cells to phagocytose FITC-conjugated *M. tuberculosis* and FITC-conjugated *E. coli* was compared to human monocytes and monocyte-derived macrophages (but not THP-1 cells) and evaluated using flow cytometry (Fig. 2.6). The results demonstrate that both human monocytes and macrophages have higher phagocytic ability than U937 cells differentiated with PMA, rhIFN-γ or 1,25-$(OH)_2$-vitamin D_3. While monocytes showed the highest phagocytic ability for *E. coli* (3-fold greater than macrophages and 5-fold greater than PMA-treated U937), they phagocytosed *M. tuberculosis* with similar efficiency to macrophages and with a 3-fold greater efficiency than PMA-differentiated U937. Undifferentiated U937 showed poor ability to phagocytose both *E. coli* and *M. tuberculosis*. Differentiation of U937 with rhIFN-γ, PMA or 1,25-$(OH)_2$-vitamin D_3 resulted in a 2- to 4-fold increase in the phagocytosis of both bacteria compared to untreated cells.

(a)

(b)

(c)

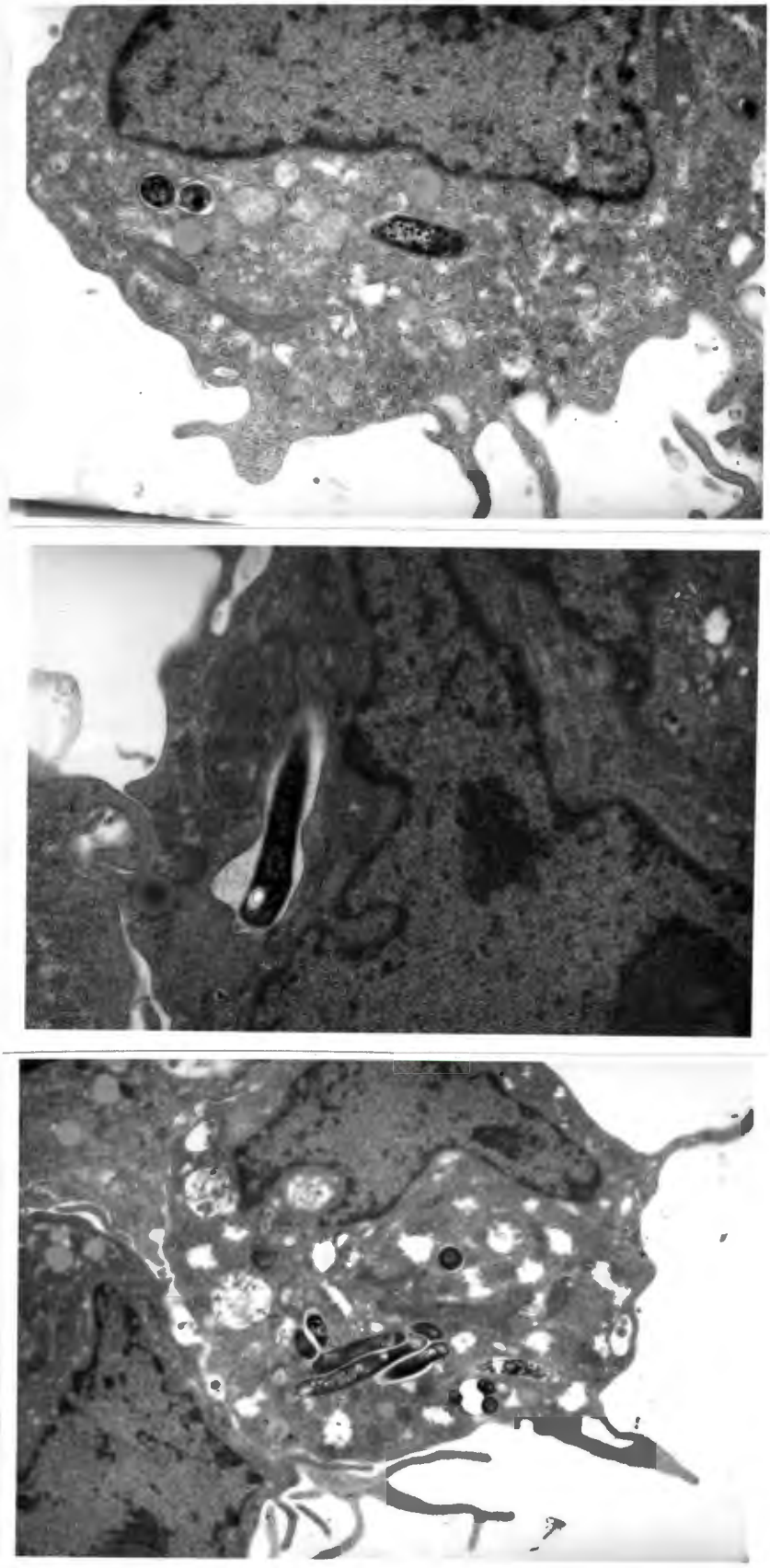
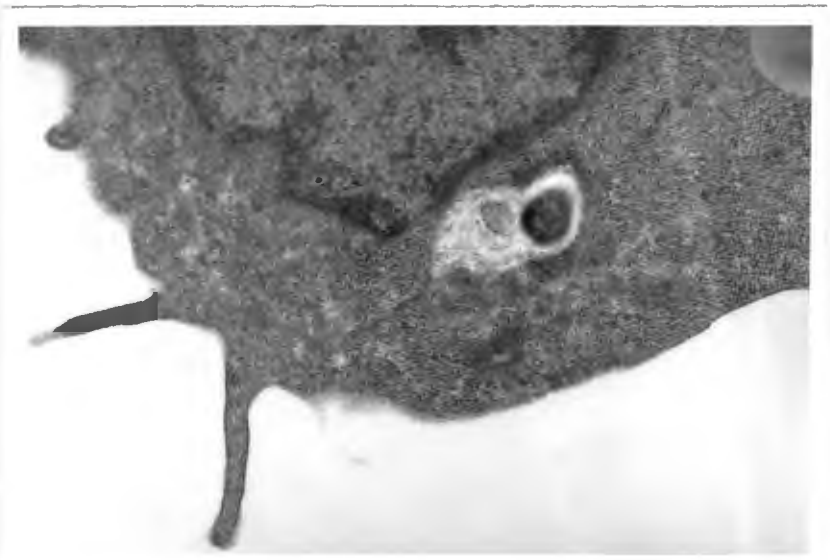
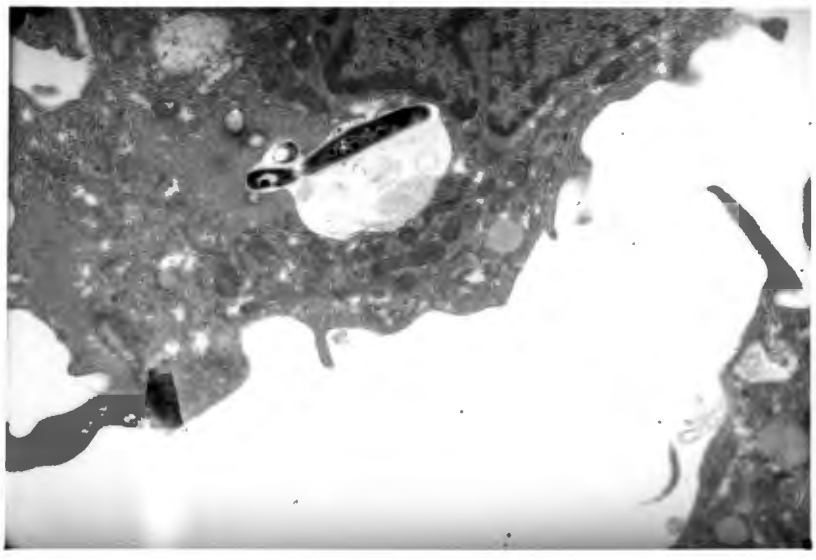


FIGURE 2.5. TEM photomicrographs of PMA-differentiated U937 cells (5 $ng.ml^{-1}$) infected with *M. tuberculosis* (50 $CFU.ml^{-1}$; 5000x magnification). The bacilli were contained within tightly apposed phagosomes (a-c) or within larger vacuoles (d-e). (f) Histogram illustrating the distribution of *M. tuberculosis* bacilli contained in either individual or communal vacuoles within infected U937 cells.

(d)



(e)



(f)

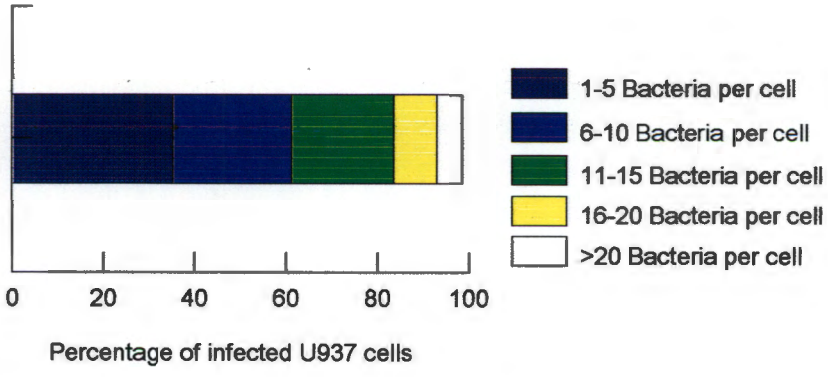


FIGURE 2.5. Continued.

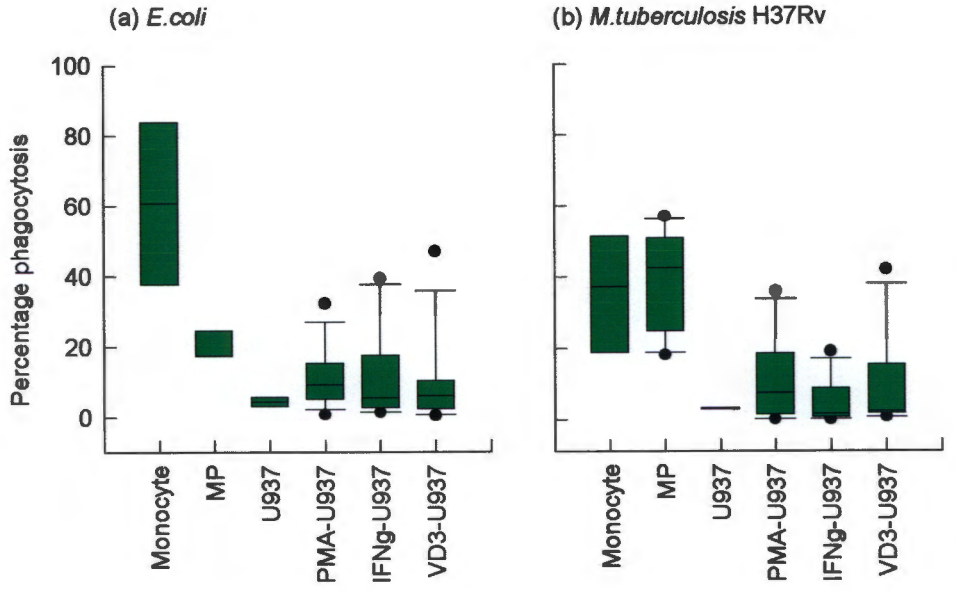


FIGURE 2.6. Flow cytometric evaluation of phagocytosis of FITC-conjugated (a) *E. coli* (1000 CFU.ml^{-1}) or (b) *M. tuberculosis* H37Rv (100 CFU.ml^{-1}) by human monocytes, macrophages, undifferentiated U937 cells or U937 cells treated with PMA (5 ng.ml^{-1}), rhIFN-γ (200 iu.ml^{-1}), or 1,25-$(OH)_2$-vitamin D_3 (10^{-7} M) for 48 hours at 37°C. Each box-and-whisker plot shows the distribution, median, and 10th and 90th percentile of at least 4 independent experiments. (•) Indicate possible outliers.

The level of phagocytosis measured by flow cytometry was significantly lower than the mycobacterial binding levels shown in Fig 2.4. There are a number of possible explanations for the discrepancies between these two methods. Firstly, conjugating the bacteria with FITC may alter cell recognition and therefore uptake of these organisms. Not only may FITC-labeling obscure surface structures on the bacilli that are targets for either antibody or complement binding but may also affect the interaction of these organisms with their specific macrophage entry receptors (complement receptors or Fc receptors, for example). Previous studies have demonstrated, however, that FITC-labeling does not alter the ability of human macrophages or polymorphonuclear cells to phagocytose other intracellular pathogens (Cantinieaux *et al.*, 1989; Drevets and Campbell, 1991). Secondly, it may be that many more mycobacteria (5-fold) bind to differentiated U937 than are subsequently incorporated by phagocytosis. It may be that most of the associated organisms become non-specifically bound to the surface of U937 cells and only those bacilli that have associated with phagocytic receptors become internalized. However, the strong correlation found between mycobacterial binding (Fig. 2.4) and TEM-determined infection efficiency (Fig. 2.5) argues against this possibility. Thirdly, it is possible that *E. coli*

and *M. tuberculosis* were not uniformly or adequately labeled with FITC resulting in differential fluorescence at the single cell level. Because the TEM results confirmed the mycobacterial binding results, it is reasonable to conclude that flow cytometric assessment of intracellular *M. tuberculosis* is a less sensitive approach resulting in measurable under-estimations of true phagocytic ability for U937 cells.

2.3.3 Respiratory burst activity following infection

Consistent with their immature phenotype, undifferentiated U937 and THP-1 cells were unable to reduce NBT in response to stimulation with PMA or infection with *M. tuberculosis* (Fig. 2.7 a-b). Following differentiation with rhIFN-γ, 1,25-$(OH)_2$-vitamin D_3 and to a lesser extent PMA, however, the ability of differentiated cells to reduce NBT in response to PMA-activation was measurably increased. Despite showing moderate to strong respiratory burst activity following PMA-stimulation, however, both THP-1 and U937 cells showed uniformly poor oxidative burst activity following infection with *M. tuberculosis*. By comparison, freshly isolated human monocytes showed a much stronger respiratory burst response following infection with both *E. coli* and *M. tuberculosis* than terminally differentiated macrophages or U937 cells (Fig. 2.7 c-d).

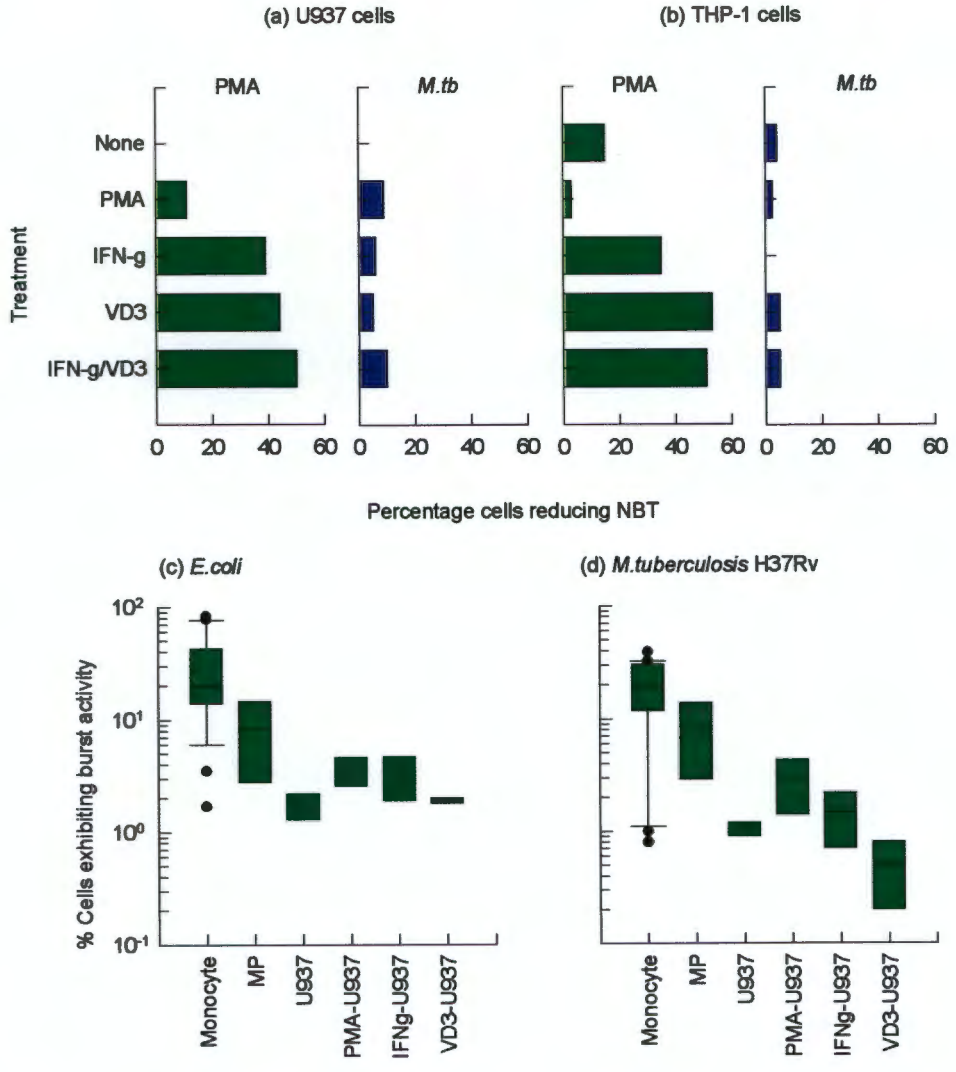


FIGURE 2.7. The ability of U937 to mount a respiratory burst response following PMA-stimulation or infection with *M. tuberculosis* was demonstrated by (a-b) NBT reduction and (c-d) flow cytometric evaluation of dihydrorhodamin-123 reduction and compared with either THP-1 or human monocytes and monocyte-derived macrophages. (a-b) U937 and THP-1 cells were either untreated or induced to differentiate with PMA (5ng.ml^{-1}), rhIFN-γ (200 iu.ml^{-1}), or 1,25-$(OH)_2$-vitamin-D_3 (10^{-7} M) for 48 hours as described in Methods and Materials. Untreated, PMA-, rhIFN-γ-, and 1,25-$(OH)_2$-vitamin-D_3-differentiated U937 and THP-1 cells were then re-stimulated with PMA (10 μg.ml^{-1}) or infected with *M. tuberculosis* (100 CFU.cell^{-1}) for a further 1 hour at 37°C. Each bar represents the mean of two independent experiments. (c-d) Fresh human monocytes were derived from heparinized whole blood while monocyte-derived macrophages were differentiated for 4-6 days at 37°C. U937 cells were induced to differentiate as described above. Cells were infected with either *E. coli* (1000 CFU.ml^{-1}) or *M. tuberculosis* (100 CFU.ml^{-1}) for 10 minutes at 37°C. Dihydrorhodamin-123 substrate was added to each sample for a further 10 minutes at 37°C. Respiratory burst activity was then quatified using an Epics Profile II flow cytometer. Each box-and-whisker plot shows the distribution, median, and 10th and 90th percentile of at least 3 independent experiments. (•) Indicate possible outliers.

2.3.4 Intracellular survival of *M. tuberculosis* H37Rv and BCG

The influence of PMA-, rhIFN-γ- and 1,25-$(OH)_2$-vitamin D_3-induced differentiation on the permissiveness of U937 cells to intracellular mycobacterial growth and survival was compared with THP-1 cells in order to determine if observed differences in phenotype and function affect the anti-mycobacterial effects of these cells (Fig. 2.8). Regardless of the agent used to induce maturation, terminally differentiated U937 cells were found to be permissive to the growth of both virulent *M. tuberculosis* H37Rv and, to a lesser extent, non-virulent BCG with bacterial cell numbers increasing by approximately one log unit over an infection period of 5 days. IFN-γ-differentiated U937 cells were found to be the most permissive to mycobacterial growth compared with cells similarly differentiated with PMA or 1,25-$(OH)_2$ vitamin D_3 (Table 2.2). By comparison, differentiated THP-1 cells were less permissive to the intracellular growth of both *M. tuberculosis* and BCG than U937 cells. The kinetics of the intracellular infection differed quite significantly from those observed for U937 with bacterial growth proceeding linearly rather than exponentially in THP-1 cells. Like U937, however, rhIFN-γ-differentiated THP-1 cells were found to be the most permissive to intracellular mycobacterial growth compared with cells differentiated with PMA or 1,25-$(OH)_2$ vitamin D_3. Both U937 and THP-1 cells were better able to restrict the growth of BCG than H37Rv (Table 2.2).

Table 2.2 *Comparative doubling time of M. tuberculosis H37Rv and BCG within U937 and THP-1 cells*

Cell Line	Treatment	Doubling time (hours) [a]		Relative doubling time (H37Rv:BCG)
		H37Rv	BCG	
U937	PMA	37.2	79.0	2.1:1
	rhIFN-γ	54.4	66.1	1.2:1
	Vitamin D_3	40.7	57.9	1.4:1
THP-1	PMA	491.9	797.7	1.6:1
	rhIFN-γ	101.2	238.5	2.4:1
	Vitamin D_3	94.9	365.5	3.9:1

[a]Doubling times were calculated from best-fit straight line through logarithmic plot of [^{3}H]-Uridine incorporation (cpm) versus time (Fig. 2.9).

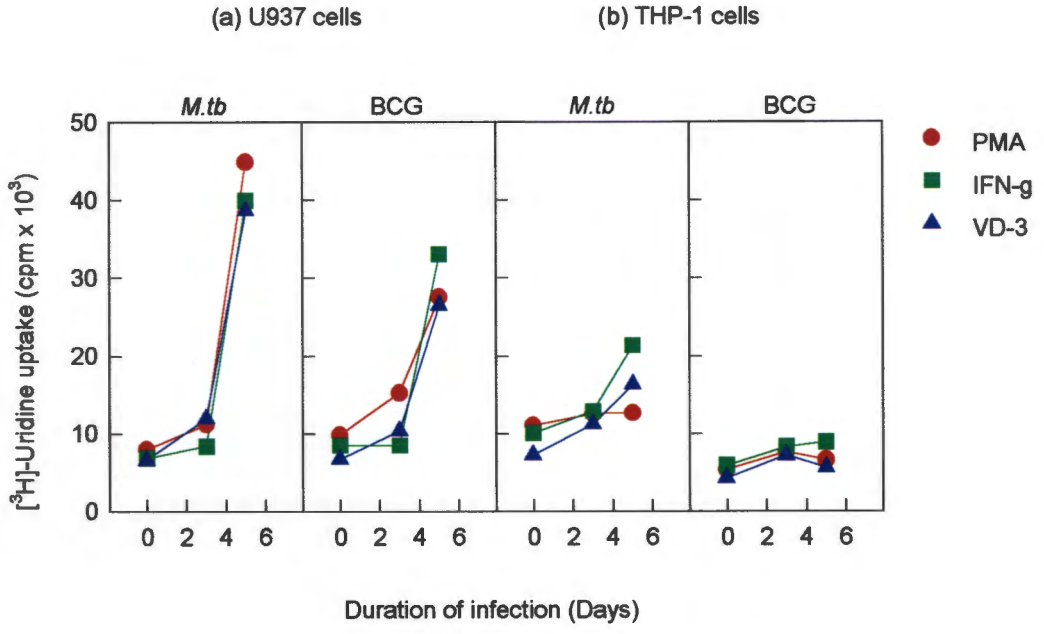


FIGURE 2.8. Effect of differentiation on the ability of U937 and THP-1 cells to control the intracellular growth of *M. tuberculosis* and BCG. (a) U937 and (b) THP-1 cells, induced to differentiate with (●) PMA (5 ng.ml^{-1}), (■) rhIFN-γ (200 iu.ml^{-1}) or (▲) 1,25-$(OH)_2$-vit D_3 (10^{-7} M), were investigated for their permissiveness to intracellular mycobacterial growth. Differentiated cells were infected with *M. tuberculosis* H37Rv or BCG (0.1 CFU.cell^{-1}) and intracellular mycobacterial growth was determined immediately following infection (day 0), and at 3 and 5 days post-infection. Mycobacterial growth was measured by [^{3}H]-Uridine (specific activity 185 MB; 1 μCi.well^{-1}) incorporation. Pooled cell lysate and supernatant (100 μl.well^{-1}) were incubated together with [^{3}H]-Uridine in 6-well replicates for 10 days at 37°C, 5% CO_2. Each data point represents the mean of two independent experiments.

2.3.5 Influence of *M. tuberculosis* infection on HLA class I and II expression

The expression of HLA class I and class II molecules on the surface of U937 cells were monitored following infection with *M. tuberculosis* (Table 2.3). While the relative level of surface HLA class I (measured by MCF) on the *M. tuberculosis*-infected but not the BCG-infected U937 cells was significantly up-regulated ($p<0.05$) compared with uninfected cells, no change was observed in HLA class II expression by infected U937 cells. Although HLA class I and II expression levels were monitored for 5 days following infection, no change in either the percentage of cells expressing HLA surface antigens or in the relative level of expression on individual cells (measured by MCF) was observed (data not shown).

Table 2.3. *Effect of mycobacterial infection on U937 cell surface HLA class I and class II expression.*

HLA molecule	Treatment	Percentage (± SD) of U937 cells expressing cell surface antigen		MCF[a] (± SD) of U937 cells expressing cell surface antigen	
		Isotypic control[b]	Specific Ab[c]	Isotypic control[b]	Specific Ab[c]
HLA class II	Uninfected	2.3 (± 1.0)	3.2 (± 2.6)	34.4 (± 20.9)	30.3 (± 18.1)
	BCG-infected	2.1 (± 0.6)	2.5 (± 1.3)	27.5 (± 11.2)	29.7 (± 15.9)
	M.tb-infected	2.0 (± 0.7)	2.2 (± 1.2)	35.0 (± 10.7)	32.8 (± 9.4)
HLA class I	Uninfected	2.1 (± 1.9)	96.6 (± 2.6)	12.6 (± 13.4)	27.0 (± 12.1)
	BCG-infected	2.7 (± 1.7)	96.7 (± 2.1)	18.2 (± 19.5)	28.2 (± 8.9)
	M.tb-infected	3.3 (± 2.4)	94.3 (± 1.3)	23.8 (± 3.5)	**59.5 (± 9.7)**[d]

[a]Mean channel fluorescence (MCF). [b]Isotypic control for non-specific antibody binding. [c]Specific antibody (Ab) directed against HLA class II and class I, respectively. [d]*M. tuberculosis*-infected U937 cells expressed significantly higher levels of HLA class I (as measured by MCF) than uninfected cells ($p<0.05$; Wilcoxon ranks test for paired non-parametric data).

A summary of the results presented in Fig. 2.1 to 2.9 is given in Table 2.4.

Table 2.4 *Phenotype of U937 and THP-1 cells*[a]

	Cell Lines				Human monocytes[b] or monocyte-derived macrophages[c]
	U937		THP-1		
	Constitutive	Inducible	Constitutive	Inducible	
Late Differentiation Markers					
CD14	-	+	-	+	+[c]
CR3	(+)	+	(+)	+	+[c]
HLA class II	-	-	-	+	+[c]
Phagocytosis	-	+	+	+	+[b/c]
Respiratory burst					
PMA	-	+	-	+	nd[d]
M. tuberculosis	-	-	-	-	+[b/c]
E. coli	-	-	nd	nd	+[b/c]
Intracellular survival					
BCG	nd	(+)	nd	(+)	nd
M. tuberculosis	nd	+	nd	+	nd

[a]Summary of results presented in Fig. 2.3 to Fig. 2.9. A + indicates that the various phenotypic or functional properties were detected in a cell line or normal monocyte. A (+) indicates that the levels of expression/growth were low. A – indicates that the properties being assessed were not detected. [b]Experiments were carried out using freshly isolated human monocytes. [c]Experiments were carried out using monocyte-derived human macrophages following 4-6 days *in vitro* differentiation. [d]nd (not determined).

2.3 DISCUSSION

The macrophage-like cell line U937 was investigated as a model for macrophage function in mycobacterial infections. U937 cells were inducible towards more mature macrophage phenotypes by the addition of PMA, rhIFNγ, or 1,25-$(OH)_2$-vitamin D_3. The efficiency of monocytic differentiation was demonstrated by the cessation of autonomous proliferative ability, growth arrest in the G_0 phase of the cell cycle, cellular adherence under certain conditions, and the acquisition of monocyte-specific surface receptors (CD11b, CD16, and CD14 to a lesser extent but not HLA class II). Although all of the agents used to induce differentiation resulted in cellular maturation, each exhibited differing effects on these individual characteristics. While PMA was the most potent inducer of growth arrest in G_0 of the cell cycle, reduced proliferation and cellular adhesion, 1,25-$(OH)_2$-vitamin D_3 showed the most marked effect on cell surface marker expression (CD14 and CD11b). These differences may reflect differences in the signal transduction pathways used by these different agents [with IFN-γ acting via specific cytokine receptors, 1,25-$(OH)_2$-vitamin D_3 via steroid receptors and PMA acting via protein kinase C; Auwerx, 1991]. The most significant phenotypic differences demonstrated by U937 cells compared with THP-1 was the constitutive ability of U937 but not THP-1 to express CD16 and the ability of THP-1 but not U937 cells to express significant levels of HLA class II following IFN-γ differentiation (Table 2.5).

U937 cells have been used extensively as models for macrophage function in a wide range of bacterial and viral infections. They have been shown to be capable of both pinocytosis and phagocytosis, to demonstrate selective oxidative metabolism, and to release cytokines in response to infection (Abshire & Neidhardt, 1993; Bianchi *et al.*, 1997; Caron *et al.*, 1994a, 1994b, 1994c, 1994d; Husmann & Johnson, 1992; Iwamoto *et al.*, 1997; King *et al.*, 1991; Pearlman *et al.*, 1988). While U937 cells have only been used fairly recently and comparatively rarely to investigate the role of human macrophages in mycobacterial infections (Lederman *et al.*, 1994; Ghassemi *et al.*, 1995; Jagannath *et al.*, 1998), THP-1 cells have been used extensively to study the interaction of a wide variety of mycobacterial species with human macrophages (Beimnet *et al.*, 1996; Friedland *et al.*, 1992; Hayashi *et al.*, 1997; Knutsen *et al.*, 1998; Lee & Horwitz, 1995; Lim *et al.*, 1997; and Lopez-Ramirez *et al.*, 1994). These studies have investigated issues ranging from mycobacterial modulation of

macrophage adhesion molecules, heat shock protein (HSP) expression and host gene manipulations following infection. The majority of these studies have empirically used these cell lines without investigating whether they are truly representative of human monocyte or macrophage function. While several studies have proposed that U937 cells share important similarities to human alveolar macrophages, the first cells to be encountered by inhaled *M. tuberculosis* (Gilbert *et al.*, 1985; Sheth *et al.*, 1988), this is the first study to investigate their usefulness compared with other models for human macrophage function in mycobacterial infections (THP-1 cells, human monocytes and differentiated macrophages).

The phagocytic function of macrophages is a central element in host defense against mycobacterial infection and uptake is generally accepted to be a macrophage-determined event initiated by direct mycobacterial binding to specific macrophage receptors (Ernst, 1998). Results presented in this chapter demonstrate that the efficiency of *M. tuberculosis* phagocytosis by U937, and to a lesser extent THP-1, is highly dependent on both the agent used to induce differentiation and the presence of serum opsonins. Differentiation with PMA exerted the most potent influence on phagocytic ability for both cell lines. Although THP-1 cells, human monocytes and differentiated macrophages generally showed greater phagocytic ability compared to U937 cells, U937 cells were found to incorporate *M. tuberculosis* quite efficiently following PMA but not IFN-γ or 1,25-$(OH)_2$-vitamin D_3 differentiation (Fig. 2.5-2.7). Complement receptors, mannose receptors, surfactant receptors, scavenger receptors and CD14 have all been implicated in mycobacterial binding to human macrophages. Because significant differences in phagocytic ability exist between U937 and THP-1, it would be interesting to investigate their respective 'favoured' routes of mycobacterial uptake. While both cell lines show similarly inducible levels of CD11b, THP-1 cells show slightly enhanced (but still relatively low) CD14 expression following differentiation. By comparison, CD16 was constitutively expressed by U937 but not by THP-1 cells. Unfortunately, little is known about the cell surface expression of mannose receptors, surfactant receptors, and scavenger receptors on U937 and THP-1 cells. Regardless of the underlying reason for the differences in phagocytic ability, in this particular respect the results indicate that THP-1 cells may be functionally more mature than U937 cells.

Electron microscopy of *M. tuberculosis*-infected U937 cells, however, clearly showed that a significant proportion of the cells contained intracellular bacilli within membrane bound

vacuoles. Schaible *et al.* (1998) recently demonstrated that, while resting macrophages predominantly incorporate bacilli into individual vacuoles (as shown for U937 cells in this study), there is a marked trend towards larger communal vacuoles containing 2 or more mycobacteria following activation of the host macrophage. Resting macrophages show an increase in individual vacuoles as the bacteria enter exponential growth, suggesting that the "efficiency" of the infection is indicated by the percentage of vacuoles containing only single bacilli. While the majority of infected U937 cells contained bacilli within individual vacuoles, a small proportion of the U937 population also contained communal vacuoles 2 hours post infection.

The production of respiratory burst via activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is one of the most important effector mechanisms in the bactericidal activity of phagocytes (Crawford *et al.*, 1994). In spite of this, the sensitivity of mycobacterial species to toxic oxygen species remains controversial (Walker & Lowrie, 1981; Chan *et al.*, 1992) and studies have shown that *M. tuberculosis* actively employs a number of mechanisms to evade the toxic effects of reactive oxygen intermediates (Brennan *et al.*, 1990; Wright & Silverstein, 1983). Differentiation of myeloid cells is none-the-less associated with the acquisition of a functional capacity to generate reactive oxygen species, such as superoxide, hydrogen peroxide, hypohalous acid and hydroxyl radicals (Rosen *et al.*, 1995). In this study the capacity of U937 cells to perform membrane-associated oxidative burst following infection with *M. tuberculosis* or activation with PMA was compared with THP-1 cells, human monocytes and macrophages and detected by the reduction of NBT and dihydrorhodamine-123 dyes. In response to PMA-activation, NBT reduction was stimulated similarly in both U937 and THP-1 cells cultured with 1,25-$(OH)_2$-vitamin D_3 and less efficiently with IFN-γ. Synergism was demonstrated following simultaneous exposure to 1,25-$(OH)_2$-vitamin D_3 and IFN-γ. PMA differentiated cells, in contrast, showed only limited ability to reduce both NBT and dihydrorhodamine-123. Despite relatively efficient phagocytosis of *M. tuberculosis*, both U937 and THP-1 cells failed to exhibit significant respiratory burst activity in response to infection (Fig. 2.5 & 2.8). Compared with freshly isolated human monocytes and terminally differentiated macrophages, U937 cells demonstrated reduced ability to release oxidative metobolites (as determined by dihydrorhodamine-123 reduction) in response to infection with *E. coli* and *M. tuberculosis*.

While human monocytes have demonstrated the ability to produce reactive oxygen and nitrogen intermediates (the latter being detected much less frequently) in response to mycobacterial infection, numerous reports have shown that within *in vitro* cultured human cells these mechanisms are inadequate to control the intracellular growth and survival of both the virulent H37Rv strain of *M. tuberculosis* (Silver *et al.*, 1998) and the avirulent strains H37Ra and BCG (Molloy *et al.*, 1994; Paul *et al.*, 1996). The ability of human monocytes to differentiate between mycobacterial species on the basis of virulence has been described but appears to depend on the maturational status and period of *in vitro* differentation of the infected phagocyte (Douvas *et al.*, 1986; Jagannath *et al.*, 1996; Silver *et al.*, 1998). The present study investigated (i) whether U937 and THP-1 cells could support the intracellular growth of *M. tuberculosis*, (ii) whether these cells were able to discriminate between mycobacterial species on the basis of virulence and (iii) whether the mechanism used to induce differentiation had any impact on their ability to control intracellular infection. U937 cells were better able to support intracellular mycobacterial growth than THP-1 cells. Like human monocytes (Jagannath *et al.*, 1996), both cell lines were approximately 1.5- to 4.0-fold more permissive to the growth of virulent than attenuated mycobacterial strains, irrespective of the agent used to induce differentiation (Table 2.4). Similarly, IFN-γ-differentiated U937 and THP-1 cells were more permissive to the growth of both *M. tuberculosis* and BCG than cells differentiated with PMA or 1,25-$(OH)_2$ vitamin D_3 demonstrating that maturational status does influence the ability of these cells to control intracellular mycobacterial growth.

Several studies have demonstrated that intracellular pathogens can affect the antigen-presenting and accessory cell functions of monocytes due to changes in the expression of molecules important for their interaction with T cells, presumably by distortion of signaling pathways that activate monocytes (Reiner, 1994). In *M. tuberculosis* infection, Pancholi *et al.* (1993) demonstrated that chronically infected macrophages stimulated T cell proliferation poorly compared to cells infected for shorter periods of time. It has since been reported that altered antigen presentation following mycobacterial infection is associated with reduced expression of cell surface HLA class II (Gercken *et al.*, 1994; Waddee *et al.*, 1995; Hmama *et al.*, 1998), reduced expression of B7 and increased expression of ICAM-1 (Saha *et al.*, 1994; Lopez-Ramirez *et al.*, 1994). Hmama and coworkers (1998) recently found that IFN-γ induced expression of HLA-DR by THP-1 cells was markedly reduced in cells infected with *M. tuberculosis* due to defective transport and processing of HLA class II molecules through the

endosomal/lysosomal system. The present study found that although chronic mycobacterial infection of PMA-differentiated U937 cells had no detectable effect on HLA class II expression, the level of HLA class I expression on cells infected with virulent *M. tuberculosis* H37Rv but not BCG was significantly increased. The inability of mycobacterial infection to influence HLA class II expression on U937 cells is not surprising since this cell line has previously been shown to lack the ability to express HLA class II as a result of hypomethylation of the relevant gene (Peterlin *et al.*, 1984). The present study confirmed that no cell surface HLA class II molecules were inducible using IFN-γ, PMA, GM-CSF or 1,25-$(OH)_2$ vitamin D_3 (Fig. 2.4 & Table 2.1). On the other hand, the increased cell surface expression of HLA class I following infection with virulent but not attenuated mycobacterial strains is of particular interest since experiments described in the following chapter (Chapter 3) have investigated the ability of U937 cells to present mycobacterial antigens to HLA class I-restricted T cells. Although the role of HLA class I presentation in mycobacterial infections is contentious, there have been isolated reports indicating that infection with virulent *M. tuberculosis* does facilitate the escape of exogenous antigens into the HLA class I processing pathway (Mazzaccaro *et al.*, 1996) and that mycobacterial antigens are able to gain access to the alternate HLA class I pathway (Canaday *et al.*, 1999). Given the ability of *M. tuberculosis* to evade normal HLA class II-restricted antigen presentation (Gercken *et al.*, 1994; Waddee *et al.*, 1995; Hmama *et al.*, 1998), it may be important that HLA class I processing and HLA class I-restricted cytotoxic T lymphocytes contribute to detection of intracellular, 'hidden' *M. tuberculosis*. A recent report by Kurts *et al.* (1998) demonstrated that high antigenic loads are strongly associated with increased 'cross presentation' of exogenous antigens by HLA class I molecules. This may be relevant to this particular study since U937 cells were found to be particularly permissive to the intracellular growth of mycobacteria compared with THP-1 cells (Fig. 2.9), a factor which could possibly favour increased mycobacterial antigen loading into the HLA class I pathway.

This study has demonstrated that, in many respects, the U937 cell line represents a useful model for a variety of different aspects within the framework of mycobacteria-macrophage interactions. Comparing the phenotypes and functional abilities of U937 with THP-1 cells, human monocytes and macrophages, some maturational differences were evident. Although U937 cells were found to be capable of phagocytosing *M. tuberculosis* efficiently following differentiation, their phagocytic ability was reduced compared with THP-1, human monocytes and terminally differentiated macrophages. More importantly, following

phagocytosis, U937 cells were found to be more permissive to the intracellular growth of *M. tuberculosis* than THP-1 cells and chronically infected U937 cells were found to express significantly increased levels of cell surface expression of HLA class I compared with uninfected cells.

The inability of U937 cells to constitutively and inducibly express HLA class II despite expressing high levels of cell surface HLA class I (Sundstrom & Nilsson, 1976; Peterlin *et al.*, 1984) has provided a unique opportunity to investigate the ability of human macrophages to process and present mycobacterial antigens to T cells in the absence of a functional HLA class II presentation pathway (a subject which will form the major focus of Chapter 3). This characteristic in particular, together with the ability of these cells to phagocytose *M. tuberculosis* relatively efficiently, to readily support the intracellular growth of these organisms and to show significantly increased cell surface HLA class I expression following chronic infection, makes the U937 cell line a very attractive model for further investigations into the presentation of mycobacterial antigens to HLA class I-restricted CTLs.

CHAPTER 3

EVALUATION OF U937 AS A HUMAN MACROPHAGE MODEL FOR HLA CLASS I-RESTRICTED PRESENTATION OF MYCOBACTERIAL ANTIGENS

3.1 INTRODUCTION

The importance of $CD4^+$ T cell-mediated immunity is well-documented in tuberculosis (Barnes *et al.*, 1994). The role of $CD8^+$ T cells in mycobacterial infections has proven more difficult to demonstrate. Evidence from murine models of tuberculosis showed that $CD8^+$ CTL lines were cytolytic towards macrophages infected with *M. tuberculosis* (De Libero *et al.*, 1988), β_2-microglobulin deficient mice were reported to be more susceptible to mycobacterial infection than their wild-type littermates (Flynn *et al.*, 1992), and mice with a targeted disruption in the gene for CD8 were found to be highly susceptible to infection with *M. tuberculosis* (D'Souza *et al.*, 1998). Until very recently, little success had been achieved in determining the role of HLA class I-restricted $CD8^+$ T cells in human immunity to tuberculosis with mycobacteria-responsive $CD8^+$ T cells only rarely having been isolated from patients with tuberculosis (Rees *et al.*, 1988).

Generally, HLA class I-restricted $CD8^+$ T cells respond to microbial antigens present in the cytoplasm of infected antigen-presenting cells. These antigens are processed via the classical HLA class I processing pathway, in which cytoplasmically situated protein antigens are degraded into peptide fragments by proteasomes. These peptides are transported into the ER, where they become associated with HLA class I molecules. The resulting peptide/HLA class I complexes are then routed through the Golgi complex and transported to the cell surface (Heemels & Ploegh, 1995; York & Rock, 1996).

The mechanism by which mycobacterial antigens derived from intracellular but phagosomally-situated *M. tuberculosis* might gain access to the HLA class I presentation pathway is slowly emerging. The ability of exogenous bacterial and particulate antigens to gain access to the HLA class I processing pathway has been described previously (Rock *et al.*, 1990; Kovacsovics-Bankowski *et al.*, 1993; Pfeifer *et al.*, 1993; Norbury *et al.*, 1995; Harding, 1996) and there is now evidence to suggest that infection with *M. tuberculosis* is able to facilitate such an exchange of antigens between phagosomes and the cytoplasm (Mazzaccaro *et al.*, 1996). Furthermore, a recent report by Canaday *et al.* (1999) has demonstrated that *M. tuberculosis*-derived antigens are capable of accessing the HLA class I pathway by an alternative route that does not require proteosomal processing or trafficking through the ER. The efficiency of the phagosome-to-cytosol HLA class I processing pathway

for presentation of exogenous antigens remains controversial, however, and does not seem to be a constitutive characteristic of all professional antigen presenting cells (Reis e Sousa and Germain, 1995).

With the emergence of more sophisticated immunological techniques, and a corresponding renewed interest in the role of $CD8^+$ CTLs in tuberculosis, a number of studies have been published very recently demonstrating the existence of classical HLA class I-restricted *M. tuberculosis*-responsive human $CD8^+$ T cells (Turner and Dockrell, 1996; Tan *et al.*, 1997; Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998; Mohagneghpour *et al.*, 1998; Canaday *et al.*, 1999). Many of these studies have focused on the ability of these $CD8^+$ T cells to produce cytokines or proliferate in response to stimulation with defined mycobacterial peptides (Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998; Canaday *et al.*, 1999). Those reports that have demonstrated the existence of cytotoxic $CD8^+$ T cells capable of lysing *M. tuberculosis*-infected target cells have used very defined priming conditions to facilitate the generation of $CD8^+$ CTLs. Tan *et al.* (1997) reported that mycobacterial-specific $CD8^+$ CTL activity was only demonstrable following *in vitro* co-culture with specific growth factors, while Mohagheghpour *et al.* (1998) made use of mycobacterial peptide pulsed dendritic cells to efficiently prime $CD8^+$ CTL effector cells.

The present chapter describes a relatively simple, robust, and easily adaptable *in vitro* model that makes use of the human macrophage cell line U937 (Sundstrom and Nillson, 1976) to present mycobacterial antigens to human HLA class I-restricted $CD8^+$ CTLs. U937 cells were selected because they constitutively express high levels of cell surface HLA class I molecules while expressing undetectable and uninducible levels of HLA class II both at the mRNA level and at the cell surface (Chapter 2; Peterlin *et al.*, 1984). In addition, U937 cells have been shown to have a relatively efficient phagosome-to-cytosol pathway for delivery of exogenous antigens to the HLA class I processing pathway (Harris *et al.*, 1995). Results presented in this chapter demonstrate that *M. tuberculosis*-infected U937 target cells are more rapidly and strongly lysed by $CD8^+$ CTLs than infected autologous macrophages. U937 cells provided not only a useful *in vitro* human macrophage model for selective evaluation of HLA class I-restricted $CD8^+$ CTL function in mycobacterial infections but were also shown to be a sensitive indicator for $\gamma\delta^+$ CTL activity.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines and culture conditions

U937, Daudi, and K562 were maintained routinely in suspension culture in RPMI-1640 (Flow Laboratories) supplemented with 10% (v/v) heat-inactivated FCS (Delta Bioproducts), 2mM L-glutamine, and 10mM HEPES at 37°C in a humidified atmosphere of 5% CO_2. Passage was performed every 2-3 days. Cells used in all experiments were in the logarithmic phase of growth and viability exceeded 95% as measured by Trypan blue exclusion.

3.2.2 Induction of differentiation

Differentiation was induced by treating U937 cells (5 x 10^5 cells.ml^{-1}; 5ml.well^{-1} in 6-well plates) with PMA (5 ng.ml^{-1}; Sigma; Hewison *et al.*, 1992), 1,25-$(OH)_2$-vitamin D_3 (10^{-7} M; Roche Products Ltd; Zuckerman *et al.*, 1988), or recombinant human IFN-γ (100-200 U.ml^{-1} rhIFN-γ; Cetus; Roberts *et al.*, 1991) for at least 48 hours at 37°C, as described in Chapter 2.

3.2.3 Mycobacterial growth conditions

M. tuberculosis H37Rv and *M. bovis* BCG were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% OADC (State Vaccine, Cape Town, South Africa) and 0.02% Tween-80 (Merck, Darmstadt, Germany) at 37°C, 5% CO_2. Mid-log phase cultures were snap frozen in liquid nitrogen and stored at -70°C. Mycobacterial viability was monitored by determining colony forming units (CFU) of serial 10-fold dilutions of the bacterial preparations on Middlebrook 7H10 agar (Difco) supplemented with 10% OADC. Frozen mycobacterial cultures were thawed immediately before use and clumps disrupted by repeated passage through a 25-gauge needle. In all experiments, bacterial preparations were coated with serum opsonins by incubating thawed aliquots with an equal volume of fresh human serum for 30 minutes at 37°C.

3.2.4 Isolation of human PBMC

Peripheral blood mononuclear cells (PBMC) were obtained from healthy adult volunteers by centrifugation of heparinized venous blood over Ficoll-Hypaque density gradients (Sigma) as previously described (Boyum *et al.*, 1968).

3.2.5 Selection of human subjects and tissue typing

U937 cells were shown to express HLA-A3, -B18, -B51, and -Cw1 (Chapter 2). Twelve suitable HLA-matched or –mismatched healthy adult employees at Groote Schuur Hospital and Western Province Blood Transfusion Centre were recruited for this study. HLA typing of U937 and human PBMC was performed using the microdroplet lymphocyte cytotoxicity test as described by Bodmer *et al.* (1977) and was determined according to phenotype.

3.2.6 Flow cytometry

An Epics Profile II flow cytometer (Coulter) was used to perform immunophenotyping. Histograms were gated on the PBMC populations by forward scatter (FS) versus log side scatter (LSS). Monoclonal antibodies were from Coulter (Hialeah, FL) or Becktin-Dickinson (San Jose, CA) and were directed against T cell phenotypic markers CD4, CD8, γδ, and CD3, and the T cell activation markers IL-2 receptor (CD25) and HLA-DR. All the monoclonal antibodies were directly conjugated with FITC or RD1 (PE). Isotypic controls were used in all cases to set cursors to allow 2% false positives and antibodies were used as the concentrations suggested by the manufacturer.

3.2.7 T cell rosetting and monocyte-depletion

Adsorbed FCS was prepared by incubating 50 ml FCS (Delta Bioproducts) with 25 ml washed sheep red blood cells (SRBC's) for 1 hour at 37°C, followed by 16 hours at 4°C. The FCS-SRBC suspension was centrifuged, the FCS collected, filtered and stored at –20°C. Freshly isolated PBMC were re-suspended at 5 x 10^6 cells per ml in 40% adsorbed FCS PBS. The SRBC's used for rosetting (5-10 ml) were washed three times with PBS. An equal volume of freshly isolated PBMC suspended in 40% absorbed FCS PBS was added to 3%

packed SRBC in 40% absorbed FCS PBS. This was under-layed with Ficoll-Hypaque (5ml) and incubated on ice for 45 minutes. The PBMC-SRBC gradient was finally centrifuged for 30 minutes at 900g. The supernatant was aspirated and the pellet containing SRBC-T cell rosettes was treated with NH_4Cl to lyse the SRBC's. The rosetted T cells were then washed three times with 10% AB RPMI-1640 and adjusted to the desired concentration. T cells purified using this method yielded populations which were approximately 85-95% $CD3^+$ by flow cytometry.

For depletion of monocytes, T cells were purified by two cycles of SRBC rosetting. Purified T cells were further depleted of monocytes by an overnight incubation at 37°C, following which the non-adherent population was removed and re-plated for a further 2 hours at 37°C to allow for any further monocyte adherence. Non-adherent cells were then removed, washed once and adjusted to the desired concentration in 10% AB RPMI-1640. Measurement of the percentage of purified cells expressing monocyte-specific markers $CD14^+$ and $HLA\text{-}DR^+$ was used as an indicator of residual monocyte contamination. The proportion of cells staining positive for either $CD14^+$ or $HLA\text{-}DR^+$ were at the detection limit of the flow cytometer (<1%, respectively).

3.2.8 Stimulation of mycobacterial-specific CTLs and isolation of T cell subsets

Conventional mycobacterial priming

PBMC (1×10^6 cells.ml^{-1}) were stimulated with PPD (3 μg.ml^{-1}; Central Veterinary College, Weybridge, UK), *M. tuberculosis* H37Rv or BCG (1 CFU.$cell^{-1}$) for 6 days at 37°C. On day 6, the primed CTLs were adjusted to the desired concentration to be used in the cytotoxicity assays (described below).

In experiments requiring isolation of individual T cell subsets, the separation was done on day 5 using Minimacs magnetic bead separation (Miltenyi Biotec, CA) and the cells were allowed to recover overnight. Purification was done according to the manufacturer's directions (Miltenyi Biotec). $CD4^+$ and $CD8^+$ T cells were selected using directly-conjugated magnetic beads while $\gamma\delta^+$ T cells were enriched by sequential $CD4^+/CD8^+$ T cell-depletion or isolated using indirectly-conjugated γδ mAb (Becton-Dickenson) and goat anti-mouse IgG_1

magnetic beads (Miltenyi Biotec). Briefly, PBMC (1×10^7), re-suspended in 80 µl separation medium [PBS containing 5 mM EDTA, 0.5% bovine serum albumin (BSA)] were incubated together with 20 µl CD4-, CD8- or γδ-coated magnetic beads for 15 minutes at 4°C. Immediately prior to use, a MS^+ separation column (Miltenyi Biotec) was washed with 500 µl separation medium and placed onto the Minimacs magnet. The labelled cells were pipetted onto the column and allowed to run through. The column was washed once with 500 µl separation buffer and the effluent collected as the negative fraction. The column was washed a further three times with 500 µl separation buffer before the column was removed from the magnet. The column was placed into a capture tube and the positively labelled (attached) cells flushed out in 1 ml separation buffer using a plunger. These cells were collected as the positive fraction. For sequential CD4/CD8-depletion, a flow resistor (provided by the manufacturer) was attached to the MS^+ column to maximise the efficiency of CD4 or CD8 retention on the column. The purity of the $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cell fractions was determined by flow cytometry. The purity of the positively selected $CD4^+$ and $CD8^+$ T cell subsets ranged between 95-99% with the $CD4^+$ and $CD8^+$ purified T cell populations showing less than 1 % contamination with CD8 or CD4 T cells, respectively. The purity of the $\gamma\delta^+$ T cell fractions ranged between 85-90% but showed less than 1 % contamination with either $CD8^+$ or $CD4^+$ T cells. On day 6, the purified T cell populations were adjusted to the desired concentration to be used in ^{51}Cr release assays or used to generate T cell lines or clones.

To investigate the influence of culture conditions on the efficiency of $CD8^+$ CTL priming, several alternative approaches were assessed for their ability to prime *M. tuberculosis*-specific $CD8^+$ CTLs. The first two approaches manipulated culture conditions at the level of the CD8 T cell by addition of growth factors or the early separation of CD8 T cell effectors. The last two approaches manipulated culture conditions at the level of the antigen presenting cell by either shocking mycobacterial antigens into the HLA class I processing pathway or by using U937 cells (which are HLA class I^+/class II^- and have an efficient phagosome-to-cytosol delivery system) to present antigen.

Supplemented with low dose rIL-2

PBMC were primed with *M. tuberculosis* (1 $CFU.ml^{-1}$) or PPD (3 $\mu g.ml^{-1}$) as described above for 5 days but rhIL-2 (10 $iu.ml^{-1}$; Cetus) was added at the initiation of the experiment

(adapted from Tan *et al.*, 1997). $CD8^+$ T cells were isolated on day 5 using Minimacs magnetic bead separation (described above; Miltenyi Biotec) and the purified cells allowed to recover for at least 16 hours prior to assessment of their cytolytic activity.

Early CD8 selection and rIL-2 stimulation

PBMC were primed with *M. tuberculosis* (1 $CFU.ml^{-1}$) for 24 hours following which the $CD8^+$ CTL subset was isolated using Minimacs magnetic bead separation (described above; Miltenyi Biotec) and cultured in the presence of rIL-2 (50-100 $iu.ml^{-1}$) for the remaining 5 days. No additional antigen presenting cells were added to the purified $CD8^+$ T cells. On day 6, CTLs were adjusted to the desired concentration to be used in the cytotoxicity assays.

Osmotic lysis

To determine whether poor $CD8^+$ CTL priming was the result of inefficient HLA class I presentation of mycobacterial antigens, osmotic shock was investigated as an alternative method for loading of mycobacterial antigens into the HLA class I pathway. Macrophage target cells were prepared according to the method described by Moore *et al.* (1988). Autologous human macrophages were prepared by culturing PBMC (1×10^6 $cells.ml^{-1}$; 10% assumed to be macrophages) in 6-well tissue culture plates. After 24 hours, non-adherent cells were removed and the monolayers washed three times with warmed 10% AB RPMI. The final wash medium was removed from the macrophage monolayers and replaced with 1ml pre-warmed hypertonic medium [0.5 M sucrose, 10% w/v polyethylene glycol-1000, 10mM HEPES in RPMI-1640, pH 7.4] containing either *M. tuberculosis* (1×10^5 $.ml^{-1}$) or PPD (3 $\mu g.ml^{-1}$) for 10 minutes at 37°C. This was diluted to 10 ml with pre-warmed hypotonic medium (60% RPMI-1640, 40% dH_2O) and incubated for a further 2-3 minutes at 37°C. The target cells were washed three times with pre-warmed 10% FCS RPMI-1640 and overlaid with monocyte-depleted PBMC in 10% AB RPMI-1640 to give a final concentration of 10 responder cell per osmotically shocked target cell. On day 5, $CD8^+$ T cells were isolated using Minimacs magnetic bead separation (described above; Miltenyi Biotec) and allowed to recover for at least 16 hours prior to the cytotoxicity assay.

U937 as antigen presenting cells

U937 cells were induced to differentiate with PMA for 48 hours. Differentiated cells were either (i) uninfected and then paraformaldehyde (PFA) fixed; (ii) infected with *M. tuberculosis* H37Rv (2 hours, 5 CFU.cell^{-1}) and then PFA fixed; or (iii) PFA fixed and then co-cultured with *M. tuberculosis* (6 days, 5 CFU.cell^{-1}). For the PFA-fixation steps, adherent U937 cells were EDTA-detachment and then fixed with 0.75% paraformaldehyde (PFA) in PBS using the methods described by Pfeifer *et al.* (1993) and Moreno and Lipsky (1986). Briefly, the cells were washed three times to remove excess serum, and re-suspended in PBS containing a final concentration of 0.75% PFA (Merck, Darmstadt, Germany) at 37°C for 5 minutes. The reaction was stopped by the addition of 0.4M lysine (Sigma). Cells were washed once with PBS and twice with 10% AB serum RPMI-1640 and re-suspended in 10% AB RPMI-1640. "Leaching" of any remaining PFA was accomplished by incubation at 37°C for at least 60 minutes, after which time the cells were washed once, re-suspended in fresh 10% AB RPMI-1640 and adjusted to desired concentration.

For these experiments, the ability of U937 cells to present antigen to and prime HLA class I-matched (monocyte-depleted) PBMC proliferative ability was investigated. Monocyte-depleted PBMC (1 x 10^5 cells.well^{-1}) were cultured together with (i) uninfected (and then fixed); (ii) *M. tuberculosis*-infected (and then fixed); or (iii) fixed and then *M. tuberculosis*-infected U937 cells (1 x 10^4 U937 cells.well^{-1}) in triplicate wells for 6 days. [^{3}H]-thymidine (1 μCi.well^{-1}) was added to each well for the last 18 hours of the assay. To inactivate any residual infectious *M. tuberculosis*, cells in each well were fixed by the addition of an equal volume of 1% PFA for 1 hour. Cells were harvested using an automated cell harvester (Titertek^R 630) and radioactivity (cpm) was measured using a liquid scintillation counter (Tricarb 4640).

3.2.10 Cytotoxicity assays

Adherent target cytotoxicity assay

The adherent target cytotoxicity assay using autologous macrophages has been described previously by Lorgat *et al.* (1992). Monocyte-derived macrophages (day 6; plated at 10^5

PBMC.well^{-1} in microtitre wells; 10% assumed to be macrophages) were infected with *M. tuberculosis* H37Rv (5 CFU.cell^{-1}), pulsed with the irrelevant streptococcal antigen streptokinase-streptodornase (SK-SD; 250 iu.ml^{-1} SK; 62.5 iu.ml^{-1} SD; Lederle Laboratory), or left uninfected and concurrently labelled with ^{51}Chromium (6 μCi.well^{-1}; ^{51}Cr; Amersham) for 16 hours at 37°C. The target cells were washed three times with pre-warmed 10% FCS PBS. *M. tuberculosis*-primed PBMC effector cells were serially diluted and added to triplicate wells at varying concentrations to achieve final effector:target ratios of 10:1, 3:1 and 0.3:1. Wells containing medium alone were used to determine spontaneous ^{51}Cr release. The effector and target cells were incubated for either 4 or 16 hours (as indicated in the figure legends) at 37°C, 5% CO_2. The contents of each well was removed and counted to determine the amount of spontaneous ^{51}Cr-release (cpm). Triton X-100 (5% in PBS; 100μl) was added to each well for an additional 4 hours, removed and counted to determine the maximum amount of ^{51}Cr released. The spontaneous:maximum ^{51}Cr-release never exceeded 15 %. Specific target cell lysis for triplicate wells was calculated as follows: [mean test cpm/(mean test cpm + mean maximum cpm)] and expressed as a percentage.

Non-adherent target cytotoxicity assay

U937 cells were induced to differentiate with PMA, IFN-γ or 1,25-$(OH)_2$-vitamin D_3 for 48 hours prior to the assay as described previously. The cells were either infected with *M. tuberculosis* H37Rv (5 CFU.cell^{-1}), pulsed with SK-SD (250 iu.ml^{-1} SK; 62.5 iu.ml^{-1} SD; Lederle Laboratory), or left uninfected for16 hours at 37°C. The cells were subsequently labelled with 250 μCi ^{51}Cr for 60-90 minutes, washed three times with cold 10% FCS PBS and adjusted to the 1 x 10^5 cells per ml. *M. tuberculosis*-primed PBMC effector cells were serially diluted and added to triplicate wells at varying concentrations to achieve final effector:target ratios of 10:1, 3:1, 1:1 and 0.3:1. The effector and target cells were co-incubated for 4 hours at 37°C, 5% CO_2. Specific target cell lysis was calculated from the mean of triplicate wells using the following equation: [mean test cpm-mean spontaneous cpm]/[mean maximum cpm-mean spontaneous cpm] and expressed as a percentage. Spontaneous ^{51}Cr release was determined from sextuplicate wells of ^{51}Cr-labelled targets incubated with medium alone while maximum ^{51}Cr-release was measured from sextuplicate wells of targets treated with 5% Triton X-100 for the duration of the assay. The spontaneous:maximum ^{51}Cr-release never exceeded 25 %.

3.2.11 Generation of T cell clones

T cell clones were generated by limiting dilution as previously described from *M. tuberculosis*-primed bulk PBMC cultures, or from short-term $\gamma\delta^+$ T cell lines (Bach, 1983). Cells to be cloned were washed in PBS and re-suspended at 10^6 cells.ml^{-1} in fresh culture medium containing RPMI-1640 supplemented with 10% autologous serum, 2-mercaptoethanol (5 x 10^{-5} M; BDH Chemicals, Poole, England), non-essential amino acids (10mM; Highveld Biologicals, Johannesburg, South Africa), L-glutamine (2mM; Highveld Biologicals), sodium pyruvate (1mM; Highveld Biologicals) (cloning medium). Cells were then serially diluted as required in cloning medium. Cloning was done in Terasaki plates and responder cells were seeded at 1.0, 0.3 or 0.2 cells per well. Feeder cells used were autologous irradiated (40 Gy) PBMC (10^4 cells.well^{-1}). Cells were stimulated with rhIL-2 (100 iu.ml^{-1}) and either PHA (1.1 x 10^{-2} mu.ml^{-1}) or *M. tuberculosis* (0.1 CFU.feeder^{-1}), in a final volume of 20 µl. Plates were incubated at 37°C, 5% CO_2. Cell growth was determined by visual inspection using an inverted microscope (Nikon TMS, Japan) after 8-10 days. Wells were scored positive if greater than one forth of a particular well surface was covered with cells.

3.2.12 Expansion and maintenance of clones

Positively scored wells were selected on day 9 to 10 by transferring the contents of individual positive Terasaki wells to U-bottomed 96-well microtitre plates. Re-stimulation was performed using irradiated autologous PBMC as feeder cells (10^5 cells.well^{-1}), rIL-2 (100 U.ml^{-1}) and either PHA (1.1 x 10^{-2} mu.ml^{-1}) or *M. tuberculosis* H37Rv (0.1 CFU.feeder cell^{-1}) depending on the stimulus used for the initial priming, in a final volume of 100 µl per well of cloning medium. The growing cultures were supplemented with 100 µl fresh cloning medium containing rIL-2 (100 U.ml^{-1}) 3-4 days after transfer and cells were re-suspended regularly to avoid formation of large clusters. Seven days after transfer, clones were split into two wells and re-stimulated with feeders, antigen, and rIL-2 as indicated above. Cells were transferred to 24 well tissue culture plates, usually by the end of the second passage in microtitre wells, and re-stimulated as previously described in a final volume of 1 ml. Cultures were re-suspended regularly, supplemented with fresh cloning medium every 3-4

days and passaged weekly. Once sufficient cells had been obtained, aliquots of clones were cryo-preserved and the remainder of the cells analysed as indicated below.

3.2.13 Selection and characterisation of clones

The T cell clones were selected firstly on the basis of their cytolytic function against *M. tuberculosis*-infected U937 target cells, and secondly by phenotype and mycobacterial-specific proliferative ability.

Cytotoxicity

The clones were screened for their cytolytic activity against *M. tuberculosis*-infected U937 target cells in a 4 hour ^{51}Cr-release assay (described above). The initial assessment of cytotoxic ability was performed using 50-100 µl of each respective clone at unknown cell concentration against either uninfected or infected PMA-differentiated U937 cells (10^4 cells.well^{-1}). Clones that were able to kill U937 target cells in a mycobacterial-specific manner (*M. tuberculosis*-specific kill >5% above background was chosen as an arbitrary cut-off point) were selected for further analysis. Cytotoxic function of the "positive-killer clones" was then confirmed in triplicate and at multiple effector to target ratios. Cytolytic activity of the T cell clones was measured on day 2 or 3 following re-stimulation with feeders, antigen and rIL-2.

Phenotypic analysis

Phenotypic analysis of the clones obtained was performed by flow cytometry as previously described using 3-colour fluorescence (CD4-FITC/CD8-PE/CD3-PI). CD3$^+$ T cell clones that were both CD4- and CD8-negative were then screened using γδ-FITC/CD3-PE dual colour fluorescence.

Proliferation

T cell clones were screened for their antigen reactivity by stimulating resting clones (1×10^6 cells.ml^{-1}) with fresh irradiated autologous feeders (1×10^6 cells.ml^{-1}) and with either PHA (1.1×10^{-2} mu.ml^{-1}), PPD (3 μg.ml^{-1}), or *M. tuberculosis* H37Rv (0.1 CFU.feeder^{-1}) for 40-48 hours, 37°C, 5% CO_2. Proliferation to the various antigens was measured by ^{3}H-thymidine incorporation. ^{3}H-Thymidine (1 μCi.well^{-1}; specific activity 185 MB; Amersham International) was added to triplicate wells for the last 8 hours of the assay. The clones were fixed by the addition of an equal volume of 1% PFA for 1 hour to inactivate any infectious *M. tuberculosis* before being harvested using an automated cell harvester (Titertek^R 630). The radioactivity (cpm) was measured using a liquid scintillation counter (Tricarb 4640).

3.2.14 Statistical analysis

Statistical analyses were performed using the Wilcoxon ranks test for paired non-parametric data and the Mann-Whitney *U*-Test for unpaired non-parametric data (Kaplan, 1987).

3.3 RESULTS

3.3.1 Selection of HLA class I-matched donors

U937 cells were shown to express HLA-A3, -B18, -B51, and –Cw1 (Chapter 2). Twelve suitable healthy adult employees at Groote Schuur Hospital and the Western Province Blood Transfusion Centre were recruited for this study on the basis of their HLA-typing. Six of the donors were selected because of their HLA class I-compatibility to U937 [4 A3- and 2 B51-matched]. The remaining donors were HLA class I-mismatched to U937 cells. All of the selected donors showed strong proliferative responses to the purified protein derivative (PPD) of *M. tuberculosis*. The HLA-typing and PPD response of the selected donors has been summarised in Table 3.1.

Table 3.1 *HLA-typing of class I-matched and -mismatched donors*

HLA-histocompatibility	Donor	SI[a]	HLA class I-typing			HLA class II-typing	
Cell Line	U937	-	A3,-	B18,51	Cw1,-	-	-
HLA class I-matched	RG	58.8	A2,31	**B51**[b],62	Cw3,-	DR4,11	DQ3,-
	BR	7.0	A2,24	**B51**[b],7	Cw3,6	DR2,13	DQ1,-
	TS	125.4	**A3**[b],2	B35,44	Cw4,5	DR1,7	DQ1,2
	PB	77.3	**A3**[b],28	B7,42	Cw7,-	DR2,11	DQ1,7
	ES	89.1	**A3**[b],28	B35,7	Cw4,-	DR2,-	DQ1,-
	EC	91.8	**A3**[b],30	B7,65	Cw7,8	DR2,11	DQ1,7
HLA class I-mismatched	MH	12.3	A1,28	B37,62	Cw3,6	DR2,13	DQ1,-
	NP	22.9	A11,30	B13,58	Cw6,7	DR2,17	DQ1,7
	SG	52.3	A28,34	B16,40	Cw-,-[c]	DR2,14	DQ1,-
	SJ	23.4	A24,31	B7,8	Cw7,-	DR2,17	DQ1,2
	MF	40.5	A26,33	B58,-	Cw3,6	DR4,17	DQ2,8
	WM	193.5	A30,68	B42,53	Cw4,-	DR8,18	DQ4,7

[a] SI (stimulation index to PPD) was calculated as follows: [cpm following PPD stimulation] ÷ [cpm of unstimulated cells]. [b]HLA class I-matched to U937 cells. [c]Cw-,- indicates that no HLA-Cw surface antigen was detected.

3.3.2 NK and LAK activity against U937

In order to investigate the feasibility of using U937 cells to present mycobacterial antigens to HLA class I-matched T cells, the relative contribution of natural killer (NK) cell or lymphokine activated killer (LAK) cell activity was investigated (Fig. 3.1). Undifferentiated U937 cells were found to be as susceptible to NK activity as K562 (the classical NK indicator cell line). And following rIL-2 stimulation of LAK effectors (6 days), U937 cells were killed with similar efficiency as Daudi cells (the classical LAK indicator cell line). Differentiation of U937, however, significantly abrogated their sensitivity to NK- and LAK-mediated cytotoxicity.

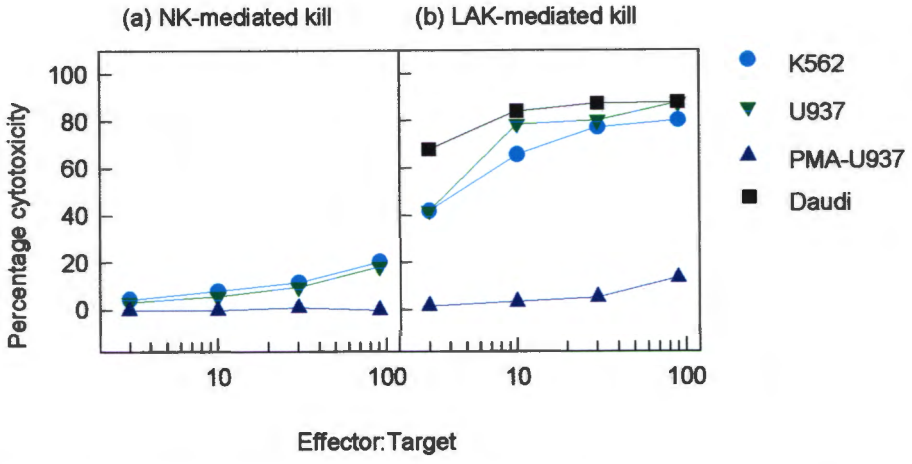


FIGURE 3.1. NK (a) and LAK (b) cell-mediated kill against untreated U937 cells (▼), PMA-differentiated U937 cells (▲), K562 (●), and Daudi (■). Cytotoxicity was determined after 4 hours. Each data point represents the mean percentage kill of duplicate experiments.

3.3.3 Effect of differentiation on cytolysis of U937 cells

To determine whether differentiated U937 cells could be used as targets for human CTL activity and whether the agent used to induce differentiation had an influence on their susceptibility to T cell-mediated cytolysis, the ability of this cell line to present mycobacterial antigens to *M. tuberculosis*-primed, HLA class I-matched CTLs was investigated (Fig. 3.2). U937 cells were treated with PMA, IFN-γ, or 1,25-$(OH)_2$-vitamin D_3 for 48 hours and then infected with *M. tuberculosis* H37Rv and used as target cells in a 4 hour ^{51}Chromium release assay. The *M. tuberculosis*-primed (day 6) CTLs used in these experiments were HLA-B51-matched to U937 target cells. Following differentiation, PMA-treated U937 cells were found to be more susceptible to mycobacterial antigen-specific CTL activity [19.7 (± 9.7) % mycobacterial-specific] than IFN-γ- [7.8 (± 4.4) % mycobacterial-specific) or 1,25-$(OH)_2$-vitamin D_3-differentiated cells [9.2 (± 4.1) % mycobacterial-specific) and their susceptibility to lysis correlated well with their respective phagocytic abilities (Fig. 2.5; r = 0.89). PMA-differentiated U937 cells were therefore used in all subsequent experiments.

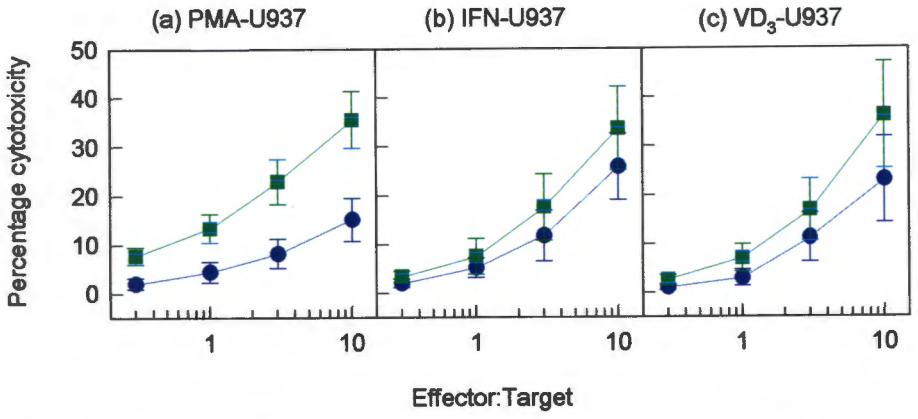


FIGURE 3.2. Comparison of *M. tuberculosis*-specific cytolysis generated against (a) PMA-, (b) IFN-γ-, and (c) 1,25-$(OH)_2$-vitamin D_3-differentiated U937 target cells. The CTLs used in these experiments were derived from a single donor (RG; Table 3.1) HLA-matched to U937 at –B51. Target cells were either infected with *M. tuberculosis* (■) or pulsed with an irrelevant streptococcal antigen SK-SD (●). Cytotoxicity was measured after 4 hours. Each data point represents the mean percentage kill of at least two independent experiments (± SEM).

Taken together, the results presented in Fig. 3.1 and 3.2 indicate that the cytolysis generated against *M. tuberculosis*-infected, PMA-differentiated U937 target cells is antigen-specific and not influenced by either NK- or LAK-mediated cytolytic activity.

3.3.4 Cytolysis of U937 and autologous macrophages

Fig. 3.3 shows the mean susceptibility of *M. tuberculosis*-infected U937, compared with autologous macrophage targets, to CTL activity. To investigate the HLA-restriction of the mycobacterial antigen-specific cytolysis generated against U937 targets, the CTLs used in these experiments were HLA-A3-matched, HLA-B51-matched or HLA-mismatched to U937 target cells. Both HLA-A3- and –B51-matched CTLs showed significantly greater ability ($p<0.05$, Wilcoxon ranks test) to lyse *M. tuberculosis*-infected U937 cells than SK-SD-pulsed cells (Fig. 3.3a & b, respectively). The HLA-B51-restricted CTLs in particular mediated 3-fold stronger mycobacterial antigen-specific cytolysis against U937 than against autologous macrophage targets. The magnitude of the mycobacterial-specific response was influenced by the HLA-restriction of the CTLs since the HLA-A3-restricted CTL population showed reduced cytolysis against antigen-pulsed U937 target cells as compared with autologous macrophage targets. Mycobacterial-specific CTLs generated from donors which were HLA-mismatched to U937 cells did not demonstrate any significant ability to lyse U937 targets ($p>0.2$), but showed cytolytic activity against autologous macrophage targets (Fig. 3.3c).

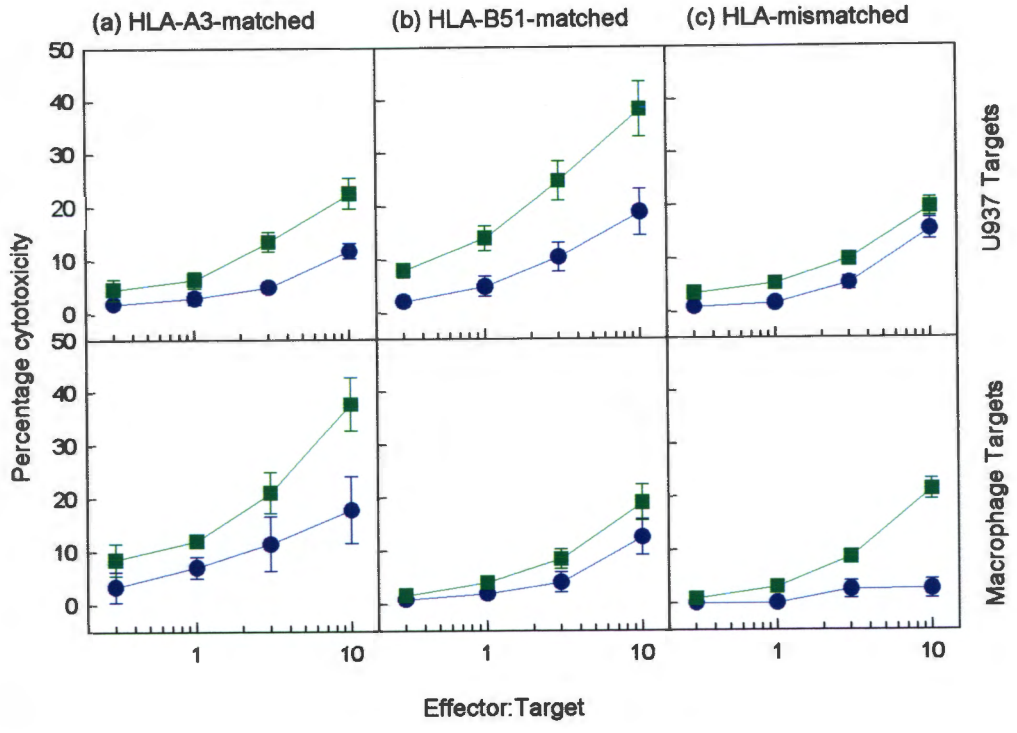


FIGURE 3.3. *M. tuberculosis*-specific cytolysis generated by (a) HLA-A3-matched, (b) HLA-B51-matched, and (c) HLA-mismatched donor CTLs against PMA-treated U937 (top panel) and autologous macrophage targets (bottom panel). Target cells were infected with *M.tuberculosis* (5 CFU.cell^{-1}) (■) or pulsed with an irrelevant antigen, SK-SD (●). Cytotoxicity against U937 and autologous macrophage targets was measured after 4 hours. Each data point represents the mean percentage cytolysis (± SEM) of at least 3 independent experiments.

Lysis by the HLA class I-matched CTL populations was found to be restricted to live organisms since both *M. tuberculosis* H37Rv- and BCG-infected (but not PPD-pulsed) U937 targets were recognised by the *M. tuberculosis*-primed CTLs and lysed with similar efficiency (Fig. 3.4). The *M. tuberculosis*-primed CTLs were, however, capable of recognising and lysing autologous macrophage targets pulsed with PPD. The inability of PPD-pulsed U937 target cells to be recognised in this context is consistent with the absence of a functional HLA class II-processing pathway in the U937 cell line.

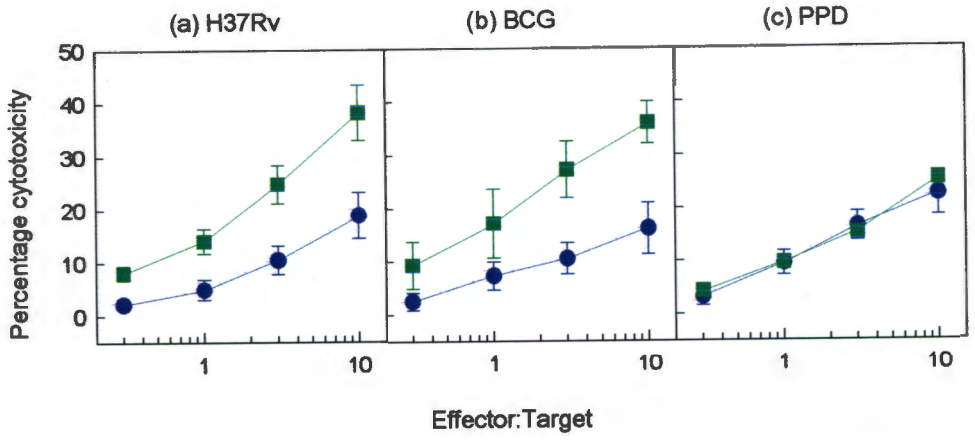


FIGURE 3.4. Mycobacterial antigen-specific cytolysis generated by HLA-B51-matched CTLs against U937 targets infected with (a) *M. tuberculosis* H37Rv, (b) BCG, or (c) pulsed with the soluble mycobacterial extract PPD. Cytotoxicity was measured against target cells infected/pulsed with mycobacterial antigens (■) or with an irrelevant antigen, SK-SD (●). Cytotoxicity was measured after 4 hours. Each data point represents the mean percentage cytolysis (± SEM) of at least 2 independent experiments.

3.3.5 T cell subset purification

To directly determine the contribution of the different T cell subsets to mycobacterial antigen-specific cytolysis generated against U937 cells, $CD8^+$, $CD4^+$ and $\gamma\delta^+$ T cells were isolated from *M. tuberculosis*-primed PBMC by positive selection and, in the case of $\gamma\delta^+$ T cells, by sequential negative selection with anti-CD8- or anti-CD4-coated magnetic beads. As shown in Fig. 3.5 (top panel), *M. tuberculosis*-activated, purified $CD8^+$ T cells were cytolytic towards HLA class I-matched *M. tuberculosis*-infected U937 but not cells pulsed with the irrelevant streptococcal antigen, SK-SD (15.7 ± 5.0 % mycobacterial-specific cytolysis, $p<0.05$). The $CD8^+$ CTL activity was HLA class I-restricted since $CD8^+$ cells from HLA class I-mismatched donors (Fig. 3.5, bottom panel) showed no significant ability to lyse *M. tuberculosis*-infected U937 targets ($p>0.1$). No cytolysis was obtained with purified $CD4^+$ T cells from both the HLA class I-matched and HLA-mismatched donors. Interestingly, the $\gamma\delta^+$-enriched CTL population showed the most significant ability to lyse *M. tuberculosis*-infected U937 target cells (17.9 ± 9.3 % mycobacterial-specific cytolysis, $p<0.02$). Although clearly mycobacterial antigen-specific, these cells were found not to be restricted to the classical HLA class I or class II molecules because $\gamma\delta^+$ CTLs from both HLA class I-matched and the HLA-mismatched donors showed strong cytolytic activity against infected U937 targets. This study therefore found that although U937 target cells are

selectively lysed by $CD8^+$ rather than $CD4^+$ CTLs, the T cell population which demonstrated the strongest mycobacterial antigen-specific cytolytic activity against U937 targets was in fact $\gamma\delta$-TCR^+.

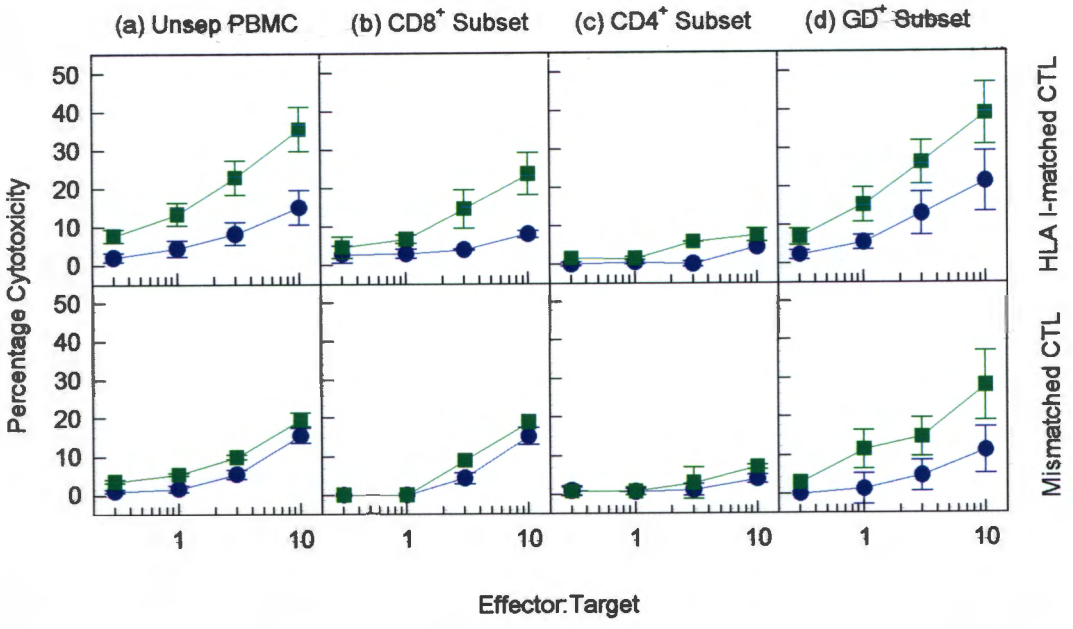


FIGURE 3.5. Characterisation of the cytolytic T cell subset generating mycobacterial-specific cytolysis against U937 target cells. The *M. tuberculosis*-primed HLA-B51-matched CTL (top panel) and the HLA-mismatched CTL (bottom panel) populations were fractionated into $CD8^+$, $CD4^+$ or $\gamma\delta^+$ T cell subsets and assessed for their respective cytolytic abilities against U937 target cells. U937 target cells were either infected with *M. tuberculosis* (5 $CFU.cell^{-1}$) (■) or pulsed with an irrelevant antigen, SK-SD (●). Cytotoxicity was measured after 4 hours. Each data point represents the mean percentage cytolysis (± SEM) of at least 3 independent experiments.

3.3.6 U937 cells and autologous macrophages as targets

Differentiated U937 cells were more rapidly and strongly lysed by $CD8^+$ T cells than autologous macrophage targets (Fig. 3.6). Although all target cells were infected with *M.tuberculosis* for the same period of time and under identical conditions, macrophages required longer to be efficiently lysed by $CD8^+$ CTLs. By comparison, infected U937 target cells were lysed far more rapidly by $CD8^+$ CTLs with the level of mycobacterial-specific cytolysis reaching a maximum within 4 hours (data not shown).

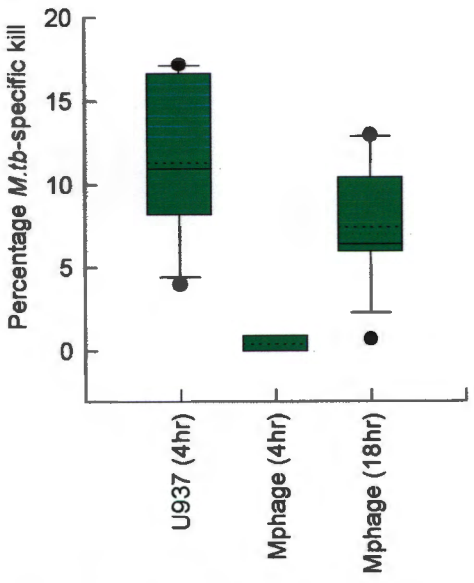


FIGURE 3.6. Comparison of $CD8^+$ CTL activity against U937 versus autologous macrophage targets. Cytotoxicity was measured after 4 hours for U937 targets, and after 4 and 18 hours for macrophage targets (as indicated). The CTLs used in these experiments were derived from a single donor (RG; Table 3.1) HLA-B51-matched to U937. Mycobacterial-specific lysis was calculated as follows: [% cytolysis against *M. tuberculosis*-infected targets]-[% cytolysis against SK-SD-pulsed targets]. Each box-and-whisker plot shows the distribution, median (solid line), mean (dotted line), 10^{th} and 90^{th} percentile of 6 independent experiments. (●) Represent possible out-liers.

3.3.7 $CD8^+$ T cell activation

Phenotypic characterization of the $CD8^+$ T cell population showed that under conventional priming conditions (*M. tuberculosis*-priming of bulk PBMC in the absence of additional growth factors) only 22.9 (± 14.4) % of the CD8 cells expressed IL-2 receptor (CD25) while 16.0 (± 11.4) % expressed HLA-DR (Table 3.2). Although $CD8^+$ CTLs were not selectively expanded following exposure to mycobacterial antigens [relative expansion ratio of 0.9 (day 0:day 7)], there was no significant reduction in proportion of CD8 T cells (CD4:CD8 ratio) following stimulation.

Table 3.2. *Phenotype and activation marker expression by the M. tuberculosis-primed CD8$^+$ CTL populations*

CTL Donor	CD4:CD8[a]		Relative Expansion of	$CD8^+$ Activation Marker expression (%)	
	Day 0	Day 7	$CD8^+$ T cells[b]	IL2-R^+	HLA-DR^+
RG	1.8	1.5	0.9	24.8	17.4
RG	2.2	0.8	0.8	14.6	8.9
RG	2.6	1.3	0.8	3.4	9.2
TS	nd[c]	1.8	nd	20.4	9.7
MA	nd	1.5	nd	61.7	46.7
SG	1.5	2.9	0.7	19.2	15.4
SC	1.0	1.0	0.8	17.3	11.9
EC	0.9	2.0	0.7	19.4	9.5
MH	1.5	1.4	1.0	28.7	24.3
SJ	2.5	2.5	1.1	20.0	15.8
BR	2.7	2.5	1.0	22.9	6.6
Mean (± SD)	1.9 (± 0.7)	1.7 (± 0.7)	0.9 (± 0.2)	22.9 (± 14.4)	16.0 (± 11.4)

[a] CD4:CD8 ratios were calculated from absolute % cells expressing CD4 or CD8 as determined by flow cytometry. [b] Relative expansion was calculated from % $CD3^+$ cells expressing CD8 TCR at day 0 compared with at day 6. [c]Not determined.

Because it has been well established that conventional mycobacterial priming preferentially activates and expands $CD4^+$ and possibly $\gamma\delta^+$ T cells, it has been proposed that more defined culture conditions may be necessary for optimal activation of *M. tuberculosis*-specific $CD8^+$ CTLs *ex vivo* (Tan *et al.*, 1997; Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998). For instance, Tan and colleagues (1997) reported that $CD8^+$ CTL activity was only evident in human alveolar lymphocytes and PBMC cultured with mycobacterial antigens in the presence (but not in the absence) of rIL-2. In order to define the optimal conditions necessary for $CD8^+$ CTL priming in mycobacterial infections, the effect of various alternative strategies on CD8 activation marker expression and cytolytic ability were investigated (Fig. 3.7). The results showed that priming of bulk PBMC with the virulent H37Rv strain of *M. tuberculosis* did not result in any significant enhancement in $CD8^+$ CTL activation or function compared with the stimulation with the attenuated vaccine strain BCG. In contrast, PBMC cultured together with a combination of *M. tuberculosis* and low doses of rIL-2 (10 iu.ml^{-1}) showed increased expression of IL-2 receptor on $CD8^+$ CTLs but no concomitant enhancement of mycobacterial-specific CD8 T cell cytolytic activity. An alternative approach using short-term bulk stimulation of PBMC with *M. tuberculosis* (24 hours) followed by $CD8^+$ CTL subset isolation and rIL-2 (50-100 iu.ml^{-1}) activation was used to investigate whether early

exclusion of $CD4^+$ T cells enhanced mycobacterial-specific $CD8^+$ CTL activity. The results showed, however, that this approach provided no significantly enhanced $CD8^+$ CTL activation or cytolytic activity compared with conventional *M. tuberculosis* priming used throughout this study. Another approach investigated in this study was the use of osmotic shock or lysis to facilitate the release of exogenous antigens into the HLA class I processing pathway (Moore *et al.*, 1988). Although priming of PBMC effector cells using osmotically shocked, *M. tuberculosis*-infected target cells resulted in increased expression of IL-2 receptor on $CD8^+$ T cells compared with conventional priming, these CD8 cells did not demonstrate any enhanced cytolytic ability.

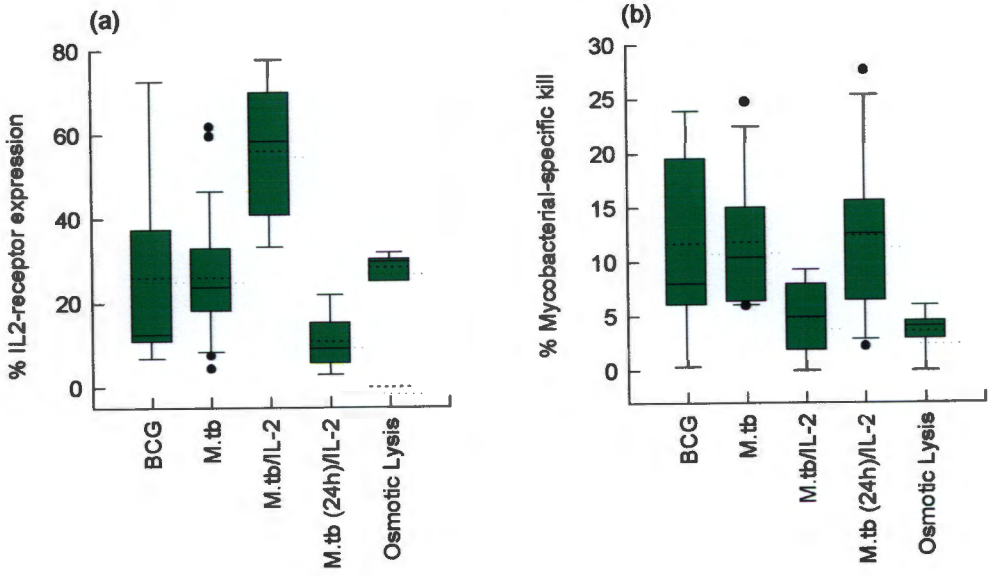


FIGURE 3.7. The effect of priming conditions on mycobacterial antigen-specific $CD8^+$ CTL cytolytic activity. (a) $CD8^+$ CTL activation marker expression (IL-2R) and (b) mycobacterial-specific cytolytic activity was assessed following conventional BCG- and *M. tuberculosis* H37Rv-priming, following addition of exogenous low-dose rIL-2, following early isolation and rIL-2 stimulation, or following osmotic lysis of *M. tuberculosis*-infected stimulator cells (described in Materials and Methods). IL-2 receptor expression (a) has been presented as the percentage of $CD8^+$ T cells expressing this activation marker. Cytotoxicity against autologous macrophage targets was measured after 18 hours. Mycobacterial-specific cytolysis was calculated as follows: [% cytolysis against *M. tuberculosis* infected targets]-[% cytolysis against SK/SD pulsed targets]. Each box-and-whisker plot shows the distribution, median (solid line), mean (dotted line), 10^{th} and 90^{th} percentile of 6 independent PBMC donors. (•) Represent possible out-liers.

Taken together, the experiments described in this Chapter have demonstrated that U937 cells can present antigen to conventionally primed $CD8^+$ CTLs in standard cytotoxicity (^{51}chromium release) assays (Fig. 3.2-3.6). The ability of *M. tuberculosis*-infected, PMA-differentiated U937 cells to activate $CD8^+$ T cells was subsequently investigated (Fig. 3.8). Because U937 cells do not express HLA class II (Peterlin *et al.*, 1984) and have an efficient phagosome-to-cytosol delivery system for exogenous antigens (Harris *et al.*, 1995), the ability of these monocytic cells to preferentially prime *M. tuberculosis*-specific, HLA class I-restricted $CD8^+$ T cells was of particular interest. *M. tuberculosis* infected U937 cells showed good ability to present antigen to monocyte/macrophage-depleted HLA-B51-matched PBMC. This ability was abrogated if U937 were fixed prior to *M. tuberculosis* infection indicating that antigen uptake and processing was a requirement for antigen recognition.

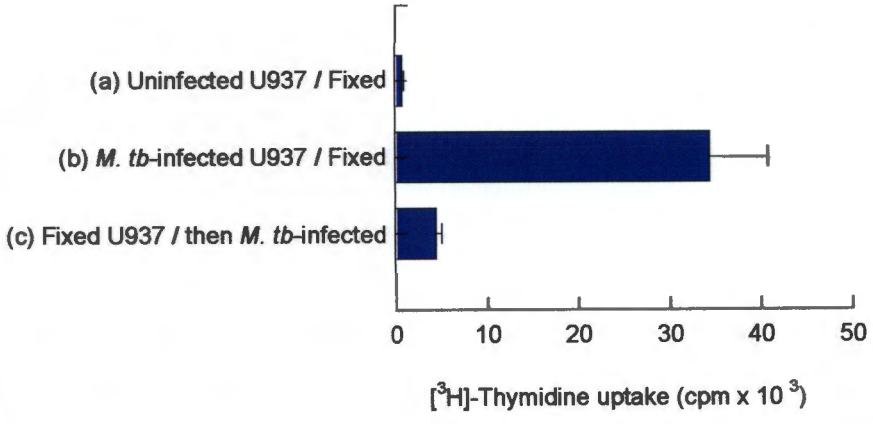


FIGURE 3.8. Antigen presentation by *M. tuberculosis*-infected U937 cells. Monocyte-depleted, HLA-B51-matched PBMC were co-cultured for 6 days with PMA-differentiated U937 presenting cells that were either (a) uninfected and then paraformaldehyde-fixed; (b) *M. tuberculosis*-infected (2 hours; 5 CFU.cell^{-1}) and then fixed; or (c) fixed and then co-cultured with *M. tuberculosis* (6 days; 5 CFU.cell^{-1}). The inability of U937 cells that were fixed prior to the addition of *M. tuberculosis* to present mycobacterial antigens served to control for antigen carry-over or presentation by residual, non-depleted autologous monocytes. Each bar represents the mean of at least 3 independent experiments (± SD).

While *M. tuberculosis* infected U937 cells were capable of stimulating mycobacterial antigen-specific PBMC proliferation, antigen presentation by U937 cells activated $CD8^+$ T cells poorly compared with autologous macrophage APC's (Table 3.3). $\gamma\delta^+$ T cells, in contrast, were strongly activated and preferentially expanded.

Table 3.3. *Comparison of U937 versus macrophage CD8 and γδ T cell priming*

T cell subset	Relative expansion following priming with U937 cells[a]	Activation marker expression (%)[b]			
		IL-2 receptor		HLA-DR	
		U937	Macrophage	U937	Macrophage
CD8	0.7	7.7 (± 7.1)	18.9 (± 12.8)	6.5 (± 0.6)	12.7 (± 4.3)
γδ	17.2	68.9 (± 6.4)	93.1 (± 4.6)	70.4 (± 15.9)	56.4 (± 11.5)

[a]Relative expansion was calculated from % $CD3^+$ cells expressing CD8 or γδ TCR at day 6 compared with at day 0.

[b]Assessed by flow cytometry using 2-colour fluorescence [CD8-FITC/IL2R-PE or DR-PE; and γδ-FITC/IL2R-PE or DR-PE]. Activation marker expression has been presented as the percentage of $CD8^+$ or $\gamma\delta^+$ T cells expressing IL-2R or HLA-DR. The data represents the mean percentage (± SD) of 2 to 4 independent experiments.

3.3.8 T cell clones

T cell clones were generated from *M. tuberculosis*-primed HLA-B51-matched PBMC and selected on the basis of their cytolytic activity against U937 target cells. Table 3.4 summarises the cloning strategy used and the cloning efficiency obtained. Because no significantly enhanced $CD8^+$ CTL cytolytic activity was generated by implementing various *in vitro* culture manipulations (Fig. 3.7), *M. tuberculosis*-reactive $CD8^+$ CTL clones were generated from short-term PBMC lines primed under conventional conditions with *M. tuberculosis*. The $\gamma\delta^+$ T cell clones were generated from short-term *M. tuberculosis*-primed CD4/CD8-depleted ($\gamma\delta^+$-enriched) or $\gamma\delta^+$ T cell lines.

Initial experiments made use of *M. tuberculosis* as the primary cloning stimulus (Table 3.4). The T cell clones generated under these conditions (87.9% $CD8^+$, 12.1% $\gamma\delta^+$) showed poor ability to lyse *M. tuberculosis*-infected U937 targets and, in the case of the $\gamma\delta^+$ T cell clones, poor subsequent *in vitro* survival. To overcome these problems and to improve the efficiency of $\gamma\delta^+$ T cell cloning, subsequent experiments were carried out in the presence of PHA rather than *M. tuberculosis*. Using this strategy, 48 $CD8^+$ and 8 $\gamma\delta^+$ T cell clones capable of lysing *M. tuberculosis*-infected U937 targets were generated.

Table 3.4. *Summary of limiting dilution cloning of T cells*

Cloning stimulus	T cell line	Cells per well	Positive wells per total wells	Negative wells (%)[a]	Cloning Efficiency (%)[b]	*M.tb*-spec. CTL clones (%)[c]
M. tb	*M.tb*-stim. PBMC	0.3	63/2500	97.5	8.4	9.0
	CD4/8-depleted	0.3	143/2400	94.0	19.9	1.6
		1.0	48/600	92.0	8.0	0.0
		1.0	66/1800	96.3	3.7	12.0
	$\gamma\delta^+$ T cell line	0.3	20/2100	99.0	3.2	0.0
		1.0	4/600	99.3	0.7	0.0
		1.0	64/1800	96.4	3.6	33.0
PHA	*M.tb*-stim. PBMC	0.2	124/600	79.3	100.0	17.0
	CD4/8-depleted	0.3	246/600	59.0	100.0	15.6
	$\gamma\delta^+$ T cell line	0.3	137/300	54.3	100.0	12.5

[a] Positive wells are likely to be monoclonal according to Poisson statistics (95% probability) when >37% negative wells are obtained (Bach, 1983). [b] Cloning efficiency has been defined as the proportion of seeded cells that give rise to a growing clone (Stemme & Kallberg, 1992). [c] Percentage of total clones screened that were able to kill U937 target cells in a mycobacterial-specific manner (with an arbitrary cytotoxicity "cut-off point" of >5% above background).

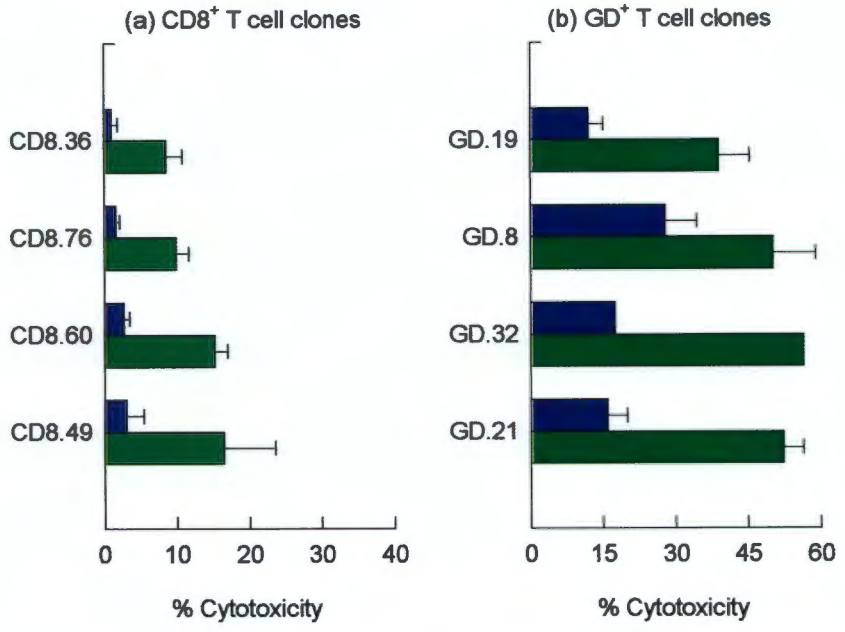


FIGURE 3.9 A representative panel of mycobacterial antigen-specific $CD8^+$ (a) and $\gamma\delta^+$ (b) CTL clones mediating cytolytic activity against U937 cells. U937 target cells were either infected with *M. tuberculosis* (5 $CFU.cell^{-1}$) (green bars) or pulsed with an irrelevant antigen, SK-SD (blue bars). Cytotoxicity against U937 targets was measured after 4 hours. Each data point represents the mean percentage cytolysis (± SD) of at least 3 independent experiments.

The $CD8^+$ CTL clones exhibited lower levels of mycobacterial antigen-specific cytolysis (13.2 ± 8.1%) against U937 cells (Fig. 3.9a) compared with the $\gamma\delta^+$ CTL clones (31.2 ± 7.8%) (Fig. 3.9b). The $CD8^+$ T cell clones were also cytolytic towards *M. tuberculosis*-infected autologous macrophage targets but the level of cytolysis was reduced compared with U937 target cells (5.4 ± 3.3% mycobacterial-specific). All of the $CD8^+$ T cell clones tested proliferated strongly to live *M. tuberculosis* and less vigorously to soluble PPD (Fig. 3.10). The $\gamma\delta^+$ T cell clones proved very difficult to maintain *in vitro* with only 3 of the 8 $\gamma\delta^+$ clones ($\gamma\delta$.8, $\gamma\delta$.19 and $\gamma\delta$.32) surviving for longer than 4 passages and only 2 of these 3 clones ($\gamma\delta$.8 and $\gamma\delta$.19) maintaining their cytolytic function.

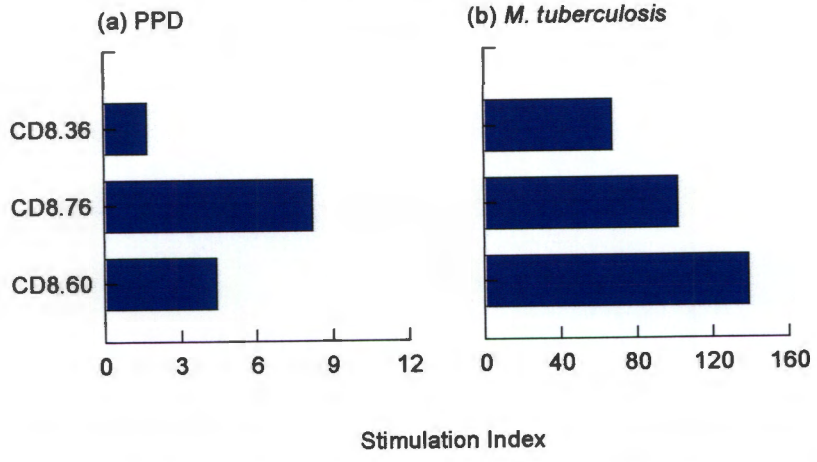


FIGURE 3.10. Proliferative responses of $CD8^+$ T cell clones to (a) soluble PPD or (b) *M. tuberculosis*. $CD8^+$ T cell clones were incubated with no antigen, PPD (3 $\mu g.ml^{-1}$) or *M. tuberculosis* (5 $CFU.feeder^{-1}$) in triplicate wells in the presence of autologous irradiated (40 Gy) PBMC feeders as APC's. After 40 hours, cultures were pulsed with $[^3H]$-thymidine (1$\mu Ci.well^{-1}$) for 8 hours. Results are expressed as stimulation indices and were calculated as follows: [mean cpm of antigen stimulated wells] ÷ [mean cpm of unstimulated wells].

3.4 DISCUSSION

The present study investigates the human macrophage cell line U937 as an *in vitro* model for HLA class I-restricted presentation of mycobacterial antigens to human cytolytic T cells. PMA-differentiation of U937 cells completely abrogated their susceptibility to both NK- and LAK-mediated anomolous cytotoxicity, and U937 target cells infected with *M. tuberculosis* were effectively lysed by *M. tuberculosis*-specific HLA class I-matched human CTLs. Cytolysis against U937 target cells was directed against mycobacterial antigens and mediated by HLA class I-matched but not HLA-mismatched CTLs. The HLA class I-matched CTL activity was shown to be restricted to live mycobacterial organisms (H37Rv and BCG) but

not to soluble PPD. Finally, purified $CD8^+$ CTL lines and $CD8^+$ T cell clones from HLA class I-matched but not HLA-mismatched donors showed mycobacterial antigen-specific cytolysis against infected U937 target cells. Given the absence of HLA class II (Chapter 2; Peterlin *et al.*, 1984) and CD1 (Kasinrerk *et al.*, 1993) expression on U937 cells, the data supports the conclusion that antigens derived from *M. tuberculosis* can access the HLA class I processing pathway.

Many of the more recent studies describing mycobacterial-specific $CD8^+$ CTLs have focused on their ability to produce cytokines or to proliferate in response to stimulation with defined mycobacterial antigens (Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998; Canaday *et al.*, 1999). Those studies that have demonstrated the existence of $CD8^+$ CTLs able to lyse *M. tuberculosis*-infected target cells have used very defined conditions to facilitate efficient CTL priming. For instance, Tan *et al.* (1997) found that mycobacterial-specific $CD8^+$ CTL activity was only demonstrable following *in vitro* co-culture together with IL-2. While Mohagheghpour *et al.* (1998) used dendritic cells pulsed with defined mycobacterial HLA class I peptide epitopes to prime strong $CD8^+$ CTL activity. It has been suggested that for investigation of CTL activity using standard ^{51}Cr release assays is not a very sensitive approach for detecting CTL activity in cells under conditions of low precursor frequencies (Lalvani *et al.*, 1998). In the present study, we demonstrated $CD8^+$ CTL activity against U937 cells under conventional priming conditions, both at the bulk and clonal level. Although U937 target cells shared only a single HLA class I haplotype match (HLA-A3 or –B51) with the CTL donors, they were more rapidly and strongly lysed than autologous macrophage targets. The most direct interpretation of these results is that U937 cells have a more efficient phagosome-to-cytosol delivery system for HLA class I-restricted presentation of exogenous antigens compared with macrophages. A recent report by Harris and colleagues (1995) provides strong support for this interpretation. These authors directly isolated and identified HLA class I-associated peptides from HIV *nef*-transfected U937 cells and found that, in addition to the anticipated HIV *nef*- and endogenously-derived peptides, a significant proportion (10% or 20 pmol) of peptides isolated from the HLA-A, B, and C pools were derived from identifiable exogenous proteins. Although Harris *et al.* (1995) showed relatively good yields of exogenously-derived HLA class I-associated peptides, they calculated that HLA class I presentation of endogenously derived peptides was still 200-fold more efficient than presentation of peptides derived from exogenous proteins.

The difference in kinetics and efficiency of CTL-mediated cytolysis against U937 versus macrophage target cells may, however, reflect either a fundamental difference in the interaction of these targets with the CTL effectors or differential susceptibility of the target cells to CTL-mediated cytolytic mechanisms. A recent study demonstrated, however, that U937 cells, either untreated or terminally differentiated, were no more sensitive to perforin-mediated cytotoxicity than human monocytes (Jones and Morgan, 1994). Unlike human monocytes which can be induced to express cell surface Fas (CD95), U937 cells are considered to be a Fas^- cell line and would therefore not be susceptible to Fas-mediated cytolysis (Zeine *et al.*, 1998). The differential sensitivity of U937 cells and macrophages to mycobacterial-specific cytolysis could reflect other fundamental differences in the mechanism of cytolysis against these disparate target cells, an intriguing possibility that would require further investigation.

Despite the finding that U937 cells are more sensitive to mycobacterial-specific $CD8^+$ CTL activity than autologous macrophages, the level of CD8 CTL-mediated cytolysis of U937 targets was lower than observed for $\gamma\delta^+$ CTLs. The CTL effectors used in this study were generated using conventional priming conditions in that *M. tuberculosis*-infected monocyte-derived autologous macrophages were used for the initial priming and $CD8^+$ CTLs were generated in bulk in the absence of any additional cytokines or growth factors. To address whether the low levels of $CD8^+$ cytolysis generated against target cells during the effector phase of the CTL activity was due to restricted HLA class I presentation of mycobacterial antigens by target cells or due to poor initial priming of the CTL population, we investigated the effects of varying culture conditions on CTL generation. Although a few studies have shown mycobacterial-specific $CD8^+$ cytolytic activity following conventional priming (Turner & Dockrell, 1996), the majority of authors have found that $CD8^+$ CTL functional activity is difficult to demonstrate *in vitro* and required very specific manipulation (Lewinshon *et al.*, 1998; Tan *et al.*, 1997; Lalvani *et al.*, 1998; Monagneghpour *et al.*, 1998). We were unable to demonstrate enhanced $CD8^+$ T cell cytolytic activity following the various culture manipulations assessed in this study, as compared with conventional priming using either *M. tuberculosis* or BCG. It seems likely that low cytolytic capacity demonstrated by $CD8^+$ CTLs in mycobacterial infections is due to the combined effects of inadequate initial priming of this T cell subset (with CD8 CTLs being represented at very low precursor frequency), together with the relative inefficiency of the pathway allowing HLA class I-

presentation of exogenously-derived bacterial antigens (Reis e Sousa and Germain, 1995).

T cell activation in response to antigen requires at least two types of signals to be provided by an antigen-presenting cell (APC) (Schwartz, 1992). The first is the interaction between the T cell receptor with peptide that is associated with HLA molecules on the surface of the APC (Schwartz, 1985). As a result of this interaction, the TCR-associated CD3 complex mediates intracellular signals that are necessary but not sufficient for T cell clonal expansion (Barber *et al.*, 1989; Weiss *et al.*, 1994). The second critical signal is mediated by receptors on the T cell that interact with co-stimulatory molecules on the surface of the APC (Jenkins & Johnson, 1993; Linsley *et al.*, 1991). The type of co-stimulatory signal that is delivered is dependent on several factors, including the type and activation status of the APC (Thomas *et al.*, 1993), the type of T cell (CD4 versus CD8), and whether the T cells are naïve or memory phenotype (Byrne *et al.*, 1988). Several cell surface co-stimulatory interactions have been implicated in T cell activation. The most thoroughly investigated are those between CD80 (B7.1)/CD86 (B7.2) on the APC and CD28/CTLA-4 on T cells (Jenkins & Johnson, 1993; Linsley *et al.*, 1991; Norton *et al.*, 1992; Moudgil & Sercarz, 1993). Although CD80 (B7.1)/CD28 co-engagement between T cells and APC has been demonstrated to be critical during the inductive phase of the T cell response, this interaction has been shown to be less important during the subsequent effector phase of CTL activity (Harding & Allison, 1993). Previous studies have reported that CD80 (B7.1) is absent on U937 cells, while CD86 (B7.2) is present at very low levels (Palmer & van Seventer, 1997; Stonehouse *et al.*, 1999). Despite this, U937 cells have been shown to provide efficient co-stimulatory signals for the priming of Th_1-like cells by a CD80/CD86-independent pathway and that the co-stimulatory signal is as potent as the one provided by $B7^+$ human monocytes (Johnson and Jenkins, 1994; Palmer & van Seventer, 1997; Stonehouse *et al.*, 1999). Consistent with their strong HLA class I expression but lack of HLA class II expression, a recent study confirmed that U937 cells preferentially provide co-stimulatory activity for $CD8^+$ rather than $CD4^+$ T cell responses (Stonehouse *et al.*, 1999).

It is noteworthy that both purified $\gamma\delta^+$ CTLs and $\gamma\delta^+$ T cell clones recognised and lysed *M. tuberculosis*-infected U937 target cells very efficiently. Previous reports have found that antigen-specific responses of γδ T cells are not restricted by classical HLA class I or II, or non-classical CD1a, CD1b or CD1c antigen presenting molecules and that antigens may be

presented directly on the surface of infected cells since no apparent antigen processing appears necessary (Morita *et al.*, 1995). γδ T cells have consistently been implicated in mycobacterial infections and have been shown to produce Th_1-type cytokines as well as being strongly cytolytic (Balaji and Boom, 1998). The present study has clearly demonstrated that U937 cells provide a very selective model for evaluating the mycobacterial-specific cytolytic abilities of this T cell subset and could therefore provide a useful tool for further investigations into the protective role of $\gamma\delta^+$ CTLs in mycobacterial pathogenesis.

Despite being strongly cytolytic towards *M. tuberculosis*-infected U937 targets, the purified γδ T cell lines and γδ clones generated in the present study were very difficult to maintain *in vitro*. The problems associated with generating and maintaining mycobacterial antigen-specific γδ T cell clones have been widely experienced (De Libero, personal communication; Holoshitz, personal communication) and appear to be a recurrent theme in published reports on γδ T cell activity in a variety of infectious models. Earlier studies found that the majority of γδ T cells underwent apoptosis following priming and that the remaining population became functionally anergic to re-stimulation (Janssen *et al.*, 1991; Spaner *et al.*, 1993; Ferrarini *et al.*, 1995; Rovere *et al.*, 1996). These studies showed that, despite expressing constant amounts of Fas (CD95) throughout their *in vitro* propagation, γδ T cells became prone to Fas-FasL triggered apoptosis only when chronically stimulated (Ferrarini *et al.*, 1995). More recently, Manfredi *et al.* (1998) and Li *et al.* (1998) provided an explanation for these findings by demonstrating that the engagement of the γδ TCR by mycobacterial antigens induced the expression of Fas ligand (FasL) by chronically activated $Fas^+/FasL^-$ γδ T lymphocytes. Activation-induced cell death (AICD) of γδ T cells has been proposed to represent a negative feedback mechanism for controlling their own expansion and may contribute to explain the limited time span of γδ T cell expansion during an infection, even in situations where the pathogen has not been fully eliminated (Ferrarini *et al.*, 1995). γδ T cell expansion takes place during the acute phases of most infections, a characteristic that has contributed to these cells being considered to be a first line of defence against many microbial and viral infections (Boismenu and Havran, 1997). Their expansion is crucial in controlling multiplication of microorganisms at a time when no specific αβ T cells are yet available. Later, the AICD of antigen-stimulated γδ T cells may provide a feedback mechanism, avoiding the potentially harmful persistence of activated γδ T cells when the

more protective antigen-specific αβ T cells were expanded enough to eradicate the infection. Despite being of obvious biological benefit to the host, AICD of γδ T cells creates significant problems for the study of this T cell subtype in culture.

The results presented in this Chapter have demonstrated that the human monocytic cell line U937 is a suitable *in vitro* model for HLA class I-restricted presentation of mycobacterial antigens to human cytolytic T cells. U937 cells, in addition to phagocytosing *M. tuberculosis* efficiently and supporting the intracellular growth of this organism (Chapter 2), present *M. tuberculosis*-derived antigens to HLA class I-matched $CD8^+$ CTLs more effectively than monocyte-derived macrophages. This study also demonstrated that this cell line provides a highly selective indicator for mycobacterial-specific $\gamma\delta^+$ CTL cytolytic activity.

There have been several recent reports linking the precise mechanism of cytolysis utilised by cytotoxic $CD8^+$ T cells towards *M. tuberculosis*-infected target cells to their ability to directly restrict intracellular mycobacterial survival (Stenger *et al.*, 1997; 1998). Similar investigations into the mechanism of cytolysis favoured by the $CD8^+$ T cell lines and $CD8^+$ CTL clones described in this Chapter and their effect on intracellular mycobacterial survival were not feasible due to the consistently low cytolytic capacity demonstrated by these cells against *M. tuberculosis*-infected targets. The establishment of strongly cytolytic human *M. tuberculosis*-reactive $\gamma\delta^+$ CTL clones, however, afforded a unique opportunity to investigate more thoroughly the protective role of this T cell subset in human mycobacterial infections. To date, there have been no published reports on the mechanism/s of cytolysis utilised by cytotoxic $\gamma\delta^+$ T cells in mycobacterial infection and no reports on the effect of $\gamma\delta^+$ T cell-mediated cytolysis on intracellular mycobacterial survival. For these reasons, the following Chapter (Chapter 4) has specifically focused on these issues.

CHAPTER 4

MECHANISM OF HUMAN $\gamma\delta^+$ T CELL CYTOTOXICITY AGAINST *M. TUBERCULOSIS*-INFECTED U937 CELLS

4.1 INTRODUCTION

Although almost a decade has past since the identification of T cells bearing the γδ TCR, the biological significance of this unique T cell subset still remains uncertain. It is clear, however, that $\gamma\delta^+$ T cells contribute to protective immunity in mycobacterial infections. $\gamma\delta^+$ T cells are markedly expanded during mycobacterial infections (Kabelitz *et al.*, 1990; Pfeffer *et al.*, 1990; De Libero *et al.*, 1991; Kabelitz *et al.*, 1991; Panchamoorthy *et al.*, 1991; Pechhold *et al.*, 1994) and accumulate at the site of pathology (Modlin *et al.*, 1989; Griffin *et al.*, 1991). It is also clear that there are several features that distinguish this T cell subset from $\alpha\beta^+$ T cells. $\gamma\delta^+$ T cell responses precede those of $\alpha\beta^+$ T cells (Ferrick *et al.*, 1995); they home to distinct physiologic locations; they express a much more limited repertoire of TCR variable genes (Bluestone *et al.*, 1991) and, perhaps the most striking distinction, they recognise low MW, phosphorylated, non-peptidic mycobacterial antigens in a non-HLA-restricted manner (Constant *et al.*, 1994; Tanaka *et al.*, 1994; Tanaka *et al.*, 1995; Morita *et al.*, 1995; Lang *et al.*, 1995; Kabelitz *et al.*, 1991; reviewed by Porcelli *et al.*, 1996).

One of the most important effector functions of mature activated $\gamma\delta^+$ T cells is their HLA-unrestricted cytolytic activity against infected target cells. Accordingly, a significant amount of attention has focused on their ability to destroy cells infected with both bacterial and viral pathogens (Munk *et al.*, 1990; Malkovsky *et al.*, 1992a; Bukowski *et al.*, 1994; Tsukaguchi *et al.*, 1995). Few studies, however, have examined the mechanism of $\gamma\delta^+$ T cell cytolytic activities against infected target cells or the impact of this cytolysis on the survival of infecting pathogens.

Two major mechanisms have been described for the successful cytolysis of target cells by cytotoxic T lymphocytes (summarized in Fig. 4.1; Kagi *et al.*, 1994; Lowin *et al.*, 1994; Atkinson & Bleackley, 1995; Henkart *et al.*, 1995). The first mechanism involves granule exocytosis and is mediated by perforin and granzymes (reviewed recently by Page *et al.*, 1998). Following TCR-dependent recognition of the target cells, a number of signals, which include protein tyrosine phosphorylation and increased intracellular calcium levels, are generated within the CTL. These signals result in the transcription of effector proteins and eventually the appearance of electron-dense granules within the CTL. These effector proteins

are the mediators of CTL function and are stored in the granules until required. Subsequent interaction with a target cell results in directed exocytosis of the CTL granules towards the point of contact with the target cell (Geiger *et al.*, 1982; Kupfer and Dennert, 1984; Kupfer *et al.*, 1985; Yannelli *et al.*, 1986). During granule-mediated cytotoxicity, the cytolytic proteins released from the granules result in a "lethal hit" on the target cell and the cell dies. Perforin contained within the CTL granules causes osmotic damage due to its binding of phosphorylcholine headgroups, polymerisation, and subsequent pore formation in the lipid bilayer of the target (Lichtenheld *et al.*, 1988; Shinkai *et al.*, 1988; Tschopp and Nabholz, 1990). Granzymes are the major protein components of CTL granules and are postulated to synergise with perforin to trigger an internal disintegration pathway within the target cell that is mediated by caspases and apoptosis (Page et al., 1998).

The second major mechanism by which T lymphocytes lyse target cells is receptor mediated by Fas-Fas ligand (Fas-FasL) interaction. A number of cell types express a surface protein called Fas/APO-1/CD95 which contains a "death domain" in its cytoplasmic region (Nagata and Goldstein, 1995). During CTL activation, levels of a protein named Fas ligand (FasL) are upregulated on the CTL surface. FasL can cross-link target cell Fas receptors, and the oligomerized receptors can then transduce a death signal to the target cell through a number of associated proteins (Cleveland and Ihle, 1995; Fraser and Evan, 1996). Following the initial interaction, which results in FasL expression, no further specific recognition of the target cell through TCR is required for Fas-mediated cytotoxicity. Thus, a FasL bearing CTL is able to destroy any Fas^+ cell, whether it has been specifically recognised or not (Page *et al.*, 1998). The available evidence suggests that granule exocytosis is the predominant mechanism involved in the elimination of infected cells (Kagi *et al.*, 1994), while Fas-mediated lysis may be reserved for homeostatic control of activated peripheral T cells (Ramsdell *et al.*, 1994; Singer & Abbas, 1994; Nagata & Goldstein, 1995).

While human $\gamma\delta^+$ T cells constitutively possess cytoplasmic granules containing cytolytic mediators (perforin and serine esterases; Nakata *et al.*, 1990; Koizumi *et al.*, 1991), they have also been shown to express FasL (Nagata & Golstein, 1995; Suda *et al.*, 1995; Vincent *et al.*, 1996), demonstrating their potential ability to use either mechanism of cytotoxicity.

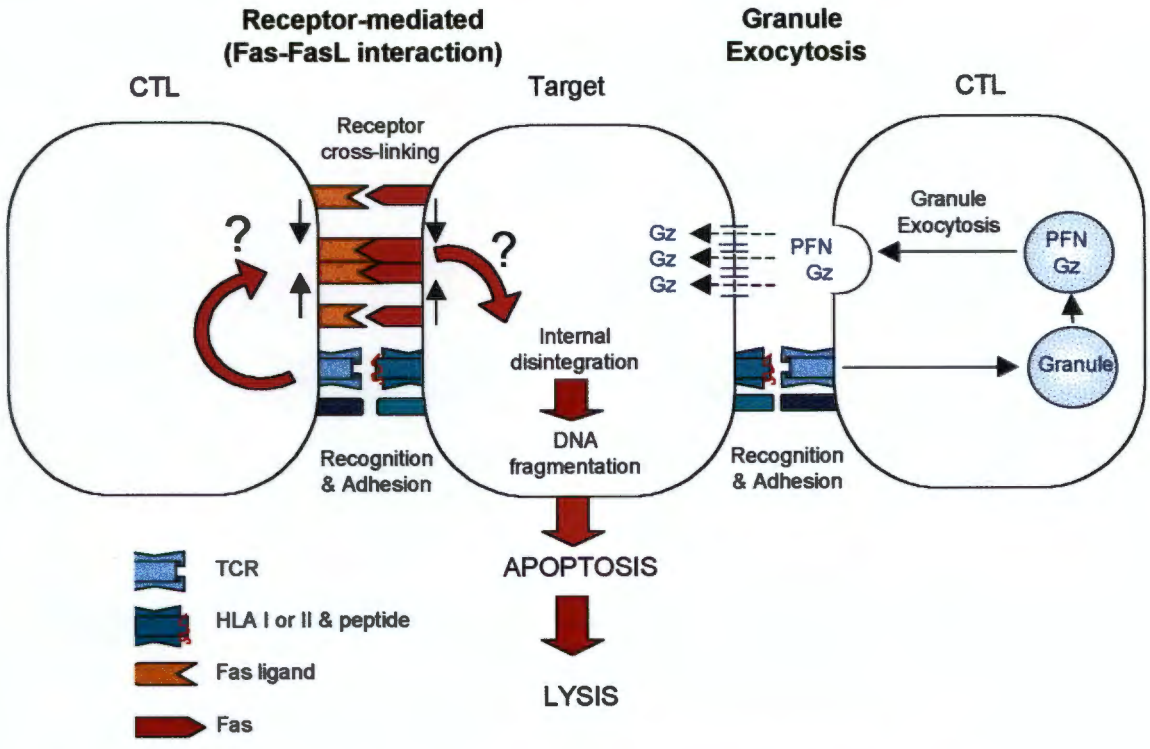


FIGURE 4.1 The two proposed mechanisms of cytotoxicity (adapted from Berke, 1994).

The precise role of CTL's in controlling the spread of intracellular pathogens such as *M. tuberculosis* remains uncertain and this intriguing question has been the focus of several recent reviews (Tschopp and Hofmann, 1996; Mazzaccaro *et al.*, 1998; Stenger and Modlin, 1998; Kaufmann, 1999). One postulate that has been favoured in the past is the ability of T cell mediated cytolysis to release live bacteria from inefficiently activated macrophages, allowing these bacilli to be taken up and killed by newly recruited and freshly activated macrophages (De Libero *et al.*, 1988; Kaufmann, 1988). The ability of T cell-mediated cytolysis to directly impact on the viability of intracellular pathogens was considered highly controversial. However, recent findings by Stenger and colleagues (1997; 1998) have suggested that such a role for CTL-induced lysis does exist. Moreover, this ability of CTL's to reduce the intracellular survival of *M. tuberculosis* was found to be restricted to effector cells utilising the granule-exocytosis pathway (Stenger *et al.*, 1997; Stenger *et al.*, 1998).

Experiments presented in this chapter investigated two important issues. Firstly, which cytolytic mechanism was used by $\gamma\delta^+$ CTL clones against *M. tuberculosis*-infected U937 target cells, and secondly, did $\gamma\delta^+$ CTL-mediated cytolysis impact on intracellular mycobacterial viability? The $\gamma\delta^+$ T cell clones were shown to mediate strong cytolytic activity against *M.*

tuberculosis-infected target cells that was dependent on cell-to-cell contact. Cytolysis was Ca^{2+}-dependent and completely inhibited by de-granulation of the $\gamma\delta^+$ effector T cells (using Sr^{2+}) indicating that the predominant mechanism of target cell lysis was granule exocytosis. Despite mediating significant target cell cytolysis, however, the $\gamma\delta^+$ CTL clones did not result in a detectable reduction in intracellular mycobacterial survival.

4.2 MATERIALS AND METHODS

4.2.1 Mycobacterial growth conditions

M. tuberculosis H37Rv cultures were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% OADC (State Vaccine, Cape Town, South Africa) and 0.02% Tween-80 (Merck, Darmstadt, Germany) at 37°C, 5% CO_2. Mid-log phase cultures were snap frozen in liquid nitrogen and stored at -70°C until needed. Frozen mycobacterial cultures were thawed immediately before use and clumps were disrupted by repeated passage through a 25-gauge needle. In all experiments, bacteria were coated with serum opsonins by incubating thawed aliquots with an equal volume of fresh human serum for 30 minutes at 37°C. Mycobacterial viability, was measured by determining colony forming units (CFU) of serial 10-fold dilutions of bacterial preparations on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 10% OADC.

4.2.2 Isolation of *M. tuberculosis*-reactive $\gamma\delta^+$ T cell lines

Peripheral blood mononuclear cells (PBMC) were obtained from healthy PPD^+ BCG-vaccinated adult volunteers by centrifugation of heparinized venous blood over Ficoll-Hypaque density gradients (Sigma) as previously described (Chapter 3; Boyum *et al.*, 1968). PBMC (1×10^6 cells.ml^{-1}) were stimulated with *M. tuberculosis* H37Rv (1 CFU.$cell^{-1}$) for 6 days at 37°C to prime and expand *M. tuberculosis*-responsive $\gamma\delta^+$ T cells. $\gamma\delta^+$ T cells were isolated by sequential $CD4^+/CD8^+$ T cell-depletion either using directly conjugated $CD4^+$ and $CD8^+$ Minimacs magnetic beads or by positive selection using $\gamma\delta^+$ mAb (Becton-Dickenson; San Jose, CA) indirectly conjugated to goat anti-mouse IgG_1 Minimacs magnetic beads (Miltenyi Biotec, CA). Short term $\gamma\delta^+$ T cell lines were maintained by re-stimulating cells

weekly with irradiated (40 Gy) autologous PBMC feeders (1 x 10^6 cells.ml^{-1}), *M. tuberculosis* H37Rv (1 CFU.cell^{-1}) and bi-weekly with rIL-2 (100 iu.ml^{-1}). The phenotype of γδ$^+$ T cell lines was monitored weekly using flow cytometry and 2-colour fluorescence (γδ-FITC/CD3-PE). The γδ$^+$ T cell lines were difficult to maintain beyond 3-4 passages using *M. tuberculosis* as the antigenic stimulus, and this finding coincided with the lines rapidly becoming functionally anergic and loosing their cytolytic ability.

4.2.3 Generation of γδ$^+$ T cell clones

The γδ$^+$ T cell clones were generated by limiting dilution as described by Bach (1983) from bulk *M. tuberculosis*-primed PBMC or γδ$^+$ T cell lines (Chapter 3; Table 3.4). Briefly, the cells were seeded at 0.2 cells.well^{-1} into Terasaki wells in fresh cloning medium [RPMI-1640 supplemented with 10% autologous serum, 2-mercaptoethanol (5 x 10^{-5} M; BDH Chemicals, Poole, England), non-essential amino acids (10mM; Highveld Biologicals, Johannesburg, South Africa), L-glutamine (2mM; Highveld Biologicals), sodium pyruvate (1mM; Highveld Biologicals)]. Cells were stimulated with PHA (1.1 x 10^{-2} mu.ml^{-1}, Murex Biotec; Dartford; England), rhIL-2 (100 iu.ml^{-1}) and fresh, irradiated (40 Gy) autologous PBMC feeders (1 x 10^6 cells.ml^{-1}) in a final volume of 20 µl.well^{-1}. Plates were incubated at 37°C, 5% CO_2. Cell growth was determined by visual inspection using an inverted microscope (Nikon TMS, Japan) after 10-12 days. Wells were scored positive if greater than one fourth of a particular well surface was covered with cells.

Positively scored wells were selected between days 10 and 12 by transferring the contents of individual positive Terasaki wells into U-bottomed 96-well microtitre plates. Re-stimulation was performed using irradiated autologous PBMC as feeder cells (10^5 cells.well^{-1}), rIL-2 (100 U.ml^{-1}) and PHA (1.1 x 10^{-2} mu.ml^{-1}) in a final volume of 100 µl per well of cloning medium (described above). The growing cultures were supplemented with 100 µl fresh cloning medium containing rIL-2 (100 U.ml^{-1}) 3-4 days after transfer and cells were re-suspended regularly to avoid formation of large clusters. Seven days after transfer, clones were split into two wells and re-stimulated with feeders, PHA, and rIL-2 as indicated above. By the end of the second passage in microtitre wells, the cells were transferred to 24 well tissue culture plates and re-stimulated as previously described in a final volume of 1 ml. Cultures were re-

suspended regularly, supplemented with fresh cloning medium every 3-4 days and passaged weekly.

The mycobacterial-responsive $\gamma\delta^+$ T cell clones were selected on the basis of their cytolytic function against *M. tuberculosis*-infected U937 target cells (described in more detail in Chapter 3). The initial assessment of cytotoxic ability was performed using 50-100 µl of each respective T cell clone at unknown cell concentration against either *M. tuberculosis*-infected or SK-SD-pulsed PMA-differentiated U937 cells (10^4 cells.well^{-1}). Clones that were able to kill U937 target cells in a mycobacterial antigen-specific manner were selected for further analysis (*M. tuberculosis*-specific kill >5% above background was chosen as an arbitrary cut-off point). Cytotoxic function of the "positive-killer clones" was then confirmed in triplicate wells and at multiple effector to target ratios.

Both the $\gamma\delta^+$ CTL lines and clones were extremely difficult to maintain *in vitro*. Despite a total of 8 $\gamma\delta^+$ T cell clones having been generated, only 3 of the 8 clones survived for longer than 4 passages (γδ.8, γδ.19 and γδ.32). Only 2 of these 3 $\gamma\delta^+$ T cell clones (γδ.8 and γδ.19) maintained long term cytolytic function against *M. tuberculosis*-infected U937 cells and have been investigated extensively in the present chapter.

4.2.4 Flow cytometry

An Epics Profile II flow cytometer (Coulter) was used to perform immunophenotyping. Histograms were gated on the PBMC populations by forward scatter (FS) versus log side scatter (LSS). Monoclonal antibodies were obtained from Coulter (Hialeah, FL) or Beckton-Dickinson (San Jose, CA) and were directed against CD4, CD8, γδ, and CD3. All the monoclonal antibodies were directly conjugated with FITC or RD1 (PE). Isotypic controls were used in all cases to set cursors to allow 2% false positives and antibodies were used as the concentrations suggested by the manufacturer. Five percent colour compensation was used for samples analysed using 2-colour immuno-fluorescence.

4.2.5 Cell lines

PMA-differentiated U937 cells were used widely in the present study as target cells for $\gamma\delta^+$ T cell mediated cytolytic activity. U937 cells were maintained routinely in suspension culture in RPMI-1640 (Flow Laboratories, Irvine, Scotland) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa), 2 mM L-glutamine, and 10 mM HEPES at 37°C in a humidified atmosphere of 5% CO_2. Passage was performed every 2-3 days. They were induced to differentiate using PMA (5 $ng.ml^{-1}$; Sigma, St. Louis, MO) for 48 hours (as described extensively in Chapter 2 and 3) before infection with *M. tuberculosis* and use as targets in cytotoxicity assays. Daudi B cell lymphoma cell line and Jurkat T cell lines were also used in this study to investigate various functional properties of $\gamma\delta^+$ CTL activity. Cells used in all experiments were in the logarithmic phase of growth and viability exceeded 95% as measured by trypan blue exclusion.

4.2.6 Cytotoxicity assay

PMA-differentiated U937 cells were used as targets for assessment of $\gamma\delta^+$ T cell cytolytic ability. Target cells were either infected with *M. tuberculosis* H37Rv (5 $CFU.cell^{-1}$), pulsed with the irrelevant streptococcal antigen streptokinase-streptodornase (SK-SD; 250 $iu.ml^{-1}$ SK; 62.5 $iu.ml^{-1}$ SD; Lederle Laboratory, Wayne, NJ), or left uninfected for16 hours at 37°C. On the day of the experiment, adherent U937 cells were EDTA-detached, labelled with 250 μCi ^{51}Cr for 60-90 minutes, washed 3 times with cold 5% FCS PBS and finally adjusted to the desired concentration in 10% AB serum RPMI.

The cytolytic activity of the $\gamma\delta^+$ T cell clones was assessed three days following the last addition of feeder cells or 2 days following the last addition of rhIL-2 (section 4.2.5). The $\gamma\delta^+$ T cell clones were incubated in 10% AB serum RPMI (containing no IL-2) for 8-24 hours prior to cytotoxicity assay. The $\gamma\delta^+$ T cell clones were finally adjusted to the desired concentration and incubated together with the ^{51}Cr-labelled target cells at indicated effector:target cell ratios for 4 hours at 37°C. The percentage specific target cell lysis was calculated from the mean of triplicate wells using the following equation: [mean test cpm- mean spontaneous cpm]/[mean maximum cpm- mean spontaneous cpm] x 100. Spontaneous

^{51}Cr release was determined from ^{51}Cr-labeled targets incubated with medium alone while maximum ^{51}Cr-release was measured from targets treated with 5% Triton X-100 for the duration of the assay.

4.2.7 Prevention or disruption of cytotoxicity

Prevention of cell-cell contact

Prevention of cell-cell contact was carried out as previously described by Lang *et al.* (1995). Briefly, the $\gamma\delta^+$ T cell clones and *M. tuberculosis*-infected U937 targets were either centrifuged (50g for 5 minutes) at the beginning of the cytotoxicity assay or maintained in suspension throughout the entire 4-hour incubation period by gentle pipetting every 15 minutes.

Disruption of granule exocytosis

Three alternative approaches were used to disrupt granule exocytosis of the $\gamma\delta^+$ CTL clones (Fig. 4.1): (i) Previous studies have demonstrated that extracellular Ca^{2+} ions are necessary for granule exocytosis and perforin-based cytotoxicity, but not for the interaction of Fas-FasL (Berke, 1994; Nagata and Goldstein, 1995). In the present study, the Ca^{2+} chelator EGTA/Mg^{2+} (6mM/3mM, Saarchem Ltd., South Africa) was added to remove extracellular Ca^{2+} from the culture medium. (ii) Cyclosporin A (CsA) has previously been shown to interfere with the biochemical events in the later stages of Ca^{2+}-dependent granule exocytosis that follows the binding of calmodulin to cytoskeletal or cytoplasmic calmodulin binding proteins (Trenn *et al.*, 1989). The $\gamma\delta^+$ CTL clones used in this study were pre-incubated with 100-200 $ng.ml^{-1}$ CsA (Sandoz, Switzerland) for 30 minutes and then added to ^{51}Cr-labelled target cells in the absence or presence of 100-200 $ng.ml^{-1}$ CsA. (iii) Strontium ions (Sr^{2+}) have previously been found to result in de-granulation of CTL's and release of cytotoxic granule contents thereby transiently inhibiting CTL lytic activity (Neighbour and Huberman, 1982; Stenger *et al.*, 1997). In the present study, the $\gamma\delta^+$ CTL clones were pre-incubated for 10-24 hours in the presence of 25 mM $SrCl_2$ (Sigma, St. Louis, MO) and then added to ^{51}Cr-labelled target cells in the absence or presence of 25 mM $SrCl_2$.

Prevention of Fas-FasL interaction

To prevent the interaction of Fas with FasL, ^{51}Cr-labelled target cells (Fas$^+$ Jurkat cells) were pre-incubated for 1 hour with 0.5-2 μg.ml^{-1} anti-Fas monoclonal antibody (ZB4; Immunotech, Westbrook, ME) prior to the addition of the γδ$^+$ CTL clones.

Table 4.1 *Summary of the agents used to prevent or disrupt cytolysis*

Cytolytic Pathway	Agent	Concentration	Mechanism	Reference
Granule exocytosis	Mg_2/EGTA	3 mM/6 mM	Removes Ca^{2+} from the culture medium.	MacLennan *et al.* (1980)
	$SrCl_2$	25 mM	Causes degranulation and release of granule contents in the absence of additional immunologic stimuli.	Neighbour *et al.* (1982)
	Cyclosporin A	100-200 ng.ml^{-1}	Inhibits biochemical events in the late stages of Ca^{2+}-dependent granule exocytosis that follow binding of calmodulin to cytoskeletal or cytoplasmic calmodulin binding proteins	Trenn *et al.* (1989)
Fas-FasL	ZB4 (Anti-Fas mAb)	0.5-2 μg.ml^{-1}	Inhibits the physical interaction of Fas-FasL	Mori *et al.* (1997)

In control experiments, EGTA/Mg^{2+}, CsA, $SrCl_2$, and ZB4 did not cause lysis of either the target cells or the γδ$^+$ CTL clones at the concentrations used in this study (as measured by trypan blue exclusion and the spontaneous:maximum ratio of the ^{51}Cr-release assay).

4.2.8 Effect of γδ$^+$ CTL clone cytotoxicity on intracellular mycobacterial survival

To investigate the effect of γδ$^+$ T cell clone cytotoxicity on intracellular mycobacterial survival, *M. tuberculosis*-infected U937 targets cells were cultured in either the presence or absence of the γδ$^+$ T cell clones (at an E:T of 10:1) for 4 hours in parallel with the ^{51}chromium labelled target cells in the standard cytotoxicity assay (described above). At the end of the incubation period, γδ$^+$ T cell clones and target cells were pelleted by centrifugation at 200 g for 10 minutes. The supernatant was decanted and the remaining cell pellet solubilised using 0.25% SDS PBS. The cell lysate and corresponding supernatant were pooled to compensate for mycobacteria released into the surrounding medium as a result of cell lysis. Mycobacterial

survival in the absence or presence of the $\gamma\delta^+$ CTL clones was measured by determining colony forming units (CFU) of serial 10-fold dilutions of bacterial preparations on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 10% OADC.

4.3 RESULTS

4.3.1 $\gamma\delta^+$ T cells are expanded and activated following stimulation with *M. tuberculosis*

$\gamma\delta^+$ T cells were found to represent only a small percentage of the $CD3^+$ cells (5.9 ± 3.9 %) present in freshly isolated PBMC from the donors evaluated in this study. They became significantly expanded following stimulation with *M. tuberculosis* with a mean of 43.2 (± 18.4) % of *M. tuberculosis*-primed PBMC expressing γδ TCR (Table 4.2). The *M. tuberculosis*-stimulated $\gamma\delta^+$ T cell population was found to be strongly activated with 79.7 (± 17.7) % of $\gamma\delta^+$ cells expressing IL-2 receptor and 60.9 (± 20.1) % expressing HLA-DR.

Table 4.2. *$\gamma\delta^+$ T cells are preferentially expanded and activated following stimulation with M. tuberculosis*

Donor	Percentage $\gamma\delta^+$ T cells following *M.tb*-priming[a]	Relative expansion[b]	$\gamma\delta^+$ activation marker expression (%)[c]	
			$IL\text{-}2R^+$	$HLA\text{-}DR^+$
RG	38.1	14.7	85.1	100.0
JP	33.5	12.9	100.0	58.6
MW	31.8	12.2	91.2	65.5
TS	65.1	25.0	90.0	66.0
MA	69.5	8.2	55.6	66.5
SG	20.3	2.4	65.7	56.7
SC	33.0	3.9	98.1	74.9
EC	23.3	4.3	85.4	42.4
MH	50.4	5.9	77.5	56.0
BR	66.7	7.8	48.1	23.0
Mean (± SD)	43.2 (± 18.4)	9.7 (± 6.8)	79.7 (± 17.7)	60.9 (± 20.1)

[a]Expressed as a percentage of $CD3^+$ cells

[b]Expressed as a ratio of $\gamma\delta^+$ T cells at day 6 versus day 0

[c]Results have been expressed as a percentage of $\gamma\delta^+$ T cells expressing IL-2R or HLA-DR following 6 days of *in vitro* priming with *M. tuberculosis*.

4.3.2 $\gamma\delta^+$ T cell-mediated lysis of *M. tuberculosis*-infected U937 cells

This study has demonstrated that PMA-differentiated U937 cells provide a highly selective indicator for mycobacterial-specific $\gamma\delta^+$ CTL cytolytic activity (Chapter 3) and $\gamma\delta^+$ T cell lines and $\gamma\delta^+$ T cell clones capable of specifically lysing *M. tuberculosis*-infected U937 cells have been generated. To further investigate the functional activity of this T cell subset in tuberculosis, two of the $\gamma\delta^+$ CTL clones (γδ.8 and γδ.19) were studied in more detail. Fig. 4.2 (a-b) shows that γδ.8 and γδ.19 CTL clones were preferentially cytolytic towards *M. tuberculosis*-infected U937 cells as compared with cells pulsed with an irrelevant streptococcal antigen, SK-SD. In all experiments, the $\gamma\delta^+$ CTL clones were found to be similarly cytolytic towards the control, uninfected U937 target cells and cells pulsed with an irrelevant streptococcal antigen, SK-SD (data not shown). To allow clear comparison between the mycobacterial antigen-specific and non-specific cytolysis generated by the $\gamma\delta^+$ T cell clones, only SK-SD-pulsed (irrelevant antigen) U937 target control has been included in figures throughout this chapter.

The cytolytic ability of γδ.8 and γδ.19 T cell clones was not restricted exclusively to target cells infected with *M. tuberculosis*, however, since both $\gamma\delta^+$ T cell clones were also cytolytic towards Daudi Burkett's lymphoma cell line (Fig. 4.2c). The cross-reactivity between mycobacterial antigen- and Daudi-reactive $\gamma\delta^+$ T cell subsets is well described and studies have shown that, although the stimulatory ligands within these antigen preparations differ (Kaur *et al.*, 1993; Constant *et al.*, 1994; Tanaka *et al.*, 1995), the $\gamma\delta^+$ T cell responses against these two antigens are nonetheless closely related and strictly overlapping (Davodeau *et al.*, 1993; Bukowski *et al.*, 1995).

4.3.3 Cellular contact requirement for $\gamma\delta^+$ T cell cytotoxicity

When the $\gamma\delta^+$ CTL clones were kept in suspension throughout the assay, the level of cytotoxicity generated against *M. tuberculosis*-infected U937 cells was greatly reduced (75.9% inhibition) compared with parallel experiments in which $\gamma\delta^+$ CTL clones were kept in close contact with ^{51}Cr-labelled targets (Fig. 4.3). These results suggest that lysis of U937 target cells was dependent on cell-to-cell contact.

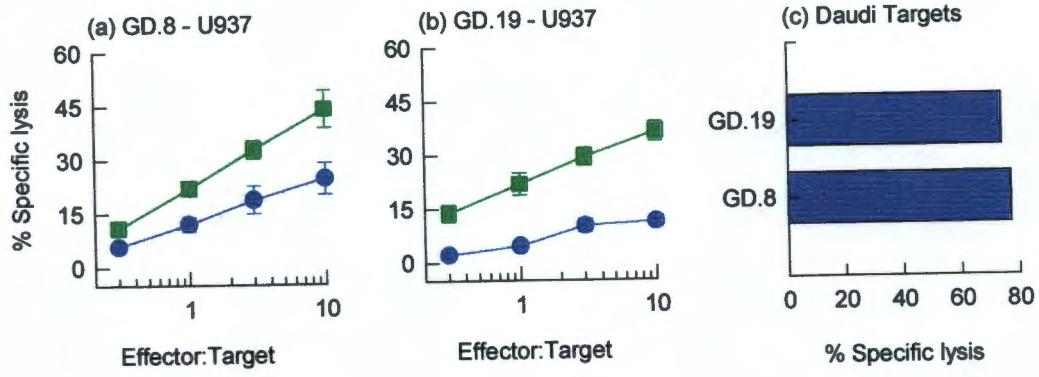


FIGURE 4.2. *M. tuberculosis*-specific cytolysis generated by $\gamma\delta^+$ CTL clones (γδ.8 and γδ.19) against infected U937 target cells. (a) γδ.8 and (b) γδ.19 CTL clones were evaluated for cytolytic activity against U937 targets either infected with *M. tuberculosis* (5 CFU.cell^{-1}) (■) or pulsed with an irrelevant streptococcal antigen, SK-SD (●). Each data point represents the mean percentage cytolysis (± SEM) of at least 3 independent experiments. (c) In a separate set of experiments, the *M. tuberculosis*-reactive $\gamma\delta^+$ CTL clones were used as effectors against Daudi target cells at an E:T of 10:1. In all experiments, cytotoxicity was evaluated after 4 hours.

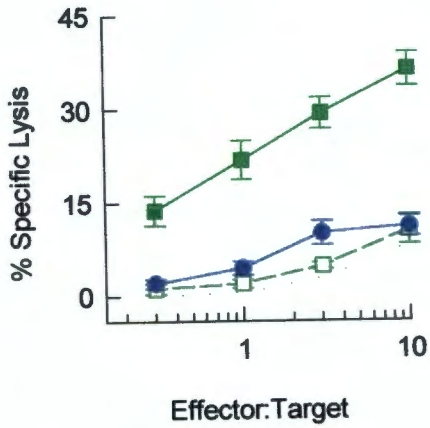


FIGURE 4.3. Prevention of $\gamma\delta^+$ CTL-mediated cytolysis by impeding cell-to-cell contact. Clone γδ.19 and *M. tuberculosis*-infected U937 targets were either allowed close effector-to-target cell contact (■; solid line) or kept in suspension by repeated gentle pipetting every 15 minutes throughout the course of the experiment (□; dashed line). U937 target cells pulsed with an irrelevant antigen, SK-SD (●; solid line) were included as an antigen-specificity control for the γδ CTL-mediated cytolysis. Each data point represents the mean (± SEM) of at least 2 independent experiments. Cytotoxicity was measured after 4 hours.

4.3.5 Mechanism of cytotoxicity mediated by $\gamma\delta^+$ CTL clones

To examine the cytolytic mechanism/s used by the $\gamma\delta^+$ CTL clones (γδ.8 and γδ.19), various inhibitors of granule exocytosis (EGTA/Mg^{2+}; Strontium; Cyclosporin A) or Fas-FasL mediated cytolysis (ZB4) were used to systematically disrupt key points in these major cell death pathways.

Calcium and granule exocytosis requirements of $\gamma\delta^+$ CTL cytotoxicity

Three alternative approaches were used to disrupt granule exocytosis of the $\gamma\delta^+$ CTL clones: (i) The release of soluble mediators from cytoplasmic granules is mediated by Ca^{2+} release and granule exocytosis can be blocked using the Ca^{2+} chelator EGTA/Mg^{2+}. Fig. 4.4 demonstrates the effect of removing Ca^{2+} using EGTA prior to the addition of ^{51}Cr-labelled target cells. The presence of EGTA/Mg^{2+} completely abrogated mycobacterial antigen-specific cytolysis for both $\gamma\delta^+$ CTL clones (Fig. 4.4, left panel). (ii) Cyclosporin A (CsA) has previously been shown to interfere with biochemical events in the later stages of Ca^{2+}-dependent granule exocytosis which follow the binding of calmodulin to cytoskeletal or cytoplasmic calmodulin binding proteins (Trenn *et al.*, 1989). In the present study, CsA only partially inhibited mycobacterial antigen-specific cytolysis by 46.6% for γδ.8 and 23.0% for γδ.19 (Fig. 4.4, middle panel). (iii) Previous studies have shown that Sr^{2+} induces a non-specific and transient degranulation of granule-containing cells, rendering them unable to lyse susceptible target cells (Neighbour and Huberman, 1982; Quan *et al.*, 1982). Fig. 4.4 (right panel) demonstrates the effect of pre-incubating the $\gamma\delta^+$ CTL clones in the presence of Sr^{2+} on $\gamma\delta^+$ T cell-mediated cytolysis. Degranulation of $\gamma\delta^+$ CTL clones using Sr^{2+} completely abrogated mycobacterial antigen-specific cytolysis against U937 target cells. Taken together, these results suggest a predominant role for the Ca^{2+}-dependent/perforin-based mechanism in $\gamma\delta^+$ T cell cytotoxicity in the present system.

Both $\gamma\delta^+$ T cell clones generated an element of non-specific cytolysis against U937 targets pulsed with the irrelevant antigen, SK-SD. Treatment of the clones with EGTA and, to a lesser extent, $SrCl_2$ also abrogated this non-specific component of cytolysis.

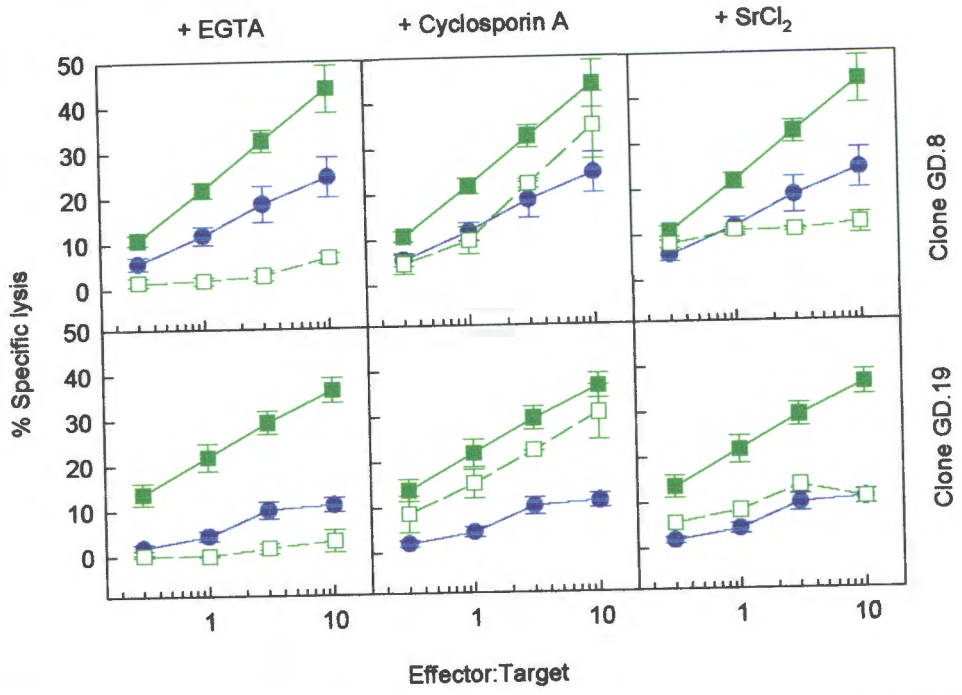


FIGURE 4.4. Characterisation of the effector mechanisms used by γδ.8 (left panel) and γδ.19 (right panel) CTL clones against U937 target cells. Mycobacterial-specific cytolytic activity against U937 target cells was investigated using EGTA (left panel), CsA (middle panel), and $SrCl_2$ (right panel) to block granule-mediated effector mechanisms. U937 target cells were either infected with *M. tuberculosis* (■; solid line) or pulsed with an irrelevant antigen, SK-SD (●; solid line). Alternatively, cytotoxic activity against *M. tuberculosis*-infected U937 cells was determined in the presence of either EGTA, CsA or $SrCl_2$ (□; dashed line). Each data point represents the mean percentage cytolysis (± SEM) of at least 3 independent experiments.

Fas-based mechanisms are not required for γδ⁺ CTL mediated cytolysis

Even though many Fas-negative targets (such as Daudi and U937 cells; Zeine *et al.*, 1998) are lysed by $\gamma\delta^+$ T cells, it is still possible that Fas-mediated cytolysis occurs. In order to evaluate the importance of Fas-FasL interactions, the present study made use of the strongly Fas-positive Jurkat target cell line that has previously been shown to be susceptible to $\gamma\delta^+$ T cell-induced cytotoxicity even in the absence of Ca^{2+} (Haeker and Wagner, 1994). Both γδ.8 and γδ.19 CTL clones showed strong cytolytic activity against Jurkat cells (Fig. 4.5.). Pre-activation of the $\gamma\delta^+$ CTL clones using PMA and ionomycin did not augment the efficiency of cytolysis against Jurkat target cells (data not shown). Figure 4.5 shows that pre-incubation for 1-2 hours at 37°C with an anti-Fas mAb (clone ZB4; Immunotech) at concentrations recommended by the manufacturer to neutralize Fas-mediated apoptosis (0.5-2 $\mu g.ml^{-1}$) did little to interfere with Jurkat cell lysis by the $\gamma\delta^+$ CTL clones. Indirect immuno-fluorescence

confirmed that pre-incubation of the Jurkat cells with ZB4 under these conditions resulted in strong staining of these cells for Fas as assessed by flow cytometry [with 98.9 (± 0.3) % staining positive for Fas, mean channel fluorescence of 2.9 (± 0.3)] down to a dilution of 0.125 $\mu g.ml^{-1}$. Cytolysis of Fas^+ Jurkat target cells was found to be sensitive to Mg^{2+}EGTA (47.0% inhibition), however, suggesting that the $\gamma\delta^+$ T cell clones preferentially use a perforin-based mechanism of cytolysis, even when given the opportunity to use a Fas-based system.

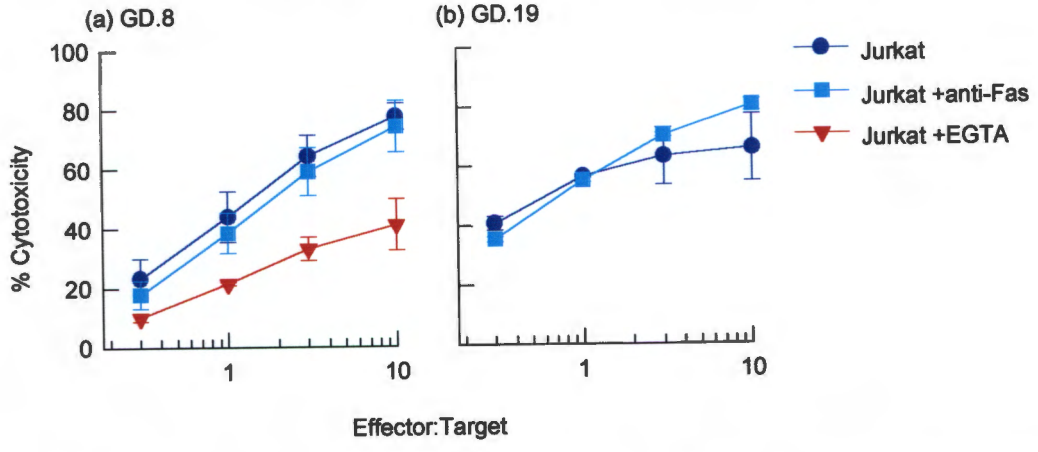


FIGURE 4.5. The role of Fas in mediating $\gamma\delta^+$ CTL clone cytotoxicity against Jurkat target cells. ZB4, an anti-Fas monoclonal antibody (500 ng.ml-1), does not interfere significantly with the lysis of Fas^+ Jurkat cells by γδ.8 and γδ.19 CTL clones. Cytolysis was measured either in the absence (●) or presence of inhibitors of either Fas-FasL interaction [ZB4 (■)] or granule exocytosis [EGTA/Mg^{2+} (▼)]. Each data point represents the mean percentage cytolysis (± SEM) of at least 3 independent experiments.

4.3.6 Effect of $\gamma\delta^+$ CTL cytolysis on intracellular mycobacterial survival

The precise role of CTL's in the control of intracellular pathogens *in vivo* remains uncertain. One possibility is that cytolysis of infected macrophages by CTL's directly impacts on the viability of the pathogens. This effect has been demonstrated with mycobacteria-specific CTL's (DeLibero *et al.*, 1988; Silva *et al.*, 1996; Stenger *et al.*, 1997; Stenger *et al.*, 1998). The present study set out to investigate whether the $\gamma\delta^+$ CTL clones, shown to use predominantly granule-dependent cytotoxicity mechanisms (Fig. 4.4), exerted any effects on survival of intracellular *M. tuberculosis*. No reduction in mycobacterial viability was observed with either of the $\gamma\delta^+$ CTL clones despite their ability to induce significant cytolysis of *M. tuberculosis*-infected U937 target cells (Fig. 4.6).

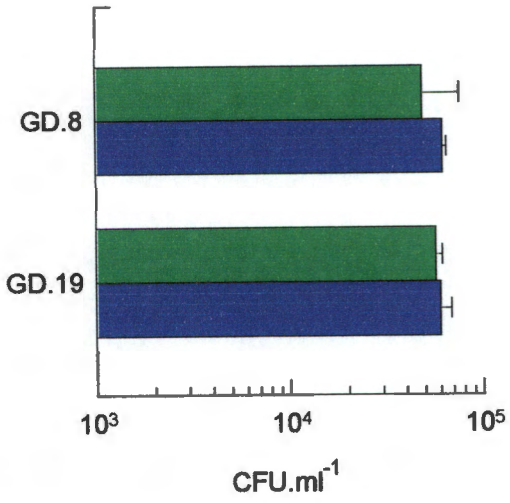


FIGURE 4.6. Granule-dependent cytolysis by $\gamma\delta^+$ CTL clones does not restrict intracellular mycobacterial growth. *M.tuberculosis*-infected U937 targets cells were incubated either in the absence (blue bars) or presence (green bars) of γδ.8 and γδ.19 CTL clones at an E:T ratio of 10:1 for 18 hours. Following incubation, U937 targets and clones were lysed with detergent and released bacilli were serially diluted and plated onto Middlebrooks agar to determine colony forming units (CFU). Each bar represents the mean percentage $CFU.ml^{-1}$ (± SD) of 2 independent experiments.

4.4 DISCUSSION

The present study demonstrates that $\gamma\delta^+$ CTL's are capable of specific and rapid cytolysis of *M. tuberculosis*-infected U937 target cells. Lysis of infected U937 cells was dependent on cell-to-cell contact and was mediated by the granule exocytosis pathway. Accordingly, the ability of the $\gamma\delta^+$ CTL clones to lyse infected targets was found to be Ca^{2+}-dependent, was significantly reduced in the presence of CsA (a specific inhibitor of granule exocytosis), and completely abrogated following de-granulation of the $\gamma\delta^+$ T cell effectors using Sr^{2+}. Despite being strongly cytolytic against infected U937 cells, however, the $\gamma\delta^+$ CTL clones did not have any impact on the survival of intracellular *M. tuberculosis*.

Although the mycobacterial antigen-responsive $\gamma\delta^+$ CTL clones described in this study were shown to selectively lyse *M. tuberculosis*-infected U937 but not cells pulsed with the streptococcal antigen SK-SD (Fig. 4.2), the ability of $\gamma\delta^+$ T cells to mediate antigen-specific cytolysis is a contentious issue. Initial reports describing the responses of cytolytic $\gamma\delta^+$ T cells in tuberculosis showed that the lytic activity was restricted to target cells infected with *M. tuberculosis* because cells similarly infected with group A streptococci or *L. monocytogenes*

were not sensitive to $\gamma\delta^+$ T cell-mediated cytolysis (Munk *et al.*, 1990). More recent studies have found, however, that the $\gamma\delta^+$ T cell response is directed against "infected" cells rather than specific pathogens. $\gamma\delta^+$ T cells derived from herpes simplex virus (HSV)-stimulated PBMC were cytotoxic towards both HSV-infected target cells as well as cells infected with unrelated viruses (Bukowski *et al.*, 1994). Moreover, $\gamma\delta^+$ T cells have been shown to lyse cells infected with human immunodeficiency virus (Wallace *et al.*, 1996), HSV (Bukowski *et al.*, 1994), simian immunodeficiency virus (Malkovsky *et al.*, 1992a; Malkovsky, 1992b) and human herpes virus-6 (Lusso *et al.*, 1995) without previous exposure to corresponding viral antigens. In light of this evidence, it has been postulated that $\gamma\delta^+$ T cells function early in viral and bacterial diseases as front line defenders to remove infected or otherwise stressed cells (Janeway *et al.*, 1988) and that the antigens recognised are likely to be of cellular origin (Bukowski *et al.*, 1994). $\gamma\delta^+$ T cells are therefore poised, as are macrophages, to recognise structures presented by both micro-organisms and stressed cells but not by normal cells (Tanaka *et al.*, 1995; Porcelli *et al.*, 1996; Havran *et al.*, 1994).

The *M. tuberculosis*-responsive $\gamma\delta^+$ T cell clones described in this chapter were found to be strongly cytolytic towards Daudi Burkitt's lymphoma cells (Fig. 4.2c). This cross-responsive property of both mycobacterial antigen- and Daudi-derived $\gamma\delta^+$ T cells has been extensively described in earlier studies (Fisch *et al.*, 1990a; Fisch *et al.*, 1990b; De Libero *et al.*, 1991; Fisch *et al.*, 1992; Davodeau *et al.*, 1993). Interestingly, while it is well established that mycobacterial antigens and Daudi Burkitt's lymphoma cells are both potent stimulators of human $\gamma\delta^+$ T cells (Davodeau *et al.*, 1993; Bukowski *et al.*, 1995; Marx *et al.*, 1997), the stimulatory ligands within these antigen preparations have been found to differ. The major mycobacterial $\gamma\delta^+$ T cell-stimulating ligands were found to be non-proteinaceous, low MW, phosphate-containing molecules (Constant *et al.*, 1994; Tanaka *et al.*, 1995; Schoel *et al.*, 1994; Morita *et al.*, 1995), while the target antigens on the surface of Daudi cells appear to be heat shock proteins (Kaur *et al.*, 1993). Although the ability of $\gamma\delta^+$ T cells to recognise structurally unrelated antigens seems to indicate that recognition must be TCR-independent, several studies have demonstrated the contrary (De Libero *et al.*, 1991; Davodeau *et al.*, 1993; Tanaka *et al.*, 1994).

In order to examine the cytotoxic mechanism/s utilised by the *M. tuberculosis*-primed $\gamma\delta^+$ CTL clones described in this study, a series of key steps along the major cell death pathways were sequentially disrupted. $\gamma\delta^+$ T cells have been shown to have the ability to mediate cytolysis by either granule exocytosis (Koizumi *et al.*, 1991; Nakata *et al.*, 1990; Gan and Malkovsky, 1996; Zeine *et al.*, 1998) or Fas-FasL interaction (Nagata and Goldstein, 1995; Suda *et al.*, 1995; Vincent *et al.*, 1996). This study found that cytolysis by the *M. tuberculosis*-reactive $\gamma\delta^+$ CTL clones against infected U937 target cells was exquisitely sensitive to EGTA, demonstrating that target cell lysis was strongly Ca^{2+}-dependent. Ca^{2+} is not only essential for granule exocytosis but also for binding of perforin to target cell membranes (Ishiura *et al.*, 1990). By comparison, several reports have demonstrated that Fas-mediated apoptosis is Ca^{2+}-independent (Odake *et al.*, 1991; Esser *et al.*, 1996; Anel *et al.*, 1994; Lowin *et al.*, 1994; Rouvier *et al.*, 1993; Mori *et al.*, 1997). The ability of specific inhibitors of granule exocytosis, such as CsA, and de-granulation of the $\gamma\delta^+$ CTL clones using Sr^{2+} to abolish the cytolysis of infected U937 targets (Fig. 4.4) provided further evidence that the $\gamma\delta^+$ CTL clones mediated cytolysis by the granule exocytosis pathway. More directly, because U937 do not express detectable levels of cell surface Fas (Zeine *et al.*, 1998), it is improbable that Fas-FasL interaction is involved in $\gamma\delta^+$ CTL-mediated cytolysis of U937 target cells.

Fas^{high} Jurkat cells have been widely used as susceptible targets for investigating Fas-FasL-mediated cytolysis (Gan and Malkovsky, 1996; Mori *et al.*, 1997). Despite their sensitivity to Fas-mediated cytolytic mechanisms, the present data (Fig. 4.5) and previous reports (Haeker and Wagner, 1994; Zeine *et al.*, 1998) have demonstrated that $\gamma\delta^+$ CTL's preferentially kill Jurkat targets by the granule exocytosis pathway and that cytolysis was strongly inhibited in the presence of even low concentrations of EGTA. These results suggest that $\gamma\delta^+$ T cells prefer the granule exocytosis pathway for mediating cytolysis even when presented with the opportunity to use a Fas-based mechanism. Similarly, CD8 T cells and NK cells have also been shown to preferentially utilise a Ca^{2+}-dependent pathway for cytolysis (Kagi *et al.*, 1994; Lowen *et al.*, 1994; Mori *et al.*, 1997).

Since $\gamma\delta^+$ T cells are capable of killing targets cells using either mechanism of cytolysis (granule exocytosis or Fas-FasL interaction), the relative contribution of each mechanism and the trigger dictating which mechanism is favoured has become the focus of several recent

studies (Haeker and Wagner, 1994; Gan and Malkovsky, 1996; Zeine *et al.*, 1998). While some studies have demonstrated that $\gamma\delta^+$ T cells are able to utilise both the Ca^{2+}-dependent and the Fas-based pathways depending on the target cell being recognised (Haeker and Wagner, 1994; Vincent *et al.*, 1996), others have found that granule exocytosis or the perforin-based pathway was predominant (Zeine *et al.*, 1998; Gan and Malkovsky, 1996). It is becoming apparent that intrinsic properties of the target cell may dictate whether cytolytic $\gamma\delta^+$ T cells utilise granule exocytosis, the Fas pathway or both pathways (Berke, 1991; Haeker and Wagner, 1994). The underlying mechanism that preferentially triggers one or the other pathway remains to be established.

The significance of cytolytic T cells in the control of intracellular pathogens *in vivo* remains unclear. One possibility is that T cell mediated cytolysis facilitates the release of live bacteria from inefficiently activated macrophages, thereby allowing these bacilli to be taken up and killed by newly recruited and freshly activated macrophages (De Libero *et al.*, 1988; Kaufmann, 1988). Until recently, the ability of T cell-mediated cytolysis to directly affect the viability of intracellular pathogens was considered highly controversial. Several studies have since demonstrated that cytolytic $CD8^+$ T cells are able to restrict the intracellular growth of *M. tuberculosis* by virtue of their ability to lyse infected target cells (Silva *et al.*, 1996; Stenger *et al.*, 1997; Stenger *et al.*, 1998). Stenger *et al.* (1997) found that $CD8^+$ CTL's that induced target cell lysis by granule exocytosis were able to reduce intracellular bacterial survival but that similarly cytotoxic $CD4^-CD8^-$ (double negative) T cells that lysed target cells by the interaction of Fas-FasL were not. The ability of these $CD8^+$ CTL's to kill intracellular *M. tuberculosis* was found to be dependent on the presence of a critical effector molecule, granulysin, within their cytolytic granules (Stenger *et al.*, 1998). In contrast, Oddo *et al.* (1998) described the ability of FasL-induced apoptosis to reduce the viability of intracellular *M. tuberculosis*. Although this study artificially induced target cell lysis using soluble recombinant Fas ligand, it serves to demonstrate that this mechanism of cytolysis can also be directly linked with anti-mycobacterial activity.

A similar mycobactericidal role for $\gamma\delta^+$ T cells in mycobacterial infections was thus investigated. Although the $\gamma\delta^+$ CTL clones investigated in the present study demonstrated a significant ability to lyse *M. tuberculosis*-infected U937 target cells by granule exocytosis, they

failed to effect mycobacterial survival (Fig. 4.6). It is likely that some key differences exist in the complement of cytolytic molecules contained within the granules of the mycobacterial antigen-specific $CD8^+$ CTL's described by Stenger *et al.* (1997, 1998) and the $\gamma\delta^+$ CTL clones described in this Chapter. While it remains to be established whether granulysin is present within the cytotoxic granules of $\gamma\delta^+$ CTL's, this protein has been detected within cytotoxic granules of both $CD8^+$ CTL's and NK cells (Jongstra *et al.*, 1987; Pena *et al.*, 1997).

The present study brings us closer to understanding the exact physiological role of $\gamma\delta^+$ T cells in tuberculosis. It demonstrates that $\gamma\delta^+$ T cells are significantly expanded and strongly activated following exposure to mycobacterial antigens, that they demonstrate a striking propensity for killing *M. tuberculosis*-infected cells, that they preferentially mediate cytolysis by the granule exocytosis pathway but that they fail to demonstrate any direct microbicidal effects of intracellular mycobacteria. It is plausible that the unique role of $\gamma\delta^+$ CTL's in tuberculosis stems from their ability, on the one hand, to become activated and produce significant amounts of IFN-γ and IL-2 very early following infection (Tsukaguchi *et al.*, 1995); and, on the other hand, to facilitate the release of intracellular mycobacteria from inadequately primed "first line of defence" phagocytes, thereby allowing the invading bacilli to be taken up by more effectively activated and mycobactericidal macrophages.

CHAPTER 5

CONCLUSIONS AND GENERAL DISCUSSION

The aim of the research presented in this thesis was to examine the use of the human macrophage cell line U937 as an *in vitro* model for human macrophage function in mycobacterial infections. This involved evaluation of the ability of U937 cells to phagocytose *M. tuberculosis*, to control the intracellular replication of this organism, and present mycobacterial antigens to human HLA class I-matched CTLs. In this concluding chapter, I present my evaluation of the utility that this cell line may have in the study of mycobacterial infections.

U937 as a model for human macrophage function in tuberculosis

This study has demonstrated that, in many respects, the U937 cell line is useful for the study of a variety of different aspects of mycobacterial-macrophage interactions. Differentiation of U937 cells using IFN-γ, 1,25-$(OH)_2$ vitamin D_3, or PMA was shown to significantly enhance their ability to phagocytose *M. tuberculosis* but failed to influence their ability to mount a subsequent respiratory burst response. Following infection, U937 cells were found to be highly permissive to the intracellular growth of both the virulent H37Rv strain of *M. tuberculosis* and the attenuated vaccine strain of *M. bovis* BCG. U937 cells have been shown to constitutively express high levels of cell surface HLA class I while producing undetectable levels of HLA class II both at the mRNA level and at the cell surface. HLA class II expression was neither up-regulated following infection with *M. tuberculosis* nor inducible using IFN-γ, 1,25-$(OH)_2$ vitamin D_3, PMA, GM-CSF or a combination of these agents. In contrast, chronic infection of U937 cells with virulent H37Rv *M. tuberculosis* but not with BCG resulted in the cell surface expression of HLA class I being significantly up-regulated. Taken together, these characteristics made U937 cells a very attractive model for further investigations into their ability to present mycobacterial antigens to human HLA class I-restricted CTLs.

A comparison of the phenotypic and functional characteristics of U937 cells with another human monocytic cell line THP-1, human monocytes and macrophages revealed some interesting

maturational differences. THP-1 but not U937 cells could be induced to express cell surface HLA class II. CD14 expression was more readily inducible on THP-1 cells compared with U937. U937 cells, on the other hand, expressed CD16 (Fcγ receptor) constitutively while THP-1 cells showed reduced expression of this antigen at the cell surface. Functionally, THP-1 cells, human monocytes and terminally differentiated macrophages were better able to phagocytose *M. tuberculosis* than U937 cells. Following phagocytosis, however, U937 cells were more permissive to the intracellular growth of *M. tuberculosis* than THP-1 cells.

This represents the first study to thoroughly investigate and compare the phenotypic and functional characteristics and utility of U937 and THP-1 cells in the context of mycobacterial infection. Although distinct phenotypic and functional differences between U937 and THP-1 cell lines have been identified in this study, the impact of these differences on later events in mycobacterial infection is an exciting area that still needs to be examined. Future experiments aimed at exploiting these differences would contribute significantly to understanding of the ways in which phenotypically distinct phagocytes initially interact with *M. tuberculosis*, how differing routes of mycobacterial entry influence subsequent intracellular processing, and the influence of disparate HLA expression on mycobacterial antigen presentation to human T cells.

U937 as a model for HLA class I presentation of mycobacterial antigens

The inability of U937 cells express HLA class II while expressing high levels of cell surface HLA class I, together with their ability to phagocytose *M. tuberculosis* and support the intracellular growth of this pathogen made this particular monocytic cell line very attractive for further investigations into the presentation of mycobacterial antigens to HLA class I-restricted CTLs. Results presented in this study demonstrated that *M. tuberculosis*-primed CTLs from HLA class I-matched donors lysed *M. tuberculosis*-infected U937 target cells in an antigen-specific manner and with a similar efficiency to autologous macrophage targets. This cytolytic activity was restricted to live organisms since only U937 cells infected with *M. tuberculosis* and BCG but not those pulsed with soluble PPD were lysed by the HLA class I-matched effector cells. Furthermore, *M. tuberculosis*-stimulated but HLA-mismatched CTLs failed to lyse infected U937 cells in an antigen-specific manner. T cell subset fractionation of the HLA class I-matched *M. tuberculosis*-primed CTL population

and limiting dilution cloning demonstrated that the cytolytic activity was mediated by $CD8^+$ cytolytic T cells and confirmed that $CD4^+$ T cells showed no significant ability of lyse infected U937 target cells. Although *M. tuberculosis*-infected U937 targets shared only a single HLA class I haplotype match (either HLA-A3 or -B51) with the CTL donors, they were more rapidly and strongly lysed by $CD8^+$ CTLs than similarly infected autologous macrophage targets.

A panel of *M. tuberculosis*-reactive $CD8^+$ CTL clones was generated which proliferated strongly in the presence of virulent *M. tuberculosis* and less vigorously to soluble PPD but demonstrated only low levels of cytolytic activity against *M. tuberculosis*-infected U937 targets. Although there has been an isolated report of moderate mycobacterial antigen-specific $CD8^+$ cytolytic activity following conventional antigenic priming (Turner & Dockrell, 1996), the majority of published reports describing the cytolytic capacity of $CD8^+$ T cells in mycobacterial infections have used sophisticated technology or defined *in vitro* culture conditions to demonstrate even low levels of $CD8^+$ CTL functional activity (Tan *et al.*, 1997; Lalvani *et al.*, 1998; Lewinshon *et al.*, 1998; Monagneghpour *et al.*, 1998; Canaday *et al.*, 1999). It seems likely that the low cytolytic capacity demonstrated by $CD8^+$ CTLs in mycobacterial infections is the result of a number of closely related factors. It is recognized that 'cross-presentation' of exogenously-derived bacterial antigens by HLA class I molecules is relatively inefficient with only an estimated 10% of phagocytes being capable of this kind of presentation (Reis e Sousa & Germain, 1995; Harris *et al.*, 1995). Furthermore, it has been suggested that the standard ^{51}Cr release assay used to determine cytolytic activity is not sensitive enough to detect cells with low precursor frequencies (Lalvani *et al.*, 1998). Unfortunately, and as a result of these limitations, many of the more recent studies have focused instead on the ability of *M. tuberculosis*-reactive $CD8^+$ T cells to produce cytokines or proliferate in response to stimulation with very defined mycobacterial peptides or antigens (Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998; Canaday *et al.*, 1999). Although providing valuable insight into various other aspects of $CD8^+$ T cell function in mycobacterial infections, these studies have done little to further our understanding of classical HLA class I-restricted $CD8^+$ CTL lytic activity in tuberculosis.

The present study, using U937 as an *in vitro* model to directly investigate *M. tuberculosis*-reactive $CD8^+$ T cell cytolytic activity, has provided not only an innovative but also a relatively simple and robust approach to a difficult problem. Furthermore, it is easily amenable to further development by the introduction of genes encoding other HLA class I molecules as well as co-stimulatory molecules. Future studies should be directed at more intensive investigations into the conditions required to enhance $CD8^+$ CTL lytic activity in response to *M. tuberculosis* stimulation. With the establishment of *M. tuberculosis*-specific $CD8^+$ T cells demonstrating greater lytic ability, this model could facilitate more defined evaluation of the mechanisms of HLA class I presentation of mycobacterial antigens to $CD8^+$ CTL, investigations into the mechanism of cytolysis favoured by these cells, the effects of cytolysis on intracellular mycobacterial viability, and identification of mycobacterial epitopes responsible for priming $CD8^+$ CTL.

U937 provide a good indicator for $\gamma\delta^+$ CTL activity in mycobacterial infections

This study further demonstrated that *M. tuberculosis*-infected U937 were highly sensitive to mycobacterial antigen-specific cytolysis mediated by $\gamma\delta^+$ CTL. Mycobacterial-specific $\gamma\delta^+$ CTLs consistently showed stronger cytolytic activity against infected U937 target cells than $CD8^+$ CTL but were not restricted to classical HLA class I or class II molecules. Although $\gamma\delta^+$ CTLs proved exceptionally difficult to maintain in long term *in vitro* culture, the establishment of a panel of strongly cytolytic human *M. tuberculosis*-reactive $\gamma\delta^+$ CTL clones provided a unique opportunity to investigate more thoroughly the role of $\gamma\delta^+$ CTL lytic activity in human mycobacterial infections. This study found that cytolysis mediated by the $\gamma\delta^+$ T cell clones was dependent on cell-to-cell contact and was mediated by the granule exocytosis/perforin pathway. Accordingly, the ability of the $\gamma\delta^+$ CTL clones to lyse infected targets was found to be Ca^{2+}-dependent, was significantly reduced in the presence of cyclosporine A (a specific inhibitor of granule exocytosis), and completely abrogated following de-granulation of the $\gamma\delta^+$ T cell effectors using Sr^{2+}.

The significance of cytolytic T cells in the control of intracellular pathogens *in vivo* remains unclear. It has been suggested that T cell mediated cytolysis facilitates the release of live bacteria from infected but inadequately activated macrophages that have failed to either control or destroy their intracellular quarry (De Libero *et al.*, 1988; Kaufmann, 1988). This

in turn would allow these bacilli to be engulfed and killed by newly recruited and freshly activated macrophages. The ability of T cell-mediated cytolysis to directly effect the viability of intracellular pathogens was considered, until fairly recently, to be highly controversial. Several studies have since confirmed that non-classical CD1-restricted $CD8^+$ CTLs are able to directly restrict the intracellular growth of *M. tuberculosis* by virtue of their ability to lyse infected target cells (Stenger *et al.*, 1997; Stenger *et al.*, 1998). Stenger *et al.* (1997) found that $CD8^-$ CTLs that induced target cell lysis by granule exocytosis were able to reduce intracellular bacterial survival but that similarly cytotoxic $CD4^-CD8^-$ (double negative) T cells that lysed target cells by the interaction of Fas-FasL were not. Granulysin, contained within the cytolytic granules of these $CD8^+$ CTLs, was identified as the cytolytic molecule responsible for the mycobactericidal activity (Stenger *et al.*, 1998).

Despite being significantly expanded and strongly activated following exposure to mycobacterial antigens, demonstrating a striking propensity for killing *M. tuberculosis*-infected cells, and preferentially mediating cytolysis by the granule exocytosis pathway, the $\gamma\delta^+$ CTL clones investigated in this study failed to exhibit any direct microbicidal effects on intracellular mycobacteria. It remains to be established whether granulysin is present within the cytotoxic granules of $\gamma\delta^+$ CTLs and this would obviously provide a future direction for this study.

From the results presented in this study, however, it seems likely that the role of $\gamma\delta^+$ CTL's in mycobacterial infections stems not from their ability to directly restrict mycobacterial growth but rather from their ability, on the one hand, to become activated very early following infection and produce significant amounts of IFN-γ and IL-2 (Tsukaguchi *et al.*, 1995) and, on the other hand, to facilitate the release of intracellular mycobacteria from inadequately primed "first line of defence" phagocytes, thereby allowing the invading bacilli to be taken up by more effectively activated and mycobactericidal macrophages. This study represents the first examination of the mechanism of cytolysis utilized by cytolytic $\gamma\delta^+$ T cells in mycobacterial infection and the first to investigate the effect of $\gamma\delta^+$ T cell-mediated cytolysis on intracellular survival.

In conclusion, the present study has demonstrated that there is definite potential for the use of the human monocytic cell line U937 as an *in vitro* model for macrophage function in

mycobacterial infections. U937 cells engulfed *M. tuberculosis* efficiently, clearly supported the intracellular growth of this organism, and presented *M. tuberculosis*-derived antigens to HLA class I-matched $CD8^+$ CTL more effectively than monocyte-derived macrophages. Furthermore, this study has demonstrated that this cell line provides a highly selective indicator for mycobacterial-specific $\gamma\delta^+$ CTL cytolytic activity.

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