Characterisation of cytolytic CD4+ and suppressor CD8+ activity of T lymphocyte clones derived from tuberculous pleuritis

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This thesis is dedicated to my late father, Helmuth Heinrich Mathias Glashoff, and to my mother, Jean Margaret Glashoff

Declaration

I, Richard Helmuth Glashoff, hereby declare that this thesis is my own unaided work, and that neither the whole nor any part thereof has been, or is to be submitted for another degree at this or any other University. Parts of this thesis have been presented at The 3rd International Congress on the Pathogenesis of Mycobacterial Disease, Stockholm, Sweden, 1996; The EMBO Workshop Meeting on T Lymphocyte-mediated Cytotoxicity, Kerkrade, The Netherlands, 1997; and The 3rd Federation of African Immunology Societies Congress, Cape Town, South Africa, 1997.

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Abstract

Despite the availability of effective treatment strategies, tuberculosis remains a leading cause of death world-wide. A major problem in the attempt to eradicate the disease has been the reliance on long-term multi-drug antibiotic treatment. The development of novel anti-tuberculosis treatment and possibly of a vaccine is essential for successful eradication of the disease. An understanding of the host cellular immune response to *Mycobacterium tuberculosis* infection is pivotal in the development of any new treatment programme. The cellular immune response following infection with *M. tuberculosis* is multi-factorial and complex. The granulomatous lesion within the lung is the outcome of the co-ordinated interaction of macrophages and T lymphocytes. The interplay between these two cell types is generally successful in limiting the infection to the lung. How these cells effect an eradication of infecting mycobacteria remains unclear. The present study has examined T lymphocyte activity in tuberculous pleuritis. The tuberculous pleural effusion affords a unique opportunity to study the mononuclear cell population of a *M. tuberculosis* disease-associated site. The mononuclear cells derived from the effusion displayed unique day 5 peak proliferation to PPD, a pattern intermediate between previously described accelerated day 4 and normal day 7 responses. Induction of *in vitro* cytotoxic activity was also intermediate to normal day 6 and accelerated day 3 kinetic patterns. The responder cell frequency in the effusion population was elevated as compared to peripheral blood-derived mononuclear cells, however LDA data suggested an element of suppressor activity as well. Although the CD4+ T lymphocyte population was expanded in the effusion, CD8+ cells were also detected. T lymphocyte clones of both CD4+ and CD8+ cell populations were generated. Mycobacterial antigen-specific CD4+ T lymphocyte clones were all of a type-1 phenotype, producing high levels of interferon-γ (> 10500 pg/ml), but undetectable levels (< 16 pg/ml) of interleukin-4. The clones all proliferated in response to mycobacterial antigen stimulation. The majority of the clones were cytolytic and the lysis of infected macrophages by CD4+ cytolytic clones resulted in a reduction in intracellular mycobacterial viability. Mean reduction in CFU counts of between 31% and 42% were detected. Mycobactericidal activity was associated with macrophage apoptosis, however apoptosis itself could not account for mycobacterial killing. A major defining feature in CD4+ mycobactericidal activity was the existence of a predominant granule exocytosis mechanism of killing. Clone cytolytic activity was strongly calcium-dependent, and inhibition of granule exocytosis by EGTA, cyclosporin A, concanamycin A and strontium resulted in a significant reduction of cytolytic activity. In addition to the effusion-derived clones, an effusion-derived T lymphocyte line, which also possessed a predominant granule-mediated killing pathway, could similarly induce mycobacterial death. A peripheral blood-derived CD4+-enriched T lymphocyte line derived from the same patient and which possessed a predominant Fas killing pathway, was unable to effect any change in mycobacterial viability. The occurrence of a granule-mediated cytolytic killing pathway in mycobacterial antigen-specific CD4+ T cells may represent an

important component of protective cell immunity and may explain organism clearance at disease sites. An antigen-specific CD8+ clone was unique in that it produced both interferon-γ and interleukin-4. In addition, the clone displayed no antigen-specific cytolytic activity. Proliferative responses were also poor. The clone lacked CD28 and was able to suppress mycobacterial-specific proliferation of autologous PBMNC and CD4+ clones. Mean levels of suppression of PBMNC of between 78% and 92% were observed. The suppression was partially mediated by IL-4 and IL-10 and was dependent on contact between suppressor cell, antigen presenting cell and responder cell. The activity of the suppressor CD8+ clone appeared to be HLA-DR-restricted. Suppressor activity mediated by CD8+ T cells may account for certain immunosuppressive features observed in TB patients. In conclusion, the major novel findings of this study were the characterisation of both effusion-derived mycobactericidal CD4+ type-1 CTL clones, and a suppressor CD8+ type-2/type-0 T cell clone. The activity of these cell types has not previously been described in tuberculosis.

List of abbreviations

AFB	-	Acid fast bacilli
AIF	-	Apoptosis-inducing factor
APC	-	Antigen presenting cell/s
ATCC	-	American Tissue Culture Collection
ATP	-	Adenosine triphosphate
BCG	-	Bacillus Calmette-Guerin
Bcl	-	B cell lymphoma-associated gene/protein
BFA	-	Brefeldin A
CD	-	Cluster of differentiation
CHX	-	Cycloheximide
CMA	-	Concanamycin A
CMI	-	Cell-mediated immunity
cpm	-	Counts per minute
CR	-	Complement receptor
CsA	-	Cyclosporin A
CTL	-	Cytotoxic T lymphocyte
dUTP	-	Deoxy-uridine triphosphate
ELISA	-	Enzyme-linked immunosorbent assay
EMNC	-	Effusion mononuclear cell/s
FACS	-	Fluorescence-activated cell sorting
FADD	-	Fas-associated death domain
FasL	-	Fas ligand
FCM	-	Flow cytometry
FCS	-	Foetal calf serum
g	-	Gravitational force
GM-CSF	-	Granulocyte-macrophage colony-stimulating factor
Gy	-	Gray
Gz	-	Granzyme
HIV	-	Human immunodeficiency virus
HLA	-	Human leukocyte antigen
ICE	-	Interleukin-1 converting enzyme
IFN-γ	-	Interferon-gamma
Ig	-	Immunoglobulin
IL	-	Interleukin
IU	-	International units
KD	-	kiloDalton/s
LAM	-	Lipoarabinomannan
LDA	-	Limiting dilution analysis

MBP	-	Myelin basic protein
MBq	-	MegaBecquerel
MDR	-	Multi-drug resistant
MHC	-	Major histocompatibility complex
MMR	-	Macrophage mannose receptor
mRNA	-	Messenger RNA
NK	-	Natural killer
PBMNC	-	Peripheral blood mononuclear cell/s
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PHA	-	Phytohaemagglutinin
PI	-	Propidium iodide
PIM	-	Phosphatidylinositol mannoside
PMA	-	Phorbol myristate acetate
PPD	-	Purified protein derivative of *M. tuberculosis*
PS	-	Phosphatidyl serine
RNI	-	Reactive nitrogen intermediate/s
ROI	-	Reactive oxygen intermediate/s
SDS	-	Sodium dodecyl sulphate
SF	-	Suppressor factor
SK-SD	-	Streptokinase-streptodornase
SNF	-	Supernatant fluid
Sp	-	Surfactant protein
TB	-	Tuberculosis
Tc	-	Cytotoxic T cell
TCR	-	T cell receptor
TdT	-	Terminal deoxy-transferase
TGF-β	-	T cell growth factor-beta
Th	-	Helper T cell
TNF-α	-	Tumour necrosis factor-alpha
TNF-R	-	Tumour necrosis factor receptor
Ts	-	Suppressor T cell
TUNEL	-	TdT-mediated dUTP nick end labelling

Chapter 1.

Introduction and review of the literature

1.1. Tuberculosis

1.1.1. Overview of the problem

Tuberculosis (TB) is the leading cause of death due to a single infectious human pathogen (Bloom and Murray, 1992). The disease remains a global problem of enormous magnitude despite the fact that effective treatment strategies are available. It has been estimated that one third of the human population is infected with *Mycobacterium tuberculosis* (Raviglione *et al.*, 1995). Chemotherapy, involving long-term combination antibiotic treatment regimens with rifampicin, isoniazid, streptomycin and other drugs, has greatly reduced the mortality rate of TB. As a result, pre-chemotherapy mortality rates of 50-60% have been drastically reduced in countries where the population has access to adequate treatment. Despite the much improved situation since the implementation of drug therapy, approximately 3 million people die from TB annually (Brennan, 1997).

Globally TB causes over 25% of avoidable adult deaths, and 7% of all deaths (Enarson and Murray, 1996). The great majority of the world's population and therefore also of infected individuals occurs in developing countries. Since the disease is often associated with poverty, over-crowding, malnutrition and poor hygiene it has always been a major burden in areas where these conditions flourish (Murray *et al.*, 1990). South Africa is no exception to these trends, indeed the incidence of TB infection in South Africa is one of the highest in the world (Strebel and Seager, 1991). The lack of effective diagnostic procedures, particularly in rural areas, is a major contributory factor in the severity of the problem since early diagnosis is essential for successful treatment. In addition, limited access to anti-tuberculous drugs, and more importantly, problems with the monitoring of drug administration compound an already serious health problem in these regions. Although the impact of TB on the population is highest in the third world, the incidence of the disease in industrialised nations has been increasing (Brennan, 1997). The false notion that the disease was under control in Europe and North America following the implementation of drug therapy and bacillus Calmette-Guerin (BCG) vaccination programmes has been shattered with the occurrence of human immunodeficiency virus (HIV)-related TB and the emergence of multi-drug resistant (MDR) strains of the organism. Between 1985 and 1992, the number of TB cases in the US increased by approximately 20%, a substantial proportion of which were HIV-related (Bloom

and Murray, 1992). TB accounts for approximately 40% of deaths related to HIV worldwide (Elliner, 1997).

The development of acquired immune deficiency syndrome (AIDS)-related TB and the occurrence of MDR-tuberculosis has exacerbated the already serious and complex nature of *M. tuberculosis* infection. The problem of HIV and TB co-infection in sub-Saharan Africa is enormous, due to the high level of HIV sero-positivity in the population, as well as exposure to TB at an early age (Benatar, 1991a). AIDS poses a major risk for the re-activation of dormant TB, a problem readily observed in this region (Narain *et al.*, 1992). Drug resistance, on the other hand, is a problem resulting from mutations in the causative *M. tuberculosis* organism. Already treatment strategies involve a multi-drug treatment regimen to try and alleviate the problem of resistance to single drugs (Snider *et al.*, 1994). The development of MDR-TB is a very serious problem in that it is difficult to treat effectively. Treatment time is prolonged and the infected individual poses a threat due to an extended period of infectivity (Benatar, 1991b; Brennan, 1997).

The serious burden of tuberculosis on the human population may suggest that the problem is a new one. On the contrary, TB is an ancient disease which is intrinsically linked to processes of urbanisation, industrialisation and colonisation. The earliest conclusive evidence of TB comes from Egypt. The well-preserved mummy of a child showing evidence of both pulmonary and vertebral TB has been dated back to 3400BC (Metcalf, 1991). The scourge of tuberculosis has thus been pathogenically linked to humankind since the dawn of civilisation. The earliest evidence of TB in Europe comes from a skeleton found in Denmark and dating back to between 2500 and 1500 BC. Historical data on tuberculosis are erratic due to the fact the different forms of the disease were for a long time not recognised as a single condition (Metcalf, 1991). Only with the isolation of the causative organism *M. tuberculosis* in 1882 by Robert Koch (1843-1910) did diagnosis and treatment of tuberculosis become more coherent. Despite this early breakthrough in the isolation and cultivation of *M. tuberculosis*, the rate of advancement in understanding of both bacteriological and clinical aspects of this disease has been slow. The misinformed conclusion that the introduction of chemotherapeutic treatment in the 1940's and 1950's basically eradicated the problem of TB led to the cessation of much research into the disease (Benatar, 1991b). With the recent re-emergence of the disease in Europe and North America, a new focused and intensive research programme has now been developed.

Without a concerted and aggressive intervention strategy the already serious world-wide situation will deteriorate rapidly. Any strategy which hopes to address the eradication of this disease must therefore take into consideration the restrictions under which developing countries operate. Due to the high cost of current treatment regimens and the compounding problem of extended treatment time, future anti-tuberculosis programmes need to focus on a

successful preventative approach (Young and Duncan, 1995). Previous preventative approaches, such as the use of BCG vaccination, have been controversial in that their efficacy has not been clearly shown. A well co-ordinated and multi-factorial anti-TB vaccine programme could lead to successful eradication of this disease. However, due to the complex nature of disease pathogenesis and a remarkable survival strategy employed by the causative organism, such a programme is proving difficult (Kaufmann and Andersen, 1998). A clearer understanding of the pathogenesis of tuberculosis remains a priority for the development of effective therapies and/or vaccines to eradicate the disease. Of particular importance is a more precise knowledge of the immunopathogenesis of this disease, since preventative and curative measures directed at the host immune response could circumvent the problem of mycobacterial antibiotic resistance (Kaufmann and Andersen, 1998).

1.1.2. Characteristics of *Mycobacterium tuberculosis*

Mycobacterium is the only genus of the family Mycobacteriaceae (Wayne and Kubica, 1986). The majority of mycobacteria are non-pathogenic and occur in varied environmental conditions from soil and water species to those occurring as natural human symbionts (Wolinsky, 1979). The pathogenic species of the genus Mycobacterium can be divided into 4 main groups: the tuberculous mycobacteria (*M. tuberculosis, M. bovis* and *M. africanum*); the lepromatous mycobacterium *M. leprae*; the non-tuberculous mycobacteria (*M. avium* complex, *M. kansasii, M. marinum, M. scrofulaceum, M. ulcerans, M. xenopi, M. szulgai* and *M. simae*); and the rapid growing potential pathogens (*M. fortuitum* and *M. chelonei*) (Hopewell, 1990).

A defining feature of all mycobacteria is the complex cell wall that is composed of cross-linked peptidoglycan molecules with attached polysaccharide (arabinogalactan) side chains. These arabinogalactan side chains are esterfied at their distal ends by high molecular weight fatty acids (mycolic acids) (Brennan and Draper, 1994). Mycolic acids are large (70 to 90 carbon atoms in length) and complex fatty acids which may contribute to the ability of mycobacteria to survive within macrophage phagocytic vacuoles. Other important wall associated components are the disaccharide trehalose (cord factor), lipoarabinomannan (LAM) and phosphatidylinositol mannosides (PIMs), all which have been implicated in the virulence and/or survival strategies of the mycobacteria (Fenton and Vermeulen, 1996). The definitive wall structure, which is high in lipid content, accounts for the acid fastness of the mycobacteria, a feature demonstrated by the Ziehl-Neelsen staining procedure.

M. tuberculosis is an acid-fast rod that that is typically beaded or stains unevenly, is somewhat curved, and approximately 0.3 to 0.6μm in width and 1 to 4 μm in length (Runyon *et al.*, 1974). In Gram-stained preparations the organism may appear Gram-positive, however staining is generally weak and varied. The staining properties of *M. tuberculosis* are

shared by all mycobacteria - therefore it is impossible to distinguish between *M. tuberculosis*, the non-tuberculous mycobacteria or *M. leprae* by staining characteristics alone (Hopewell, 1990).

Cultivation of the organism and examination of colonial appearance and determination of biochemical characteristics allows separation of *M. tuberculosis* from non-tuberculous mycobacteria. Mycobacteria are strictly aerobic, hydrophobic and generally slow growing. The strong aerophilic nature of *M. tuberculosis* accounts for the lung being the primary site of infection, an area with a constant supply of oxygen. Under optimum conditions, laboratory strains of *M. tuberculosis* undergo one replication in approximately 18 hours (Wayne, 1994). Colonies become visible on agar-based media (e.g. Middlebrook 7H10) by 2 weeks, and on egg-based media (e.g. Löwenstein-Jensen) by 3 weeks. Colonies of *M. tuberculosis* are buff coloured and rough surfaced. The lack of pigmentation and slow growth rate enables *M. tuberculosis* to be distinguished from most non-tuberculous mycobacteria. The niacin test allows for distinguishing *M. tuberculosis* and other slow-growing mycobacteria, particularly *M. avium* complex, only *M. tuberculosis* showing a positive result. Confirmation of the niacin test can be made by nitrate reduction assays which also give a strongly positive result with *M. tuberculosis* (Roberts *et al.*, 1991).

1.1.3. Pathogenesis of tuberculosis

The vehicle for transmission of the tubercle bacillus is the droplet nucleus (Toossi and Ellner, 1992). During the process of exhalation water droplets are expelled. Once outside of the mouth, the water content of the drop soon evaporates leaving a solid nucleus. In the case of persons harbouring a *M. tuberculosis* infection, tubercle bacilli may be a part of the nucleus. Coughing is the most effective means of generating droplet nuclei (Hopewell, 1990). For this reason, the more symptomatic a person with pulmonary TB, the greater the infectious potential. Apart from coughing, the processes of speech, sneezing and even normal breathing can generate droplet nuclei. The infectious potential of an individual is also related to the number of organisms that have access to the airway, and thus are liable for inclusion in the droplet nuclei. The concentration of organisms in the air is not only determined by the number being expelled but is also related to the volume of air into which the organism is being released. Small, poorly ventilated areas are thus more conducive to the spread of disease than large, well-aerated areas. The droplet nucleus remains in suspension for a short while only, but if inhaled by another individual within a few minutes it can initiate a new tuberculous infection (Hopewell, 1994).

The fate of the inhaled droplet nucleus and its associated tubercle bacilli depends on a number of factors, an important criterion being the size. Large particles (>8-10μm) are trapped in the upper airway by barriers in the nasal cavity and nasopharynx. Particles

between 5 and 10μm enter the conducting airways, land on the mucociliary blanket - which extends to the level of the terminal bronchiole - and are swept into the oropharynx. A substantial number of particles smaller than 5μm penetrate beyond the ciliated epithelium and are thus retained in the lung (Riley *et al.*, 1995; Hopewell, 1994).

Once the tubercle bacillus is within the alveolus it encounters a second line of defence - the alveolar macrophage. Tubercle bacilli are chemotactic and attract alveolar macrophages to the site of implantation. An inflammatory response is generated at the site of initial infection, the extent of the response being related to the number and virulence of the invading organisms (Dannenberg and Rook, 1994). Simultaneously there is a process of systemic haematogenous dissemination of organisms. This bacillemia does not cause symptoms but results in the seeding of other portions of the lung and other organs with the bacilli. Sufficient proliferation of organisms, either within the lung or at extra-pulmonary sites may cause clinically evident disease at this stage. Generally, however, the multiplication of organisms is held in check until the development of cellular immunity within 30 to 50 days of initial implantation (Toossi and Ellner, 1992). The development of an effective cellular immune response is marked by a cutaneous reactivity to tuberculin. Sensitised T-cells produce cytokines following exposure to infected antigen presenting macrophages. The cytokines activate resident macrophages and recruit further macrophages to the site of inflammation. The activated macrophages are more competent in their ability to control the proliferation of the bacilli, but generally complete clearance does not occur (Dannenberg and Rook, 1994). Once acquired, the infection may reactivate later in life, a process known as endogenous reactivation. The reason for the dormant foci reactivating are unknown but seems to be linked to factors which depress delayed type hypersensitivity responses (Doenhoff, 1998).

The pattern of tuberculosis infection has been divided into 5 stages based on extensive studies of the disease in rabbits (Dannenberg and Rook, 1994; Dannenberg, 1991). The first stage is *onset*, which begins with inhalation of the bacillus into an alveolus, the uptake of the bacillus by the alveolar macrophage and the subsequent destruction of the organism. Generally successful destruction is not accomplished at this early stage and the second stage, *symbiosis*, begins. Here the infected macrophage is lysed and bacilli are taken up by other macrophages recruited to the infection site by chemotaxis. A state of symbiosis ensues and bacilli multiply readily within the macrophages without any associated pathology. Some macrophages move to the draining lymph nodes and T lymphocytes are expanded following antigen recognition. The T lymphocytes relocate to the initial focus of infection. The dynamic interaction of T lymphocytes and macrophages results in the formation of the tubercle. The third stage is termed *caseous necrosis*. This stage begins with the cessation of bacillary multiplication. Tuberculin negative patients become tuberculin positive and the lesions undergo a characteristic necrosis with extensive cellular destruction at the core of the granuloma. Closely linked to this stage is the fourth stage, which involves a dynamic

equilibrium of *tissue-damaging and macrophage activating immune responses*. The cellular immune response is predominant at this point, with the associated activity of helper- and cytolytic-T lymphocytes. The fifth and final stage is *liquefaction and cavity formation*. Extensive destruction of granuloma tissue occurs due to enzymatic activity. The bacilli are released from the disease focus into the alveolus or into the blood stream. From the alveolus the bacilli are expelled to the exterior and spread to new hosts. If bacilli gain entry to the bloodstream, dissemination and multi-organ involvement may ensue. In this model of tuberculosis pathogenesis, the sequential progression of disease may be halted at any stage, since entry into a subsequent stage is dependent on the failure of the previous one. Since the majority of infected individuals do not proceed to clinical disease, the later stages of infection are not observed.

1.1.4. Clinical manifestations of tuberculosis

Clinical manifestations of tuberculosis span a broad range from a subtle, indolent process to an explosive, life-threatening or fatal illness. The clinical expression of infection with *M. tuberculosis* is quite varied and depends on a number of factors including the age and immune status of the host, the virulence of the organism, and the sites of disease involvement (Hopewell, 1994). Primary tuberculosis is seldom associated with significant clinical symptoms. Post-primary TB, which is generally responsible for the majority of symptoms leading towards positive diagnosis, may be due to either reactivation or exogenous re-infection. Most cases of adult tuberculosis represent reactivation of previous dormant foci.

Among healthy individuals infection with *M. tuberculosis* is generally likely to be asymptomatic. It has been estimated that the lifetime risk of developing clinically evident TB after being infected is 10% - with a 90% likelihood of the disease remaining latent (Comstock, 1982). In specific sub-populations - e.g. in immunocompromised individuals - the proportion which develop evident TB is much higher. BCG immunisation is believed to minimise the risk of early disseminated TB in these groups and especially in children.

The most important factor influencing the clinical features of TB is the site of involvement. In non-HIV related TB, approximately 85% of cases are limited to the lungs, the remainder involving non-pulmonary sites or both (Farer *et al.*, 1979). The situation in HIV co-infection is skewed towards extra-pulmonary or dual pulmonary and non-pulmonary involvement (Small *et al.*, 1991). This involvement of multiple sites in HIV related TB is an indication of the serious effects of limited immune function on the containment of infection.

Systemic manifestations of the disease include fever, malaise and weight loss - symptoms predominantly mediated by cytokines, especially tumour necrosis factor (TNF)-α. Fever is the

most easily quantified and has been found to occur in between 37-80% of patients (Kiblawi *et al.*, 1981). In addition to generalised effects there are remote manifestations which are not a result of anatomic site of involvement. Amongst these the most commonly encountered are haematological abnormalities (especially leukocytosis and anaemia), hyponatremia and psychological disorders (Hopewell, 1994).

1.1.4.1. Pulmonary TB

The clinical manifestations of reactivation of pulmonary tuberculosis are very variable and include both generalised systemic symptoms (e.g. malaise, lethargy, fever, weight loss and night sweats) and localised symptoms (Toossi and Ellner, 1992). Cough is by far the most common localised symptom of pulmonary TB. Early in infection the cough may be non-productive, but subsequently as inflammation and tissue necrosis ensue, sputum is generally produced. Inflammation of lung parenchyma adjacent to the pleural surface may cause pleuritic pain. Spontaneous pneumothorax may occur causing chest pain and dyspnea. In addition severe respiratory failure sometimes also occurs. Haemoptysis may also be a presenting symptom but is not necessarily indicative of active TB (Hopewell, 1990).

Pulmonary TB nearly always causes abnormalities on chest radiographs (Benatar, 1991b). In primary TB occurring as a result of recent infection, the process is generally seen as a middle or lower lung zone infiltrate. Tuberculosis that develops as a result of endogenous reactivation of latent infection usually causes abnormalities in the upper lobes of one or both lungs. Cavitation (destruction of lung tissue) is common in this form of TB. Healing of the tuberculous lesions usually results in development of a scar with loss of lung parenchymal volume and calcification. Erosion of a parenchymal focus of tuberculosis into a blood or lymph vessel may result in dissemination of the organism.

Definitive diagnosis of tuberculosis can be established only by isolation of tubercle bacilli in culture or via identification of *M. tuberculosis* DNA using polymerase chain reaction (PCR) techniques. In pulmonary TB sputum is the diagnostic specimen of choice. Detection of acid fast bacilli (AFB) in sputum smears is indicative of active TB. Sputum negativity (i.e. no detection of AFB) must be followed up with bacteriologic culture which generally confirms clinical diagnosis (Roberts *et al.*, 1991).

1.1.4.2. Extra-pulmonary TB

Extra-pulmonary TB presents more of a diagnostic and therapeutic problem in that it is a less common manifestation of TB and therefore less familiar to the clinician (Weir and Thornton, 1985). Extra-pulmonary TB involves relatively inaccessible sites and due to the nature of the sites fewer bacilli are able to cause greater damage. Low numbers of bacilli and difficulty in isolation from infection sites make positive diagnosis more problematic than in pulmonary TB. Extra-pulmonary TB manifests at certain localised regions. The most common regional

manifestations are lymphatic (27% of all extra-pulmonary TB), pleural (22%), genitourinary (16%), miliary/disseminated (10%), skeletal (9%), meningeal (4%) and peritoneal (4%) (Hopewell, 1990). Although miliary or disseminated TB usually involves the lungs - there is generally multi-organ involvement, hence the inclusion in the extra-pulmonary category. Other less common sites of infection include abdominal and pericardial TB. A detailed discussion of all of these manifestations is beyond the scope of this thesis, however pleural tuberculosis will be discussed due to its relevance in this study.

Pleural tuberculosis was traditionally considered to almost always be a manifestation of primary tuberculosis. Today it is recognised as a manifestation in older patients with concomitant pulmonary TB. The epidemiology of pleural TB parallels that of the overall pattern of TB (Benatar, 1991b). The disease is more common in males and anincrease in incidence occurs with increasing age between 5 and 45. There are two mechanisms by which the pleural space becomes involved in tuberculosis and the difference in pathogenesis results in different clinical presentations, diagnostic approaches and treatment. These two forms are tuberculous pleuritis and tuberculous empyema. The latter form is much rarer, involves large numbers of organisms and appears to be caused by rupture of adjacent tuberculous cavities in the lung. Further discussion is focused on classical tuberculous pleuritis.

In the course of a tuberculous infection a few organisms may gain access to the pleural space and in the presence of cell-mediated immunity (CMI), cause a hypersensitivity response (Ellner, 1978). Normally this occurrence goes unnoticed and the process spontaneously resolves. In some patients, however, tuberculous involvement of the pleura is manifested as an acute illness with fever and pleuritic pain. Effusions are generally small and usually unilateral, but sometimes large effusions are produced causing dyspnea.

Diagnosis of pleural tuberculosis is generally established by analysis of pleural fluid or by pleural biopsy. Pleural fluid is nearly always straw coloured with cell counts of between 100 - 5000/μl (Jay, 1985). Mononuclear cells predominate in most cases. The fluid is exudative with a high protein concentration. Pleural fluids rarely give positive smear results due to low numbers of organisms. Culture positivity is also low, with only 20-40% of patients with proved tuberculous pleuritis being culture positive. Confirmation of diagnosis is often via needle biopsy of pleura.

1.1.5. Pathogenesis and Immune response in tuberculosis

1.1.5.1. Overview

Host defences against *M. tuberculosis* depend on cell-mediated rather than humoral immunity. Persons with defective cell-mediated immunity, such as those with HIV infection and chronic renal failure, are markedly at risk for TB, whereas those with defective humoral immunity, such as those with sickle cell disease or multiple myeloma, show no increased disposition to the disease (Barnes and Modlin, 1996). Experimental evidence indicates that anti-mycobacterial defence mechanisms are mediated primarily by T lymphocytes and macrophages. Macrophages act as effectors and T lymphocytes act as inducers of protection (Kaufmann, 1998b). Adoptive transfer of resistance against tuberculosis in animal models is dependent on T cells, a fact which implicates these cells as the mediators of disease resolution (Orme and Collins, 1984).

Macrophages, in addition to being mediators of cell-mediated immunity (CMI), also act as the preferred host cells of *M. tuberculosis.* Their role in tuberculosis is thus contradictory - promoting not only protection against the disease but also survival of the pathogen. T cells similarly have a dual role, being pivotal for protective immunity, but also contributing to pathogenesis. A co-ordinated interaction of T cells and macrophages is essential for optimum protection (Barnes *et al.*, 1994). This interaction is best achieved in the granulomatous lesion, which provides the tissue site for defence against tuberculosis. Despite the localised, co-ordinated T cell-macrophage interaction, full eradication of organisms is seldom achieved and individuals usually remain infected without developing disease. Later imbalances in immune function may promote mycobacterial re-emergence and the development of clinical disease.

A major factor in successful protective immunity is the production of cytokines by both infected macrophages and T cells. The initial pattern of cytokine production by the macrophages appears to be pivotal in the establishment of a protective Th1 type T cell response. The T cells in turn produce cytokines which activate other T cells (interleukin-2) as well as macrophages (interferon (IFN)-γ and TNF-α), and which may also impart anti-mycobacterial activity.

1.1.5.2. Macrophages and macrophage-derived cytokines

Murine macrophages have for a long time been known to have anti-mycobacterial activity (Rook and Bloom, 1994). Later work identified interferon (IFN)-γ as the key endogenous activating cytokine that triggers the anti-mycobacterial activity of macrophages (Rook *et al.*, 1986a, 1986b; Flesch and Kaufmann, 1987). In addition to IFN-γ, TNF-α, although ineffective alone, is able to synergise with IFN-γ to induce *in vitro* anti-mycobacterial effects in murine macrophages (Flesch and Kaufmann, 1990). Other cytokines have been implicated in macrophage defence against *M. tuberculosis* - although their roles are less well established.

Interleukin (IL)-4 and IL-6 both have anti-mycobacterial effects if added to infected macrophage cultures (Flesch and Kaufmann, 1990). Various cytokines involved in granuloma formation including IL-8, IL-1, IL-2, IL-4 and IFN-γ also contribute indirectly in anti-mycobacterial activity. IL-12 is a key pro-inflammatory cytokine and appears to play a major role in activating macrophages as well (Zhang *et al.*, 1994). In contrast, the cytokines IL-10 and transforming growth factor (TGF)-β have been shown to reduce macrophage anti-mycobacterial activity (Bermudez and Champsi, 1993; Bermudez 1993).

Protective immunity in murine models occurs via a strong T helper cell type 1 (Th1) process. The CD4+ cells, and to a lesser extent the CD8+ cells, produce high levels of interferon-γ, which in turn correlates with protective immunity (Kawamura *et al.*, 1992, Huygen *et al.*, 1992). In humans the pattern of cytokine production of T cell clones derived from TB patients has been shown to include interferon-γ, TNF-α, and IL-10. This is indicative of a Th0 pattern, but it appears that the pro-inflammatory cytokines predominate (Barnes *et al.*, 1993).

Infection of macrophages with *M. tuberculosis* results in the production of a characteristic pattern of cytokines that have the potential to exert potent immunoregulatory effects and to mediate many of the clinical manifestations of TB (Barnes *et al.*, 1992). The most important of these include IL-1, TNF-α, IL-6, IL-10, T cell growth factor (TGF)-β, and IL-12.

IL-1 is produced upon stimulation of human monocytes with *M. tuberculosis*, and with the mycobacterial cell wall component lipoarabinomannan (Barnes *et al.*, 1992; Zhang *et al.*, 1993). IL-1 is an endogenous pyrogen and may contribute to the fever that is characteristic of TB. IL-1 may also enhance the inflammatory response by inducing macrophages to produce other cytokines e.g. TNF-α, and by stimulating T cell proliferation. Interestingly, IL-1 has also been linked to immunosuppression, since patients with depressed proliferative responses to *M. tuberculosis* produce high levels of IL-1 (Ellner, 1978).

Human mononuclear cells including alveolar macrophages produce high levels of TNF-α in response to *M. tuberculosis*, LAM and mycobacterial proteins of molecular masses of 20, 44, 58 and 65kD (Barnes *et al.*, 1990, 1992). The expression of the CD14 molecule is also necessary for TNF-α production (Zhang *et al*, 1993). Clinical and experimental data suggest that TNF contributes both to protection against tuberculosis and to immunopathology. TNF enhances antimycobacterial activity of macrophages *in vitro* (Flesch and Kaufmann, 1990). In addition, TNF appears to be involved in successful granuloma formation, since the administration of anti-TNF antibodies interferes with granuloma formation and mycobacterial clearance in mice (Kindler *et al.*, 1989). TNF levels are elevated at the site of disease in tuberculous pleuritis patients (Barnes *et al.*, 1990), and patients with chronic refractory pulmonary TB have lower levels of TNF than newly diagnosed patients, which indicates the importance of this cytokine for effective disease clearance. The role of TNF in

immunopathology has been demonstrated by the administration of TNF to animals – a procedure that results in fever and wasting (Beutler and Cerami, 1987). In TB patients with cachexia and high fever, levels of TNF are higher than in patients with less severe symptoms (Cadranel *et al.*, 1990). Thalidomide abrogates the functional activity of TNF, and in the process alleviates symptoms such as anorexia and fever (Tramontana *et al.*, 1995). TNF thus has a dual role in TB, as an important anti-mycobacterial mediator within the localised granuloma, but also as a mediator of tissue necrosis, fever and cachexia.

IL-6 reduces the binding of TNF to macrophages and reduces the anti-mycobacterial activity of TNF in mycobacteria-infected macrophages (Bermudez *et al.*, 1992). Addition of IL-6 enhances intracellular and extracellular mycobacterial growth, and may therefore be important in down regulating the inflammatory response (Shiratsuchi *et al*, 1991).

IL-10 and TGF-β are both anti-inflammatory cytokines produced by macrophages following exposure to *M. tuberculosis* (Barnes *et al*, 1992). IL-10 inhibits cytokine production by monocytes, inhibits the microbicidal activity of murine macrophages and suppresses antigen-specific T-cell proliferation by down-regulation of macrophage MHC class II expression (Oswald *et al*, 1992; De Waal Malefyt *et al*, 1991). Neutralisation of IL-10 with anti-IL-10 significantly reduces bacterial load in mice (Bermudez and Champsi, 1993). IL-10 also reverses the anti-mycobacterial activity of TNF. In TB patients the neutralisation of IL-10 enhances IFN-γ production by PBMC, by enhancing IL-12 production by monocytes (Gong *et al.*, 1996). IL-10 may thus play a role in anergic responses to *M. tuberculosis*, but may also be important in moderating what could become an uncontrolled pro-inflammatory response leading to sever tissue damage.

TGF-β inhibits cytokine production by macrophages and inhibits MHC class II expression (Ruscetti *et al*, 1993). This cytokine also inhibits IL-2 dependent T-cell proliferation and IL-2 receptor expression (Ortaldo, 1991). TGF-β also appears to de-activate macrophages, and is produced at the highest levels in macrophages infected with the most virulent mycobacterial strains. In the presence of anti-TGF-β, the anti-mycobacterial effects of IFN-γ are reduced (Toossi *et al*, 1995)

IL-12 is a macrophage derived cytokine which plays a pivotal role in the induction of a Th1 type response and is discussed below.

1.1.5.3. Binding and uptake of *M. tuberculosis*

M. tuberculosis is a facultative intracellular pathogen and has developed numerous mechanisms for entering human macrophages. The receptors involved in entry processes are numerous and diverse, and include (i) complement receptors, (ii) mannose receptor, (iii)

surfactant protein receptors, (iv) CD14, (v) scavenger receptors, and (vi) Fc receptors (Ernst, 1998).

M. tuberculosis bacilli are thought to enter the macrophage by specific binding to several distinct cell surface molecules, and the precise route of entry is likely to determine the fate of the bacilli within the macrophage. Direct binding of *M. tuberculosis* to the macrophage is possible via complement receptors (CRs) and the macrophage mannose receptor (MMR). The MMR is involved in non-opsonic binding and phagocytosis through recognition of terminal mannose residues on the invading bacilli. MMRs seem to be important in phagocytosis of virulent strains of *M. tuberculosis* in the absence of serum (Schlesinger, 1993). Mannose-dependent binding accounts for only a small proportion of total *M. tuberculosis* phagocytosis. CD14 has also been implicated in non-opsonic binding and uptake (Peterson *et al.*, 1995). Opsonic binding involves heat-labile serum components and the complement receptors CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18). The role of these components is well established, with adherence and ingestion of *M. tuberculosis* being strongly inhibited (up to 84%) by antibodies to the various CRs (Schlesinger and Horwitz, 1991). Complement component 3 (C3) is important in the facilitation of *M. tuberculosis* binding and uptake (Schlesinger *et al.*, 1990). Fc receptors, however, are unlikely to play a major role due to their initiation of reactive oxygen intermediates (ROI) following engagement.

Surfactant proteins are abundant in the alveoli of the lung and therefore a role for surfactant protein (Sp) receptors in the pathogenesis of *M.tuberculosis* has been proposed. It appears that Sp-A enhances binding and uptake of *M. tuberculosis*, but the exact mechanisms remain unclear. The binding and entry of *M. tuberculosis* via CD14, scavenger receptors and Fc receptors is less well characterised than CR-mediated entry (Ernst, 1998).

M. tuberculosis seems to have exploited multiple receptors to ensure successful entry into the macrophage. Why do mycobacteria target macrophages? Several hypotheses have been proposed. The most commonly cited reasons include the idea that the macrophage provides a safe haven for replication since the organism has succesfully developed strategies that protect it from both the normal macrophage anti-bacterial properties and from the cellular immune response (Schaible *et al.*, 1999). Macrophages may also enable the organism to spread accross pulmonary epithelial barriers. Also, *M. tuberculosis* may gain from being within in the macrophage in the sense that pathogenicity may be enhanced in this location (Fenton and Vermeulen, 1996).

1.1.5.4. Intracellular survival of *M. tuberculosis*

Following attachment and subsequent phagocytosis, sustained intracellular bacterial growth depends on the ability to avoid destruction by lysosomal enzymes, ROI and reactive nitrogen intermediates (RNI). These macrophage defence mechanisms can be avoided if the mycobacterium-containing phagosomes are prevented from fusing with the lysosomes. Virulent *M. tuberculosis* appears to be able to prevent phagosome-lysosome fusion by disrupting normal functioning of phagosomes and preventing them from developing acidic hydrolase-rich compartments. Mycobacterium-containing vesicles fail to fuse with endosomal vesicles containing other ingested material e.g. electron-dense colloids (Rastogi *et al.*, 1992). The restricted ability of the mycobacterium-containing phagosomes to fuse suggests an alteration in their biochemical composition - either preventing delivery of mycobacteria into the lysosomal compartment or blocking phagosome association with anti-bacterial molecules. The latter possibility has been confirmed in studies that revealed that vacuolar membranes surrounding the bacilli lacked a proton ATP-ase, which may be responsible for phagosomal acidification (Sturgill-Koszycki *et al.*, 1994). Continued containment of mycobacteria within these specialised vesicles may also reduce the ability of mycobacterial antigens to be processed, associated with MHC class II, and transported to the surface of the cell (Pancholi *et al.*, 1993).

1.1.5.5. Microbicidal activity of macrophages

The assumption that activated human macrophages can kill *M. tuberculosis* has not been demonstrated unequivocally *in vitro*, especially in the case of human monocytes and macrophages. Douvas *et al.* (1986) reported that 3-day-old macrophages showed suppression of growth of virulent *M. tuberculosis*. Furthermore, human monocytes activated by IFN-γ and TNF-α have been reported to possess strong mycobactericidal activity (Dennis, 1991). Later studies have disputed these findings as artefactual, since treatment of macrophages with these cytokines may render them more sensitive to the toxic effects of the mycobacteria (Warwick-Davies *et al.*, 1994).

Were mycobacterial killing to take place it would occur within macrophage phagolysosomes. Toxic constituents within this acidic vesicle include lysosomal hydrolases, ROI e.g. H_2O_2 and O_2^-, RNI e.g. NO and NO_2^-. RNI production is an important effector mechanism against a variety of pathogens in murine macrophages. NO and other RNI are derived from L-arginine via an enzymatic pathway which is controlled by an inducible nitric oxide synthase (iNOS) (Nathan and Xie, 1994). Cytokines are powerful mediators of RNI synthesis. The pro-inflammatory cytokines TNF-α and IFN-γ are potent activators of iNOS, while IL-4, IL-10 and TGF-β suppress it. Growth inhibition of mycobacteria by cytokine-stimulated murine macrophages correlates strongly with generation of RNI (Fenton and Vermeulen, 1996).

Although well characterised in mice, the role of RNI in humans is less well understood. Production of iNOS mRNA and protein have been reported, however the absence of detectable RNI activity seems to indicate that resting macrophages require other induction signals for RNI production. Another explanation may be that infected human macrophages produce higher levels of the cytokines IL-4, IL-10 and TGF-β than their murine counterparts - thus suppressing successful RNI production (Fenton and Vermeulen, 1996).

Initial infection by *M. tuberculosis* leads to the rapid activation of alveolar macrophages, the induction of cytokines which serve to limit the growth of ingested organisms, and the recruitment of additional leukocytes from the peripheral circulation. Enhancement of mycobactericidal function can be exerted by T cells, especially αβ+ CD4+ T cells which secrete IFN-γ and IL-2 (Orme, 1993). Although IFN-γ alone seems unable to activate macrophages sufficiently to kill *M tuberculosis*, combined cytokine exposure (with e.g. TNF-α) may lead to successful macrophage anti-mycobacterial activity. TNF is essential for both granuloma formation and mycobactericidal activities of macrophages in the murine model (Kindler *et al.*, 1989). Other macrophage-derived cytokines that may activate human macrophages to kill mycobacteria are granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-12.

1.1.5.6. Interactions between macrophages and T cells

The collaboration of T cells and macrophages at the disease site is essential for clearance of *M. tuberculosis* infection. This occurs via antigen specific DTH responses. Initial presentation of antigens to T cells in the lymph nodes is primarily carried out by dendritic cells, which migrate from infection sites to draining lymph nodes. Antigen presentation by either dendritic cells (in the lymphoid tissue) or macrophages (in the granulomatous lesion at the disease site) occurs in association with MHC class I or class II proteins or with non-polymorphic MHC-like proteins e.g. CD1. Macrophage-derived cytokines play critical roles in the stimulation of antigen-specific T cells. Subsequently to activation, cytokines produced by T cells further modulate macrophage function, although the final outcome may be either to augment or inhibit intracellular *M. tuberculosis* growth.

The Th1-type cytokines (e.g. IL-2 and IFN-γ) may enhance antigen presentation and stimulate antimicrobial responses within the macrophage itself. The presence of both antigen-specific T cells and activated macrophages together with their associated cytokines within the granuloma provides for long term containment of infected macrophages. In murine models of TB, this initial Th1-type response is followed by a Th2-type response which limits inflammation and minimises tissue damage, but also suppresses the DTH reaction (Orme *et al.*, 1993). IL-12 may be a pivotal cytokine in the initiation of the Th1-type pro-inflammatory DTH response. IL-12 enhances production of IFN-γ which facilitates the production of Th1 cells, augments cytotoxicity of antigen-specific T cells and natural killer cells (Brunda, 1994).

Elevated levels of IL-12 have been observed in pleural fluids of tuberculous pleuritis patients (Zhang *et al.*, 1994). In contrast, the Th2-type responses may contribute to the immunosuppression often observed in advanced disease. This type of response is particularly apparent in HIV and TB co-infection, where Th1-type responses are diminished. Macrophages are likely to contribute to these responses via the production of immunosuppressive cytokines e.g. IL-10 and TGF-β (Zhang *et al.*, 1994). The cytokine network therefore controls a complex set of responses that may be growth modulatory to *M. tuberculosis*, pro-inflammatory, or immunosuppressive.

Due to the protected environment within host macrophages that pathogenic strains of *M. tuberculosis* create, tuberculosis can be considered as a prototype of an infection controlled through the cellular immune response. Without the activity of functional cellular immunity *M. tuberculosis* would be able to replicate and spread rapidly, initiating systemic infection and death. The cellular immune response is responsible for the containment of the disease observed in the majority of infected individuals.

1.1.5.7. T lymphocyte-mediated protection in *M. tuberculosis* infection

The T cell receptor (TCR) and the non-covalently linked CD3 molecule on the surface of peripheral T cells are responsible for the transduction of the antigenic signal received from the antigen-presenting cell. The TCR is a disulphide heterodimer which is composed either of an α and β chain, or a γ and a δ chain. In human peripheral blood approximately 90% of T cells express the αβ TCR, with generally <10% expressing the γδ TCR.

Macrophages are professional antigen-presenting cells (APC) and in tuberculosis it is the T cell-macrophage interaction which is pivotal for successful control of the disease (Orme *et al.*, 1993). Antigen is processed and then presented by antigen presenting cells in one of two ways. Endogenous antigens are processed via the MHC class I pathway and are classically presented to CD8+ T cells. Exogenous antigens are processed via the MHC class II pathway and are presented to CD4+ T cells. Exceptions to this standard pattern have been observed, and it now also recognised that non-peptide antigens may also be presented to T cells, but not via MHC-linked pathways, but rather through the CD1 complex of cell surface molecules (Porcelli *et al.*, 1992).

The αβ T cells which express the CD4 marker are able to recognise portions of phagocytosed protein presented by the APC in combination with the MHC class II molecule. The CD8-expressing αβ T cells recognise peptides derived from proteins of cytosolic origin which are presented in combination with MHC class I molecules. Functionally, CD4+ T cells are involved in the amplification of the immune response (helper function), whilst some CD4+ and the majority of CD8+ T cells are involved in lysis of infected target cells (cytotoxicity).

Based on their cytokine production profile, CD4+ T cells in the mouse have been subdivided into two groups. Th1 cells produce IL-2 and IFN-γ, which support their role as regulatory and effector cells in the cellular immune response. Th2 cells produce IL-4, IL-5, IL-6 and IL-10, and provide help to B-cells for the production of immunoglobulin. Th2 cells seem to predominate later in tuberculosis infections in the mouse, and are linked with the down-regulation of the pro-inflammatory response. Both subsets have been found to secrete the cytokines IL-3, TNF-α and GM-CSF (Del Prete *et al.*, 1994).

Adoptive transfer studies in mice have shown that protective immunity is reliant upon functional CD4+ or CD8+ T cells, as well as monocytes/macrophages (Orme and Collins, 1984). The continued exposure of T cells to mycobacterial antigens induces a significant state of memory immunity, which in most cases leads to a delayed type hypersensitivity (DTH). In the murine system the T cell-mediated response can amplify the power of the macrophages to kill and digest the bacilli. The degree of T cell activation and the local concentration of antigens influences the outcome of the disease. A strong T cell response and a small antigen load generates a highly organised granuloma consisting of lymphocytes, macrophages, giant cells and fibroblasts in dynamic equilibrium. Once the infected macrophages are lysed, either directly or via the action of CTL, escaping bacteria are subsequently ingested and destroyed by surrounding macrophages activated by T cells. A necrotic caseous centre is formed in the tubercle. Such a tubercle is termed a proliferative focus and is a very successful tissue reaction, since it can arrest the infection and in some cases even leads to sterilisation of the caseous tissue (Toossi and Ellner, 1992). An over-reactive T cell response with high antigen load brings about a less well-organised tubercle. In this case there is liquefaction of the caseous tissue due to lytic enzymes released from degenerating and dying macrophages. Mycobacterial growth is not contained in such a scenario, and destruction of pulmonary tissue can occur leading to the formation of a cavity adjacent to the bronchi, permitting the mycobacteria to spread to other tissues or to the environment. Clinical tuberculosis therefore seems to be directly associated with mycobacterial T cell reactivity, which controls the local concentrations of tubercle bacilli and their antigens (Munk and Emoto, 1995).

1.1.5.8. T cell subsets involved in *M. tuberculosis* infection

Of the different subsets of T cells for which a role has been defined in *M. tuberculosis* i.e. CD4+, CD8+, γδ, and double negative, CD4+ T cells have been most thoroughly investigated. Their role is two-fold since they are involved both in the production of cytokines (helper function) and in cytotoxic activity.

1.1.5.8.1. CD4+ T cells

The bulk of experimental and clinical data suggest a central role for CD4+ cells in immune defence against tuberculosis. Mice depleted of CD4+ T cells prior to infection with *M. bovis* or

M. tuberculosis are unable to control mycobacterial growth (Pedrazzini *et al.*, 1987; Muller *et al.*, 1987). MHC class II-deficient mice which lack conventional CD4+ effector cells suffer severely from both *M. bovis* BCG and *M. tuberculosis*, disease ultimately leading to death (Ladel *et al*, 1995). Adoptive transfer of CD4+ cells from sensitised animals confers protection against tuberculosis (Orme, 1987; 1988). In the human, CD4+ cells are expanded at the site of disease in patients with tuberculous pleuritis. HIV infection, which depletes CD4+ T cells, markedly increases susceptibility to both primary and reactivation tuberculosis (Barnes *et al.*, 1991). In addition, HIV infected tuberculosis patients demonstrate a progressive increase in clinical indicators of severe disease (e.g. extra-pulmonary involvement, mycobacteremia and acid fast smears) concomitant with a decline in CD4+ cell count (Jones *et al.*, 1993). The severity of this change is illustrated by the fact that mycobacteremia was observed in 4% of patients with a CD4+ cell count of >200/μl, but increased to 49% in those with 100 or less CD4+ T cells/μl.

1.1.5.8.2. Th1 and Th2 subsets in human mycobacterial infection

Human T cells can exhibit dichotomous patterns of cytokine production similar to those of murine Th1 and Th2 cells. The startling difference between the murine and human groups is the production of IL-10. This cytokine is only produced by Th2 mouse T cells, but by both Th1 and Th2 groups in the human (Del Prete *et al.*, 1993). In leprosy, the two divergent forms of disease are due to the predominance of either a Th1 population (tuberculoid leprosy), or a Th2 population (lepromatous leprosy). Patients displaying the latter form of the disease have IL-4 and IL-10 predominating within the lesions, there is an ineffective immune response and bacillary burdens are enormous (Yamamura *et al.*, 1991).

The relative amounts of either Th1 or Th2 cytokines occurring at the site of pathology in *M. tuberculosis* infections, or being secreted by PBMNC or T cell clones, have been reported by several groups. Contradictory patterns have been reported. Certain reports have noted that CD4+ *M. tuberculosis*-reactive T cell clones are Th1-like, in that they produce high levels of IFN-γ, but no IL-4 or IL-5 (Del Prete *et al.*, 1991). Other groups have indicated that *M. tuberculosis*-reactive T cells secrete a broad range of cytokines including IFN-γ, IL-2, IL-4, IL-5 and IL-10 (Boom *et al.*, 1991; Barnes *et al.*, 1993). Evaluation of PBMNC from both tuberculosis patients and healthy tuberculin responders, stimulated with *M. tuberculosis in vitro,* showed that levels of IL-4 production and mRNA expression were similar for both groups. However, the PBMNC from the tuberculosis patients had reduced IL-2 and IFN-γ production and mRNA expression compared to healthy tuberculin reactors (Zhang *et al.*, 1995). This would suggest susceptibility to TB being associated with a depressed Th1 response, but not an enhanced Th2 response. Other investigators have reported the frequency of IL-4 producing cells being higher in *M. tuberculosis*-stimulated PBMC from patients, than those from healthy tuberculin reactors - whereas the frequency of IFN-γ-producing cells was similar in both groups (Surcel *et al.*, 1994). These disparate results may

result from different experimental procedures and may reflect actual events *in vivo*. These findings emphasise the importance of assessment of actual response at the site of disease. In patients with tuberculous pleuritis, expression of mRNA for the Th1 cytokines IFN-γ and IL-2 is greater in the pleural fluid than in the blood, with concentrations of IFN-γ being 15-fold higher than in serum (Barnes *et al.*, 1993). Levels of mRNA expression of IL-4, a Th2 cytokine, are lower in the pleural fluid than in the blood. Lymphocytes isolated from the pleural fluid and stimulated with *M. tuberculosis*, produce more IFN-γ and IL-2 than do peripheral blood lymphocytes. These findings suggest that there is a selective concentration of Th1 like cells at the site of disease with patients showing a resistant immune response. Th1 cells are thus important in anti-mycobacterial defences.

The capacity of IFN-γ to augment mycobacterial killing in human macrophages remains controversial (Douvas *et al.*, 1985; Shiratsuchi *et al.*, 1990). However, this cytokine has been shown to stimulate human macrophages to produce TNF and 1,25-dihydroxy-vitamin D, both of which facilitate mycobacterial clearance (Rook *et al.*, 1986a, 1986b; Bermudez and Young, 1988). IFN-γ administration to patients with disseminated *M. avium* complex disease, resulted in marked improvement (Holland *et al.*, 1994). IL-2, another classic pro-inflammatory Th1-type cytokine, is also beneficial in that it expands populations of antigen-reactive T cells, simultaneously increasing the level of macrophage-activating factors secreted by the same T cells. IL-2 has been reported to reduce the bacillary burden in lepromatous leprosy (Kaplan, 1991). In contrast to Th1 cytokines, IL-4 deactivates macrophages (Lehn *et al.*, 1989; Ho *et al.*, 1992). IL-4 also blocks T cell proliferation by down-regulating IL-2 receptor expression (Martinez *et al.*, 1990). In contrast to the effects of IFN-γ and IL-2, IL-4 has the capacity to suppress the immune response of tuberculosis patients.

IL-12 plays a central role in defining the development of a protective Th1 type response in tuberculosis. IL-12 is a macrophage derived heterodimeric cytokine which strongly augments pro-inflammatory Th1 type responses (Barnes *et al.*, 1994). IL-12 is concentrated at the site of disease in patients with tuberculosis pleuritis, and the cytokine is produced by pleural fluid leukocytes on stimulation with *M. tuberculosis* (Zhang *et al.*, 1994). IL-12 production is enhanced in leprosy patients who develop a Th1 predominant response (Sieling *et al.*, 1994). IL-12 also enhances cytotoxicity and augments proliferation of antigen-specific cytolytic T cells and NK cells (Gately *et al.*, 1992; Bertagnolli *et al.*, 1992). An intriguing property of IL-12 is the fact that its capacity to induce proliferation of cytolytic T cells is dependent on co-stimulation of the TCR with antigen or anti-CD3. This contrasts strongly with other proliferation inducing cytokines e.g. IL-2 and IL-7 (Bertagnolli *et al.*, 1992; Bertagnolli and Hermann, 1990). IL-12 may thus be an important candidate in controlling cytolysis and cytolytic cells in the initial immune response to microbial pathogens by inducing expansion of such cells only in the presence of antigen. IL-12 enhances cytotoxicity of CD4+ T cells against human macrophages infected with *M. tuberculosis* (Boom *et al.*, 1992). Additional

roles for IL-12 may include enhancement of mycobacterial antigen recognition, or promotion of T cell expansion, based on the ability of IL-12 to synergise with IL-2, and thus to enhance lymphocyte proliferation and result in the generation of a Th1 response (Zhang *et al.*, 1994).

1.1.5.8.3. Cytolytic activity of CD4+ cells

In addition to their role as helper T cells, CD4+ T cells also contribute to immune defence through direct cytolysis of macrophages and non-phagocytic cells infected *with M. tuberculosis*. Human *M. tuberculosis*-specific cytolytic T cells cultured *in vitro* are predominantly CD4+ (Ottenhoff and Mutis, 1990). In addition the cytolytic activity of CD4+ cells is enhanced at the site of pathology, as compared with peripheral blood (Lorgat *et al.*, 1992a). Many macrophages infected with *M. tuberculosis* are thought to have low anti-mycobacterial potential, allowing the bacilli to evade host defences (Kaufmann, 1988b). Cytolytic T cells that specifically recognise mycobacterial antigens can lyse these macrophages, releasing bacilli to be engulfed and killed by macrophages with greater anti-mycobacterial activity. It has been suggested that cytolytic T cells could directly reduce the bacillary load by causing mycobacterial death simultaneous to host macrophage death. Mycobacterial antigen-specific cytolytic CD4+ T cells generated *in vitro* have not been able to reduce bacillary load (Fazal *et al.*, 1994). Recently however, CD8+ mycobacterial antigen-specific cytolytic T cells have been able to reduce mycobacterial numbers (Stenger *et al.*, 1997). In the current study we show that cytolytic CD4+ T cells do indeed reduce mycobacterial numbers. These findings implicate CD4+ CTL directly in the reduction of bacillary load and in a favourable outcome for the disease process. It should, however, be noted that a diametrically opposed hypothesis has been suggested, in which cytolytic T cells serve to cause immunopathology by releasing toxic products following the destruction of host macrophages. These toxic products would serve to induce immunopathological conditions leading to caseous necrosis (Dannenberg, 1991)

1.1.5.8.4. CD8+ T cells and human mycobacterial disease

CD8+ T cells are the major cytolytic population in defenses against many intracellular pathogens. In animal models of tuberculosis, CD8+ T cells appear to play an important role. Adoptive transfer and cell depletion studies have implicated these cells in the control of *M. tuberculosis* infection (Orme and Collins, 1984; Orme 1987; Muller *et al.*, 1987). Murine CD8+ T cell lines and clones lyse *M. tuberculosis*-infected macrophages in an antigen-specific manner, and restrict the growth of the bacilli within the macrophages (De Libero *et al.*, 1988). The most striking evidence for a role for CD8+ T cells comes from studies with β2-microglobulin deficient mice. These mice are unable to express functional MHC Class I molecules and thus cannot develop a CD8+ T cell response. On infection with *M. tuberculosis*, 70% of the β2-microglobulin deficient mice died, whereas normal mice did not (Flynn *et al.*, 1992). Although granuloma formation was still intact, the granulomata contained tenfold more AFBs (Flynn *et al.*, 1992). These findings suggest that (in the mouse at least)

CD4+ T cells and NK cells are not sufficient for protection. The most likely explanation for these phenomena are that mycobacterial antigens can and are presented to CD8+ T cells via the MHC class I route. The 65kD mycobacterial heat shock protein has been shown to be presented in this way (Silva and Lowrie, 1994). Despite the importance of CD8+ T cells in the murine system, their role in human tuberculosis has remained uncertain. Human *M. tuberculosis*-specific cytolytic T cells are not CD8+. There is no expansion of this population of cells at the site of diseases (Barnes *et al.*, 1989) and the severity of tuberculosis in HIV-infected patients is unaffected by the CD8+ count (Jones *et al.*, 1993). Despite these facts, CD8+ T cell clones have been generated which respond to *M. tuberculosis* antigens and which are MHC class I restricted (Rees *et al.*, 1988). Cytolytic MHC class I-restricted CD8+ clones have also recently been described (Lalvani *et al.*, 1998).

1.2. Cytotoxicity

1.2.1. Introduction

Cytotoxic T cells are an important component of the cellular immune response. All cytotoxic activity was originally attributed to either natural killer (NK) cells or CD8+ lymphocytes (Kägi, *et al.* 1996). Intensive research in the area of cytotoxicity has subsequently demonstrated that CD4+ lymphocytes (Tite and Janeway, 1984; Erb *et al.*, 1990), as well as γδ cells (Munk *et al.*, 1990) and double negative (CD4-CD8-) cells (Stenger *et al.*, 1997) possess cytotoxic capabilities. T cell-mediated cytotoxicity has long been recognised as pivotal for the elimination of both virus-infected cells and transformed (oncogenic) cells. This elimination is mediated by CD8+ T cells which respond to MHC class I-associated antigen (Smyth and Trapani, 1998). Since MHC class I molecules are expressed on almost all somatic cells, the surveillance by CD8+ T cells for foreign antigen is extensive. Until recently, the model of CD8+ cytolytic activity included the processes of: (i) recognition of foreign antigen, (ii) activation of effector cells, (iii) release of cytolytic granules (including granzymes and perforin), and, (iv) target cell lysis (Berke, 1994). Target cell lysis was regarded as a form of necrosis. This model has subsequently been modified to incorporate not only phenotypically different effector cells involved in cytotoxicity, but also divergent mechanisms of induction of target cell death (granule- vs. receptor-mediated), and different forms of target cell death itself (apoptosis vs. necrosis/oncosis).

1.2.2. Mechanisms of cell death: Necrosis (Oncosis) and Apoptosis

There are two mechanisms of cell death. *Necrosis* is a cell death process which involves extensive intracellular organelle breakdown, loss of membrane integrity, cell swelling and finally disintegration (Majno and Joris, 1995). Necrosis is rapid and results in localised inflammation - the dying cell releases cellular components which may trigger activation of nearby immune cells. Necrotic death has also been termed accidental cell death - and this process seems to occur when a cell is subjected to severe injury or undergoes an extreme

alteration in normal physiological functioning. Necrosis was for a long time the only recognised form of cell death, however in the last decade or so another major mechanism of cell death, *apoptosis*, has been exhaustively studied. Apoptosis is a regulated death process, which has been termed physiological cell death, programmed cell death or cellular suicide. Apoptosis is fundamentally different from necrosis in that it involves a highly regulated sequence of events unlike the random destruction observed in necrotic death (Abastado, 1996; Depretaere and Golstein, 1998). Apoptosis usually involves nuclear shrinkage and densification, which is then followed by nuclear fragmentation. In addition the cytoplasmic component (cytosol) shrinks and the cell breaks down into distinct apoptotic bodies. During this process the cell membrane remains intact and it is only at the later stages of apoptosis that membrane integrity is lost (Green, 1998). This stage of apoptosis is termed secondary necrosis. The process of secondary necrosis is generally averted *in vivo*, since apoptotic bodies are rapidly and efficiently removed by phagocytes (neutrophils and macrophages), which recognise phosphatidyl serine (PS) moieties exposed on the surface of apoptotic cells (Zwaal and Schroit, 1997). Due to the confusion that the term secondary necrosis raises, it has been proposed that *oncosis* be used as an alternative to classical necrosis (Majno and Joris, 1995).

Apoptosis is triggered by a variety of signals. These include irradiation, low level oxidative stress, toxins, glucocorticoids, ATP, drugs, cytolytic granules (perforin, granzymes, granulysin etc.) and specific receptor-ligand interactions i.e. Fas receptor (CD95/Apo-1) and FasL, TNF receptor (TNF-R) and TNF-α interactions (Abastado, 1996; Vaux and Strasser, 1996). Of particular interest in T cell mediated cytotoxity are the cytolytic granules and the Fas-mediated pathway, which account for the majority of T cell-mediated cytotoxicity (Kägi *et al.*, 1994: Trapani, 1998).

1.2.3. CD4+ and CD8+ cytotoxic T cells

T lymphocytes of the CD8 type possess cytolytic granules (Shresta *et al.*, 1998). They are able to kill target cells via release of these components. Depending on the level of onslaught, the target cell may die via necrosis or apoptosis (Berke, 1995). High level attack leads to loss of membrane integrity and rapid death. Cytolytic granules are however also able to induce apoptosis. This occurs via cytolytic granule component (granzyme)-mediated activation of the apoptotic signal cascade which involves the interleukin 1 converting enzyme (ICE)-related family of cysteine proteinases termed caspases (Shresta *et al.*, 1998). Although granule-mediated killing is predominant in CD8 cells, killing of target cells via FasL ligation with Fas has also been reported (Esser *et al.*, 1997; Ju *et al.*, 1994).

The recognition that CD4 cells can also be cytotoxic led to an intensive investigation into the mechanism of action of these cells (Kägi *et al.*, 1996). CD4 cells were for a long time accepted as being devoid of cytolytic granules (Hahn *et al.*, 1995). The mechanism of

cytolytic activity of these cells was reported to operate via receptor-ligand (Fas-FasL) interaction, resulting in target cell apoptosis (Erb *et al.*, 1990; Rahelu *et al.*, 1993; Stalder *et al.*, 1994; Ashany *et al.*, 1995). It has since been reported that certain CD4 lymphocytes do possess cytolytic granules and they can induce target cell death via the secretory pathway (Lewinsohn *et al.*, 1998; Sevilir-Williams and Engelhard, 1996). The development of cytolytic granule-mediated killing by CD4 cells appears to be an adaptation for the containment of infections which involve intracellular bacteria or other parasites (Sevilir-Williams and Engelhart, 1997). The occurrence of multiple mechanisms of cytolysis induction in both CD4 and CD8 cells points toward functional diversification. Granule mediated killing is generally associated with the removal of infected or transformed cells (Page *et al.*, 1998). Fas-FasL-mediated apoptosis does not have this association, and appears to be more generally linked with immunomodulation and clearance of host cells from areas of immune activity (Takayama *et al.*, 1995; Depretaere and Golstein, 1997). Fas-mediated target cell apoptosis is dependent on the expression of the Fas molecule on the target cell (Lewinsohn *et al.*, 1998; Liles *et al.*, 1996). Since a large number of cell types do not express Fas, or do so only at low levels, optimal killing via this pathway is prevented. The presence of an alternative mechanism of lysing infected cells would ensure their elimination, even in the absence of Fas expression.

1.2.4. General features of CTL activity

Irrespective of the predominant mechanism of CTL killing, the sequential stages in the process appear similar. In all cases of T cell-mediated killing, the initial events involve T cell receptor (TCR)-MHC-peptide interactions (Berke, 1994). This stage is also termed *conjugation*. The formation of the CTL-target conjugate also involves adhesion molecules and co-receptors. Following TCR engagement, the CTL is activated via a TCR signal transduction cascade. The *activation* of CTL by definition involves the preparation of the T cell for cytotoxic activity. The principal mode of activity may be via granule release (secretory or exocytotic kill), or via receptor-ligand interaction, principally Fas-FasL (non-secretory or receptor-mediated kill). The activation of CTL which kill via exocytosis, involves the functional maturation of cytolytic granules and their localised release (Page *et al.*, 1998; Griffiths, 1997). In CTL which operate via Fas-FasL interaction, the up-regulation of FasL on the CTL membrane is the pivotal activation event (Takayama *et al.*, 1995). Following the binding (conjugation) and activation of the CTL, the CTL delivers the *lethal hit*. This is the effector phase of the process and involves the release of cytolytic granules, or the engagement of FasL with its receptor molecule. The lethal hit initiates the target cell death (Berke, 1994). Once the lethal hit has been delivered, the CTL detaches and is free to recycle and initiate a new cytolytic event. The fate of the target cell is generally death via *apoptosis*, however if the onslaught is extreme (high numbers of CTL or concentrated release of granule contents, particularly perforin) *necrosis* can also occur (Berke, 1995b; Podack, 1995).

1.2.4.1. Conjugate formation and CTL activation

The simplified model of lymphocyte-target cell adhesion mediated solely by intercellular binding of the TCR/CD3 complexes with MHC class I or II molecules has been expanded following the recognition of the important role that auxiliary adhesion molecules play (Berke, 1994). CTL-target cell conjugation is dependent on temperature, metabolic energy and Mg^{2+}, features not observed in events solely dependent on TCR-MHC interactions.

In the case of secretory cytolysis, the formation of a stable CTL-target conjugate appears to be dependent on contact between large surfaces of the cells involved, TCR-MHC-peptide interaction and a "zipping-up" process. The "zipping-up" involves auxiliary CTL determinants e.g. CD2, CD4, CD8, CD28, CD43 and LFA-1 molecules, and target cell determinants e.g. B7 components, CD22, ICAM-1, ICAM-2 and LFA-3 molecules (Berke, 1994). Minimal cross-linking of the TCR leads to activation, however granule release only occurs once firm adherence between CTL and target has occurred. Conjugate formation in Fas-mediated cytolysis is similar, with up-regulation of FasL expression being initiated at this stage (Yagita *et al.*, 1995).

Conjugation is a rapid event. Activation of the CTL and delivery of the lethal hit is dependent on the status of the effector cell. If already primed, the CTL can deliver the lethal hit within minutes, since the cytolytic granules have been recruited and/or FasL has been expressed on the cell membrane. Following initial priming of the CTL via TCR engagement, the CTL is able to to recycle i.e. disengage from the initial target cell and move on to a new one. For granule exocytosis this involves a new sequence of conjugate formation, but in FasL-mediated killing, the CTL is able to initiate apoptosis in any target cell expressing Fas on its surface. This fundamental difference between the two major mechanisms of cytolysis has important implications (Trapani, 1998). FasL-expressing CTL have the ability to lyse any cell which expresses Fas, including other T cells (Nagata and Golstein, 1995). This is not a feature of exocytosis-mediated kill, since each lethal hit requires TCR-MHC engagement which ensures that only infected/transformed target cells are eliminated. This is a protective mechanism of the host, since elimination of all cells presenting foreign antigen would hinder normal immune function which is dependent on antigen-presenting cells for initiation (Kägi *et al.*, 1996).

1.2.4.2. Lethal hit delivery

1.2.4.2.1. Secretory mechanism of lethal hit

The fact that both NK cells and certain CTL contain cytoplasmic lytic granules and their target cells display complement-like lesions on their membranes led to the proposal that both these cell types employed a mechanism of cytotoxicity involving secretion of lytic components. This process was believed to result in pore formation on the target cell membrane (Tschopp and Nabholz, 1990). This model of killing involves conjugate formation

followed by localised Ca^{2+}-dependent degranulation. The origin of the pore forming material is the cytoplasmic granules which contain perforin and granzymes (i.e. granule enzymes) (Tschopp and Nabholz, 1990; Page *et al.*, 1998).

The requirement for extracellular Ca^{2+} is absolute and multi-levelled. Ca^{2+} is required for (i) the regulated secretion of lytic granule constituents, such as perforin and proteases, (ii) binding of perforin to the target cell membrane, and (iii) polymerisation of perforin to polyperforin which is believed to be important in pore formation (Mac Lennan *et al.*, 1980; Berke, 1994). The discovery that certain CTL can lyse target cells in the absence of extracellular Ca^{2+} led to the elucidation of the non-secretory mechanism of cytolysis which was confirmed by the lack of perforin or BLT-esterase activity in certain CTL (Kägi *et al.*, 1996).

Cytoplasmic granules are present in most CTL (Page *et al.*, 1998). They are present at early activation and proliferation stages of immune responses to viruses or allografts *in vivo*, and in cells cultured with IL-2 or stimulated with antigens or mitogens *in vitro*. The cytolytic granules contain perforin (cytolysin) a 65kD lytic protein. In addition the granules contain hydrolytic lysosomal enzymes, serine proteases and proteoglycans. Both perforin and serine esterases have been located within the same granule (Henkart *et al.*, 1987). The cytoplasmic granule consists of a dense core and a multi-vesicular cortex. The dense core contains secretory proteins such as perforin and chondroitin sulphate proteoglycans. The core is also rich in mannose-6-phosphate (Man-6-P) receptor, a marker present in early endocytic compartments. Granzymes A and B possess Man-6-P residues, which enable specific recognition and targeting by the Man-6-P receptor for concentration in the lytic granules (Trapani, 1998; Griffiths, 1997). The multi-vesicular cortex of the granules contains lysosomal proteins such as acid phosphatase, α-glycosidase, and cathepsin D. The pH of the cortex is acidic like cortices of endosomes and lysosomes. Acidification of granules is an essential process for functional granule activity (Kataoka *et al.*, 1996). The granules are complex and unique and much remains unknown about their formation, maturation and release.

Although perforin is structurally similar to the complement components C6-C9, and complement-like pores have been observed in target cells, complement and perforin differ in that perforin does not have a membrane bound receptor but rather binds to phosphorylcholine residues of target cell membranes (Krahenbuhl and Tschopp, 1991). Both perforin and complement do induce pores or channels in the target cell membrane. In the case of perforin, polyperforin-induced channels appear to be similar to other ion channels. There is increased conductance across such channels, however whether they are crucial in target cell lysis itself remains controversial (Liu *et al.*, 1995). It seems unlikely that influx of

water resulting in swelling and osmotic lysis occurs, since exposure of cells to perforin resulted in net contracture of the cell prior to lysis (Liu *et al.*, 1995).

Perforin is a 70kD protein which is stored in the cytoplasmic granules of killer lymphocytes (Liu *et al.*, 1995). Perforin is water-soluble and shares many features with a broad family of pore forming proteins/peptides produced widely in nature. Binding and polymerisation of perforin appear to be separate events, however both are dependent on Ca^{2+} ions. The perforin pore is in the range of 5-20nm in diameter and has a conductance of 0.4-6 nanoSiemens (nS) (Trapani, 1998).

The perforin pore has a maximal permissive conductance size of approximately 8kD. This would preclude massive efflux of cellular constituents, but would allow movement of small intracellular molecules such as ATP and K^+ ions which could perturb intracellular homeostasis. Perforin could contribute to target cell death by two mechanisms: (i) by creating an osmotic stress sufficient to induce colloid osmotic lysis, (ii) by triggering an influx of potentially lethal extracellular molecules (Liu *et al.*, 1995). Very few (3-4) perforin monomers are required to form a functional ion channel, while 10-20 are necessary to produce the larger pores observable on EM. These larger pores may only occur at high perforin concentrations. It seems unlikely that the smaller pores would allow entry of other cytolytic granule components such as granzyme B. Indeed, it has now been demonstrated that granzyme B can enter cells independently of perforin, however perforin is essential for activation of granzyme B and for translocation to the nucleus (Froehlich, *et al.*, 1996).

Why are CTL resistant to their own cytolytic products? CTL have been shown to be resistant to the activity of other killer cells which would nonetheless recognise them as targets either in an antigen-specific manner or via lectin or monoclonal antibody conjugation. These cells are also resistant to isolated cytolytic granules or purified perforin. It thus seems that an innate mechanism of protection exists. Protectins have been postulated as CTL-specific proteins which mediated resistance to perforin killing (Young and Cohn, 1988). The activity of these molecules is envisaged to occur via blocking or inactivation of docked perforin monomers - thus stopping polymerisation. Other possible protection mechanisms may involve extensive repair mechanisms - involving shedding or endocytosis of bound perforin monomers. Recent work has suggested that perforin alone is unable to lead to cell death, therefore the insertion of perforin into the membranes of CTL would not be problematic unless concomitant uptake of other cytolytic components such as granzyme B occurred. It has been proposed that binding and uptake of granzyme B is via a specific receptor. If CTL lack the receptor for granyme B they would be protected from granule induced apoptosis (Froehlich *et al.*, 1998)

Perforin is important in CTL function, a fact that has been demonstrated in perforin knockout mice which are markedly impaired in cytolytic activity (Shresta *et al.*, 1998). But is the major

role of perforin the lysis of the target cell or does it operate as an entry mediator/carrier or activator of granzymes? Recent work has led to a novel hypothesis that the role of perforin in target cell death is predominantly one of co-operation with granzymes, ensuring delivery of a directional lethal hit but preventing self-annihilation (Froehlich *et al.*, 1998). In this model, perforin as well as granzymes (particularly granzyme B) bind to the target cell. Specific granzyme receptors have been proposed, but as yet remain unidentified. It has been demonstrated that CTL clones are more resistant to lysis by other CTL, lytic granules or perforin, than to complement-mediated lysis. This may be indicative of a receptor-mediated process. Following the binding of these molecules to the target cell a process of endocytosis occurs which results in internalisation of both perforin and granzymes within endosomal compartments or vesicles (Froehlich *et al.*, 1996). Whether this internalisation is triggered by receptor engagement (GzB-GzBR) or merely represents a repair mechanism by the host cell which is trying to counteract the perforin perturbation of membrane homeostasis remains unknown. Once internalised the perforin then polymerises and the polyperforin channels allow the release of granzymes into the target cell cytoplasm. This model accounts for the intriguing finding that granzyme B can enter a target cell independently of perforin. Proof of this model of endosomal release via perforin has not been provided, however it has become apparent that the internalised granzymes are dependent on perforin for translocation to the nucleus and for succesful induction of apoptosis (Jans *et al.*, 1996; Froehlich *et al.*, 1998). The previously held belief that perforin pores allowed the entry of granzymes now seems less likely based on the fact that perforin pores are often very small ion-exchange channels which would not readily permit entry of large proteins. Even though granzyme B can independently enter a target cell, it is unable to induce target cell death without the presence of perforin (or similar membranolytic components). This suggests that internalised granzyme B is "entrapped" within the target cell without perforin present to facilitate release. Another proposed granzyme-activation mechanism is the perforin-induced modification of cytoplasmic pH, however this model has not been confirmed either (Shestra *et al.*, 1998).

Once released, the granzyme B molecule is able to access the caspase cascade via direct activation of caspase 3/CPP-32. In addition, granzyme B is able to translocate to the nucleus, possibly via exploitation of host cytoskeletal components. Once within the nucleus, granzyme B may be able to directly cleave certain structural and/or regulatory molecules leading to the characteristic features of apoptosis (Henkart *et al.*, 1995; Andrade *et al.*, 1998; Medema *et al.*, 1998).

The granzymes are the most important constituents of cytolytic granules, being directly involved in induction of target cell apoptosis (Pham and Ley, 1997). Granzymes comprise approximately 90% of the protein content within cytolytic granules (Henkart *et al.*, 1987). Granzymes are related to the chymotrypsin family of serine proteases and demonstrate structural similarities and genetic linkage to other leukocyte serine proteases (Smyth *et al.*,

1996). A total of 8 mouse granzymes have been described and characterised (Gz A-G, and M), whereas in humans only 5 have been characterised (Gz A, B, H, M, and protease-3). Granzymes display many features in common with other chymotrypsin-like enzymes, but do possess certain defining features such as highly conserved residues at certain positions (1-4, 9-16), conserved propeptide sequences (generally G-Q, or Q-Q), 3 conserved disulphide bridges, and unusual substrate preferences (Smyth *et al.*, 1996). Numerous other cytolytic granule components exist, however their exact role in cytolysis remains unknown.

1.2.4.2.2. Receptor- (Fas-) mediated mechanism of lethal hit

The occurrence of an alternative mechanism of target cell lysis involving receptor-ligand interactions has been acknowledged for some time. The fact that exocytotic granule mediated killing could not account for all target cell death mediated by CTL necessitated an alternative cytolytic pathway. Work on apoptosis in many areas of research led to the discovery of an apoptosis-inducing molecule, subsequently termed Fas (CD95/Apo-1). Fas is a receptor expressed on the surface of numerous cell types. It is a member of the TNF receptor (TNFR) family. Several other receptors have been implicated in transducing death signals, including TNFR1 and death receptors (DR) 3, 4, and 5 (Ashkenazi and Dixit, 1998). Engagement of Fas with its ligand, Fas ligand (FasL), leads to induction of apoptosis in the Fas-expressing cell. FasL may be membrane bound or secreted. The induction of apoptosis via this mechanism is dependent on the expression of Fas on the target cell surface. Fas is not universally expressed by all cell types, and requires up-regulation under certain conditions (Vaux and Strasser, 1996).

The fact that CTL were found to induce apoptosis in the absence of perforin and proteases led to the conclusion that these components were not involved in induction of apoptosis. Subsequently, with the discovery of the Fas-mediated pathway of target cell apoptosis it has become clear that this was the mechanism responsible for granule-independent death. Since both Fas- and granzyme-mediated target cell death occurs predominantly via apoptosis, mechanistic similarities between the two processes were investigated. It has now been demonstrated that both Fas ligation and granzyme B activation lead to switching on of the central apoptotic caspase cascade, via activation of either caspase 8 (FLICE), or caspase 3 (CPP 32) (Henkart, 1996).

The Fas-mediated mechanism of target cell killing is also dependent on an initial conjugation stage. Following conjugation and TCR-MHC-peptide interaction, the effector CTL is activated and FasL is up-regulated on the lymphocyte cell surface. The delivery of the lethal hit then involves the ligation of FasL with the Fas molecule on the target cell. Although this process can occur in the absence of Ca^{2+}, processes involved in upregulation of FasL do require Ca^{2+} (Yagita *et al.*, 1995). Naturally this can only occur if Fas is expressed on the target cell. However, if the target cells do express Fas, the activated CTL is able to disengage from the

initial target and then bind to any Fas-expressing cell and trigger its apoptosis. The occurrence of such MHC-unrestricted and activated CTL could be a danger to the host in that not only infected or transformed targets are eliminated (Yagita *et al.*, 1995). Due to this possibility, it appears that the Fas-mediated mechanism of CTL activity is more important in immunoregulation. Activated T cells have to be eliminated at sites of activity to prevent massive cellular expansion. The activated T cell expressing FasL is able to act in a regulatory manner by eliminating other T cells which have been shown to express Fas as well. This ensures a regulated elimination of activated T cells capable of causing damage by both the release of pro-inflammatory cytokines and unchecked CTL activity (Nagata and Golstein, 1995; Takayama *et al.*, 1995). The exocytotic mechanism of kill is, on the other hand, preferentially employed by the host in the elimination of infected and transformed cells. This mechanism is safer for the host, in that it is dependent on repeated TCR-MHC-peptide interactions, and only targets cells expressing presenting foreign antigen will be eliminated. In addition the cytolytic granule components may be bactericidal and/or viricidal and thus aid in killing infectious organisms simultaneous to the induction of target cell apoptosis (Stenger *et al.*, 1998; Trapani, 1998).

1.2.4.3. Apoptosis: co-ordinated cell death

Most, if not all, animal cells have the ability to self-destruct by activation of an intrinsic cell suicide programme (Allen *et al.*, 1998). This usually occurs in situations where the cell is no longer needed (homeostasis) or has been seriously damaged (injury). Apoptosis plays an important role in development and homeostasis, and is also beneficial to the host in that it allows for containment and elimination of damaged or diseased cells. Errors in apoptosis may account for certain neoplastic disorders and links between apoptosis, AIDS and certain neurodegenerative diseases have been established (Lincz, 1998). The discovery that CTL activity operates via the induction of apoptosis highlighted the importance of this phenomenon in the area of cellular cytotoxicity.

1.2.4.3.1. Molecular and cellular features of apoptosis

Apoptosis is characterised by a regulated sequence of cellular changes. The nucleus and cytoplasm condense (pyknosis), without any damage to the cell membrane. Organelle structure is maintained until the later stages which involve the fragmentation of the nucleus (karyorhexis) and the "budding" of the cell into apoptotic bodies (Majno and Joris, 1995). Following apoptotic body formation, necrotic changes such as membrane leakage, swelling and lysis may occur. The regulated formation of apoptotic bodies without any leakage or lysis appears to be an adaptation by the host to avoid a localised inflammatory response (Depretaere and Golstein, 1998). Apoptotic bodies are readily phagocytosed by granulocytes and macrophages via recognition of phosphatidyl serine (PS) moieties.

In addition to the gross morphological changes induced during apoptosis, certain regulated molecular events also occur. Indeed, an area of intensive research at present is the endeavour to link the morphological changes with the molecular sequence of events which has recently been elucidated. The most characteristic event at the molecular level is the activation of nucleases which degrade chromosomal DNA, first into large (50-300kb), and subsequently very small oligonucleosomal fragments (Barinaga, 1998). It is now apparent that this DNA fragmentation is the result of a complex cascade of protease activation involving a family of cysteine proteases related to the interleukin-1 converting enzyme (ICE), termed caspases (Nicholson and Thornberry, 1997).

The process of apoptosis has four distinct stages: (i) decision by the cell to die or to attempt repair or replication, (ii) death , (iii) engulfment of apoptotic fragments by phagocytes, and (iv) degradation of engulfed corpses (Chao and Korsmeyer, 1998).

Certain major aspects of apoptosis do not require the transcription of new genes. Thus, cells possessing all the machinery required for successful apoptosis, will undergo the death process without concomitant protein synthesis (Lincz, 1998). Enucleated cells (cytoplasts) have been shown to undergo cytoplasmic apoptotic changes in the absence of nuclear material. This seems to indicate that morphological changes are not directed by nuclear events. It has now become clear that the apoptotic process is controlled by the bcl-2 family of proteins in close association with the mitochondria, and that execution of apoptosis and structural changes are mediated by the caspases. The mitochondrion is now regarded as the central co-ordinator of apoptosis, mediating the both cellular and nuclear events of apoptosis (Mignotte and Vayssiere, 1998).

The point of intersection of the mitochondria and apoptosis appears to operate in the mediation of the activity of the Bcl-2 family of anti-apoptotic proteins (Adams and Cory, 1998). The Bcl-2 proteins may remove caspases from the cytoplasm by dragging them across the mitochondrial membrane, thus preventing apoptotic events. Alternatively, the mitochondria-derived caspase activitors cytochrome c and the apoptosis-inducing factor (AIF) could be prevented from exiting the mitochondria by Bcl-2 proteins. Pro-apoptotic signals such as Fas ligation may lead to inactivation of Bcl-2 proteins, followed by activation of caspases and finally cell death (Green and Reid, 1998).

1.2.4.3.2. The caspases - regulators of apoptosis

Extensive research in the last couple of years has led to major clarification of certain aspects of the molecular processes involved in apoptosis, but in so doing have raised yet further questions. At present the accepted model for induction of apoptosis assigns a cardinal role to the caspases, a family of cysteine proteases related to ICE, and which cleave their substrates after specific aspartic acids (Cohen, 1997; Thornberry and Lazebnik, 1998).

Blockage of peptide caspases by specific inhibitors blocks apoptosis itself, a finding which implicates these proteases as the core co-ordinators of the death programme. The caspases themselves serve as substrates for other caspases and are thus activated by cleavage at specific aspartic acids resulting in a cascade phenomenon with a certain amount of redundancy (Kidd, 1998). Activation of the caspase cascade is dependent on a shift to the cytosol of certain regulatory components, i.e. cytochrome c and apoptosis-inducing factor (AIF). These components are normally sequestered within the mitochondria. (Kluck *et al.*, 1997; Krippner *et al.* 1996). It is now acknowledged that the mitochondria are pivotal in the control of apoptosis, so much so that they have been termed the "central executioners" of apoptosis. The activities initiated and controlled by the mitochondria are carried out by the caspases which cleave certain intracellular proteins, including proteins of the nucleus, cytoskeleton, endoplasmic reticulum and cytosol (Henkart and Grinstein, 1996; Barinaga, 1998). This action is believed to lead to the processes of blebbing, condensation and fragmentation, which characterise apoptosis. Exactly which proteins are involved and how their cleavage results in the morphological changes observed remains an area of intensive research (Depretare and Golstein, 1998).

The term caspase (cysteinyl aspartate-specific proteinase) embodies two distinguishing features of this group of enzymes: they are cysteine proteases and they are specific for cleavage after aspartate (Asp) residues. Accumulating evidence now suggests that the group may be subdivided into at least two subfamilies. These have been categorised as the ICE and CED-3 (*C. elegans* death gene encoded protein 3) subfamilies. The ICE subfamily appears to be predominantly involved in inflammation whereas the CED-3 subfamily is largely, if not exclusively, involved in apoptosis.

The ICE subfamily consists of caspase-1 (ICE), caspase-4 (ICE_{rel}-II, TX, ICH-2), and caspase-5 (ICE_{rel}-III, TY). The CED-3 family consists of the remaining 7 caspases including caspase-7 (Mch3, ICE-LAP3, CMH-1), caspase-3 (CPP32, apopain, Yama), caspase-6 (Mch2), caspase-8 (MACH, FLICE, Mch5), caspase-10 (Mch4), caspase-2 (ICH-1), and caspase-9 (ICH-LAP6, Mch6). This scheme of classification has been modified on the basis of tetrapeptide specificity of the caspases. This has resulted in the CED-3 family being subdivided into 2 groups. Group II contains Caspases-3, -7, and -2. Group III contains caspases-6, -8, and -9. Interestingly, granzyme B, which is a serine proteinase, displays similarities to the Group III caspases.

Numerous substrates for caspase activity have been reported. The most important of these are PARP (poly(ADP-ribose) polymerase), DNA-PK (DNA-dependent protein kinase), lamins, fodrin, actin, gelsolin, and protein kinase C (δ-isoform) (Depretaere and Golstein, 1998). The net effect of the proteolytic events mediated by caspases is to (i) halt cell cycle progression, (ii) disable homeostatic and repair mechanisms, (iii) initiate the detachment of the cell from

its surrounding tissue structures, (iv) disassemble structural components, and (v) mark the dying cell for engulfment by other cells such as macrophages. It seems unlikely that one specific cleavage event is responsible or necessary for cell death. Rather the collective effect of multiple cleavage events, but of limited key proteins, culminates in the highly ordered and systematic process of apoptotic cell death.

Regulation of caspase activity predominantly occurs at the level of proenzyme processing and maturation. Dormant caspase proenzymes are converted to catalytically competent heterodimeric proteases by cleavage at Asp-x bonds (Cohen, 1997). The active caspases then activate other dormant proenzymes into their active protease (caspase), thus leading to a cascade effect. For example, it has been demonstrated that binding of FasL to Fas (CD95) leads to the transmembrane activation of the adapter molecule FADD (Fas-associated death domain). FADD is able to trigger activation of caspase 8 proenzyme leading to caspase 8 being able to activate other proenzymes e.g. caspases-3 and -7. Following a sequence of successive cascade activations, the caspases (particularly caspase-3) then mediate the proteolysis of certain target proteins which may be structural, reparative or homeostatic. This then culminates in apoptosis (Wallach *et al.*, 1998).

1.2.4.4. Granule-mediated target cell apoptosis

Following the delivery of the lethal hit and the entry of the cytolytic components into the target cell, either via a co-ordinated receptor-ligand and endocytotic pathway, or direct entry via polyperforin pores, the target cell is induced to undergo apoptosis. How the granule-mediated mechanism of kill bypasses the apoptotic inhibitory bcl-2 family is unknown. However certain of the cytolytic granules, particularly granzyme B, have been well studied, and have been found to be structurally and functionally similar to the caspases. Therefore, once within the cytoplasm, either following direct entry, or release from an endosome via internalised polyperforin pores, the granzyme B molecules are free to initiate the caspase cascade. The outcome is the same as observed in receptor mediated target cell death (Froehlich *et al.*, 1998; Trapani, 1998).

Cytotoxic activity mediated by granule components such as granzymes A and B has conclusively been shown to intersect with the receptor-mediated apoptotic pathway at the level of caspase activity. Granzyme B is able to mediate the conversion of caspase-3 (CPP 32) proenzyme to its active form. In this sense granzyme B is similar to caspase-8 or FLICE (Lincz, 1998). In addition, granzyme B is able to cleave certain other caspase proenzymes including caspases 10, 9, 7 and 6 (Henkart, *et al.*, 1997; Thornberry, *et al.*, 1997). An intriguing finding has been that granzyme B is able to cleave certain substrates downstream of the caspase cascade independent of caspase activity (Sarin *et al.*, 1997; Talanian *et al.*, 1997). These substrates include the DNA-dependent protein kinase catalytic subunit (DNA-PK_{CS}) and the nuclear mitotic apparatus protein (NuMA) (Andrade *et al.*, 1998). Since the

cleavage of certain other substrates downstream of the caspase cascade, including poly(ADP-ribose) polymerase (PARP), U1-70kD and lamin B, is mediated by the caspases and not by granzyme B directly, there appears to be a dual pathway of granzyme B-mediated target cell apoptosis, a caspase-dependent and -independent pathway (Sarin *et al.*, 1998; Trapani, 1998).

What is the relevance of this redundancy? Various models and theories have been put forward, and one in particular seems very important in the light of CTL control of infected host cells. The Fas-mediated death pathway is strictly caspase-dependent and is under the stringent control of the various apoptotic inhibitory proteins. Ligation of Fas by FasL will not always result in target cell death as the signal may be blocked at several levels (Wallach *et al.*, 1998). Granzyme-mediated target cell apoptosis that is caspase-dependent is also subject to certain host control mechanisms - most importantly the availablity of caspase substrates, which we have seen is controlled by host cell mitochondria. If an intracellular organism (virus, bacteria or parasite) were able to enhance the activity of inhibitory proteins, it would theoretically be able to engineer a protected milieu for itself within the host cell (Trapani, 1998). A caspase-independent pathway would bypass any such protective pathway. This is particularly interesting in the case of *M. tuberculosis* infection of macrophages.

The macrophage is the chosen host cell of *M. tuberculosis* and offers the organism a protected environment within which to replicate and possibly attain heightened virulence. Induction of host cell apoptosis due to mycobacterial infection has been reported (Schluger and Rom, 1998). This may be due to the fact that H37Rv is able to down regulate Bcl-2 which results in increased apoptosis. This model makes sense if apoptosis is beneficial to the invading organism. If apoptosis does not in any way harm mycobacterial viability, apoptosis would lead to spread of the organism to additional macrophages enhancing the chances of dissemination. This simple model does not appear to be universally applicable. Induction of macrophage apoptosis by both H_2O_2 and ATP treatment, can lead to a reduction in organism viability (Molloy *et al.*, 1994; Lammas *et al.*, 1997; Laochumroonvorapong *et al.*, 1996; 1997). In addition granule-mediated apoptosis following exposure to cytolytic CD8+ lines led to a reduction in mycobacterial numbers (Stenger *et al.*, 1997). Similarly soluble FasL-induced apoptosis led to a reduction in organisms (Oddo *et al.*, 1998). Apoptosis does not seem to be a desired outcome for the infecting mycobacteria. A scenario whereby the mycobacteria enhance anti-apoptotic mechanisms would seem more likely. Perhaps whether the organism chooses pro- or anti-apoptotic signals is dependent on level of infection and virulence of organism. Host cell lysis via necrosis is not advantageous in that a localised inflammatory response would result. Perhaps, in heavily infected cells induction of apoptosis would then avoid necrosis and enable spread. On the other hand, mycobacteria within macrophages at a low level of infection may block apoptosis in order to allow for replication

to occur. How organisms induce apoptosis themselves is unknown, and similarly whether they are able to block certain apoptotic events remains conjectural (Placido *et al.*, 1997; Schluger and Rom, 1998). The granzyme-mediated model of cytolysis allows for a bypassing of host cell control mechanisms leading to death despite blocking of certain apoptotic events by the host cell. In this way the host is able to control the outcome of CTL activity. Although a recent paper has reported FasL as being able to induce apoptosis and reduce organism viability, earlier work showed that anti-Fas was not able to achieve the same result (Oddo *et al.*, 1998). In addition *M. tuberculosis* appears to be able to down-regulate Fas expression on macrophages, and dendritic cells are resistant to Fas-mediated killing (Ashany *et al.*, 1999). Without any granule-mediated CTL activity, blocking of Fas-mediated lysis would enable the organism to multiply readily within the protected environment of the macrophage phagosome. Development of a granule-based cytolytic mechanism, which is only dependent on MHC-antigen presentation, would represent insurance for the host.

1.2.4.5. Fas-mediated target cell apoptosis

The Fas-mediated mechanism of target cell death is the best characterised of all apoptotic pathways. The mechanism of Fas-induced apoptosis is similar to TNF-mediated cell death, both Fas and TNF-R being members of the TNF receptor family (which includes several other receptors e.g. CD27, CD30, and CD40). The ligation of these receptors results in a consistent sequence of events culminating in cell death.

Binding of FasL to Fas initiates a process whereby the adapter molecule FADD is linked to Fas at the cell membrane but on the cytosolic side. Complexing of Fas and FADD leads to the ability of the caspase-8/FLICE (Fas-linked ICE) proenzyme to be activated (Peter and Krammer, 1998). The activated form of caspase-8 is then able to activate other caspase precursors and initiate the cascade as discussed above. The bcl-2 family is intimately involved in this process, and it appears that initial Fas ligation and association with FADD may trigger a signal to the mitochondria and the associated bcl-2 components allowing for the release of caspases or their activation by Apaf-1 or cytochrome c (Green, 1998). Exact mechanisms are still unclear, but blocking by bcl-2 and bcl-X_L occurs at the level of FLICE/caspase-8 induction of the cascade (Ashkenazi and Dixit, 1998). In other systems the blockage may occur even further upstream. The exact links between signal reception and caspase activation in apoptosis induced by growth factor deprivation and exposure to glucocorticoids, for example, are still being assessed.

All Fas-mediated apoptotic activity is dependent on caspases, and the initiation of apoptosis is strictly monitored by the bcl-2 family of proteins. A similar situation occurs in TNF-induced apoptosis, and possibly any mediation involving receptor-ligand binding at the cell surface (Lincz, 1998).

1.2.5. Role of cytotoxic T cells in the clearance of tuberculosis infection

Fas-mediated target cell apoptosis is under strict control of the host cell. Apoptosis induced by cytolytic granules released from CTL is also under host cell control - however there is an additional caspase-independent pathway which is able to result in direct cleavage of downstream substrates and cell death (Sarin *et al.*, 1997). This may be an advantage for the host, since lysis of host cells harbouring infectious pathogens which have adapted to intracellular survival would lead to release of the invading organism and the ability to be taken up by activated and possibly more competent host macrophage cells. Lysis of the host may also affect pathogen viability, and in this way CTL function would be pivotal in aiding clearance (Oddo *et al.*, 1998). The granulomatous lesion always consists of an inner core of macrophages surrounded by a mantle of T lymphocytes. Containment of disease must therefore reside within the functional capabilities of these two cell types (Kaufmann and Andersen, 1998). The most likely scenarios are either T cell activation of infected macrophages via cytokine release resulting in containment of infection, or direct cell-to-cell contact. Reports regarding the ability of macrophages to contain mycobacterial growth are conflicting, however several recent reports have demonstrated the ability of activated macrophages to reduce the numbers of infectious organisms - either via cytokine modulation (Bonencini-Almeida *et al.*, 1998) or via interaction with T cells (Silver *et al.*, 1998). This latter report emphasises direct cell-to-cell interaction. Such interaction may well be cytolytic.

A direct role for CTL in the containment of tuberculosis is difficult to demonstrate. This is due to the occurrence of two populations of T cells mediating target lysis, i.e. CD4+ and CD8+. In addition, γδ T cells have cytolytic ability (Kaufmann and Andersen, 1998). Gene knockout studies are problematic due to this redundancy, therefore CD8+ (β2-microglobulin) knockouts, still possess CD4+ cells, which themselves may compensate for the lack of CTL activity. Lack of CD4+ cells, such as in HIV, results in high levels of TB infection, however, whether this is predominantly due to cytokine deprivation, or lack of CTL function, is unknown (Jones *et al.*, 1993). Knockouts which address particular pathways of lysis, granule- vs. Fas-mediated, also pose the problem of compensation. Mice deficient in perforin (Laochumroonvorapong *et al.*, 1997), or granzyme B (Cooper *et al.*, 1997), are not more susceptible to infection. However, these mice may have developed other mechanisms of target cell lysis e.g. a compensatory entry mechanism independent of perforin, or the use of other granzymes or granulysin, instead of granzyme B. In a similar way, Fas knockout mice, which also do not show added susceptibility to infection, may predominantly use the granule-secretion mechanism (Cooper *et al.*, 1997).

Even though a direct demonstration of the pivotal role for CTL in control of *M. tuberculosis* infection has not been possible, it seems likely that these cells are involved. This is due to the nature of the granuloma (T cell mantle always associated with the infected macrophages) and the fact that CD4+ cells which predominate in pleural effusions have enhanced cytolytic

potential (Lorgat *et al.*, 1992). The current study addresses the nature of this cytolytic activity, and how the activity of such cells may control mycobacterial spread.

It has recently become apparent that CTL may possess dual mechanisms of target cell lysis. Originally CD8+ CTL were classified as granule releasing, and CD4+ CTL as being Fas-dependent. This dichotomy was accepted due to the fact that most CD8 CTL activity was Ca^{2+}-dependent, and the CTL contained serine proteases. CD4 CTL on the other hand generally operated independently of Ca^{2+}, and serine protease activity was lacking. This clear cut differential was consistent with the idea that CD8 CTL are primarily involved in clearance of virally infected and/or transformed cells. CD4 CTL activity was assigned to immunoregulation and the control of proliferative responses at the periphery. This dichotomy has now been disputed. CD4+ CTL have been described which use a predominantly granule-mediated mechanism (Vergelli *et al.*, 1997), and CD8+ CTL which utilise Fas have also been described (Esser *et al.*, 1997; Ju *et al.*, 1994). Populations of CTL possessing both mechanisms have also come to light recently (Sevilir-Williams and Engelhardt, 1996). These findings have led to a reappraisal of CTL activity, especially within the CD4+ population.

In infections involving long term survival of intracellular organisms which are not viral (i.e. bacteria, protozoa and other parasites), presentation of antigen to classically MHC-I restricted CD8+ CTL is unlikely. In *M. tuberculosis* infection this question is keenly debated, however the fact remains that CD8+ T cells are always the minority population in this disease (Schluger and Rom, 1998). Soluble or secreted antigens of *M. tuberculosis* and other intracellular parasites would normally be presented via MHC class II (Orme *et al.*, 1993). This precludes CD8+ cells as major role players. Originally this led to a down-playing of CTL activity in this disease. With the discovery of cytolytic CD4+ cells, a reappraisal was necessary. In infections involving intracellular bacteria, MHC II-restricted CD4+ CTL could mimic the activity of CD8+ CTL in viral infections. Such a scenario is consistent with findings to date, and re-emphasises the pivotal role of CD4+ cells in TB (Sevilir-Williams and Engelhart, 1997).

Since *M. tuberculosis* preferentially infects macrophage cells, it is the lysis of these cells by CTL that is of central concern. The concept of CD4 CTL operating predominantly via Fas-FasL interaction is problematic when it comes to macrophages, since these cells express low levels of CD95 (Kiener *et al.*, 1997). Various groups have published this finding, and even with pre-activation, levels of expression <25% have been observed (Liles *et al.*, 1996). Infection with *M. tuberculosis* has been shown to decrease Fas expression (Oddo *et al.*, 1998). With Fas expression being sub-optimal on macrophages, CD4+ CTL utilising such a mechanism of activity would be restricted functionally. It thus appears that Fas-mediated killing is sub-optimal where macrophages are involved. Could cytolytic granules not play a role?

CD4+ CTL which contain cytolytic granules have been reported by several groups (Lewinsohn *et al.*, 1998; Sevilir-Williams and Engelhart, 1996). The occurrence of such cells may be an adaptation to a lack of CD8+ CTL expansion (Sevilir-Williams and Engelhart, 1997), or a specific adaptation for controlling intracellular infections such as TB. The fact that granule mediators such as granzymes can directly kill target cells independently of host control mechanisms bypasses any possible adaptation by the invading organism to enhance anti-apoptotic mediator activity.

The possibility that cytolytic granule contents themselves are microbicidal has recently been demonstrated with granulysin. Granulysin is component of cytolytic granules and has been shown to be both bactericidal and mycobactericidal (Pena and Krensky, 1997; Stenger *et al.*, 1998). Earlier reports demonstrated that lytic components of NK cells were bactericidal (Garcia-Pennarubia *et al.*, 1989). Due to homology between NK-lysin and granulysin, similar components may be responsible for the bactricidal effects in both populations. The ability of other cytolytic granule components to kill bacteria and mycobacteria needs to be investigated. The recent characterisation of granulysin emphasises the need to examine other cytolytic granule components which may also mediate microbicidal effects (Kaufmann, 1999).

The question remains - is it the lysis of the target cell (normally via apoptosis) or the granule-mediated pathway specifically, that is important in mediating microbicidal activity. A recent report showed that FasL mediated lysis of macrophages led to a reduction in viable organisms (Oddo *et al.*, 1998). This suggests that target cell apoptosis itself may be pivotal. Another study involving CD8+ CTL, showed that granule mediated lysis had the same effect. The latter study (Stenger *et al.*, 1997) implicates cytolytic granule contents, specifically granulysin, as the mediator of microbicidal activity. The current study has revealed that either mechanism of induction of apoptosis can lead to a reduction in organism viability. However, since Fas plays a lesser role, it is the predominant granule-mediated pathway that is ultimately responsible for organism clearance in this disease.

1.2.6. Potential modulation of cytotoxic immune function: prospects for disease control

Identification of the factors which lead to formation, recruitment and activation of cytolytic CD4+ cells, particularly those with granule-secreting activity, is an area of potential disease control. Factors which enhance and promote CTL activity e.g. particular antigenic determinants favouring CTL expansion, or the ability to override host cell protective mechanisms may be important in optimising CTL activity (Kaufmann and Andersen, 1998). The exact nature of such control factors needs further investigation. In addition, isolation of cytolytic factors (granzymes, perforin, granulysin) may offer additional scope for anti-tuberculous drug therapy. Linking new anti-tuberculous drugs to cytolytic agents or to CTL

themselves, may allow for exposure of organisms released following target cell lysis, thus aiding clearance of organisms remaining after apoptosis. In this way natural host defences are augmented by a successful delivery mechanism for anti-tuberculous drugs.

Lack of CTL activity may be a marker for disease severity or susceptibility. Identification of particular CTL markers, e.g. expression of serine proteases (e.g. granzyme B) in CD4+ CTL challenged with mycobacterial antigen may be important. If disease susceptibilty is linked to inadequate or inappropriate CTL activity, therapies involving enhancement strategies could be implemented. The actual signal transduction processes in both T cell activation and macrophage death need to be elucidated, which will allow for optimal manipulation of the system for successful disease control.

1.3. Immunosuppression and suppressor T cells

1.3.1. Immunosuppression and tuberculosis

Primary pulmonary infection with *M. tuberculosis* induces long-term protective immunity in the large majority of infected individuals. Lifetime risk of reactivation of latent infection is less than 5% in immunocompetent subjects (Barnes and Modlin, 1996). Alveolar macrophages are the natural hosts for *M. tuberculosis* and as discussed in detail above, it is the interaction between macrophages, T cells and various cytokines that leads to control of infection via a strong Th1 delayed type hypersensitivity (DTH) reaction.

The role of type-2 responses and humoral immunity in *M. tuberculosis* infections is considered to be marginal. Reports on both *in vivo* and *in vitro* production of type-2 cytokines are inconsistent and there is no conclusive evidence of suppression of type-1 responses via type-2 cytokines (Zhang *et al.*, 1995; Lin *et al.*, 1996). Even though Th2 or anti-inflammatory cytokines appear to play a marginal role in tuberculosis immunity, an unusual phenomenon of pulmonary TB is the fact that features of anergy or suppression occur during immune activation (Vanham *et al.*, 1998). Active pulmonary TB is characterised by signs of systemic immune activation such as polyclonal hyper-gammaglobulinemia, increased serum TNF levels and up-regulation of HLA-DR on circulating T cells (Kaplan and Freedman, 1996; Vanham *et al.*, 1996). Paradoxically there is also simultaneous evidence for antigen-specific hyporesponsiveness. Up to 20-25% of patients with non-HIV tuberculosis show a negative skin test (anergy). More accurate assessments of immune activation such as PPD -induced lymphocyte proliferative responses show reduction in values of 50% of healthy PPD reactors. In addition IL-2 and IFN-γ production is reduced (Toossi *et al.*, 1986; Vilcek *et al.*, 1986).

Several models can be proposed to explain this apparent paradox of concomitant activation and immune depression. These include (i) dysregulation of APC function - reduced antigen presentation and/or suppression of type 1 responses, (ii) immune activation during active TB

may induce refractoriness to stimulation and predisposition towards apoptosis, (iii) antigen-specific cells may be compartmentalised in the sites of infection leading to less responsive cells being recovered from the periphery (Vanham, *et al.*, 1996; van Parijs and Abbas, 1998). In addition to these models, antigen-specific suppressor T cells may be operative. Although well characterised in leprosy, such cells have hitherto not been described in TB. The mode of action of such cells may be via lysis of Th cells, competition for APC or down-regulation of APC function, induction of hyporesponsiveness in Th cells via specific receptor-mediated processes, or via the release of type-2 cytokines. These various mechanisms of anergy induction/suppression are discussed in more detail below.

Immunosuppression is a feature of tuberculosis only in certain clinical cases. The majority of persons infected with *M. tuberculosis* do not develop clinical disease. The majority of those who do develop disease are reactivation cases i.e. they have had an earlier exposure to the organism and subsequently there has been a reactivation. Most cases are localised to the lungs and treatment is successful in clearing infection. In a small minority of cases the pattern of disease development is different. There is rapid spread and dissemination of infectious particles, the patient becomes severely ill and shows signs of generalised immunosuppression (Ellner, 1997).

Why these different clinical patterns occur has been an area of great interest. The discovery of the Th1/Th2 dichotomy of cell-mediated immunity in infectious states in the mouse and subsequent parallel situations in the human led to the supposition that such a situation may apply in tuberculosis as well. Disseminated disease possibly being related to a Th2, as opposed to a Th1, pattern (Romagnani, 1992; Rook and Bloom, 1994). As discussed above, the Th1 response is normally predominant in diseases involving bacteria and viruses, whereas the Th2 response predominates in certain parasitic and helminth diseases and in allergic responses (Romagnani, 1994). The mycobacterial disease leprosy presents clinically with a very interesting picture. There are two possible outcomes for this disease - a tuberculoid form or a lepromatous form. Tuberculoid leprosy is strongly Th1 driven, the disease is localised and less severe. In lepromatous leprosy there is dissemination of infection and severe immunosuppression which is linked to severe weight loss, fever etc. The lepromatous form of leprosy is Th2 predominant (Hopewell, 1995; Bloom, 1994).. The detection of whether a form or type of disease is Th1 or Th2 driven is made from analysis of T lymphocyte function *in vitro* of cells derived from the sites of pathology.

In tuberculosis, analysis of T lymphocytes from sites of pathology (usually pleural effusions) has clearly demonstrated that Th1 cytokines are most strongly represented (Barnes *et al.*, 1993; Sieling and Modlin, 1994, Lin *et al.*, 1996). These analyses are generally performed on patients presenting with classical tuberculosis. Tuberculosis patients who are immunosuppressed have not been assessed as a group. An interesting hypothesis has been

that initiation of either a Th1 or Th2 pathway of cellular immunity will affect disease outcome. Research in this area has now revealed that IL-12 is pivotal in this regard. Infection of macrophages with *M. tuberculosis* usually results in release of IL-12. This cytokine appears to recruit and/or activate Th1-type T cells which in turn produce IFN-γ which activates macrophages. Activated macrophages then also produce TNF-α and other cytokines, all of which seem important in the containment of disease (Sieling *et al.*, 1994). In a Th2 response this sequence of events would not occur. Rather, there would be predominance of Th2 cytokines such as IL-4, IL-5 and IL-10. These cytokines do not recruit and activate Th1 cells but instead stimulate B cells and switch off or down-regulate the inflammatory response (Del Prete *et al.*, 1994). Why would this inappropriate sequence of events occur in a disease where a Th1 response is important for infection clearance?

1.3.2. Suppressor T lymphocytes

1.3.2.1. Origin and function of suppressor T cells

The concept of suppressor T cells was first proposed by Gershon and co-workers in 1972 (Gershon *et al.*, 1972). These cells were hypothesised to play a role in the switching off of immune responses and/or in the induction of tolerance. The clearest evidence of T cell-mediated suppression derives from adoptive transfer models in transplantation. It has been noted in several experimental systems that tolerance to allogeneic tissue can be transferred from a tolerant to a syngeneic naive recipient animal if T cells are injected together with the specific antigen. Both CD4+ and CD8+ T cells have been implicated in mediating these regulatory effects in different systems (Frasca *et al.*, 1997).

How suppression operates is still open to debate. Whether the suppressive effect of the suppressor T cell is via deletion of reactive T cells by cytolysis, induction of anergy via competition for APC or IL-2, or direct immunomodulation via localised release of classical type-2 cytokines (e.g. IL-4), or an undefined receptor-ligand interaction, is still not understood (Webb *et al.*, 1994). In addition, specific suppressor factors (SF) are produced by certain suppressor T cells following exposure to antigen. These SF are distinct from other cytokines in that they are only produced by suppressor cells in response to a specific antigen (O'Hara, 1995). Classically, cytokine production is not antigen specific.

In addition to their role in normal immune function and in autoimmunity, suppressor T cells are implicated in infectious diseases as well. Mycobacterial diseases are no exception, the existence and role of suppressor T cells in leprosy has been well studied. Infection with *M. lepraemurium* in mice led to the development of CD8+ splenic lymphocytes which could inhibit proliferation of splenocytes from uninfected mice to both specific antigen and mitogen stimulation. Culture of spleen cells from infected mice produced supernatants containing IFN-γ, TNF-α and lymphotoxin/TNF-β (Lemieux *et al.*, 1990). Suppression could be reduced with antibodies to IFN-γ. *M. tuberculosis* extracts have also been shown to activate CD8+ Ts

cells *in vitro* to produce soluble mediators of suppression (Sussman and Waddee, 1991). CD8+ Ts clones derived from leprosy patients have been shown to be antigen-specific and able to block responses of CD4+ antigen-specific clones. These T cells produced IL-4, and antibody to IL-4 could abrogate the suppressive effect (Bloom *et al.*, 1992). Other diseases in which Ts cells have been demonstrated include infection with the fungus *Cryptococcus neoformans* (Masih *et al.*, 1991), and several parasitic diseases including those caused by *Toxoplasma gondii* (Pomeroy *et al.*, 1991), and *Trypanosoma cruzi* (Tarleton, 1988).

1.3.2.2. Phenotypic characterisation of Ts clones

A stumbling block in the study of suppressor T cell activity *in vitro* has been the difficulties encountered when attempting to clone such cells. These difficulties may stem from the non-responsiveness of Ts clones to antigen and the difficulty of expansion of populations of cells. Despite these difficulties various groups have reported the generation and characterisation of Ts clones with differing phenotypes. In the murine model both CD4+ and CD8+ Ts clones have been described (Utsunomiya *et al.*, 1989; Hu *et al.*, 1992). In addition, dual positive (CD3+, CD4+, CD8+) (Nanda *et al.*, 1990) and dual negative (CD3+, CD4-, CD8-) (Takahashi, 1990) Ts clones have also been reported.

In the human the phenotypic characterisation of Ts clones has yielded divergent findings as well. A dual positive (CD4+CD8+) Ts clone derived from a contact dermatitis patient has been described (Kalish and Morimoto, 1988). Of more interest to the current study are the reports of mycobacterial antigen specific Ts clones derived from leprosy patients. One series of studies showed Ts clones to be CD4+ and MHC class II-restricted (Li *et al.*, 1990). These Ts clones produced IFN-γ and lacked CD28. Leprosy patient-derived CD8+ Ts clones have been characterised and shown to be HLA-DQ-restricted and produce mainly IL-4 (Salgame *et al.*, 1991b).

Many studies have shown that CD8+ T cells can suppress immune responses, hence the term cytotoxic/suppressor T cell. Initial work in experimental animal models showed that transfer of mice T cells which had been made tolerant to sheep red blood cells would suppress the response of naive mice to the same antigen, but not to horse red blood cells or other unrelated antigens (Webb *et al.*, 1994). Subsequently it was shown that the suppressive cells were CD8+, and that CD8+ T cells from mice actively immunised with sheep red blood cells also had suppressive activity. These findings led to the formulation of the hypothesis that CD8 T cells as part of their normal function have the ability to suppress immune responses in an antigen-dependent manner. Another important animal model of suppressor activity has been demonstrated by the ability of rats which had been fed myelin basic protein (MBP) to generate CD8+ T cells which secreted TGF-β and suppressed experimental allergic encephalomyelitis (Miller *et al.*, 1992). In the human, similar cells have been described in lepromatous leprosy, where they inhibit an inflammatory response to *M.*

leprae, and thus prevent successful infection control. In both the leprosy model and the MBP model in rats, the suppressor CD8+ T cells recognise peptides bound to MHC class II molecules and secrete cytokines typical of Th2 cells. Suppression thus appears to be a manifestation of the inhibition of generation or activation of inflammatory CD4+ T cells.

Other examples of CD8+ suppressor T cells have been reported. An important feature of these cells is that in certain diseases they may be beneficial e.g. in experimental autoimmune encephalomyelitis (Miller *et al.*, 1992). Elimination of CD8+ T cells by antibody treatment or gene knockout leads to more severe disease. A similar situation may apply in human multiple sclerosis, where disease exacerbation may correlate with CD8+ T cell deficiency (Kumar and Sercarz, 1993).

1.3.2.3. Mechanisms of CD8+ T cell-mediated suppression

Many studies have indicated that suppressor cell activity is antigen-specific, however the mechanism of antigen recognition remains controversial. This is particularly apparent in lieu of the finding that CD8+ Ts clones are dependent on MHC class II components (particularly DR or DQ). In addition, it has proven very difficult to identify specific effector molecules involved in suppression, thus the way in which the suppressive effect is produced also remains uncertain. Possible mechanisms of CD8 T-cell mediated suppression include lysis of APC and/or CD4+ T cells and release of soluble suppressor factors (SF) which down-regulate effector cell responses. Other possible mechanisms include cytokine mediation (e.g. IL-4, IL-5, IL-10, TGF-β), competition for MHC molecules on APC and/or IL-2 depletion, and down-regulation of important co-receptors on APC or effector cells which would induce anergy (e.g. B7 components and CD28) (Webb *et al.*, 1994; van Parijs and Abbas, 1998).

The cytolytic model originally focussed on APC lysis. Lysis of APC by cytolytic CD8+ T cells following recognition of antigen presented via MHC class I molecules is a well established phenomenon. Lysis of APC could lead to loss of activation of CD4+ T cells and lead to a situation akin to suppression. This model does not explain how a CD4+ T cell can be directly suppressed by CD8+ cells - a phenomenon observed in the animal model of mouse responses to insulin (Webb *et al.*, 1994).

It is possible to demonstrate that CD4+ T cells can be controlled by cytotoxic CD8 T cells. Immunisation with peptides derived from TCR variable regions can elicit CD8+ T cells that recognise these peptides bound to MHC class I molecules on the surface of CD4+ T cells. The CD8+ T cells can then lyse the CD4+ T cell expressing the correct receptor. This is the only known CD4+ T cell antigen that has been used successfully as a target for CTL. Cytotoxic CD8+ T cells of this kind have been shown to suppress the autoimmune disease experimental allergic encephalomyelitis (Bloom *et al.*, 1992; Miller *et al.*, 1992). Recent

attempts to raise similar cells by immunisation of patients with multiple sclerosis have given encouraging results (Kumar and Sercarz, 1993).

Not all the observations on CD8+ T cell suppression are readily explained by proposing that they kill APC or CD4+ T cells. Early studies in suppression showed that direct binding of the suppressor cells to intact antigen molecules linked to insoluble substrates in the absence of APC or MHC molecules was possible. Soluble products released by these cells were found to mediate suppression and also to bind directly to antigen (O'Hara, 1995). Detailed biochemical characterisation of these molecules has not been forthcoming. Indirect evidence suggests that they may contain TCR α-chains. Antibodies to TCR α-chains can be used to remove suppressive activity from the supernatants of suppressor cell cultures. Although intriguing, these data are inconclusive. It seems unlikely that TCR α-chains are the sole component of the suppressive factor, since TCRs do not bind to intact antigen and a mechanism for α chain secretion has yet to be found (Kuchroo *et al.*, 1991).

Although the exact mechanism of suppression remains unknown, the presence of immunoregulatory CD8+ T cells cannot be questioned. The existence of such a population of T cells is important since manipulation of immune responses via modulation of the activity of these cells may provide a novel therapeutic strategy in many diseases. In the case of autoimmune disorders, activation of suppressor T cells would be beneficial to the host. The best-characterised model of this is multiple sclerosis (Hafler *et al.*, 1997). In infectious diseases, however, such suppressor cells would down-modulate inflammatory responses and could have dire consequences (e.g. lepromatous leprosy). A clearer understanding of the origin, function and mechanism of action of suppressor T cells is essential if such cells are ever to be manipulated so as to produce a more favourable outcome for the host in whichever particular disease.

There are basically two circumstances in which suppressor T cells have been defined. In the first, individuals fail to respond to a particular antigen when T cells are present, and responsiveness is restored by removing T cells. Original experiments by Gershon and Kondo (Gershon and Kondo, 1971) described such a phenomenon. The second circumstance is the induction of unresponsiveness in individuals otherwise capable of responding. This is achieved by introduction of antigen generally in a particular form for presentation e.g. through use of low doses of soluble antigens, chemical modification of antigens, or coupling to self-antigens. Both CD4 and CD8 T cells have in varying situations been reported to transfer suppression adoptively, but no single mechanism has emerged (Webb *et al.*, 1994).

Suppressor cells have been described in various animal models. The first human suppressor T cell (Ts) clone was reported by Bensussan *et al.* (1984), to be CD8+ and antigen-specific for ragweed pollen antigen. Subsequently human CD4+ Ts clones were also described. A

consistent finding with several reports of Ts cells appears to be the lack of CD28, the co-stimulatory receptor required for T cell activation (Li *et al*, 1990; Bloom *et al.*, 1992). All other prospective markers for Ts cells have not been consistently expressed. The confusion regarding phenotypic characterisation becomes even more complex when function is assessed. In addition, Ts cells produce an array of cytokines which makes clear-cut classification of such cells on the basis of cytokine production difficult.

The isolation of suppressor T cells from lepromatous leprosy patients may explain the lack of responsiveness of T cells in these patients. In addition, the excess of type-2 cytokines would augment any anti-inflammatory activity mediated by Ts cells. Generation of Ts clones from these patients has shown them to be MHC class II-restricted CD8+ or CD4+, CD28- cells (Modlin *et al.*, 1986; Ottenhoff *et al.*, 1986).

The restriction of CD8+ suppressor cells to MHC class II is unexpected and unusual (Bloom *et al.*, 1992). Traditionally CD4+ T cells are class II-restricted, CD8+ are class I-restricted. Both CD4+ and CD8+ Ts have been described, and both DR- and DQ-restriction have been observed. In lepromatous leprosy patients both DR- (Li *et al.*, 1990) and DQ- (Salgame *et al.*, 1991) restriction have been described for a single disease. Such divergent findings further complicate an already controversial field of research. How does MHC class II play a role in CD8+-mediated events? Do Ts cells kill APC or CD4+ T cells? Do Ts induce a state of anergy in CD4+ antigen-responsive cells?

1.3.3. Cytokines and suppression

Original work on suppression focused on antigen specific suppressor factors as the prime mediators of non-responsiveness. With the exponential growth of research on cytokines and their activities, it has become apparent that several T cell-derived cytokines are immunosuppressive and may contribute to the phenomenon of antigen-specific suppression. The major difference between type-2 or anti-inflammatory cytokines and suppressor factors is the antigen-specificity of the release of the latter (O'Hara, 1995). With more detailed understanding of cytokines, their production, receptor binding and effects on other cells, it has become apparent that certain of the effects assigned to suppressor cells may be mediated or enhanced by cytokines. Of particular interest are IL-4, IL-5, IL-10 and TGF-β (Le Gros and Erard, 1994).

TGF-β is the name applied to a family of multifunctional polypeptide growth factors with varying effects. TGF-β1 is the best characterised of the family of 5 members. This cytokine is produced by a wide range of cells, with platelets being a major source. Almost all mammalian cells possess receptors for TGF-β, and both stimulatory and inhibitory activities have been assigned to this cytokine (Wahl, 1992). The effect of TGF-β on immune cells (B and T lymphocytes, NK and LAK cells, monocytes and macrophages) is generally inhibitory. It is a

potent inhibitor of both B and T cell proliferation and has been reported to inhibit NK activity and anti-microbial activities of macrophages. Localised production of TGF-β may play a role in down-regulating the immune response, and thus in immunosuppression (Sporn and Roberts, 1992; Letterio and Roberts, 1998).

IL-4 was originally characterised as a B cell stimulatory factor. Subsequently it has been shown that IL-4 is a pleiotropic cytokine which modulates the function of diverse cell types, including T cells, mc ıocytes and macrophages. IL-4 is produced mainly by CD4+ T cells, but also CD8+ T cells, mast cells and basophils. IL-4 binds to the IL-4 receptor (IL-4R) which initiates transmembrane signalling processes. IL-4 is anti-inflammatory and is an important modulator of differentiation of CD4+ cells into either Th1- or Th2-populations. A major function is the suppression of cytokine production by Th1 cells, thus suppressing cell-mediated immunity (Keegan and Pierce, 1994).

IL-5 was originally also thought to be a B cell stimulatory factor, however it has subsequently been found to have diverse activities. IL-5 is not as well characterised as IL-4, but appears to be produced by T lymphocytes, eosinophils and mast cells. IL-5 is best characterised in its role in switching CD4+ T cell differentiation to a type-2 pattern. This is especially important in helminth infections, where eosinophils are an important component. IL-5, like IL-4, has suppressive effects on Th1 cytokine production (Takatsu, 1992).

IL-10 is produced by T lymphocytes (Th2 CD4+ predominantly), macrophages, B cells and other cell types. IL-10 operates via binding to the IL-10R. IL-10 is inhibitory to Th1 cytokine production, however this effect is indirect, operating via inhibition of accessory function and antigen presentation. In addition to inhibition of cytokine production, IL-10 inhibits proliferation of all antigen-specific T cell populations. This cytokine is therefore potently suppressive in its activity, and may thus be an essential component of the suppressor activity previously ascribed to suppressor T cells (Moore *et al.*, 1993).

1.3.4. Antigen-specific suppressor factors (SF)

The isolation and characterisation of suppressor factors produced by suppressor T cells has been an area of intensive research. The increased emphasis on cytokines as mediators of many immune functions including suppression has detracted focus from suppressor factors. SF from both suppressor T cells and APC have been reported (O'Hara, 1995).

Biochemical characterisation and purification of a specific Ts-derived suppressor factor (SF) was an area of intensive research is the early 1980's. This SF bound free antigen in the absence of MHC protein. Further study indicated that the SF and Ts cells themselves shared antigen specificity, which led to the hypothesis that SF represented a soluble form of T cell receptor (Flood *et al*, 1982). Subsequent work has shown that the link between SF and T cell

receptor is mediated by the TCR-α chain. Expression of TCR-α chain was essential for suppressor activity of T cell hybridomas (Kuchroo *et al*, 1991). The TCR-α chain required for suppression has been found not to differ from normal TCR-α chains (Kuchroo *et al*, 1991). The linking of suppression to a suppressor factor which is related to the TCR-α chain has clarified certain issues regarding this factor. However, the mechanism whereby a TCR chain binds free antigen independent of MHC processing is unknown (O'Hara, 1995). Of major concern is how a soluble TCR component can act as a suppressor factor, and whether the SF (TCR-α chain) acts alone, or forms a dimeric structure with a second chain. The universality of suppression operating via a SF which is related to the TCR has also not been clarified.

1.3.5. Models of immune regulation and the role of Ts cells

Switching off of the immune response is an important process. Mechanisms whereby this occurs are slowly being elucidated. The better characterised mechanisms such as apoptosis of expanded T cells at the periphery via Fas-FasL interactions are now generally accepted. Inappropriate switching off (anergy or suppression) is less well characterised. Certain diseases are characterised by states of apparent immunosuppression, TB being a prime example (Scott *et al.*, 1994).

A model for the role of Ts cells in the termination of the inflammatory response in TB can be envisaged, with inappropriate activation of such cells being responsible for disseminated disease and anergy induction. In a normal sequence of events, following initial expansion of T cells at the site of infection and production of type-1 cytokines, activation or lysis of macrophages and localised disease containment, a switching off process is required. Apart from T cell apoptosis which accounts for reduction of clonally expanded populations (Ellner, 1997), Ts cells could be envisaged to play an active role. Ts cells are antigen specific, however their antigen requirements differ from those needed for immune activation. Ts cells respond to sub-optimal levels of antigen presentation, or modified forms of antigen. When the disease has been contained, levels of antigen are decreased. Such a reduction in antigen presentation may induce activation of Ts cells. This activation could involve the release of SF or cytokines, or the deletion of T cells or APC. This is a hypothetical model of suppressor cell activity in a disease such as TB. To date no suppressor cell activity has been characterised in TB, even though the presence of such cells has been proposed. In the current study a Ts clone was isolated from a TB patient. The fact that the patient presented with a classical reactivation type picture of the disease with associated pleural effusion was not indicative of Ts cell activity. However, if normal immune responses are dependent on such cells for switching off, this finding is not as unexpected as it may seem. Inappropriate activation of such cells in severe forms of the disease would confirm the hypothetical model of Ts cell activation being an important component of such a unfavourable outcome.

The current study describes the generation and characterisation of a CD8+ suppressor T lymphocyte clone from a tuberculosis patient. Similarities and differences between this clone and clones derived from leprosy lesions are outlined and discussed in relation to the important role such cells may play in the induction of anergy or unresponsiveness in certain situations.

1.4. Aims of the study

The aims of the current study were to characterise T lymphocyte-mediated function in tuberculosis. Although much is known about the importance of T lymphocytes in this disease, there are areas still not clearly understood. These include the lack of consensus on any definitive role for cytotoxic CD4+ T lymphocytes, and an absence of any reported suppressor cell activity in TB, despite a clinical course which would suggest the occurrence of these cells.

The majority of work in human tuberculosis has used healthy PPD-positive or -negative subjects as donors of lymphocytes. There are limitations in both the use of healthy donors and peripheral blood as a source of lymphocytes for *in vitro* studies. Due to this fact, this study has focused on using lymphocytes derived from a disease-associated site (tuberculous effusion) of a tuberculosis patient. The inherent problems of using bulk populations of antigen-primed lymphocytes has also been circumvented by the use of clones.

A panel of clones was generated from the mycobacterial-antigen stimulated population of effusion lymphocytes. All the CD4+ clones obtained were strongly Th1, and thus capable of mediating protective immunity. The majority of these cells were found to be cytolytic and the cytolytic activity of the CD4+ T cell clones was found to reduce mycobacterial viability. The mycobactericidal CD4+ CTL clones possessed a predominant granule exocytosis killing mechanism. No antigen-specific C8+ cytolytic activity was detected. Both type-1 and type-2 cytokines were produced by a CD8+ clone which appeared unresponsive to antigenic stimulation. This clone was able to inhibit autologous T cell proliferation via a cytokine- and receptor-dependent suppressor mechanism.

This study has clarified a role for CD4+ CTL in tuberculosis and has also demonstrated the occurrence of suppressor cells, which may account for the non-responsiveness observed in certain disease situations.

Chapter 2.

Comparison of cellular immune activity of peripheral blood- and effusion-derived mononuclear cells and generation and characterisation of effusion-derived T lymphocyte clones

2.1. Introduction

The primary site of infection in tuberculosis is the lung (Rook and Hernandez-Pando, 1996). The majority of clinical cases are reactivation TB, and this often involves extra-pulmonary sites (Dannenberg and Rook, 1994). Tuberculous pleuritis which accounts for 28% of all extra-pulmonary cases, involves the invasion of the pleural space by infectious material from the granulomatous lesion (Hopewell, 1994; Toossi and Ellner, 1992). The pleural effusion-derived mononuclear cell population is readily accessible by pleural aspirate, thus affording an opportunity to assess cellular function at a disease-associated site.

Tuberculous pleural effusions generally contain increased numbers of T lymphocytes, particularly of the CD4+ lineage (Barnes *et al.*, 1989). Previous studies have indicated that pleural effusion-derived lymphocyte populations from a majority of patients with active tuberculosis display accelerated proliferation kinetics to mycobacterial antigens (Lorgat *et al.*, 1992a). In addition, accelerated kinetics of *in vitro* induction of antigen-specific cytotoxic activity as well as activation marker expression and cell cycle progression have also been reported (Lorgat *et al.*, 1992b; Lukey *et al.*, 1996). Limiting dilution analysis (LDA) of pleural effusion populations has revealed increased responder cell frequency (Lukey *et al.*, 1998). Together these findings suggest that antigen-specific T cells are selectively expanded in the pleural effusion, and that these cells are antigenically primed *in vivo*.

Although the phenomenon of accelerated proliferation kinetics has been observed in a majority of tuberculosis patients, this pattern of response is not universal. Effusion-derived lymphocyte populations have been divided into three groups based on the kinetics of proliferation, namely, non-responders, normal (non-accelerated) responders, and accelerated responder populations (Lukey *et al.*, 1996, 1998). No significant correlation between responder cell frequency and kinetics of response has been observed, suggesting that other factors are involved in determining the kinetics of response (Lukey *et al.*, 1998). Characteristic v-shaped LDA curves have been noted in some non-responder populations, a finding which suggests the activity of antigen-specific suppressor cells within the pleural effusion (Lefkovitz and Waldman, 1984; Dozmorov *et al.*, 1996; Lukey *et al.*, 1998).

Therefore, although several features of immune activation have been noted in effusion-derived lymphocytes, evidence of active suppression of immune responses has also been observed. The patient in the current study was unique because elements of both immune activation and suppression were observed in the same pleural effusion. At the level of immune activation there was an expansion of the CD4+ component of T lymphocytes within the effusion as well as an enhanced frequency of PPD responsive cells in the effusion as compared to the peripheral blood. Proliferation kinetics were more rapid than normal, but did not reflect the classical pattern of accelerated kinetics. Accelerated induction of cytotoxic responses *in vitro* was observed in the effusion, but not the peripheral blood. The LDA of the effusion, however, suggested the activity of suppressor cells. The activity of such suppressor cells may also have accounted for the unique proliferation kinetics of the effusion. Based on these findings, a more detailed characterisation of cellular activity in the effusion was carried out at the clonal level. Both CD4+ and CD8+ clones were generated. The majority of CD4+ clones were cytolytic, whereas no antigen-specific cytolytic CD8+ clones were generated. The CD8+ clones were only weakly responsive to mycobacterial antigens, and one particular clone uniquely produced the cytokine IL-4. These findings led to further assessment of the relevance of CD4+ T cell-mediated cytotoxicity and CD8+ T cell-mediated suppression in tuberculosis (chapters 3 – 5).

This chapter presents a comparison of the activity of effusion- and peripheral blood-derived mononuclear populations. The generation and characterisation of the T lymphocyte clones derived from the effusion of the patient are also described.

2.2. Materials and Methods

2.2.1. Isolation of mononuclear cells from peripheral blood and pleural fluid

Peripheral blood and pleural fluid were collected from a TB patient presenting with a pleural effusion who was admitted to Groote Schuur Hospital, Cape Town. Venous blood was collected in 10 ml blood collection tubes (Becton Dickinson, Meylan, France) to which 0.5 ml of heparin (final concentration 5 IU/ml of blood, (Calciparine, Sanofi, Winthrop, France)) had been added. A total of 50 ml of blood was collected prior to chemotherapy, from which the mononuclear population was used in the generation of the clones. All subsequent peripheral blood was collected following chemotherapy. Pleural fluid was collected in 50ml tubes (Bibby Sterilin, Stone, Staffordshire, UK) containing 2.5 ml of heparin. The pleural fluid was assessed for cellular density and viability by trypan blue exclusion staining prior to mononuclear cell isolation. Mononuclear cells were isolated from both peripheral blood and pleural fluid by density gradient centrifugation using Hypaque-Ficoll (Sigma, St. Louis, USA).

Peripheral blood separation: Peripheral blood mononuclear cells (PBMNC) were isolated as previously described (Lorgat *et al.*, 1992b). Fresh heparinised venous blood was centrifuged at 300 x gravitational attraction (g) for 12 minutes in the blood collection tubes, to obtain leukocyte buffy layers. The buffy layers (4ml) were removed and resuspended in an equal volume (4ml) of phosphate buffered saline (PBS; Dulbecco, Oxoid, Unipath Ltd., Hampshire, England) and gently layered onto 4ml of Hypaque-Ficoll (Sigma, St. Louis, Mo.) in round-bottomed centrifuge tubes (Bibby Sterilin, Stone, Staffordshire, UK). The gradient was then centrifuged at 400 x g for 30 minutes. The mononuclear cell interfaces were removed and transferred to conical centrifuge tubes (Bibby Sterilin). PBS was added to each tube, and the mononuclear fraction was washed three times by repeated centrifugation (200 x g for 10 minutes) and resuspension in PBS. The washed mononuclear cell fractions were finally resuspended in RPMI 1640 medium (Flow, ICN Biomedicals, Costa Mesa, CA, USA) containing 20mM HEPES, 100 U/ml penicillin G and 100 µg/ml streptomycin sulphate (Sigma). RPMI 1640 medium was supplemented with 10% pooled, heat inactivated (56°C for 1 hour) human AB serum (Western Province Blood Transfusion Services, Cape Town). The mononuclear cells were enumerated by staining with Turks fluid (0.02% crystal violet (Sigma), 7% glacial acetic acid (Merck) in distilled water). The cell concentration was adjusted to 10^6 cells/ml.

Pleural fluid separation: The fresh heparinised pleural fluid (8ml) was layered directly onto Hypaque-Ficoll (4ml) without dilution in PBS. The separation of pleural effusion monuclear cells (EMNC) was then carried out exactly as for peripheral blood.

Cryopreservation of PBMNC and EMNC: Freshly isolated PBMNC and EMNC were aliquoted and frozen for later use. Mononuclear cells were resuspended in human AB serum containing 10% DMSO (Merck, Darmstadt, Germany) at a concentration of 10 x 10^6 cells/ml.

Aliquots (1ml) were added to cryopreservation tubes (Nunc, Denmark) and frozen at -80°C. After 24 hours frozen cells were transferred to liquid nitrogen.

2.2.2. Phenotyping of PBMNC and EMNC populations

Freshly isolated PBMNC and EMNC cells were assessed phenotypically by flow cytometry. Cells (100 µl of 10^6/ml) were incubated for 10 minutes with 5µl of FITC- (fluorescein isothyocyanite) or RD- (rhodamine) coupled monoclonal antibodies or isotype controls (Coulter Corporation, Hialeah, FL, USA). A list of antibodies and their isotypic controls is presented in Table 2.1. The cells were then fixed in 1% fresh paraformaldehyde for at least 1 hour (Merck, Darmstadt, Germany) and analysed on a flow cytometer (Coulter, Epics Profile II). A total of 10000 events were analysed.

Table 2.1. Mouse anti-human monoclonal antibodies used for phenotyping PBMNC and EMNC populations

Marker	Isotype	Conjugate	Marker	Isotype	Conjugate
CD2	IgG1	FITC	CD19	IgG1	FITC
CD3	IgG1	FITC	CD25	IgG2a	RD1
CD4	IgG1	FITC	CD28	IgG1	FITC
CD4	IgG1	RD1	CD29	IgG1	RD1
CD8	IgG1	FITC	CD56	IgG1	RD1
CD8	IgG1	RD1	HLA-DR	IgG2b	FITC
CD16	IgG1	FITC			

2.2.3. Proliferative responses of pleural effusion- and peripheral blood-derived lymphocytes

Proliferation kinetics were assessed by examining the proliferative responses of the mononuclear cell fractions from both the effusion and peripheral blood over a seven day period. The mycobacterial antigen PPD (purified protein derivative of *M. tuberculosis*, Central Veterinary College, Weybridge, England) was used at 3µg/ml. Streptokinase/streptodornase (SK-SD, Lederle laboratory, Wayne, NJ) was used at 250U/ml streptokinase and 62.5U/ml streptodornase. SK-SD was heated at 56°C for 1 hour to remove enzymatic activity. The mitogen phytohaemagglutinin (PHA, Wellcome Reagents, Beckenham, England) was used at a concentration of 5.75×10^{-3} mitogenic units/ml. Both PBMNC and EMNC proliferations were assessed in triplicate in round-bottomed 96-well microtitre plates (Greiner, Frickenhausen, Germany), each well containing 200µl of medium including 10^5 mononuclear leukocytes, with or without antigen or mitogen.

After incubation at 37°C in a 5% CO_2 humidified incubator for the specified time period, 2µCi of ^{3}H-thymidine (185Mbq/mmol; Amersham International, Aylesbury, UK) was added to each well for the last 18 hours of culture. Mononuclear leukocytes were harvested onto filter paper (Macherey-Nagel, Germany) using a cell harvester (Titertek, Flow Laboratories). Dried filter paper discs were placed into plastic tubes (Zinsser Analytic, Frankfurt, Germany), 1ml of

scintillation fluid (Insta-Gel II, Packard, USA) was added to each tube and the ^{3}H-thymidine incorporation was measured in a liquid scintillation β-counter (Tricarb Analyser 1900CA, Packard). Results are expressed as Δcpm (delta counts per minute) which represents the antigen or mitogen stimulated count minus count in absence of mitogen or antigen.

2.2.4. Limiting dilution analysis (LDA) of PBMNC and EMNC

LDA was carried out according to the methodology of Lukey *et al.* (1998). Cryopreserved PBMNC and EMNC (obtained at time of patient diagnosis) were thawed and viability was assessed by trypan blue exclusion staining. Cells with a viability >95% were then cultured in round-bottomed 96 well microtitre plates (Greiner). Growth medium (10% human AB serum in RPMI 1640) was supplemented with 5U/well recombinant human IL-2. The addition of IL-2 ensured that the only limiting factor was the presence or absence of an effector cell (Sharrock *et al.*, 1990). Freshly isolated autologous PBMNC were irradiated (30Gy) and added at 5 x 10^4/well as antigen-presenting cells. PBMNC and EMNC cells were plated over a range of cell concentrations (20000/well to 10/well) in replicates of 16 wells per each cell concentration. LDA was performed both in the presence and absence of antigen (PPD at 3μg/ml, and SK-SD at 250U/ml streptokinase, 62.5U/ml streptodornase). Each well contained a total volume of 200μl. After 7 days of culture, ^{3}H-thymidine was added (2μCi/well) for the last 18 hours of culture. Cells were harvested and thymidine incorporation determined by scintillation counting as described above. LDA was performed twice to confirm reproducibility of estimated responder frequency.

Statistical analysis of LDA: Responder frequencies were calculated according to Fazekas de St Groth (1982), using the maximum likelihood solution and expressed as multiplicity (i.e. the inverse of frequency). A culture well was considered positive when the counts per minute (cpm) measured for that well exceeded the mean plus 3 standard deviations of its corresponding control (cultures with the same cell concentration and IL-2 but without antigen). Responder frequencies were considered significantly different if their 95% confidence limits did not overlap. χ^2 values were >0.05 and were consistent with single-hit kinetics (Fazekas de St Groth, 1982). A computer programme was designed to assist in calculation of responder cell frequencies. This was done with the help of the Department of Statistics, University of Cape Town.

2.2.5. Cytotoxic activity of PBMNC and EMNC populations

Both PBMNC and EMNC were assessed for cytotoxic activity by determining their ability to lyse autologous adherent macrophages exposed to various mycobacterial antigens. Effector cells were generated by incubating PBMNC and EMNC (10ml of 10^6/ml) at 37°C for 6 days in the presence of PPD (3μg/ml). PBMNC adherent macrophages derived from patient PBMNC prior to chemotherapy were used as antigen presenting cell (APC) targets for both effector populations. Macrophages were cultured in round-bottomed 96-well microtitre plates

by plating 100µl of 10^6/ml PBMNC per well at the same time that effector cultures were initiated. On day 5 (120 hours after plating), the adherent macrophage cells were labelled with 6µCi per well of ^{51}Cr (Sodium chromate; Amersham, Aylesbury, UK) in the presence or absence of antigen. Mycobacterial antigens included PPD (3µg/ml), *M. bovis* BCG (Trudeau Institute, Seranac Lake, USA) at 10^5 CFU/ml (equivalent to 1 organism per macrophage), and *M. tuberculosis H37Rv* (ATCC #27294, Rockville, MD, USA) at 10^5 CFU/ml. Following overnight incubation, labelled target macrophages were washed thoroughly with warm PBS (supplemented with 5% FCS) to remove non-adherent cells and unincorporated label. The day 6 (144 hours) antigen (PPD)-primed EMNC and PBMNC were pelleted by centrifugation (200 x g), enumerated by trypan blue exclusion staining (0.02% trypan blue (Riedel-de-Haen, Seelze, Germany) in distilled water), and added to the target adherent macrophages at effector to target ratios of 10:1, 3:1 and 0.3:1. All effector to target ratios were set up in triplicate wells. Spontaneous ^{51}Cr release was measured in wells containing target cells, but no effector cells. Following 16 hours of incubation in a 37°C CO_2 incubator, the supernatants were harvested and placed into disposable Durham tubes (Greiner) and levels of radioactivity were determined using a γ-counter (Tricarb, Packard). Maximal ^{51}Cr release was assessed by lysis of the remaining target cells with 5% triton X-100 (Merck, Darmstadt, Germany) for 4 hours. The lysates were harvested and counted in the same way as the supernatants. Percentage killing for the mean of triplicate wells was calculated as follows: percentage specific lysis = [mean test cpm/(mean test cpm + mean cpm after triton X treatment of same wells)] x 100% - percentage spontaneous release. The percentage spontaneous release was calculated as follows: mean cpm in spontaneous release wells/(mean cpm in spontaneous release wells + mean cpm after triton X treatment of same wells) x 100%. Spontaneous release was always <20% of maximal release.

The kinetics of *in vitro* induction of cytotoxic activity was assessed by determining cytolytic activity of day 4 and day 6 PPD-primed effector cells. Experiments were carried out as above, except that two effector cell cultures were initiated for each population. Day 4 effectors were initiated 48 hours after day 6 effectors. Target adherent macrophages were cultured and radiolabelled as described above and all cytotoxicity assays were performed simultaneously.

2.2.6. Generation of CD4+ and CD8+ mycobacteria-specific EMNC T cell lines

Mycobacterial antigen-specific EMNC-derived effector cells were generated by incubating fresh EMNC (10ml of 10^6/ml) with *M. bovis* BCG (10^5 CFU/ml) at 37°C for 7 days, followed by the addition of recombinant human IL-2 (Cetus, Emeryville, USA) at 100 IU/ml for an additional 3 days. CD4+ and CD8+ lymphocyte subsets of the expanded population of antigen-specific EMNC were isolated using antibody-coupled magnetic beads (Minimax Separation System, Miltenyi Biotec, Bergisch Gladbach, Germany). EMNC (10^7 in 80µl of PBS containing 5 mM EDTA and 0.5% BSA) were mixed with 20µl of freshly washed

microbeads (i.e. either anti-CD4- or anti-CD8-coupled magnetic beads) and incubated for 15 minutes in a refrigerator (5°C). The separation column (Minimax) was washed through with 500µl of PBS-EDTA-BSA buffer (described above) and then placed on the magnetic stand (Minimax). Labelled cells (100µl) were then layered onto the washed column. The effluent (negative fraction) was collected in a 5ml tube. The column was then washed through with three 500µl aliquots of buffer. Effluent from the first wash was pooled with the negative fraction, and the remaining two wash fractions were discarded. The positive fraction (consisting of the cells attached to the magnet via the bound beads) was extracted from the column using a plunger to force 1ml of buffer through the column after removal from the magnet. Flow cytometric assessment of the purity of the CD4+ and CD8+ fractions was carried out prior to cloning. The monoclonal antibodies (anti-CD3, anti-CD4, anti-CD8, anti-CD56) and their isotypic controls are listed in Table 2.1.

2.2.7. Limiting dilution cloning of CD4+ and CD8+ EMNC fractions

Following separation of activated effector cells into $CD4^+$ and $CD8^+$ fractions and assessment for population purity by flow cytometry, the EMNC cells were cloned by limiting dilution. Cloning was carried out in RPMI 1640 medium (Flow Laboratories) to which the following was added: 2mM L-glutamine (Highveld Biologicals, Johannesburg, SA), 10mM non-essential amino acids (Highveld Biologicals, Johannesburg, SA), $5x10^{-5}$M 2-mercaptoethanol (BDH Chemicals, England), 1mM Sodium Pyruvate (Highveld Biologicals, Johannesburg, SA). This cloning medium was supplemented with 10% autologous serum. Autologous feeder cells (PBMNC) were prepared from fresh heparinised venous blood, diluted to a concentration of 10^6/ml and irradiated at 40 Gray. *M. bovis* BCG was added to the feeder cells at a ratio of 1 bacteria per cell or approximately 10 bacteria per macrophage (10^6CFU/ml). 100 IU/ml recombinant human IL-2 (Cetus) was also added to the feeder cells. Aliquots (10µl) of feeder cells were plated into Terasaki well plates (Becton-Dickinson, Mountain View, CA) using a cloning gun (Titertek multi-channel microdispenser). The T-cells (EMNC-derived CD4+ and CD8+ populations) were then plated (10µl) into the wells containing feeder cells at ratios of 5, 1 and 0.3 cells per Terasaki well. Each well contained a final volume of 20 µl. Stacks of 10 plates were wrapped in foil and some sterile distilled water was added to each wrapped stack to ensure that the wells did not dry out. Plates were incubated at 37°C in a 5% CO_2 incubator. After 10 days the plates were assessed for growth and the contents of the wells showing positive growth were expanded into 96-well round-bottomed plates (Greiner). Each well of the 96-well plates contained freshly prepared feeder cells (irradiated PBMC), BCG and rIL-2 in a final volume of 200µl/well. Following further expansion into 1ml volumes in wells of 24-well plates (Becton-Dickinson), clones were phenotyped by flow cytometry, cytokine production was assessed, and they were screened for proliferative responses (^{3}H-thymidine incorporation) and cytotoxic activity (^{51}Cr-release). Aliquots of 1-5 x 10^6 cells were cryopreserved in human AB serum containing 10% DMSO (Merck, Darmstadt, Germany) and stored in liquid nitrogen.

Clones were maintained in culture by weekly restimulation with fresh irradiated APC (PBMNC), antigen (*M. bovis* BCG or *M. tuberculosis* H37Rv) and IL-2. Clones were cultured in 24-well plates containing 500µl of 10^6/ml clone cells and 500µl of 10^6/ml irradiated feeder cells. Mycobacterial antigen (2 x 10^5 CFU/ml of *M. tuberculosis* H37Rv or 1 x 10^6 CFU/ml of *M. bovis* BCG) and recombinant IL-2 (100 IU/ml) were added to the feeder cells. After 72 hours, fresh IL-2 (100 IU/ml) was added to the clone cells together with 1ml of fresh medium. To dislodge cell aggregates, the contents of the wells were resuspended using a 1ml pipettor. Clones were used for cytotoxicity assays 48 hours after the IL-2 restimulation.

2.2.8. Phenotyping of clones and assessment of cytokine production

The phenotype of expanded clones was assessed by flow cytometry as described in section 2.2.2. Markers examined included CD2, CD3, CD4, CD8, CD16, CD19, CD29, CD56, CD25, and HLA-DR. The antibodies and their isotypic controls are listed in table 2.2.

The cytokine production profile of the clones was assessed by ELISA determination of cytokine content of clone supernatants (SNF). Levels of production of both the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 were determined. Antibodies and recombinant protein were purchased from Pharmingen (San Diego, CA, USA). Supernatants were harvested from 24-well plates, each well containing 2ml of medium consisting of 10^6 clone cells, 10^6 fresh irradiated APCs and antigen (BCG). No IL-2 was added to the cultures. Supernatants (1 ml) were harvested 48 hours after restimulation of clones with fresh irradiated APC (PBMNC) infected with BCG at a ratio of 2 bacteria to 1 macrophage (2 x 10^5 CFU/ml). Control supernatants were also collected from wells containing medium alone, clone cells alone, and irradiated feeder APC alone. Supernatants were frozen at -20°C until ELISA assays were performed. ELISA assays were carried out in flat-bottomed 96-well ELISA plates (Maxisorb, Nunc, Denmark) according to antibody manufacturer's instructions. Mouse anti-human cytokine capture antibodies were bound to the surface of the wells prior to addition of supernatants or recombinant protein standards. Biotinylated anti-cytokine detecting antibodies were then added to each of the wells, followed by the addition of streptavidin coupled with the enzyme horse radish peroxidase (HRP) (Dako, Denmark). The substrate ABTS (Sigma, St. Louis, Mo.) was finally added together with hydrogen peroxide (Merck, Darmstadt, Germany). Concentrations of antibodies, incubation times, washing steps etc. were carried out according to manufacturer's instructions. Levels of cytokine production were determined by absorbance readings at 405nm using an automated ELISA plate reader. Following an initial screen for IL-4 and IFN-γ, certain clones were assessed for the production of IL-5, IL-10 (both Pharmingen), and TGF-β1 (Promega, Madison, WI, USA). These results are presented in Chapter 5.

2.2.9. Proliferative and cytotoxic responses of CD4+ clones

Proliferation was assessed in triplicate wells of 96-well microtitre plates. Each well contained a final volume of 200µl of medium and included 10^5 clone cells (100µl of 10^6/ml) together with 10^4 irradiated feeder cells (50µl of 2 x 10^5/ml) with and without antigen or mitogen (50 µl). The mitogen phytohaemagglutinin (PHA) was used at a final concentration of 5.75 x 10^{-3} mitogenic units/ml. PPD was used at 3µg/ml and streptokinase/streptodornase (SK-SD) at 250U/ml streptokinase and 62.5U/ml streptodornase. *M. tuberculosis* H37Rv and *M. bovis* BCG were used at a ratio of 2 bacteria per macrophage (2 x 10^5 CFU/ml) and 10 bacteria per macrophage (1 x 10^6 CFU/ml), respectively. After incubation at 37°C in a 5% CO_2 humidified incubator for 2 days (42 hours), 2µCi of ^{3}H-thymidine (185Mbq/mmol, Amersham, Buckinghamshire, England) was added to each well for the last 8 hours (42-50 hours) of culture. Levels of radio-isotope incorporation were determined as described in section 2.2.3.

Cytotoxic activity of the clones was assessed in a modification of the standard 16 hour ^{51}Cr-release assay (Section 2.2.5.). Autologous adherent macrophage target cells were prepared by plating 10^5 (100 µl of 10^6 PBMNC/ml) freshly isolated PBMNC in microtitre plates as described above. On day 4 (96 hours), adherent macrophages were labelled with ^{51}Cr (6µCi/well) and antigen (PPD, SK-SD, *M. tuberculosis* H37Rv or *M. bovis* BCG) for 24 hours. Prior to addition of CTL clones, the macrophages were washed 3 times with warm PBS (supplemented with 5% FCS) to remove residual ^{51}Cr, organisms, antigens and/or detached macrophages. Effector EMNC clone cells were activated by incubation with fresh irradiated PBMNC and antigen (H37Rv or BCG) supplemented with 100 IU/ml IL-2. After 72 hours, fresh IL-2 was added to re-stimulate the cells. The activated clones were used in cytotoxicity assays 48 hours after the re-stimulation with IL-2, 120 hours after the addition of feeder cells. The clone cells were washed, counted, and added to ^{51}Cr labelled target macrophages at effector to target ratios of 10:1, 3:1 and 0.3:1 in triplicate wells. Spontaneous ^{51}Cr release was measured in wells containing target cells alone. Following 16 hours of incubation in a 37°C CO_2 incubator, the supernatants were harvested and counted. Maximal ^{51}Cr release was assessed following lysis of remaining target cells with 5% triton X-100 (Merck, Darmstadt, Germany). Percentage lysis for the mean of triplicate wells was calculated as described in section 2.2.5.

2.3. Results

2.3.1. Clinical overview of the patient

Due to the difficulty in finding patients who were prepared to donate blood weekly over an extended period, this study focused on a single, fairly young patient, who was prepared to commit himself to the project for several years. The patient was a 29-year-old male who presented with a non-productive cough, pleuritic chest pain, reduced effort tolerance, shortness of breath, and moderate night sweats. Clinical examination and chest X-ray confirmed the presence of right lung associated pleural effusion. Pleural fluid was aspirated and pleural biopsy material was removed. The pleural fluid was straw coloured and contained viable mononuclear cells (2.4×10^8 cells in 400ml of fluid). Neither the pleural fluid nor the biopsy material revealed any acid fast bacilli (AFB) by Ziehl-Nielsen (ZN) staining. The pleural fluid was also culture negative. Diagnosis of tuberculosis was made on the basis of biopsy histology. The material showed caseating granulomatous inflammation. Confirmation that the infectious agent was *M. tuberculosis* was provided by polymerase chain reaction (PCR) detection of organism DNA within the pleural fluid. The patient was HIV negative. A six-month combination anti-tuberculosis chemotherapy consisting of rifampicin, isoniazid, pyrazinamide, ethambutol and pyridoxine was prescribed. The patient responded well to treatment and has remained healthy for several years with no recurrence of illness.

The peripheral blood and effusion material from which PBMNC and EMNC populations were derived was obtained prior to the patient receiving any anti-tuberculosis treatment. The T lymphocyte clones were also generated from these populations obtained at the time of diagnosis.

2.3.2. EMNC, but not PBMNC, displayed a unique kinetic pattern of accelerated proliferation to PPD

The proliferative responses of both PBMNC and EMNC over 7 days are illustrated in Figure 2.1. Responses to mycobacterial antigen (PPD), streptococcal antigen (SK-SD) and mitogen (PHA) are illustrated. Data are presented as Δcpm (cpm in presence of antigen or mitogen minus cpm in absence of antigen or mitogen). Positive proliferation responses consistently indicated stimulation indices (SI) > 3. The pattern of accelerated EMNC PPD-reactivity peaking on day 5 in this patient is intermediate between the previously described day 3 or day 4 accelerated, or normal day 6-7 response (Lorgat *et al.*, 1992a; 1992b; Lukey *et al.*, 1998).

Accelerated proliferation of effusion-derived mononuclear cells which is indicative of *in vivo* antigen priming, has previously been described in patients with tuberculous pleuritis and pericarditis (Lorgat *et al.*, 1992a; 1992b; Lukey *et al.*, 1998). Normal antigenic proliferative responses of lymphocytes peak at day 7 (144-162 hours) after initiation, with mitogenic responses peaking at day 4 (72-90 hours). An accelerated proliferative response to antigen

theoretically would include any population of cells which reaches peak antigen response before day 6. In kinetic studies of EMNC responses a significant proportion of patients displayed maximal PPD-specific proliferative response on day 3 or day 4 of *in vitro* culture (Lorgat *et al.*, 1992a; 1992b; Lukey *et al.*, 1998). The day 3 or day 4 difference here relates to the time points at which the tritiated thymidine was added, or at which time point the proliferation assays were terminated. All these studies refer to the same pattern of accelerated kinetics.

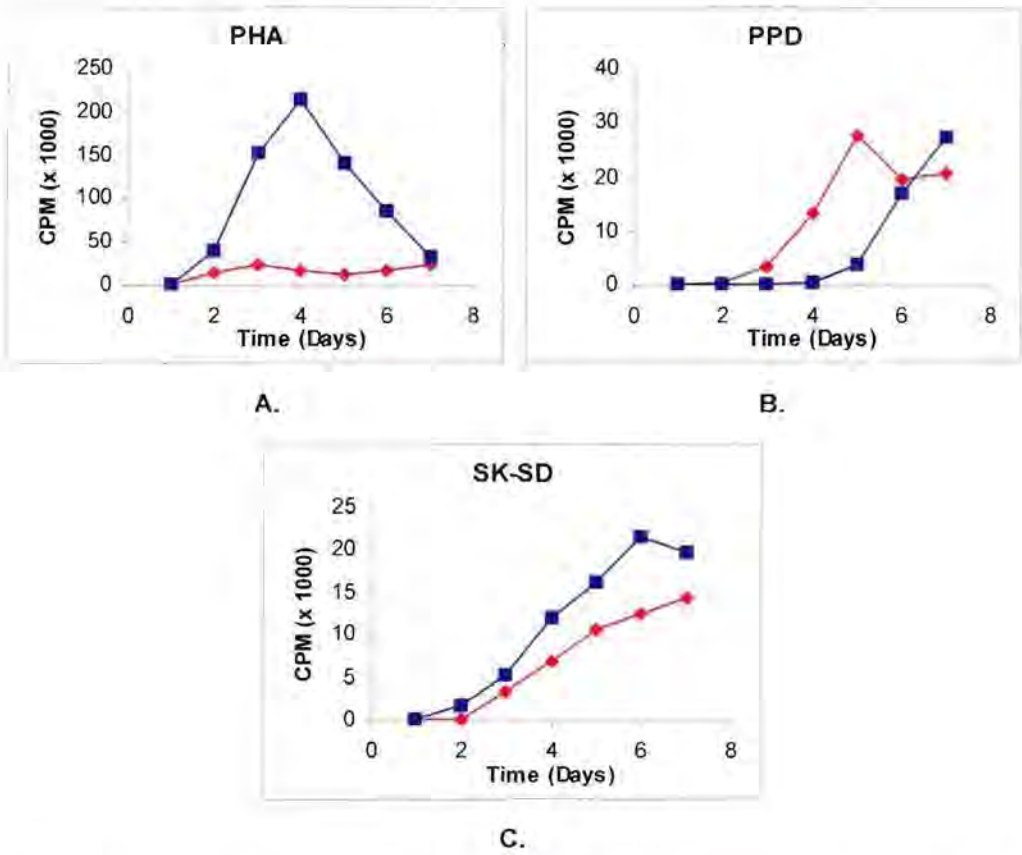


Figure 2.1. Proliferative responses of PBMNC (■) and EMNC (◆) to PHA (A), PPD (B), and SK-SD (C). Data are expressed as Δcpm (cpm with antigen - cpm without antigen). Proliferation was measured over a 7-day period. Background cpm values 9in the absence of antigen) were consistently < 2000

The tuberculosis patient in the current study showed peak PBMNC proliferation to PPD on day 7 (Δcpm = 27097), which is indicative of normal (non-accelerated) *in vitro* antigenic response kinetics. The day 4 proliferative response of PBMNC to PPD was poor ((Δcpm = 968). In the EMNC population, peak proliferative response to PPD was detected on day 5 (Δcpm = 27504). The day 4 EMNC proliferative response to PPD (Δcpm = 12409) was less than day 7 ((Δcpm = 21544), therefore this pattern did not constitute the classic day 4 accelerated response reported previously. Accelerated proliferative responses are not universal. Effusion mononuclear cells displaying either an absence of proliferative activity, or

a normal kinetic pattern of PPD-reactivity, have also been described (Lukey *et al.*, 1998). The unique kinetics of *in vitro* responsiveness to PPD observed in this patient in the EMNC population alone, whereby peak response occurred on day 5 of in-vitro culture, is a novel, intermediate pattern not previously observed. The finding is suggestive of a dual effect: elements of *in vivo* activation were present in the EMNC population, which allowed for optimal antigenic responsiveness 48 hours earlier than normally observed. However, the delayed peak response (day 5 instead of day 4) may suggest the activity of suppressor factors in the effusion, which may have inhibited an optimal accelerated response.

The kinetics of the proliferative responses to SK-SD were normal (non-accelerated) for both EMNC and PBMNC populations. The PBMNC population displayed a consistently higher response to this antigen as compared to the EMNC population. Proliferative responses of the PBMNC population peaked on day 6, whereas the EMNC population only peaked on day 7, however no significant differences in the two response patterns were observed.

The PBMNC proliferative response to mitogen (PHA) peaked at day 4 (Δcpm of 213228), as normally observed. The EMNC population produced no significant proliferation in response to mitogenic stimulation. This flat response was unique and difficult to account for in the light of strong PPD reactivity by the same effector cells. The finding was suggestive of mitogen-enhancement of suppressor activity in the effusion.

2.3.3. LDA of EMNC, but not PBMNC, produced a 'V'-Shaped Curve

LDA affords the opportunity of determining the frequency of responder cells in a population. Increased responder cell frequency in any particular part of the body suggests compartmentalised expansion of antigen-specific cells.

LDA was carried out on both EMNC and PBMNC populations in order to determine responder frequencies of cells in both groups. Frequency of responder cells was calculated using the maximum likelihood method of Fazekas de St Groth (1982), and data are shown in Table 2.2.

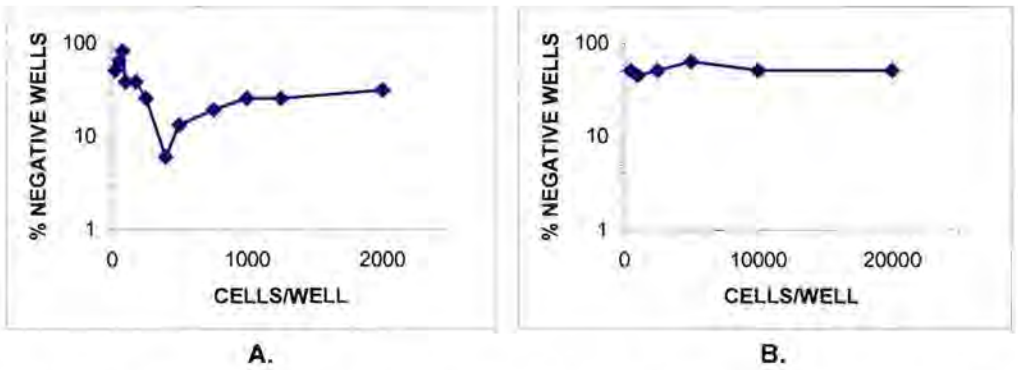
Table 2.2. Responder cell frequency of PBMNC and EMNC as determined by LDA

Population	Antigen	Frequency*	95% confidence#	Significant diff.$
PBMNC	PPD	1/9279	6529-12029	Yes
	SK-SD	1/3993	2920-5066	Yes
EMNC	PPD	1/515	392-637	Yes
	SK-SD	1/7909	5718-10105	Yes

*Note: *Frequency of responder cells expressed as inverse of mean of mutiplicity; #95% confidence interval for mean frequency value; $Significant difference between PPD and SK-SD responses of PBMNC and EMNC and between PPD and SK-SD responses of each population*

LDA was performed using both PPD and SK-SD in order to assess antigen-specificity of responder cells. Although only PPD responder cell frequency was increased in the EMNC population, differences between EMNC and PBMNC population responder cell frequencies for both PPD and SK-SD were considered significant, since there was no overlap of 95% confidence intervals. It should, however, be noted that difference in responder frequency to PPD (18 fold higher in the effusion as compared to blood) was much greater than for SK-SD (only 2 fold higher in the blood as compared to the effusion).

The LDA responses are presented graphically in Figure 2.2. Data are presented as percent negative wells for the dilution of cells plated (cells/well). Plotting of the LDA data in this way produced a classic 'V'-shaped curve for the EMNC population (Figure 2.2.A.). This pattern has previously been described, and has been ascribed to complex regulatory influences (Lefkovits and Waldman, 1979; Fazekas de St Groth, 1982; Lukey *et al.*, 1998). The LDA curve of the PBMNC population did not mirror that of the EMNC population (Figure 2.2.B.), which indicates restricted compartmentalisation within the pleural effusion.



A. B.

Figure 2.2. LDA responses to PPD of both EMNC (A) and PBMNC (B) populations. The percentage of negative wells at each leukocyte concentration was plotted. Each curve represents one experiment. Note different scales of the x-axes

2.3.4. EMNC T lymphocytes were predominantly CD4+, but displayed low levels of activation marker expression

Comparison of phenotype of EMNC and PBMNC populations was performed to assess any marked differences in the cellular composition and activation status of the two cell compartments. Flow cytometry data are presented in Table 2.3, showing both percentage positive cells and mean channel number counts (i.e. fluorescent intensity) for a number of markers.

The EMNC population showed a marked increase in the number of CD3+CD4+ positive cells as compared to the PBMNC population (50.6% vs. 33.3%). Conversely, there was a reduction in the CD3+CD8+ sub-population in the EMNC population as compared to

PBMNC population (18.1% vs. 27.9%). The CD4:CD8 ratio in EMNC was 2.8, whereas in PBMNC it was 1.2. The expansion of the CD4+ population in EMNC was confirmed by the CD3 data, which showed an increase in CD3+ cells from 63.3% in PBMNC to 75.1% in EMNC. The EMNC population did not show evidence for cell activation, with HLA-DR positivity of 20% being largely accounted for by the presence of B-cells (18.3% CD19+ cells). Both EMNC and PBMNC populations showed negligible positivity for CD4+HLA-DR+, which indicated that the CD4+ fraction did not account for the HLA-DR expression. The absence of T cell activation was confirmed by the low levels of CD25 (IL-2 receptor) observed.

Table 2.3. Flow cytometric analysis of PBMNC and EMNC, showing both percentage positive cells and mean channel number (MCN)

	Percent positive (%)		Mean channel number	
	PBMNC	EMNC	PBMNC	EMNC
CD19	6.3	18.3	4.62	4.01
CD2	86.0	86.8	25.00	25.51
CD3	63.3	75.1	8.87	7.54
CD3CD4	33.3	50.6	17.70	17.88
CD3CD8	27.9	18.1	28.91	30.09
CD29	32.5	20.7	3.09	2.99
CD4CD29	3.2	9.8		
CD16	52.5	50.4	3.62	3.07
CD56	32.4	7.5	7.25	7.46
CD16CD56	23.4	2.9		
CD25	0.3	0.6		
CD4CD25	0.1	0.2		
HLA-DR	9.6	20.0	7.11	7.84
CD4HLA-DR	0.6	1.6		

The PBMNC had a high proportion of NK cells, 32.4% of cells positive for CD56 as opposed to 7.5% in the EMNC population. The numbers of B cells were higher in the EMNC population, with 18.3% of cells positive for CD19, as compared to 6.3% in the PBMNC population. Although the percentage of cells expressing the memory cell marker CD29 was higher in the PBMNC population (32.5% as opposed to 20.7% in the EMNC population), the CD4+ memory component was enhanced in the EMNC population (9.8% as opposed to 3.2% in the PBMNC population).

Mean channel number (MCN) or mean fluorescent intensity values, which are indicative of intensity of staining and therefore of receptor marker expression, were similar for the two groups for all the markers examined.

2.3.5. Both EMNC and PBMNC displayed cytotoxic activity

Both EMNC and PBMNC displayed cytolytic activity. Levels of lysis of *M. tuberculosis* H37Rv- infected macrophages by day 3- and day 6-primed PBMNC and EMNC are illustrated in Figure 2.3.

Comparable levels of *M. tuberculosis*-specific lysis of macrophages by day 6 effectors at an effector to target ratio of 10:1 were observed with both EMNC and PBMNC populations. EMNC effectors lysed 25% of macrophages in an antigen-specific manner, and PBMNC effectors lysed 31% of target cells. Maximal levels of antigen-specific lysis were generally <35%. This was due to high levels of non-specific killing (30-40% lysis in the absence of antigen) which may have been attributable to lymphokine-activated killer (LAK) cell activity. There was some evidence of accelerated induction of cytotoxic activity in the EMNC population. At day 4, EMNC cells were able to lyse 19% of infected macrophages at an effector to target ratio of 10:1. PBMNC were only capable of lysing 14% of macrophages at the same effector to target ratio. In the EMNC population there was thus only a 6% increase in cytolytic activity from day 4 to day 6, with day 4 activity accounting for 76% of maximal day 6 activity. In the PBMNC population this increase between day 4 and day 6 was 17%, with day 4 activity accounting for only 45% of maximal day 6 activity. These findings suggest compartmentalised, partially accelerated induction of cytotoxic activity in the EMNC population.

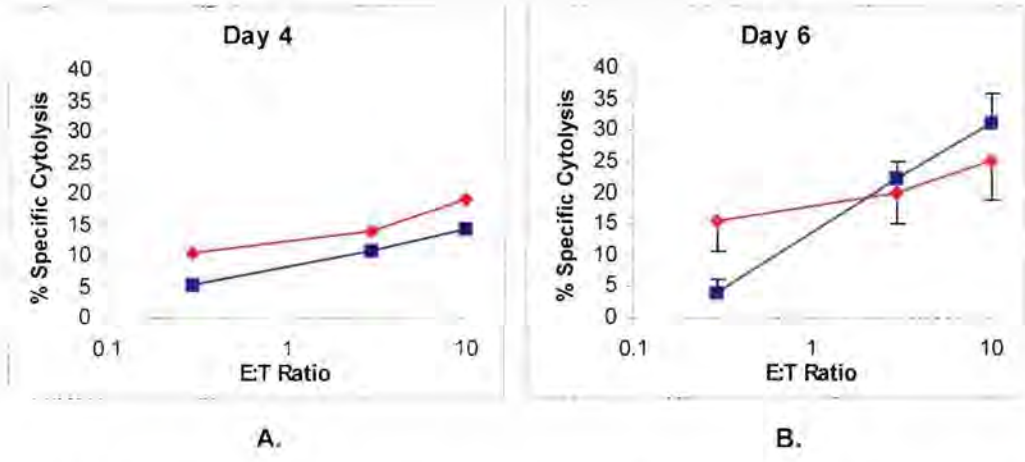


Figure 2.3. Cytolysis of *M. tuberculosis* H37Rv- infected macrophages by primed EMNC (◆) and PBMNC (■) populations. A. Antigen-specific lysis of macrophages by EMNC and PBMNC after 4 days of antigen stimulation *in vitro*. B. Antigen-specific lysis of macrophages by EMNC and PBMNC after 6 days of antigen stimulation *in vitro*. Data are shown as mean % antigen-specific lysis. Data in A are from one experiment, data in B from 3 experiments ± standard deviation

2.3.6. CD8+ EMNC clones were difficult to generate and maintain, and were not cytotoxic

Both CD4+ and CD8+ fractions of T lymphocytes from the EMNC population were cloned by limiting dilution. Although 5 CD8+ clones were expanded into microtitre plates, only 2 survived long enough for more detailed characterisation.

The cloning efficiency differed markedly for the CD4+ and CD8+ sub-populations. 15 Plates (900 wells) of each population were plated at 0.3 cells/well. Positive growth in 300 wells would represent 100% cloning efficiency. 66 wells (i.e. 22% of possible positive wells) were positive in the CD4+ group, whereas none (0%) were positive in the CD8+ group. This represents cloning efficiencies of 22% and 0% for the two populations, respectively. At a cloning dilution of 1 cell/well, 5 wells (0.6% of a total of 900 possible positive wells) of the CD8+ group were positive, which indicates a cloning efficiency of 0.6%. Cloning efficiencies of 22% and 0.6% for the CD4+ and CD8+ populations ensured clonality according to Poisson distribution. Estimation of clonality was determined according to the zero term of Poisson distribution $F_0=e^{-u}$, where Fo is the fraction of negative cultures, e is the base of the natural log, and u is the average precursor cell number. This translates to a cloning efficiency of <37% being considered indicative of clonality (Lefkovits and Waldmann, 1984).

The two surviving CD8+ clones were difficult to maintain and expand *in vitro*. Neither of the clones showed any mycobacterial- (PPD-) antigen specific cytolysis in assays that screened for cytotoxicity activity (Table 2.3) Proliferative responses were also generally poor. The cytokine production patterns differed from those of the CD4+ clones, with one CD8+ clone uniquely producing fairly high levels of the Th2 cytokine IL-4. These findings are discussed in Section 2.3.7.

2.3.7. CD4+ EMNC clones all proliferated to mycobacterial antigens, and the majority were cytotoxic

The 68 clones (66 CD4+ and 2 CD8+) were screened for proliferative and cytotoxic activity. Neither of the CD8+ displayed antigen-specific cytotoxic activity (<10% macrophage lysis). Similarly, these clones had poor proliferative responses to a range of mycobacterial antigens. These findings are discussed in detail in Chapter 5.

All the CD4+ clones displayed good proliferative responses to mycobacterial antigens (stimulation indices - i.e. cpm in presence of antigen divided by cpm in the absence of antigen - were consistently > 3). Table 2.4. illustrates the varying cytolytic ability of the CD4+ clones. The clones were arbitrarily divided into strong killers (>20% antigen-specific lysis), weak killers (10-20%), and non-killers (<10%). A majority of CD4+ clones were cytotoxic (36/66 or 54.5%). Of these, 15 (22.7%) showed antigen-specific (BCG) lysis of macrophages of >20%. Several clones from this group of 15 (3.2D, 10.4C, 11.2E, 14.6B, 14.10C, 15.3A) were selected for more detailed characterisation of both their mechanism of killing (Chapter 4) and their mycobactericidal potential (Chapter 3). Approximately 45% of the CD4+ clones did not lyse BCG-infected macrophages, suggesting that cytolytic potential was not a universal phenomenon in mycobacterial antigen-responsive CD4+ clones.

Table 2.4. Cytolytic activity of CD4+ and CD8+ clones against BCG infected macrophages

	CD4+	CD8+
Total Clones	66	2
>20% Ag-specific cytolysis	15 (22.7%)	0 (0%)
10-20% Ag-specific cytolysis	21 (31.8%)	0 (0%)
<10% Ag-specific cytolysis	30 (45.5%)	2 (100%)

2.3.8. Cytokine production profiles of CD4+ EMNC clones were strongly type-1, whereas those of CD8+ clones were either type-1 or type-0

The production of the cytokines IL-4 and/or interferon-γ by T cells is an important indicator of whether cells are type-1 (Th1) or type-2 (Th2). In the current study the terms type-1 and type-2 are used in preference to Th1 and Th2 to describe interferon-γ and IL-4 producers respectively. This is because both CD4+ and CD8+ clones were categorised according to cytokine production. The use of Th1 and Th2 to describe CD8+ clones is inaccurate. Type-0 cells or clones refers to those cells which produce both these defining cytokines.

All of the CD4+ clones (66/66) produced high levels of IFN-γ. All clones produced in excess of 10000 pg/ml, with a range of 10500 pg/ml – 59200 pg/ml being observed. Both CD8+ clones produced IFN-γ, however clone 2.9D produced higher levels (22500 pg/ml) than clone 11.5A (8000 pg/ml). None of the CD4+ clones (0/66), nor CD8+ clone 2.9D, produced detectable levels (>16 pg/ml) of IL-4. The CD8+ clone 11.5A produced 150 pg/ml of IL-4. All the CD4+ clones were thus strongly type-1 (Th1), whereas one CD8+ was type-1 (2.9D), the other type-0 (11.5A).

2.3.9. A sub-population of CD4+ EMNC showed strong cytolytic activity

Cytotoxic activity mediated by CD4+ cells has been described in EMNC populations (Lorgat *et al.*, 1992a). Cytotoxic CD4+ clones derived from EMNC populations have not however been well characterised.

The majority (55%) of CD4+ clones generated in the current study were cytotoxic. A sub-population of the cytotoxic clones (22.7%) showed strong (>20%) antigen-specific lysis of BCG-infected macrophages (Section 2.3.7.). These clones were subsequently assessed for possible mycobactericidal activity and their mechanism of target cell killing was also investigated (Chapters 3 and 4). The proliferative and cytotoxic responses of 3 such clones (3.2D, 10.4C and 15.3A) are illustrated in Figure 2.4. A - C. All of the cytolytic CD4+ clones showed strong mycobacterial antigen-specific killing ability. PPD, *M. tuberculosis* H37Rv, and *M. bovis* BCG were used as mycobacterial antigens. No killing was observed against macrophages pulsed with irrelevant antigen SK-SD. The proliferative responses for the different mycobacterial antigens varied, but generally good proliferation was observed. In certain cases, PPD proliferative responses approximated mitogenic (PHA) responses (clones 3.2D and 15.3A).

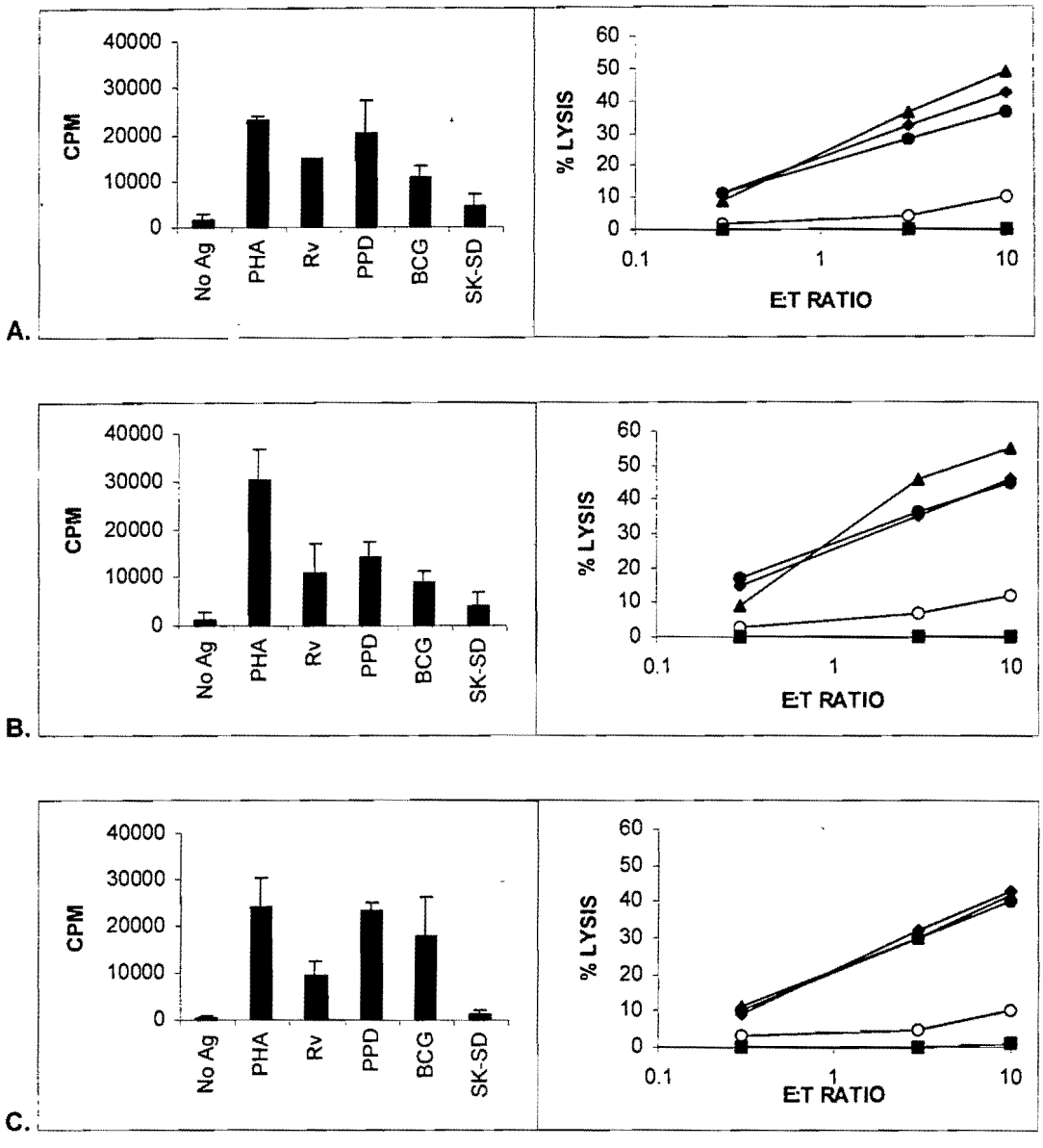


Figure 2.4. Proliferative and cytolytic responses of 3 CD4+ clones 3.2D (A), 10.4C (B), and 15.3A (C). Proliferative data are illustrated as mean cpm/min values ± SD of 3 experiments. Cytotoxicity data are illustrated as mean % lysis in the absence of antigen (■), or in the presence of SK-SD (O), BCG (●), PPD (▲), or Rv (◆). Cytotoxicity data are representative of 6 experiments.

2.4. Discussion

Comparisons of immune parameters in both pleural effusions and peripheral blood in tuberculosis patients have provided some insight into the constituents of protective immunity and also the immunopathogenesis of the disease. Previous studies have indicated that there is a compartmentalisation of immune responses to sites associated with disease (Rossi *et al.*, 1987; Fujiwara and Tsuyuguchi, 1989; Manca *et al.*, 1991). Pleural effusions contain highly PPD reactive, interferon-γ-producing (Th1) memory cells. The localised occurrence of these cells may indicate that they play an important role in the localised cell-mediated immune response to *M. tuberculosis* infection (Barnes *et al.*, 1989).

Localised expansion of T cells in pleural fluid is predominantly restricted to the CD4+ lymphocyte subset (Barnes *et al.*, 1989). This recruitment of CD4+ cells is accompanied by enhanced PPD-responsiveness and interferon-γ production, suggestive of a pivotal role for Th1 CD4+ lymphocytes in protective immunity (Fujiwara and Tsuyuguchi, 1989; Ribera *et al.*, 1988). Effusion mononuclear cells from a majority of TB patients exhibit accelerated kinetics of reactivity to PPD *in vitro* (Lorgat *et al.*, 1992a). In addition, induction of antigen-specific cytotoxic activity is also accelerated (Lorgat *et al.*, 1992b). These findings suggest that effusion-derived cells are primed *in vivo*, with accelerated *in vitro* responses representing cell re-priming.

In the present study, the effusion mononuclear cells displayed an unusual form of accelerated reactivity to PPD. Peak responsiveness on day 5 of stimulation constitutes a response intermediate to normal day 7 kinetics and accelerated day 4 kinetics (Lorgat *et al.*, 1992a; Lukey *et al.*, 1998). The effusion-derived cells also displayed unique non-responsiveness to PHA, a phenomenon not noted in previous studies (Lukey *et al.*, 1996; Lukey *et al.*, 1998). The lack of PPD-reactivity of pleural effusion cells has previously been linked to suppressor activity (Lukey *et al.*, 1998). It is possible that the unique observation of day 5 accelerated response was also indicative of concomitant suppressor activity. Pleural effusions, including the one in the present study, contain numerous cell types other than CD4+ lymphocytes. In particular, CD8+ T lymphocytes have been observed. Although these cells have been implicated in cytotoxic activity (Stenger and Modlin, 1998; Lalvani *et al.*, 1998), the lack of enrichment in CD8+ cell numbers at disease sites may suggest that these cells have a less prominent role in protective immunity. CD8+ suppressor T cells derived from lepromatous leprosy lesions have been characterised (Kaplan *et al.*, 1987), and it is possible that the activity of similar cells was responsible for the distinctive response kinetics observed in the present study. The suppression of effusion cell responsiveness to PHA was not attributable to T cell anergy, since the cells were responsive to PPD. Suppression mediated by monocytes or macrophages was also unlikely, because normal SK-SD responses were observed in the effusion mononuclear population. T cell-mediated

suppressor activity in the effusion was confirmed with the isolation of a CD8+ T cell clone capable of suppressing autologous T cell proliferation (chapter 5).

The presence of active suppression within the effusion was also confirmed by limiting dilution analysis. A characteristic v-shaped LDA curve was generated from the effusion population alone. V-shaped LDA curves have been associated with suppression in several studies (Lefkowitz and Waldman, 1979; Dozmorov *et al.*, 1995; Dozmorov and Miller, 1996; Lukey *et al.*, 1998). In the latter study, these curves were associated with non-responsive effusion cells derived from patients with active TB. The occurrence of an effusion associated v-shaped curve in the present study may help to explain the unique kinetics of proliferative response. The intermediate pattern of day 5 accelerated response to PPD may be due to simultaneous presence of enhanced T cell reactivity and active suppression. The flat PHA response of the effusion cells suggests optimal stimulation or activation of suppressor cells by PHA, such that suppressor activity was sufficient to almost completely abrogate T cell responsiveness to the mitogen. PPD may have been unsuccesful as an antigenic stimulus for suppressor cells due to the requirement of CD8+ cells for MHC class I presentation of unique mycobacterial peptides.

The effusion contained a high frequency of PPD-responsive cells. LDA indicated that 1 in 515 cells were responsive, a finding that compares favourably to the range of frequencies (mean of 1 in 519) observed in patients with accelerated kinetics of reactivity (Lukey *et al.*, 1998). Patients with normal response kinetics generally had lower frequencies of responder cells (mean of 1 in 840). The high frequency of responder cells confirms that this effusion displayed evidence of localised recruitment of antigen-responsive T cells. It also suggests that in the absence of any suppressor activity, accelerated reactivity of the classical day 4 pattern would have been detected. Induction of significant effusion cell cytotoxic activity *in vitro* was already detectable on day 4 of *in vitro* culture, a finding also previously associated with enhanced responsiveness of T cells in the effusion population (Lorgat *et al.*, 1992a). Although a detailed seven-day assessment of kinetics of induction of cytotoxic activity was not performed, the increased cytotoxic potential of the effusion cells on day 4 (compared to PBMNC) suggests increased cytotoxic T-cell reactivity.

The localised expansion of the CD4+ T cells occurred in the absence of any significant activation marker expression. This lack of activation marker expression has been found in other studies (Lukey *et al.*, 1996). Although some increase in HLA-DR expression was observed, this was largely due to B-cells, and no CD4+ T cells displayed activation markers. However, a localised increase in the CD4+ memory cell component was noted in the effusion. The accumulation of memory T cells in the absence of any detectable T cell activation may be due to low responder cell frequency. Detection of activation in a population with only 1 out of every 519 cells responsive to antigen suggests that activation

would be detected in about 0.2% of cells. In addition, factors in the effusion may have limited activation of T cells. Limiting T cell activation could minimise tissue damage caused by release of cytokines such as TNF-α.

Cloning from pleural effusions affords the opportunity to assess cellular immune function of cells derived from a disease-associated site in detail. The majority of T cell clones generated from the effusion mononuclear cell population were cytotoxic. Cytotoxic activity in pleural effusions has previously been linked to the activity of CD4+ cells (Ottenhoff and Mutis, 1990; Lorgat *et al.*, 1992a). The CD4+ cytotoxic T cell clones were all potent producers of interferon-γ and responded to a range of mycobacterial antigens. The localised increase in CD4+ T cells coupled with the findings that both *in vitro* induction of PPD-specific cytotoxic activity was enhanced and that the majority of CD4+ clones generated were cytotoxic, suggested an important role for these cells in successful infection clearance.

It has previously been suggested that cytolytic CD4+ T lymphocytes may play a role in infection clearance due to their ability to lyse infected macrophages. This lysis could be envisaged to result in either the simultaneous death of the infecting bacilli, or their release from host macrophage cells. The released organisms would then be engulfed by freshly recruited macrophages possibly more competent in killing the organisms or inhibiting their replication. Repetition of this cycle could result in a reduction of organism numbers at disease sites (Ottenhoff and Mutis, 1990; Orme *et al.*, 1992). In the current study, cytotoxic CD4+ effusion-derived T cell clones were found to reduce the numbers of viable organisms following lysis of infected macrophages (chapters 3 and 4). The finding that cytotoxic CD4+ cells are mycobactericidal is important in that it defines a pivotal functional role for the expanded CD4+ population at the disease-associated site.

Generation of CD8+ clones was more problematic. Of two CD8+ clones generated, one was unique in that it produced IL-4 and only low levels of interferon-γ. This clone was found to be poorly responsive to a range of mycobacterial antigens, but responded well to PHA stimulation. This finding, together with the detection of strong inhibition of effusion cell proliferation in the presence of PHA, led to more detailed analysis of suppressor activity mediated by this clone (chapter 5). It was found that the clone could indeed suppress autologous T cell proliferation in response to various mycobacterial antigens and to PHA.

In summary, this chapter has highlighted several important features of the effusion-derived mononuclear cell population. Evidence of a compartmentalised recruitment and priming of lymphocytes was coupled with certain unique findings suggestive of active suppression. The generation of CD4+ and CD8+ clones has enabled the assignment of important functional roles to these cells at the disease site, namely mycobactericidal cytotoxic activity and antigen-specific suppression.

Chapter 3.

Lysis of *M. tuberculosis*-infected macrophages by CD4+ CTL clones is associated with reduced mycobacterial viability

3.1. Introduction

CD4+ T lymphocytes are expanded at sites of infection in tuberculosis (Barnes *et al.*, 1989; Munk and Emoto, 1995). There is also an increase in the frequency of antigen-responsive cells at disease sites (Lukey *et al.*, 1998). The function of CD4+ T lymphocytes in tuberculosis has traditionally been linked to the production of cytokines (Schluger and Rom, 1998). CD4+ T lymphocytes derived from pleural effusions are strongly Th1, producing high levels of interferon-γ (Barnes *et al.*, 1989; Ribera *et al.*, 1990). Activation of infected macrophages by cytokines such as interferon-γ has been implicated in killing of intracellular mycobacteria. Although activated murine macrophages do appear to be mycobactericidal, human macrophages have not been found to be able to kill intracellular bacilli, even after stimulation with interferon-γ (Dannenberg and Rook, 1994). Since the granulomatous lesion is generally succesful in containing bacillary spread (Dannenberg, 1991), an alternative mechanism to cytokine-mediated macrophage activation must be operative in order for infection to be contained in the human. Indeed, successful control of mycobacterial infection has recently been shown to depend on the activity of primed lymphocytes as well as interferon-γ (Bonencini-Almeida *et al.*, 1998). This finding confirms reports that CD4+ T lymphocytes are essential for prevention of organism replication and spread (Silver *et al.*, 1998; Yoneda and Ellner, 1998).

Cytolytic activity in tuberculous pleuritis is enhanced, and has been shown to be due to the CD4+ population (Lorgat *et al.*, 1992a). In the absence of successful cytokine-mediated macrophage killing of intracellular organisms, cytotoxic activity may account for the containment of infecting organisms at disease sites. Lysis of infected macrophages by CTL could, however, result in either of two options, organism death or organism spread. Cytotoxic activity may thus represent a double-edged sword, with either beneficial or detrimental consequences for the host organism. The lysis of infected cells in the absence of concomitant organism death poses a danger for the host, unless released organisms are rapidly engulfed by adjacent macrophages.

Several studies have illustrated that induction of host macrophage apoptosis by various agents such as ATP (Molloy *et al.*, 1994; Lammas *et al.*, 1997), H_2O_2 (Laochumroonvorapong *et al.*, 1996, 1997) or soluble FasL (Oddo *et al.*, 1997), is accompanied by a reduction in mycobacterial viability. This suggests that under certain

circumstances, lysis of infected macrophages would aid in organism clearance. Although cytolytic CD4+ cells have been reported to have no effect on mycobacterial viability (Fazal *et al.*, 1994; Pithie *et al.*, 1995), CD8+ CTL have indeed been linked to organism death (Stenger *et al.*, 1998). The defining feature of mycobactericidal CD8+ CTL activity is the presence of a granule-mediated cytolytic pathway involving the activity of the granule component granulysin (Stenger *et al.*, 1997; Pena *et al.*, 1997).

The majority of CD4+ CTL activity has been associated with Fas-mediated apoptosis of target cells. Recent reports have, however, highlighted the ability of CD4+ CTL to utilise granule-mediated cytolytic pathways (Sevilir Williams and Engelhard, 1996; Vergelli *et al.*, 1997; Lewinsohn *et al.*, 1998). The occurrence of a killing mechanism in CD4+ CTL common to that utilised by mycobactericidal CD8+ CTL is interesting and begs more detailed investigation. CD4+ CTL activity in diseases involving intracellular infection by bacteria may in fact be analogous to CD8+ CTL activity in viral infections (Smyth and Trapani, 1998; Harty and Bevan, 1999).

The present chapter characterises cytolytic and mycobactericidal activity of effusion-derived CD4+ clones. The mycobactericidal activity was associated with macrophage cell death. The kinetics of target cell death and reduction in viable mycobacteria were similar, and blocking of target cell death abrogated any mycobactericidal activity. These findings are the first report of CD4+ CTL clones that lyse macrophages and concomitantly reduce mycobacterial viability.

3.2. Materials and methods

3.2.1. Growth and maintenance of *M. tuberculosis* cultures

M. tuberculosis H37Rv strain # 27294 was obtained from the American Tissue Type Collection (ATTC) (Rockville, MD, USA), and *M. bovis* BCG from the Trudeau Mycobacteria Collection (Saranac Lake, NY, USA). Stock cultures were grown in Middlebrook 7H9 broth (Difco, Detroit, Michigan) supplemented with 10% OADC enrichment media (State Vaccine Institute, Cape Town, SA) and 0.5% glycerol (Merck, Darmstadt, Germany). Cultures were grown at 37°C in a 5% CO_2 incubator with periodic shaking. Growth was monitored by daily assessment of culture absorbance readings (450nm). When cultures reached mid-log phase of growth (day 4-6), 5% of sterile glycerol was added and 100µl aliquots frozen in liquid nitrogen. The bacterial concentration was determined by plating out freshly thawed aliquots following disruption of clumps by passing the vial contents through a fine gauge syringe needle. 100µl aliquots were plated at various dilutions in 7H9 broth (10^{-1} to 10^{-8}) on 7H11 agar plates (Difco, Detroit, Michigan) supplemented with 10% OADC and 0.5% glycerol. For infection of adherent macrophages, aliquots were thawed, clumps were disrupted and mycobacteria were diluted in growth medium (RPMI 1640 supplemented with 10% pooled human AB serum) prior to the addition to the macrophage cultures.

3.2.2. Lysis of *M. tuberculosis* infected macrophages by CD4+ CTL clones

Lysis of adherent macrophages by CD4+ CTL clones was carried out in a modified version of the 16 hour cytotoxicity assay described in Section 2.2.5. Clones were stimulated with fresh irradiated autologous PBMNC, *M. tuberculosis* H37Rv (10^5 CFU/ml) and recombinant human IL-2 (100 IU/ml) for 72 hours, followed by addition of fresh IL-2 (100 IU/ml) in RPMI 1640 medium supplemented with serum. Clones were used 48 hours after second addition of IL-2, 120 hours after addition of feeder PBMNC. This ensured that clones were activated and that no significant numbers of contaminating feeder cells were present. Adherent macrophages were cultured in 96 well microtitre plates (Greiner) by plating fresh PBMNC (100µl/well of 10^6 PBMNC/ml). On day 4 (i.e. 96 hours) following initial plating, macrophages were infected with H37Rv (2 organisms per macrophage, 2 x 10^5 CFU/ml) and simultaneously labelled with ^{51}Cr (6µCi per well). The level of infection of macrophages was monitored periodically by ZN staining of H37Rv-infected macrophages cultured in chamber well slides (Nunc, Denmark). Consistently >80% of macrophages were infected with between 1 and 5 organisms. After overnight labelling, macrophages were washed 3 times with warm PBS containing 10% foetal bovine serum (FBS, Delta Bioproducts, Johannesburg, South Africa), and clone cells were added to macrophages at effector to target ratios of 10:1, 3:1 and 0.3:1. Effectors and targets were then incubated together for 16 hours. Calculation of % lysis was as described (Section 2.2.5.). In kinetic experiments macrophage lysis was assessed at 4, 8, 12, 20 and 24 hours after addition of the clones. Increased effector to target ratios (20:1 and 50:1) were used in some experiments.

3.2.3. Effect of CTL-mediated macrophage lysis on *M. tuberculosis* viability

Mycobacterial survival was determined following cytotoxicity assays as described above. Parallel sets of macrophages were cultured in 96 well microtitre plates (Greiner). One plate was used for standard cytotoxicity assays (^{51}Cr-release), the other for assessing mycobacterial survival. For the latter, the macrophages were not co-labelled with ^{51}Cr when infected with H37Rv. After overnight infection the macrophages were washed with PBS/10% FBS as described above. CTL clones (100μl) were than added to the infected macrophages at an effector to target ratio of 10:1. Following exposure of infected macrophages to CTL for 4, 8, 16 or 20 hours, the contents of each well was removed and triplicate well contents were pooled. SDS (Merck, Darmstadt, Germany) in PBS (final concentration 0.25%) was then added to each well. On dissolution of the residual macrophages (approximately 10 minutes), the well contents were removed and pooled with original well contents. In this way the total bacterial numbers were compared - with or without exposure to CTL clones. The pooled lysate was diluted (10^{-1} to 10^{-5}) in 7H9 broth and 100μl aliquots plated on 7H11 plates as described above (Section 3.2.1.). Colony counts were carried out after 3 weeks of incubation at 37°C. Standard ^{51}Cr-release assays were always carried out in parallel to the CFU experiments, so that the percentage target cell lysis could be related back to any changes in CFU counts.

In addition to CFU plating, bacterial numbers were assessed by labelling pooled lysates with ^{3}H-uridine (Amersham, Buckinghamshire, England), as previously described (Fazal *et al.*, 1994). Briefly, pooled lysates were diluted (1 in 10) in 7H9 broth. Aliquots (100μl) were added in triplicate to round bottomed 96-well microtitre plates (Greiner), and 2μCi/well of ^{3}H-uridine was added. Cultures were incubated for 6 days at 37°C. Labelled mycobacteria were harvested onto filter paper (Machery-Nagel, Germany) using a Titertek cell harvester (Flow Laboratories, Norway). The filter paper discs were placed into scintillation vials, scintillation fluid (1ml) was added (Quicksafe A, Zinsser Analytic, Frankfurt, Germany), and the amount of incorporated label was assessed in a β-counter (Tricarb 4640, Packard, Meriden, CT).

To ensure that the CTL clones were directly mycobactericidal, control experiments were performed in which H37Rv-infected macrophages were lysed by the addition of SDS (final concentration 0.25%). The lysates containing the released mycobacteria were then exposed to the CTL clones at an effector to target ratio of 10:1. CFU were assessed by plating on 7H11 agar plates before and after exposure to the clones as described above.

3.2.4. Kinetics of macrophage lysis and *M. tuberculosis* death

The kinetics of macrophage death and associated *M. tuberculosis* death were assessed by measuring ^{51}Cr-release and CFU growth and/or ^{3}H-uridine incorporation at various time points following exposure of infected macrophages to the CTL clones. Comparison of the

kinetics of these two processes enabled an assessment of which stage of target cell death was associated with mycobacterial death.

3.2.5. Generation of PBMNC and EMNC CD4+ enriched cell lines

Cell lines were generated from patient-derived EMNC and PBMNC populations. Thawed EMNC and PBMNC cells (10ml of 10^6/ml) were stimulated with *M. tuberculosis* H37Rv (2 x 10^5 CFU/ml) for 6 days. Recombinant human IL-2 (100 IU/ml) was added for an additional 3 days. The lines were then enriched for CD4+ lymphocytes by magnetic bead separation (Minimax, Miltenyi Biotec, bergisch Gladbach, Germany) as discussed in Section 2.2.5. Population purity was assessed by flow cytometry (>90% for both lines), and lines were restimulated and expanded with fresh irradiated autologous PBMNC (feeder cells), H37Rv (2 x 10^5 CFU/ml) and IL-2 (100 IU/ml). Lines were then maintained in the same way as the clones, with weekly restimulation with feeder cells, antigen and IL-2.

3.2.6. Analysis of mechanism of macrophage death

In addition to ^{51}Cr release, the mechanism of macrophage death triggered by exposure to the CTL clones was assessed by flow cytometry of annexin V and propidium iodide stained cells (Apoptosis Detection Kit, R&D, Minneapolis, MA; Annexin-V-FLUOS Staining Kit, Boehringer Mannheim, Mannheim, Germany). Following the addition of effector CTL for 4, 8, or 16 hours, adherent macrophages were washed free of CTL clones with warm PBS supplemented with 5% AB serum. The adherent monolayer was then removed by the addition of 0.25% EDTA. Macrophages which did not detach were removed by gentle scraping using the rubber end of a sterile 1ml syringe plunger (Omnican 100, Braun Petzold, Melsungen, Germany). Detached macrophages (approximately 1 x 10^6) were washed three times with cold PBS and cell pellets were labelled with annexin V and propidium iodide according to the manufacturer's protocol. This involved adding 100µl of reaction mixture containing FITC-labelled annexin V and propidium iodide in Ca^{2+} buffer (binding buffer) to cells for 10 minutes at room temperature in the dark. FCM analysis was performed with a Coulter Epics Profile II flow cytometer. Control untreated adherent macrophages were used to gate on the macrophage population. T lymphocytes did not occur within the gated macrophage region. This was confirmed by staining macrophages with FITC-labelled anti-CD3 (Coulter) after exposure to CTL clones, and determining the number of CD3+ cells within the gated macrophage region. Less than 10% of cells within the gated region were CD3+.

Annexin V staining of adherent cells was also assessed by fluorescent microscopy. Macrophages were grown for 5 days from PBMNC (10^6/ml) on chamber well slides (Nunc, Denmark). Following cytotoxicity assay period, monolayers were washed with warm PBS and labelled with staining mixture (100µl) for 15 minutes. Monolayers were washed thoroughly to

remove the staining mixture, air dried and viewed under a Fluorescent microscope (Zeiss, Axiophot, Germany).

In addition to annexin binding, apoptosis levels in macrophages were assessed by TdT-mediated dUTP nick end labelling or TUNEL (*in situ* Cell Death Detection Kit, Fluorescein, Boehringer Mannheim, Mannheim, Germany). Macrophages were then analysed by fluorescent microscopy and flow cytometry. Adherent macrophages were cultured on chamber slides (Nunc, Denmark) and infected for 24 hours with *M. tuberculosis* H37Rv. Infected macrophages were then incubated overnight in the presence or absence of CTL clones (E:T ratio of 10 to 1). Control uninfected macrophages were also used in some experiments to determine whether *M. tuberculosis* infection had any effect on detectable levels of macrophage apoptosis. Macrophages were washed vigorously with warm PBS, air dried and fixed with fresh 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes. The fixed cells were rinsed with PBS and permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. Slides were then rinsed twice with PBS and the 50µl of TUNEL reaction mixture (terminal deoxynucleotidyl transferase (TdT) together with FITC-labelled nucleotide mixture) was added. A coverslip was placed over each sample to ensure even spreading of the reaction solution, and to prevent evaporation. The slides were incubated at 37°C in the dark for 60 minutes. The stained slides were then rinsed 3 times with PBS, air dried and viewed under a fluorescent microscope (Zeiss, Axiophot, Germany).

TUNEL labelling for flow cytometry involved detachment of adherent macrophage monolayers by EDTA treatment following exposure to the CTL clones, as described above for annexin V staining. The detached cells (approximately 1 x 10^6) were washed twice in PBS/1% BSA at 4°C and then fixed with 100µl of 4% paraformaldehyde in PBS for 30 minutes at room temperature. Cells were then washed free of fixative with PBS, and the cells were permeabilised by addition of 0.1% triton X-100 in 0.1% sodium citrate for 2 minutes on ice. Prior to the addition of the TUNEL label, cells were washed twice with PBS. TUNEL reaction solution (TdT and FITC-labelled nucleotide mixture) was added at 50µl per sample and incubated for 60 minutes at 37°C. Controls were set up using label solution (FITC-nucleotide mixture) in the absence of enzyme solution (TdT). Labelled cells were finally washed twice and resuspended in PBS prior to analysis on a flow cytometer.

Annexin and TUNEL staining were also carried out on adherent macrophages which had artificially been induced to undergo necrosis or apoptosis. Necrosis was induced by exposing macrophages to sterile distilled water for 30 minutes at 37°C, or by exposure to medium heated to 56°C for 20 minutes. Apoptosis was induced by treating macrophages with H_2O_2 (1 mM) or ATP (2mM) for 90 minutes, after which they were re-incubated in complete growth medium for an additional 4 to 16 hours. (Section 3.2.7).

3.2.7. Induction of macrophage apoptosis by ATP and H_2O_2 and assessment of effects on *M. tuberculosis* viability

Adherent macrophage cultures were induced to undergo apoptosis by treatment with H_2O_2 or ATP. Dilution series of both treatments were carried out to determine concentrations and time kinetics for optimal induction of apoptosis. Optimal concentrations were then used to assess the affect of apoptosis induction by these agents on the survival of *M. tuberculosis*. For ATP treatment, adherent monolayers were pulsed with 2mM ATP (Sigma) for 90 minutes, washed with PBS and incubated for up to 16 hours in fresh RPMI 1640 medium supplemented with 10% AB serum. For H_2O_2 treatment, 1mM H_2O_2 (BDH Biochemicals) was added to adherent monolayer cutures, and apoptosis assessed at various time points up to 16 hours.

Level of macrophage lysis was determined in a modification of the standard 16 hour 51Cr-release assay (Section 2.2.4.). Adherent macrophages (day 5-7 post plating) were labelled with 6μCi/well 51Cr with or without simultaneous infection with H37Rv. After overnight incubation macrophages were treated with ATP or H_2O_2-, and at various time points specific 51Cr-release measured as described above. Levels of apoptosis were assessed by flow cytometry following annexin V and PI staining and/or by fluorescent microscopy. For fluorescent microscopy, apoptotic adherent macrophages were stained with acridine orange (1μg/ml) and ethidium bromide (1μg/ml) (both Sigma) for 10 minutes, washed, mounted and viewed.

The effect of either ATP- or H_2O_2-induced apoptosis on *M. tuberculosis* survival was assessed as described in Section 3.2.3. Instead of exposing infected macrophages to cytolytic clones, ATP or H_2O_2 was administered as described above. *M. tuberculosis* viability was monitored by both CFU counting and assessment of ^{3}H-uridine incorporation.

Infected macrophage lysates (SDS-treated) were exposed to ATP or H_2O_2 (Section 3.2.3.) to assess whether the agents were directly mycobactericidal. The number of CFU's was determined by plating lysates onto 7H11 agar plates before or after exposure to ATP or H_2O_2. Comparison of CFU counts enabled evaluation of the mycobactericidal activity of these two agents.

3.2.8. Inhibition of macrophage apoptosis and the effect on *M. tuberculosis* survival

Brefeldin A was used to inhibit apoptosis of macrophages following exposure to the cytolytic clones (Kataoka *et al.*, 1996). Brefeldin A (Sigma) was prepared by solubilisation in methanol. Aliquotted stock solutions were later thawed and diluted into growth medium at a concentration of 10μM. BFA was added to the cytotoxicity assays at the same time that the clones were added to the adherent macrophages. Solvent controls were included in initial experiments, and no effect on cytolytic activity was observed. In certain experiments clones

were pre-incubated with BFA for 3 hours to prevent both FasL expression and cytolytic granule maturation. The effect of BFA on ATP and H_2O_2 induced apoptosis was also assessed. The viability of *M. tuberculosis* following inhibition of target macrophage apoptosis by BFA was determined by both CFU counting and ^{3}H-uridine incorporation, as described above.

Possible toxic effects to the macrophages in the presence of BFA were excluded by assessing macrophage viability following treatment of macrophages for 16 hours. Trypan blue exclusion staining was carried out on EDTA-detached macrophages following BFA treatment. In order to exclude the possible BFA-mediated inhibition of macrophage antigen presentation, the ability of BFA-treated macrophages to induce CTL clone proliferation was assessed. As in cytolytic assays, BFA was added to H37Rv-infected macrophages at the same time as the clone cells. Assessment of clone cell proliferation was carried out in a modification of the standard procedure (Section 2.2.9.). Day 5 adherent macrophages (plated at 10^6 PBMNC/ml, 100µl/well) were infected with H37Rv (2 organisms per macrophage) for 24 hours. Macrophages were washed and CTL clones (100µl of 10^6/ml) were added. ^{3}H-thymidine incorporation was carried out for the final 8 hours of culture (42-50 hours).

3.3. Results

3.3.1. Lysis of *M. tuberculosis*-infected macrophages by CD4+ CTL clones was accompanied by a reduction in mycobacterial viabilty

In order to assess whether the cytolytic activity of CD4+ effusion-derived clones had any effect on intracellular mycobacteria, viable bacilli were enumerated prior to, and following, exposure of the macrophages to the clones. The effect of lysis of mycobacteria-infected macrophages on the viability of *M. tuberculosis* is presented in Figures 3.1.(A.) and 3.2.

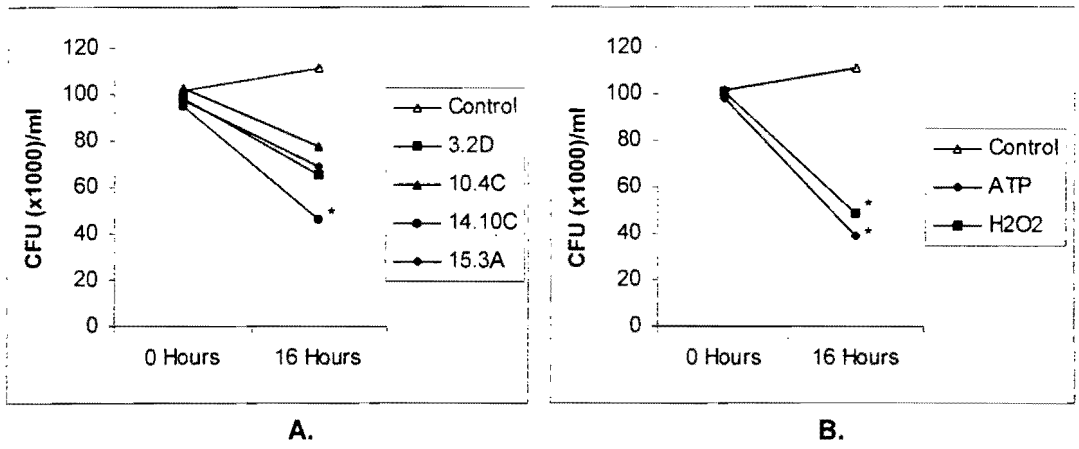


Figure 3.1. Changes in CFU counts following 16 hour exposure of infected macrophages to CD4+ CTL clones (A.), or the apoptogenic agents ATP (2mM) or H_2O_2 (1mM) (B.). Change in CFU counts in the absence of clones or apoptogenic agents is represented as control. Reduction of CFU in the presence of H_2O_2, ATP and clone 14.10C was statistically significant ($P < 0.05$). Data represent 3 experiments

The effusion-derived CD4+ CTL clones were able to potently reduce the number of viable mycobacteria. Although only clone, 14.10C, showed a statistically significant reduction ($P < 0.01$), all the clones were able to reduce CFU counts by more than 30%. This compares favourably with the optimal levels of reduction of CFU counts in the presence of the apoptogenic agents ATP and H_2O_2 (Figure 3.1.B and Figure 3.2). The percentage reduction of viable mycobacteria by the four CD4+ clones illustrated in Figures 3.1 and 3.2. was as follows: 42% (±12) for clone 3.2D, 32% (±8) for clone 10.4C, 60% (±13) for clone 14.10C, and 38% (±7) for clone 15.3A. All these values were determined using a clone (effector) to macrophage (target) ratio of 10:1. The apoptogenic agents ATP and H_2O_2 were able to effect levels of viable organism reduction of 57% (±13), and 67% (±8), respectively. The levels of reduction in CFU counts induced by macrophage exposure to both these agents was statistically significant ($P < 0.01$).

The levels of antigen-specific lysis of macrophages were comparable for all 4 clones at an effector to target ratio of 10:1 (34-42%). There was, however, no direct correlation between level of target cell lysis (^{51}Cr-release) and level of CFU reduction (Figure 3.2). The variance in ability of the clones to reduce numbers of viable mycobacterial was therefore not directly

linked to the number of macrophages which were lysed. This finding suggests that the mechanism whereby the clones lysed the infected macrophages may be important in determining mycobactericidal potential.

The CTL clones were themselves not directly mycobactericidal. Direct exposure of the clones to viable mycobacteria did not result in any reduction in CFU counts. Similarly, exposure of intracellular mycobacteria (released from macrophages by SDS treatment) to the clones had no effect on organism viability. Reduction in CFU counts was thus dependent on CTL-mediated lysis of infected macrophages, and was not mediated by clone derived cytokines (Bermudez and Young, 1988; 1992). Inhibition of macrophage apoptosis by brefeldin A (BFA) was found to abrogate the mycobactericidal activity of the clones, a finding which appears to directly implicate target cell death or an associated event with CFU reduction (Section 3.3.6.)

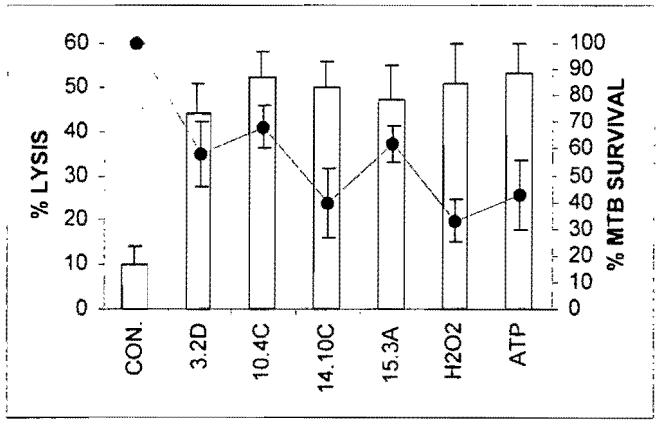


Figure 3.2. Effect of lysis of *M. tuberculosis*-infected macrophages on mycobacterial viabilty. Percentage lysis after exposure to the clones, ATP or H_2O_2 is plotted on the left y axis and is indicated by the bars. Percentage organism survival (100 - % CFU reduction) is plotted on the right y axis and is indicated by the lines. Control illustrates the level of lysis of macrophages in the absence of any treatment, with organism survival of 100%. Data represent 3 experiments showing standard deviation

3.3.2. Patient-derived CD4+-enriched EMNC line, but not PBMNC line, was also mycobactericidal

The finding that effusion-derived CD4+ clones were mycobactericidal led to an assessment of whether the site of origin of the clones (i.e. the pleural cavity) was a determining factor in their unique functional activity. CD4+-enriched EMNC and PBMNC lines were generated from patient material obtained at the time of diagnosis. These lines were then assessed for mycobactericidal activity.

Both EMNC and PBMNC CD4+-enriched lines were cytolytic. Levels of antigen-specific macrophage lysis induced by the two lines is illustrated in Figure 3.3(A). The cytolytic activity of the lines was comparable to that of the clones (mean antigen specific lysis of 25% and 31% at an effector to target ratio of 10:1 for EMNC and PBMNC, respectively). Only the

EMNC cell line was mycobactericidal, reducing organism viability by 41±17%. The PBMNC cell line was unable to reduce mycobacterial viability following lysis of infected macrophages. The number of viable mycobacteria increased marginally during the period of macrophage exposure to the PBMNC line, a pattern observed in control experiments where no CTL cells were added. These findings are illustrated in Figure 3.3(B). The ability of the EMNC line and effusion-derived clones to reduce mycobacterial viability suggests that cells at the sites of infection differ functionally from their counterparts in the peripheral blood.

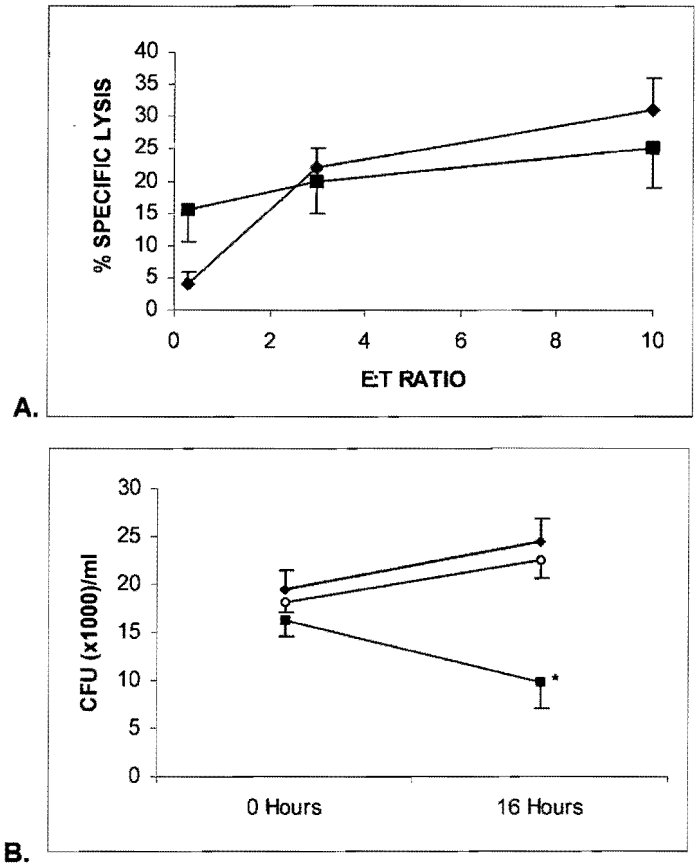


Figure 3.3. Comparison of cytolytic activity of CD4+ enriched EMNC and PBMNC cell lines (A.) and the effect of cell line-induced macrophage lysis on *M. tuberculosis* viability (B.). Mycobacterial antigen-specific lysis is illustrated at 16 hours for the PBMNC line (◆) and EMNC line (■) at 3 effector:target ratios. Mycobacterial viability was assessed by CFU counting before (0 hours) and after (16 hours) exposure to the two cell lines for 16 hours at an effector to target ratio of 10:1. The CFU counts after 16 hours in the presence of the EMNC line were statistically significantly reduced ($P < 0.01$) as compared to control (O) and PBMNC-exposed macrophages (◆). Data are representative of 3 experiments ± standard deviation

3.3.3. Slow kinetics of CD4+ CTL-induced macrophage killing was accompanied by mycobacterial death at the later stages of target cell death

The kinetics of CD4+ CTL clone cytolytic activity was investigated by determining ^{51}Cr-release following macrophage exposure to the clones over a 24 hour time period. The level of macrophage cytolysis was determined at 4, 8, 12, 16, 20, and 24 hours post exposure to the clones. The results are illustrated in Figure 3.4.

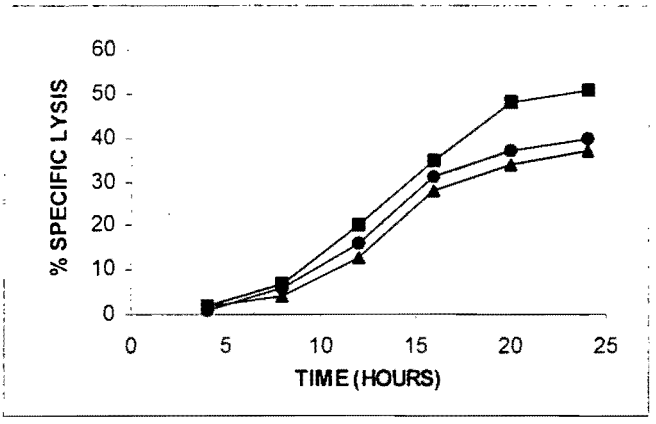


Figure 3.4. Kinetics of ^{51}Cr-release by *M. tuberculosis* infected macrophages following exposure to 3 different clones at an effector:target ratio of 10:1. ^{51}Cr-release was assessed at 4 hourly intervals over a 24 hour period. Clones illustrated include 3.2D (▲), 10.4C (■), and 15.3A (●). Data are representative of 3 experiments. Error bars (SD) omitted for clarity but did not exceed 20% of the mean at time points >12 hours

Maximal ^{51}Cr-release occurred at 24 hours for all the clones examined. The tapering off of the cytolysis curves after 16 hours was accompanied by a progressive increase in the level of non-specific macrophage lysis (spontaneous release). This was probably due to *M. tuberculosis*-induced lysis of macrophages after 40 hours (16 hours of cytolysis assay + 24 hours of labelling) of infection. For this reason 16 hours was used throughout the study in evaluating optimal levels of cytolysis. Low levels of cytolysis were observed after 4 hours (1-2% specific lysis) for all the clones.

Increasing effector to target ratios to 20:1 and 50:1 resulted in a moderate increase in cytolysis levels. For example, for clone 3.2D levels of antigen-specific cytolysis of 28% at 10:1 increased to 33% at 20:1, and 36% at 50:1. Effector to target ratios in excess of 20:1 were not considered physiological since it is unlikely that a single macrophage is exposed to more than 10 CTL in the granulomatous lesion (Dannenberg, 1991) or in the pleural space. In addition, since increased E:T ratios did not have a marked effect on percentage lysis, the E:T ratio was subsequently maintained at a maximum level of 10:1.

The kinetics of killing of *M. tuberculosis* was assessed in parallel experiments over 20 hours. Both ^{3}H-uridine incorporation and CFU counting were used to assess reduction of viable mycobacteria, with both methods giving comparable results. The kinetics of clone 3.2D-

induced killing of mycobacteria is illustrated in Figure 3.5. Similar results were obtained with all the CD4+ CTL clones examined. Levels of reduction of organism viability mediated by clone 3.2D over 20 hours were as follows: 4 hours, 4.0%; 8 hours, 25.1%; 16 hours, 53.1%; 20 hours, 51.2%. No significant increase in the levels of organism killing was observed when effector to target ratios were increased. At an effector to target ratios of 20:1, clone 3.2D induced a 54.6% reduction, and at 50:1 this only increased to 55.9%. This confirms the finding that higher E:T ratios did not significantly increase levels of lysis as measured by ^{51}Cr-release, and would appear to indicate that E:T ratios >10:1 could not significantly enhance the mycobactericidal activity of the clones.

Optimal reduction in viable bacilli as determined by both ^{3}H-uridine incorporation and CFU counting was observed at 16 hours. Although levels of ^{51}Cr-release continued to increase up to 24 hours, no additional organism killing was observed beyond 16 hours (Figure 3.5.). Optimal killing of intracellular organisms (8 to 16 hours) occurred when the increase in ^{51}Cr-release was also maximal, a finding which suggests that mycobactericidal activity was associated with the later events in target cell death such as extensive membrane leakage, and not with the initiation of the death process itself.

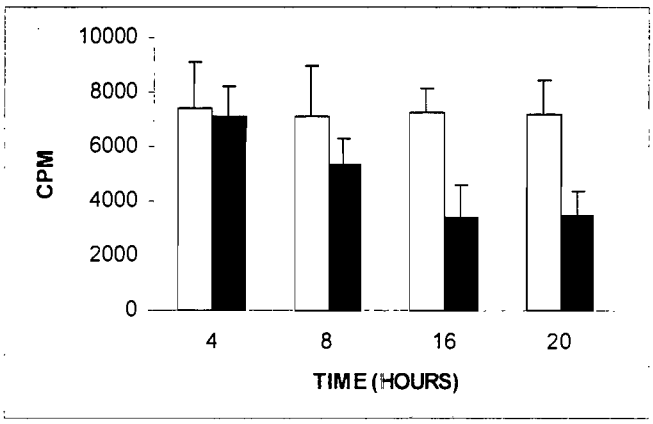


Figure 3.5. Kinetics of *M. tuberculosis* death following exposure of infected macrophages to CD4+ CTL clone 3.2D. Organism viability was determined by ^{3}H-uridine incorporation and data are expressed as counts per minute (cpm). Open bars illustrate numbers of viable intracellular mycobacteria over 20 hours in the absence of any CTL clone. Filled bars illustrate numbers of viable intracellular organisms after exposure to clone 3.2D over 20 hours. Viability of the mycobacteria was determined at 4 hourly intervals. Data are representative of 3 experiments ± standard deviation.

3.3.4. Both ATP and H_2O_2 could induce macrophage lysis and were mycobactericidal

ATP and H_2O_2, agents which have both been reported to induce macrophage apoptosis and to reduce viable intracellular mycobacteria, were used to induce target macrophage cell death (Molloy *et al.*, 1994: Laochumroonvorapong *et al.*, 1996; 1997). The ability of these agents to induce apoptosis was confirmed in the current study (Section 3.3.5). Levels of macrophage lysis induced by ATP (2mM) and H_2O_2 (1mM) as measured by ^{51}Cr-release

were 53±7% and 51±9% of macrophages, respectively (Figure 3.2). As discussed in Section 3.3.1, these levels of lysis were accompanied by mean reduction in CFU values of 57% and 67% respectively. Since both agents induce the same form of cell death (i.e. apoptosis), osmotic lysis (exposure of the cells to H_2O) and heat shock, both of which cause cell necrosis, were also assessed to determine whether the mechanism of cell death was an important determinant of mycobactericidal activity. Osmotic lysis led to the death of >90% of macrophages, but had no effect on organism viability. Similarly heat shock led to the death of >85% of cells, but no effect on organism viability was observed. Necrotic death was confirmed by propidium iodide staining.

Neither ATP nor H_2O_2 were directly mycobactericidal. Control experiments involving SDS lysis of infected macrophages prior to exposure to the apoptogenic agents showed that neither of the agents could directly kill mycobacteria. The mycobactericidal activity of these agents was thus dependent on active participation of the target cell.

3.3.5. CD4+ CTL clones, EMNC and PBMNC lines, ATP and H_2O_2 all induced macrophage apoptosis as assessed by annexin v surface membrane expression

Due to the finding that the induction of apoptosis (but not necrosis) was accompanied by a reduction in viable mycobacteria, the mechanism whereby the CTL clones killed infected macrophages was investigated further. More detailed assessment of cell death was carried out by flow cytometry and fluorescent microscopy of annexin V-propidium iodide- and also TUNEL-stained macrophages. Representative flow cytometric histograms of annexin V and propidium iodide binding by macrophages following exposure to clone 3.2D for 8 hours are illustrated in Figure 3.6.

The annexin V binding by macrophages following exposure to CTL clones was monitored over time in order to establish the kinetics of apoptosis induction. These findings, together with the kinetics of H_2O_2 and ATP-induced apoptosis induction are illustrated in Figure 3.7. The clones showed rapid induction of apoptosis, a finding which was confirmed by TUNEL staining, Table 3.1). The apoptogenic agents demonstrated a slower induction of apoptosis. The kinetic pattern of apoptosis induction by the clones differed from that of ^{51}Cr-release and also of reduction of viable organisms. Although mycobactericidal activity was associated with apoptosis, there was no correlation between the kinetics of apoptosis induction and mycobacterial death.

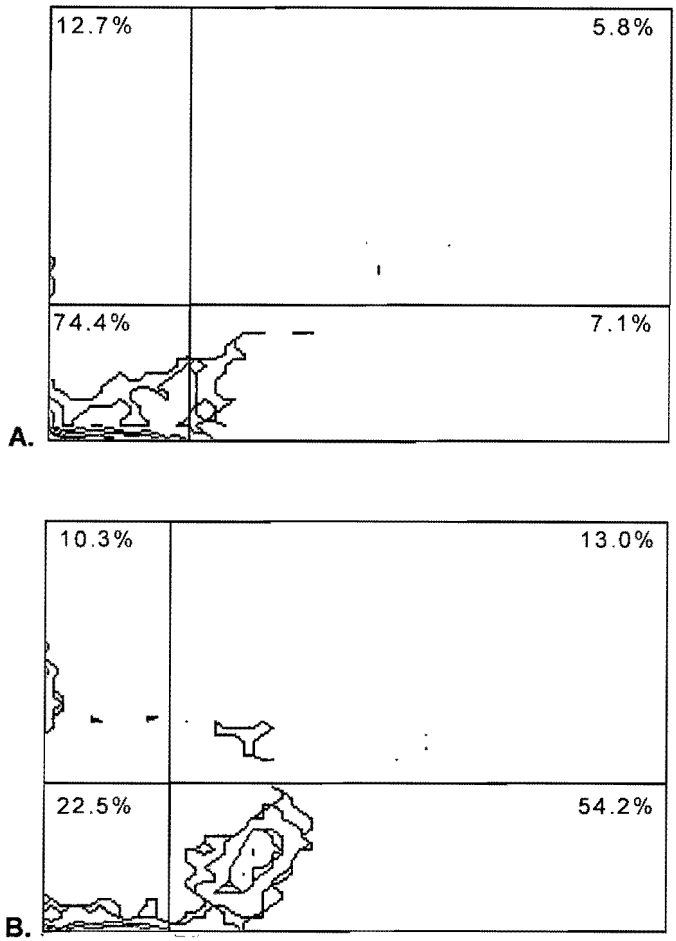


Figure 3.6. Flow cytometric analysis of Annexin V and propidium iodide stained macrophages in the absence (A.), and following exposure to clone 3.2D (E:T ratio = 10:1) for 8 hours (B.). Annexin V binding is plotted on the x-axis, and propidium iodide on the y-axis. The apoptotic population (lower right quadrant) increased from 7.1% to 54.2% following exposure to the clone

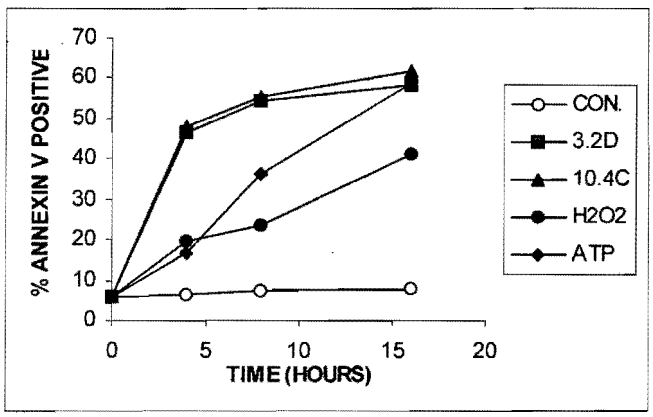


Figure 3.7. Kinetics of macrophage apoptosis induction as determined by comparison of percentage annexin V positive (apoptotic) macrophages at various time points following exposure to CTL clones (3.2D and 10.4C), H_2O_2, or ATP. Macrophages not exposed to clones or cytolytic agents are plotted as control (Con.).

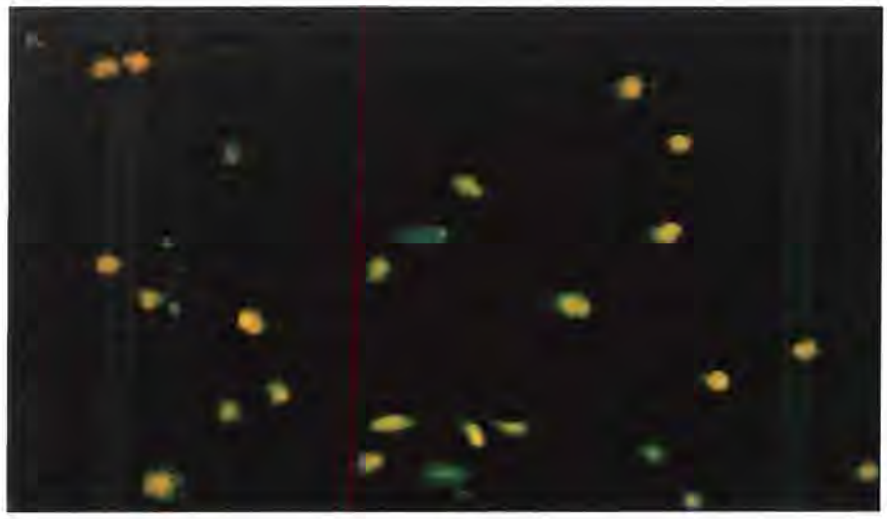
The kinetics of apoptosis induction was also examined by flow cytometric analysis of TUNEL stained cells over a 16-hour time period. The kinetics of apoptosis induction as assessed by these two methods is presented in Table 3.1. Although fairly high levels of non-specific TUNEL positivity was detected by flow cytometry at all time points (34.5-36.6%), only low levels were observed by fluorescent microscopy (Figure 3.8). This discrepancy may have been due to different staining protocols used in the preparation of the cells for the two detection methods. Consistently low levels of non-specific annexin V staining (<10%) were detected by flow cytometry. Despite differences in non-specific staining, levels of both annexin V-binding and TUNEL positivity increased following exposure of the macrophages to the clones or the apoptogenic agents. Comparison of treatment-specific staining (indicated in parentheses in Table 3.1), indicates that although high levels of annexin V staining was detected by 4 hours, TUNEL staining of comparable levels was only observed at 8 hours. This may be due to different kinetics of phosphotidylserine exposure and host cell DNA fragmentation. Annexin V binds to phosphatidyl serine (PS), which is exposed on the cell surface following induction of apoptosis (van Engeland *et al.*, 1996). Normally, PS moieties are restricted to the inner leaflet of the phospholipid bi-layer. On induction of apoptosis there is a loss of this membrane asymmetry, and PS is exposed at the cell surface. TUNEL detects DNA strand breaks, a feature associated with most forms of apoptosis. Labelled nucleotides are linked to DNA at the points of breakage via the enzyme TdT (Gavrieli *et al.*, 1992). Only apoptotic cells containing damaged DNA are labelled using this technique.

How PS exposure is related to DNA degradation is unknown (Zwaal and Schroit, 1997), however it appears that the processes involved in induction of membrane changes and the other events of apoptosis occur independently (Zhuang *et al.*, 1998). Indeed, DNA degradation seems to be caspase-dependent in most systems and involves translocation of signals from the cell membrane to the nucleus (Green, 1998). PS exposure on the other hand is an early event in apoptosis and may be linked to mitochondrial membrane potential changes which have been described as being an important regulatory step in the apoptotic programme (Depraetere and Golstein, 1998). PS exposure generally precedes DNA degradation, which in turn may be reflected in the lower levels of TUNEL positivity.

The levels of annexin V binding by macrophages after exposure to the CD4-enriched EMNC and PBMNC cell lines were also examined. After 8 hours both lines had induced levels of annexin V binding comparable to that seen with the CD4+ clones. This indicates that these lines both induced apoptosis in their target cells.

Table 3.1. Comparison of percentage of macrophage cells stained with Annexin V or by TUNEL at 3 time points following exposure to clones, H_2O_2, and ATP. Control values represent untreated macrophages. Values in parentheses indicate treatment-specific staining (control minus treatment). ND = not determined

	4 Hours		8 Hours		16 Hours	
	Annexin V	TUNEL	Annexin V	TUNEL	Annexin V	TUNEL
Control	6.6	34.5	7.1	34.7	8.0	36.6
ATP	16.5 (9.9)	46.6 (12.1)	54.2 (47.1)	80.2 (45.5)	58.3 (50.3)	83.3 (46.7)
H_2O_2	19.7 (13.1)	45.8 (11.3)	23.3 (16.7)	53.0 (18.3)	41.0 (33.0)	72.4 (35.8)
3.2D	46.3 (39.7)	49.8 (15.3)	54.2 (47.1)	79.2 (44.5)	58.3 (50.3)	ND
10.4C	48.2 (41.6)	45.4 (10.9)	55.3 (48.2)	79.7 (45.0)	61.7 (53.7)	ND
PBMNC	ND	ND	49.7 (42.6)	ND	ND	ND
EMNC	ND	ND	51.3 (44.2)	ND	ND	ND



A.

B.

Figure 3.8. Fluorescent microscopy of TUNEL stained macrophages following exposure to CTL clone 10.4C (A.) or fresh PBMNC (B.) for 8 hours. Apoptosis was observed in the macrophages exposed to the CTL clone, but not fresh PBMNC. Magnification = 400X

3.3.6. Inhibition of target cell apoptosis abrogated mycobactericidal activity of CD4+ CTL clones, ATP and H_2O_2

Brefeldin A has been reported to inhibit target cell apoptosis at both the level of the effector cell and the target cell (Kataoka *et al.*, 1996). In the effector cell BFA inhibits up-regulation of FasL and also transport and release of cytolytic granule contents. At the level of the target cell, BFA inhibits important intracellular transport processes involved in apoptosis, especially those implicated in gross morphological changes such as cell shrinkage and apoptotic body formation. The effect of brefeldin A on macrophage cell death and mycobacterial killing is presented in Table 3.2. Both percentage specific lysis and percentage reduction of viable mycobacteria are shown.

Brefeldin A inhibited lysis of infected macrophages by between 82.4% and 100%. This was accompanied by an abrogation in CFU reduction by between 90.3%and 100%. Levels of inhibition of macrophage lysis in the presence of BFA of 92.5% and 90.2% were observed for ATP- and H_2O_2-mediated cell death, respectively. Complete abrogation of ATP- and H_2O_2-mediated mycobactericidal activity was observed in the presence of BFA.

Table 3.2. Effect of brefeldin A (10μM) on ^{51}Cr-release and annexin V binding by macrophages at various time points following exposure to CTL clones, ATP, or H_2O_2. No reduction in CFU (mean value) indicated by an asterisk (*)

	% Specific lysis (^{51}Cr-release)		% Reduction (CFU)	
	-brefeldin A	+ brefeldin A	- brefeldin A	+ brefeldin A
ATP	53±7	4±4	57±13	-9±15*
H_2O_2	51±9	5±3	67±8	-1±8*
3.2D	34±7	6±3	42±7	1±9
10.4C	44±6	3±2	31±6	3±7
14.10C	39±6	2±2	37±7	-3±10*
15.3A	35±8	0±2	38±6	-12±14*

BFA did not cause loss of cell viability nor did it in any way inhibit the ability of macrophages to present antigen if only added 24 hours after macrophages were exposed to antigen. The viability of BFA-treated macrophages was assessed by trypan blue exclusion. Macrophage viability was consistently >80%. PBMNC treated with BFA after 24 hour exposure to antigen, were still capable of supporting CD4+ CTL clone proliferation. Therefore the inhibitory effect excluded toxicity toward macrophages, inhibition of antigen presentation, or interference with T cell functioning.

3.4. Discussion

CD4+ effusion-derived CTL have been demonstrated to induce apoptosis of *M. tuberculosis*-infected macrophages and to concomitantly reduce the number of viable intracellular mycobacteria. Although an effusion-derived CD4+ enriched line was found to mimic the clones in this mycobactericidal activity, a peripheral blood-derived line did not. These findings suggest that effusion-derived CD4+ cells possess unique mycobactericidal activity which may implicate these cells in organism clearance at disease-associated sites.

The mycobactericidal activity of the CD4+ CTL clones was confirmed by both CFU counting and tritiated uridine incorporation. Although the level of reduction of intracellular organisms varied between the clones, all the clones examined were able to decrease the number of viable intracellular bacteria after 16 hours by at least one third. The levels of reduction in viable organisms effected by the clones were comparable to levels induced by the strongly apoptogenic agents ATP and H_2O_2, as observed in this and other studies (Molloy *et al.*, 1994; Laochumroonvorapong *et al.*, 1996, 1997). The ability of some of the clones to decrease the number of infecting organisms by up to 50% highlighted the potency of this mycobactericidal activity. Any reduction in infecting organism numbers at this level in a localised site would be sufficient to clear the infection if the process were repeated several times (Molloy *et al.*, 1994). In the granuloma, such repeated cycles of infected macrophage lysis may indeed occur. The core of the granuloma is dominated by macrophages containing engulfed mycobacteria (Dannenberg and Rook, 1994). Induction of apoptosis of these infected macrophages by CD4+ CTL, which lie predominantly adjacent to the macrophages in the mantle zone, would result in the creation of apoptotic bodies as well as the possible release of intracellular organisms following secondary necrosis. Subsequent uptake of released organisms and/or apoptotic bodies by neighbouring macrophages could initiate another cycle of CTL-mediated lysis. If each cycle of macrophage lysis was accompanied by a significant reduction of organisms, the activity of the CTL at the disease site could successfully clear the infection.

The mycobactericidal activity mediated by the CD4+ CTL clones was also observed in a CD4+-enriched EMNC line, but not a CD4+-enriched PBMNC line. This finding suggests that the ability of the clones and EMNC line to kill mycobacteria was due to a unique feature of the pleural milieu. The inability of the PBMNC line to reduce numbers of viable organisms confirms the findings of earlier studies, where peripheral blood-derived CD4+ CTL clones had no demonstrable mycobactericidal activity (Fazal *et al.*, 1994; Pithie *et al.*, 1995). Although these studies used heterologous HLA-matched APC, the findings clearly indicated that Cd4+ CTL could not effect any changes in organism viability. The mycobactericidal activity of the clones and EMNC line in the present study was not linked to the level of macrophage cytolysis, since both cell lines displayed similar levels of cytotoxic activity. On the basis of the available data it seems that apoptosis per se can be excluded as the

mediator of mycobacteridal activity. Both of the cell lines as well as the clones induced macrophage apoptosis, as assessed by membrane phosphatidyl serine expression on the cell surface. It remains possible that later events associated with the apoptosis process could be preferentially involved in mycobactericidal events mediated by effusion lines and clones. Any downstream differences would not necessarily be mirrored by differential annexin v expression.

Late events in the macrophage apoptotic death programme were associated with the reduction of viable intracellular organisms. Although apoptosis was initiated rapidly after exposure to the CD4+ CTL clones, as demonstrated by detectable annexin V binding by 4 hours, organism death was optimal at only 16 hours. The kinetics of organism killing therefore correlated with macrophage ^{51}Cr-release rather than with the initiation of apoptosis. The slow kinetics of target cell lysis observed in this study, can be linked to the target cell type involved (adherent macrophages), and the mechanism of death (apoptosis). Necrotic cell death is always rapid since it involves cell swelling accompanied by early loss of cell membrane integrity (Majno and Joris, 1995. A hallmark feature of CD8+ CTL activity has always been the rapid death of target cells by apoptosis (Shresta *et al.*, 1998). This rapidity of apoptotic induction and subsequent cell death has also been observed with CD4+ CTL (Trapani, 1998). Critical factors in the kinetics of target cell death seem to be the type of target cell involved, and the mechanism whereby the CTL induces apoptosis (Trapani, 1998). Many *in vitro* assays of cytotoxity utilise transformed cell lines as targets. These cells divide rapidly, are non-adherent, and generally also die rapidly by apoptosis. Macrophages, on the other hand, are terminally differentiated cells (Cotter *et al.*, 1994). They do not usually replicate following differentiation, and their metabolism is slower than other cell types. Due to these cells not being in an active replicative state cell death following the induction of apoptosis appears to take longer (Hahn *et al.*, 1995). Optimal detection of macrophage cell death by ^{51}Cr-release following induction of apoptosis has previously been reported to be maximal from only 16 hours onwards (Lewis *et al.*, 1995), a finding confirmed in the present study.

The close association between infected macrophage apoptosis and killing of mycobacteria was strengthened by the ability of brefeldin A to inhibit both target cell death and mycobacterial death. Brefeldin A potently inhibited macrophage lysis by the clones and cytolytic agents. This inhibition was accompanied by abrogation of mycobacterial killing. The activity of BFA was not due to toxicity toward the macrophages or T cells, or inhibition of antigen presentation. Without apoptosis, no organism killing was observed. BFA, however, blocks all induction and execution steps of the cytotoxic process (Kataoka *et al.*, 1996). These findings do not, therefore, clarify whether inhibition of granule release from CTL, or inhibition of macrophage apoptosis was the pivotal stage in mycobacterial death.

The finding that effusion- and peripheral blood-derived cells both induced apoptosis confirms that this cell death process was not the sole mediator of organism death. It seems rather that reduction in numbers of viable mycobacteria was dependent on an apoptosis-associated event, resulting in organism death concomitant with the late stages of host cell death. Late events in the apoptotic death programme include cell shrinkage, fusion of different cellular compartments, destruction of organelles, formation of apoptotic bodies, and permeabilisation of the cell membrane (Majno and Joris, 1995; Lincz, 1998). The fusion of cellular compartments, may be an important component of anti-microbial activity. Fusion of mycobacteria-containing phagosomes with lysosomes has been observed in the later stages of apoptotic cell death (Laochumroonvorapong *et al.*, 1997). The fusion of these two cell compartments, a process which is actively prevented by pathogenic mycobacteria under normal conditions (Schaible *et al.*, 1999), would expose the organisms to potent host cell lysosomal enzymes. Although this is an attractive model which may explain apoptosis-related mycobactericidal activity mediated by agents such as ATP and H_2O_2, it does not account for the difference in anti-mycobacterial activity between the two CD4+-enriched cell lines. It remains possible that the CD4+clones and ATP have distinct mechanisms of mycobacterial killing.

Another important late apoptotic event is secondary necrosis-associated membrane permeabilisation (Majno and Joris, 1995). Changes to cell membrane permeability may also result in rupture of the membrane-bound phagosomal compartment. The mycobacteria could then be released into the cytoplasm to be exposed to various toxic cell components. Levels of mycobacterial killing resulting from exposure to cytolytic granule components may be enhanced when apoptotic fragments containing surviving mycobacteria are engulfed by adjacent intact macrophages. The uptake of *M. avium*-containing apoptotic fragments by macrophages has been reported to reduce organism viability (Fratazzi *et al.*, 1997). Surviving organisms are possibly "weakened" after exposure to toxic cell components or cytolytic granule contents. Uptake by newly recruited activated macrophages may be sufficient to kill them. Alternatively, induction of a new cycle of CTL-mediated cytolysis could be envisaged to result in clearance of organisms from the disease site.

Cytolytic granules have been found to contain a range of components, including perforin and granzymes (Page *et al.*, 1998). Although these two proteins have been extensively studied, the activity of most of the granule components has not been clarified. Of particular importance would be exposure to cytolytic granule components such as granulysin, The latter cytolytic granule component has been shown to be directly mycobactericidal (Stenger *et al.*, 1998). If granulysin or a similar mycobactericidal cytolytic granule component were present only the in effusion-derived cell line and clones, it could account for the apoptosis-associated killing of intracellular organisms observed at the late stages of macrophage death. The current study did not address whether granulysin was present in the effusion-derived cells

and clones. However, since mycobacterial antigen-responsive CD8+ CTL utilise granule exocytosis as the mechanism of apoptosis induction, suggests that a similar mechanism may may occur in CD4+ CTL exhibiting comparable mycobactericidal activity. It is likely that granulysin, or a similar as yet undefined component, was present in the effusion-derived cells in concentrations sufficient to kill more than one third of viable organisms.

The effusion-derived CD4+ cells were uniquely mycobacteridal, a finding that suggests that the site of origin was responsible for induction of this activity. Cells initially enter the pleural cavity from the lung, therefore cells in the pleural effusion are representative of cells occuring at the disease site. The pleural effusion of this patient showed a localised expansion of CD4+ cells, increased numbers of antigen-responsive cells, increased numbers of CD4+ memory cells, accelerated kinetics of response to PPD, and accelerated kinetics of induction of cytolytic activity *in vitro* (Chapter 2). All these findings were suggestive of *in vivo* priming. Cells primed *in vivo* by macrophages infected with a virulent clinical strain of *M. tuberculosis* within a unique cytokine milieu could be stimulated to produce unique cytolytic granule components, or to produce higher concentrations of such components. Re-stimulation of these cells *in vitro* may then expand an already unique population of cells. Peripheral blood-derived cells, on the other hand, were not primed *in vivo*, nor were they contained within a unique disease-associated site. Primary stimulation *in vitro* with a laboratory strain of *M. tuberculosis*, and in the absence of a unique cytokine milieu, may be insufficient to initiate production of the unique cytolytic granule component.

The findings discussed in this chapter have defined a novel role for CD4+ T cells at the disease site. The role of these cells in cytokine production for the recruitment and/or activation of other cells is well-defined (Barnes and Modlin, 1996). A probable link between the predominance of CD4+ cells at sites of disease, and the enhanced cytotoxic potential of *ex vivo*-derived cells led to an assessment of the role of cytolytic CD4+ T lymphocytes at the clonal level. The majority of CD4+ T cell clones generated in this study were cytolytic. This cytolytic activity was associated with potent mycobactericidal ability, a finding that implicates CD4+ T cells directly as key mediators of organism clearance from disease sites. The present findings then would extend the function of CD4+ cells beyond cytokine release and macrophage activation activities, to also include CTL associated mycobacterial killing as an important component of the protective cellular immune response at the site of infection.

The phenomenon of different T cell-mediated cytolytic pathways (Fas versus granule release) having different functional roles was investigated further to determine whether a particular killing mechanism could account for mycobactericidal activity. Detailed assessment of the mechanisms of cytotoxicity employed by the cytolytic CD4+ T cell clones, as well as CD4+-enriched lines, and the association between killing mechanism and mycobactericial potential are the subject of Chapter 4.

Chapter 4.

Mycobactericidal CD4+ CTL clones possess dual granule- and Fas-(CD95) mediated mechanisms for killing macrophages

4.1. Introduction

The pivotal role of CD4+ T cells in the successful containment and clearance of infection in TB is well established (Boom, 1996; Kaufmann and Andersen, 1998). The major roles for CD4+ are in the provision of cytokines for macrophage activation, and cytotoxic activity (Boom *et al.*, 1991). Although the importance of helper CD4+ cells in the control of TB is undisputed, the exact role and mechanism of action of CD4+ CTL in this disease remains somewhat speculative (Tan *et al.,* 1997; Schluger and Rom, 1998). However, the fact that *M. tuberculosis*-specific CTL cultured *in vitro* are CD4+ (Ottenhoff and Mutis, 1990) and that CD4+ cells derived from the site of disease display enhanced cytolytic activity (Lorgat *et al.*, 1992), suggest a role for such cells in protective immunity.

Cytolytic T cells are able to kill target cells by either of two major mechanisms, cytolytic granule release and receptor-ligand binding (Kägi *et al.*, 1994; Trapani and Jans, 1999). In cellular immunity, receptor-ligand killing is predominantly mediated by interactions between the Fas receptor (CD95) and its ligand (FasL). Additional receptor-ligand interactions involving TNF-α and its receptor TNF-R1 (Zheng *et al.*, 1995; Wallach *et al.*, 1998) and ATP and its purinergic receptor P2x7/P2z (Lammas *et al.*, 1997) have also been reported.

While CD4+ and CD8+ CTL both utilise these two major mechanisms of killing, granule-exocytosis appears to predominate in CD8+ CTL and Fas-FasL interactions in CD4+ CTL (Henkart, 1994; Podack, 1995; Trapani, 1998). Fas-mediated cytotoxicity is important in immunoregulation and in processes of clonal deletion (Krammer *et al.*, 1994; Winoto, 1997). Deletion of populations of cells via FasL-Fas interactions is less stringently controlled then granule exocytosis. Once an effector cell has been stimulated to express FasL, no subsequent antigen presentation pathways are required. Any cell expressing Fas can then be targeted by another cell expressing FasL (Depraetere and Golstein, 1997; Nagata, 1997). T cell populations, expanded at sites of infection or at the periphery, may in this way be rapidly deleted by one another preventing excessive cellular congestion and swelling (Krammer, 1999). The primary function of Fas-mediated T cell killing therefore seems to be in homeostasis. Granule exocytosis on the other hand appears to have evolved for the elimination of infected or transformed cells (Kägi *et al.*, 1996; Trapani and Jans, 1999). This mechanism of killing is more stringently controlled. Granule release is dependent on antigen presentation by the target cell and subsequent T cell receptor-MHC-antigen interaction is

followed by complex signalling events in the effector CTL culminating in granule release. Potentially detrimental bystander cell killing is minimised in this way (Hahn *et al.*, 1995; Darmon *et al.*, 1999).

Both mechanisms of CTL cytotoxicity result in target cell apoptosis (Shresta *et al.*, 1998). Fas-killing and granule exocytosis merge at the point of caspase cascade activation (Trapani and Jans, 1999). Interestingly, the granule exocytosis mechanism, particularly as mediated by granzyme B, also includes a caspase independent pathway (Sarin *et al.*, 1997). Granzyme B can efficiently cleave substrates downstream of the caspases, and inhibitors of caspase activity do not block apoptosis mediated by this granzyme component (Andrade *et al.*, 1998; Talanian *et al.*, 1997). The caspase-independent mechanism of target cell killing may represent an additional back-up cytolytic pathway circumventing any anti-apoptotic signalling (such as that mediated by bcl-2 and related proteins), and its presence would ensure cytolysis of the target cell (Trapani and Jans, 1999). The anti-apoptotic proteins have been found to operate in numerous apoptotic pathways, including that of Fas ligation, and they may be manipulated by invading organisms such as intracellular bacteria and viruses (Kägi *et al.*, 1996; Smyth and Trapani, 1998). Maintenance of cytolytic activity in the presence of invading organisms is essential for the host, therefore the occurrence of granule-mediated killing which could bypass host control mechanisms would represent an important component of protective immunity (Podack, 1995).

Due to the fact that the majority of CTL kill via either granule exocytosis or Fas-FasL interactions, and since both mechanisms have been implicated in mycobactericidal activity (Stenger *et al.*, 1997; Oddo *et al.*, 1998), the relative contribution of these two pathways to macrophage killing by the CD4+ CTL clones was investigated. The clones were found to possess both Fas- and granule exocytosis--mediated killing mechanisms, however the latter was predominant. Inhibition of either mechanism of killing resulted in both a reduction of target cell lysis and of associated mycobacterial death. Since granule-exocytosis was the major component of CTL killing and organism eradication, it seems that this mechanism may represent an adaption by CD4+ cells to ensure optimal clearance of intracellular organisms. These findings have major implications in prospective immunomodulatory therapies in that enhancement of mycobactericidal killing via manipulation of CTL activity could ensure rapid and efficient removal of infecting organisms.

4.2. Materials and methods

4.2.1. Assessment of the role of calcium (Ca^{2+}) in cytolytic activity of the clones

The importance of Ca^{2+} in the cytolytic activity of the clones was assessed using the Ca^{2+}-chelating agent EGTA (Sigma, St. Louis, MI, USA). Clones were passaged as discussed (Section 3.2.2.), by adding fresh irradiated antigen presenting cells (PBMNC), antigen (*M. tuberculosis* H37Rv) and IL-2. After 72 hours, fresh IL-2 was added, and 48 hours later the clones were used in cytotoxicity assays. Target adherent macrophages were cultured as discussed in Section 3.2.2. Briefly, fresh PBMNC (10^6/ml) were plated (100µl/well) in round bottomed 96-well microtitre plates (Greiner). Following an initial culture period of 96 hours the adherent macrophages were simultaneously infected with H37Rv and labelled with ^{51}Cr overnight. Growth medium containing EGTA was then used to determine the effect of Ca^{2+}-removal on clone-mediated cytolytic activity.

EGTA (6mM) and 3mM $MgCl_2$ (both from Sigma, St. Louis, Mo.) were added to RPMI 1640 medium (EGTA medium). The pH was adjusted to 7.4 and the medium was filter sterilised. The EGTA medium was supplemented with 10% pooled, heat-inactivated, human AB serum in all experiments. The effect of Ca^{2+}-removal by EGTA on cytotoxicity of the clones was examined under standard conditions of effector/clone activation, or following additional activation of the CTL clones using 20ng/ml of PMA and 400nM ionomycin (both from Sigma). Pre-activation with these agents was carried out for 4-6 hours prior to cytolytic assays to ensure optimal up-regulation of co-receptors and activation markers, including FasL. The importance of Ca^{2+} for the expression of FasL was investigated investigated by pre-incubating the clones in EGTA medium for 4 hours prior to cytolytic assays. The effect of prolonged Ca^{2+} deprivation on clonal cytolytic function was then assessed in standard 16 hour cytolytic assays, also in the absence of Ca^{2+}.

Standard 16 hour ^{51}Cr-release cytotoxicity assays were carried out for all experiments, as discussed in Section 3.2.2. Parallel control experiments were carried out in which clone viability was routinely assessed by trypan blue exclusion staining following incubation in EGTA medium for 16 hours. This ensured that any reduction in cytolytic activity was not due to any changes in clone viability.

In order to assess the effect of EGTA (Ca^{2+} removal) on the mycobactericidal potential of the clones, parallel experiments were set up in which H37Rv-infected (non-^{51}Cr-labelled) macrophages were exposed to the clones in the presence or absence of EGTA. Total macrophage-associated organism numbers were determined with or without exposure to the clones. As described in Section 3.2.3., various dilutions of the pooled lysates were plated (10^{-1} to 10^{-8}), and after 3 weeks of culture at 37°C on 7H11 agar plates, CFU numbers were determined.

4.2.2. Detection of cytolytic granule activity in the CTL clones

Cytolytic granule contents were functionally detected by an enzymatic assay for serine protease activity. 100µl of 5 x 10^5/ml activated CTL clones (48 hours after IL-2 restimulation, 120 hours after addition of fresh APC's, antigen, and IL-2) were degranulated by adding 25mM $SrCl_2$ for 10 hours. Degranulation was carried out in 96-well microtitre plates (Greiner) at 37°C. Supernatant (20µl) was then removed and incubated with 35µl of 1mM BLT (Nα-CBZ-L-Lysine Thiobenzyl ester, Sigma), 35µl of 1mM nitrobenzoic acid (Sigma) and 0.1% triton X-100. The reaction mixture was incubated at 37°C for 30 minutes, and absorbance was read at 405nm. Non-cytolytic control cells (U937) were treated in the same manner, allowing comparison of absorbance values between CTL cells and non-cytolytic cells. Additional well containing no cells were used as a negative control. Data are presented as absorbance units for the various clones and the control cell population.

The serine protease (esterase) enzymatic activity assay was also used to assess the level of granule release (degranulation) by the CTL clones following treatment with $SrCl_2$. The serine esterase activity both within the clones (cell lysates) and the supernatant fluid was determined before and after overnight (16 hour) incubation of the clones in the presence of $SrCl_2$. Comparison of levels of enzymatic activity in the two sample groups enabled an evaluation of the extent to which degranulation had occurred (Section 4.2.3.)

4.2.3. Inhibition of granule-mediated cytotoxicity by Cyclosporin A (CsA), concanamycin A (CMA) and strontium ($SrCl_2$)

The chemical and pharmacological agents CsA (Sandoz, Basel, Switzerland), CMA (Wako Chemical Co., Wako, Japan) and strontium ($SrCl_2$) (Sigma) have been reported to inhibit granule-mediated CTL killing in different ways (Trenn *et al.*, 1989; Strack *et al.*, 1990; Kataoka *et al.*, 1996; Neighbour and Huberman, 1982; Stenger *et al.*, 1997). All 3 agents were analysed for their ability to inhibit CTL killing. CsA and CMA were both solubilised in ethanol and stored as stock solutions at -20°C. Stock solutions were diluted in growth medium prior to initiation of cytolytic assays. Strontium was solubilised in sterile distilled water and stored at -20°C. Initially, dose response experiments (based on published reports) were carried out for each inhibitory agent to assess at which concentration optimal inhibition of killing occurred without affecting CTL viability. Viability of the clones prior to cytotoxicity assays was determined using trypan blue exclusion staining. Optimal inhibitory concentrations (CMA 100nM, CsA 500ng/ml, $SrCl_2$ 25mM) were subsequently used for all CTL cytotoxicity assays. Solvent controls were tested for each agent and indicated no effect on cytolytic activity.

Cytotoxicity assays were carried out as discussed in section 3.2.2. In certain experiments CTL clones were pre-activated for 4 hours with PMA and ionomycin, as discussed in section 4.2.1. Inhibition of cytotoxic function by CMA and $SrCl_2$ required pre-treatment of the clones to ensure optimal inactivation or removal of cytolytic granules. CMA pre-incubation was carried

out for 4 hours using a 1mM CMA solution prepared in RPMI 1640 medium. To ensure optimal removal of granule contents by $SrCl_2$, CTL were incubated overnight in the presence of 25mM $SrCl_2$. Viability was always monitored prior to the cytolytic assays by trypan blue staining. After treatment with $SrCl_2$ clones were also assessed for their ability to proliferate to mycobacterial antigen to ensure functional responsiveness following the potentially toxic degranulation treatment. De-granulated and normal clones were tested for their ability to proliferate to *M. tuberculosis* H37Rv as described in Section 2. With all pre-treatments, CTL clones were washed prior to the cytotoxicity assays (pelleted, resuspended in fresh RPMI supplemented with human AB serum), counted, and then incubated for the duration of the assay in the presence of the agent at optimal inhibitory concentration.

^{51}Cr-release was measured as discussed in section 3.2.2. In certain experiments the granule-exocytosis inhibitory agents were used in combination with α-Fas monoclonal antibody ZB4 which inhibits Fas-mediated lysis in order to assess the combinatory effects of the two types of inhibitory agent (Section 4.2.5).

The effect of inhibition of granule exocytosis killing by CsA, CMA and $SrCl_2$ on the mycobactericidal activity of the clones was also determined. Parallel experiments to determine the CFU counts before and after exposure to the clones were carried out as discussed in Section 3.2.3. The effect of inhibition of granule release by these 3 agents on mycobactericidal activity was determined by comparing CFU reduction in the presence or absence of each agent.

The effect of CsA, CMA, and $SrCl_2$ on the cytolytic activity and mycobactericidal potential of the CD4+-enriched PBMNC and EMNC lines was determined as for the clones. The same inhibitory concentrations were used, and the lines were maintained and activated in the same way as the clones.

4.2.4. Assessment of the contribution of the Fas pathway to CTL cytolytic activity

4.2.4.1. Functional detection of FasL on CTL clones

FasL expression was assessed functionally using Jurkat T cells (Vergelli *et al.*, 1997). Jurkat cells constitutively express Fas and are lysed by effector CTL expressing FasL. Prior to using this cell line for cytotoxicity assays, Fas expression was confirmed by flow cytometry. Jurkat cells were stained with 5μl of anti-Fas monoclonal antibody ZB4 (Immunotech, Marseilles, France), or an isotype control (normal mouse anti-human IgG1). Following washing by repeated centrifugation 100μl of goat anti-mouse FITC-conjugated IgG was added. After labelling, excess stain was removed by repeated washing steps. cells were fixed in 1% paraformaldehyde and analysed on a flow cytometer (Coulter Epics profile II). The percentage of Jurkat cells expressing Fas was > 86%.

CTL clones were assessed for their ability to lyse Jurkat cells, a process strictly dependent on the expression of FasL. Cytotoxicity assays were a modification of the standard 16 hour adherent target technique. Actively growing Jurkat cells (24 hours post passaging by dilution into fresh RPMI 1640 medium supplemented with 10% foetal bovine serum (Delta Bioproducts, Isandi, SA)), were labelled with ^{51}Cr (250μCi) for 1 hour at 37°C with regular resuspension to preventing pelleting of the cells. After labelling, the Jurkat cells were washed 3 times in fresh RPMI medium, counted and plated (100μl of 10^6/ml Jurkat cells) in round-bottomed microtitre plates (Greiner). The CTL clones (untreated or PMA / ionomycin activated) were washed, counted, and added to the Jurkat cells at ratios of 10:1, 3:1 and 0.3:1. Plates were incubated for 16 hours at 37°C. Spontaneous release was determined from wells containing Jurkat target cells alone. Maximal release was determined from wells containing Jurkat cells exposed to 1% triton X-100. Supernatants from wells (140μl of total 200μl) were carefully removed following the assay period and counted in a γ-counter. The percent specific lysis of Jurkat cells was calculated as follows: 100 x [(experimental cpm)-(spontaneous cpm)]/[(maximum cpm)-(spontaneous cpm)], where spontaneous cpm was the cpm in the absence of effector cells, and maximum cpm is the cpm in the presence of 1% triton X-100 (Sigma). Spontaneous release was consistently <20%.

The effect of both EGTA and $SrCl_2$ on CTL killing of Jurkat cells was assessed to ensure that granule-mediated killing was not operative in this system and that neither agent could affect Fas-mediated killing. EGTA-medium (6mM EGTA, 3mM $MgCl_2$) was used to determine the effect of EGTA on Jurkat cell lysis. Degranulated CTL clones were exposed to Jurkat cells in the presence of $SrCl_2$ to determine whether this treatment inhibited Fas-mediated killing. Experimental procedures were as described above. Comparison of the % lysis of Jurkat cells in the presence or absence of either EGTA or $SrCl_2$ allowed for determination of the relative contribution of granule-exocytotis killing in this system and the effect of granule-exocytosis inhibition on Fas-mediated lysis.

Anti-Fas monoclonal antibody ZB4 (Immunotec, Marseilles, France) was used to block killing of Jurkat cells. Jurkat cells were pre-incubated with 500ng/ml α-Fas for 1 hour prior to the addition of the CTL clones. Anti-Fas was then maintained in the culture wells for the duration of the assays. High levels of blocking of killing were taken as indicative of Fas being the mediator of lysis in this system.

4.2.4.2. Detection of Fas on macrophages

Fas expression on macrophages was assessed by flow cytometry following indirect labelling with ZB4. The level of Fas expression was monitored over several days (day 1 to day 7) following plating of macrophages in 24-well tissue culture plates (Becton-Dickinson). Macrophages were plated at 10^6 PBMNC/ml, 2ml per well. After incubation for 1-7 days, adherent macrophages were removed by exposure to 0.2% EDTA followed by gentle

scraping using the rubber end of the plunger of a tuberculin syringe. Labelling was carried out using 500ng/ml anti-Fas ZB4 (Immunotech), or an isotype control (IgG1). Following initial labelling, 100µl of FITC-labelled goat anti-mouse IgG (Dako) secondary antibody was used to detect the primary anti-Fas antibody. The labelled cells were fixed in 1% paraformaldhyde prior to analysis on a flow cytometer (Coulter Epics Profile II). The effect of overnight infection with H37Rv (2 organisms per macrophage, 4 x 10^5 CFU/well) on day 4 adherent macrophages was determined in order to assess whether infection (as carried out in standard cytotoxicity assays) had any effect on Fas expression. Antibody labelling of infected cells was carried out as above.

4.2.5. Inhibition of Fas-mediated killing by α-Fas

The effect of blocking Fas-FasL interaction and therefore Fas-mediated CTL killing was assessed by binding anti-Fas blocking monoclonal antibody (ZB4) to the Fas molecules on the surface of the macrophages prior to exposure to CTL clones or lines.

Adherent macrophages were labelled with ^{51}Cr and infected with H37Rv as discussed in previous sections. Prior to the cytotoxicity assay, macrophages were washed and pre-incubated with anti-Fas monoclonal antibody ZB4 (Immunotech, Marseille, France) for 1 hour. Initial dose response experiments to determine optimal blocking were carried out. Subsequently, the optimal blocking concentration (500ng/ml) was used. Control experiments using normal mouse IgG1 (isotype control) were also set up. CTL clones were added to the anti-Fas treated macrophages and standard 16 hour cytotoxicity assays were performed. Levels of ^{51}Cr-release in the presence or absence of anti-Fas were then compared. The functional activity of ZB4 was assessed by determining its ability to block Jurkat cell killing by CTL (section 4.2.4.1.), as well as the ability of ZB4 to block Jurkat cell apoptosis initiated by 1µg/ml of the pro-apoptotic monoclonal antibody CH-11 (Immunotech, Marseille, France).

Anti-Fas antibody (ZB4) was used in combination with CsA, CMA and $SrCl_2$ (as discussed in section 4.2.3.), to determine the combined effect of agents which block both granule exocytosis and Fas-mediated killing.

Parallel experiments to determine the effect of blocking of CTL killing by anti-Fas on the mycobactericidal activities of the clones were also carried out as with the inhibitors of granule exocytosis. Total CFU counts were determined both before and after exposure of the infected macrophages to the clones in the presence of anti-Fas. Total (pooled) contents of triplicate wells were plated onto 7H11 agar plates at various dilutions, as discussed in Section 3.2.3. The inhibitory effect of a combination of anti-Fas and $SrCl_2$ on total CFU counts was also determined.

All experiments on the role of Fas-mediated killing in CTL clone activity were repeated using the CD4+-enriched EMNC and PBMNC lines. All experimental procedures were identical to those used in analysis of clone function. Both the level of inhibition of lysis and the effect on CFU numbers in the presence of anti-Fas were investigated.

The role of Fas in CTL mediated lysis was further addressed by attempts to induce macrophage apoptosis using the apoptogenic anti-Fas monoclonal antibody CH-11 (Immunotech). Adherent macrophages (day 4) were infected with H37Rv and labelled with ^{51}Cr, as discussed previously. The macrophages were exposed to 1μg/ml of CH-11 in RPMI 1640 medium supplemented with human AB serum at the same time other macrophages were exposed to the CTL clones. After 16 hours the level of lysis was calculated as for a standard adherent cytolytic assay. Additional experiments using isotype control antibody (normal mouse IgG1) were set up to exclude possible non-specific antibody-mediated effects. Inhibition of anti-Fas induced lysis by the blocking antibody (ZB4) was determined by pre-incubating adherent macrophages with ZB4 for 1 hour prior to exposure to CH-11. The effect of cycloheximide on Fas-induced lysis of macrophages is discussed below (Section 4.2.8.).

4.2.6. Assessment of the role of ATP and TNF-α in CTL clone activity

In order to determine whether ATP or TNF-α played any role in CTL clone mediated cytotoxicity, the effect of blocking of macrophage lysis induced by these agents was investigated.

For ATP-mediated lysis, day 4 macrophages were labelled with 6μCi/well of ^{51}Cr, as for standard cytotoxicity assays. On day 5, the labelled macrophages were washed and exposed to 2mM ATP in RPMI 1640 supplemented with 10% human AB serum for 90 minutes. The macrophages were then incubated for 16 hours and the level of lysis determined as for a standard ^{51}Cr-release assay. ATP-mediated lysis of macrophages was blocked using the enzyme hexokinase (Sigma). Initial dose response experiments determined optimal blocking concentration at 2U/ml (i.e. optimal inhibition of ATP-induced lysis). This dose of hexokinase was then used in CTL clone assays to determine whether ATP-mediated lysis was operative, and to what extent. The effect of the addition of hexokinase to cytolytic assays was determined by comparing the amount of ^{51}Cr-release in the presence or absence of the enzyme.

TNF-α-mediated macrophage lysis was also assessed in this way, with anti-TNF-α antibody (Promega). A range of concentrations of TNF-α was used to induce macrophage lysis, however poor lysis at all concentrations (maximal 10%) was observed. To ensure that TNF-α was functionally active, U937 cells were exposed to the cytokine at the same concentrations that the macrophages were. Both macrophage lysis and U937 lysis induced by TNF-α was blocked using anti-TNF-α monoclonal antibody (Genzyme, Cambridge, MA, USA). ^{51}Cr-

labelled macrophages were pre-incubated with 500ng/ml of anti-TNF-α. Throughout the assay period (16 hours) the antibody was retained, and levels of macrophage lysis could then be compared in the presence or absence of the blocking antibody. Further experiments which address the effect of CHX on TNF-α-mediated lysis of macrophages are discussed in Section 4.2.8.

4.2.7. Assessment of the role of HLA-DR and CD1 molecules in clone cytolytic function

HLA-DR restriction of the clones was assessed using the antibody L243. Proliferation of clones in response to *M. tuberculosis* H37Rv-infection was examined with and without the anti-HLA-DR antibody L243. A dose response using mouse ascites L243 indicated strong inhibition of proliferation at dilutions up to 10^{-6}. Clone proliferation assays were carried out as described in section 2.2.9. L243 antibody, or isotypic control antibody (IgG1), was added to the PBMNC 1 hour prior to the addition of the clones. Tritiated thymidine incorporation was assessed after 2 days as described above.

CD1 expression on both infected and uninfected adherent macrophages was assessed using anti-CD1a, b, and c monoclonal antibodies (Immunotech, Marseille, France). Macrophages were examined on day 4-7 of culture, with and without overnight infection with H37Rv (at 2 organisms per macrophage). Indirect labelling using anti-Cd1a, b, and c monoclonal antibodies or isotypic controls (mouse IgG1) was carried out. A range of antibody concentrations were used (1ng/ml - 1μg/ml). The binding of anti-CD1 molecules to the macrophages was detected by a secondary FITC conjugated goat anti-mouse antibody (Dako). Levels of FITC binding were then determined by flow cytometry. The effect of IL-4 (4mM) and GM-CSF (4mM) (Porcelli *et al.*, 1992) on CD1 expression was also examined.

4.2.8. Effect of cycloheximide-mediated inhibition of protein synthesis on macrophage killing by CTL clones

CHX was assessed for its affect on clone-mediated lysis of infected macrophages. CHX was solubilised in methanol and stored at -20°C. Stock solution was diluted into growth medium prior to cytolytic assays. In standard cytotoxicity assays, CHX (1mM) was added to microtitre plate wells containing ^{51}Cr-labelled and H37Rv-infected macrophages, prior to the addition of the clones. The level of lysis in the presence or absence of CHX was compared. Solvent controls were included, with no residual effect on cytolysis being observed. The effect of CHX on TNF-α- and anti-Fas-mediated lysis was also investigated by adding CHX (1mM) to the adherent macrophages prior to exposure to these apoptogenic agents.

Enhancement of CTL clone killing of macrophages was further investigated at the level of mycobactericidal activity. Total CFU counts were monitored before and after exposure to the clones or the apoptogenic agents in the presence and absence of CHX. Whether a particular component of clone cytotoxicity (Fas- or granule-mediated) was linked to the enhanced

cytotoxicity in the presence of CHX was assessed by determining levels of CTL killing inhibition using anti-Fas and $SrCl_2$. The inhibition assays were carried out as described in sections 4.2.3. and 4.2.5.

4.3. Results

4.3.1. CTL clone-mediated lysis of infected macrophages was calcium-dependent

The effect of Ca^{2+} on the cytolytic activity of the CD4+ CTL clones was assessed using the Ca^{2+} chelating agent, EGTA. EGTA-mediated inhibition of infected macrophage killing by clone 3.2D is presented in Figure 4.1. Similar patterns of inhibition were observed in all the clones examined (n = 6).

EGTA strongly inhibited clone-mediated killing. Antigen-specific lysis of 31±6% mediated by clone 3.2D was reduced to 5±4% in the presence of EGTA at an effector to target ratio of 10:1. This was equivalent to an inhibition of 84%. Pre-treatment of the clones with PMA and ionomycin had little effect on the cytolytic potential of the clones (increasing clone-mediated lysis by only 4% at a 10:1 effector to target ratio). Inhibition of killing by EGTA was not affected by the PMA/ionomycin pre-treatment protocol. Levels of EGTA inhibition following pre-activation were comparable to levels observed in the absence of pre-treatment (83% and 84%, respectively). Pre-incubation of the clones with EGTA for 4 hours prior to the assay led to complete abrogation of killing. Incubating of clones with EGTA prior to the CTL assays may have affected both killing pathways (granule- and Fas-dependent), since Ca^{2+} is required for up-regulation of FasL expression on the cell surface, as well as for maturation, recruitment and release of cytolytic granules (Trapani, 1998).

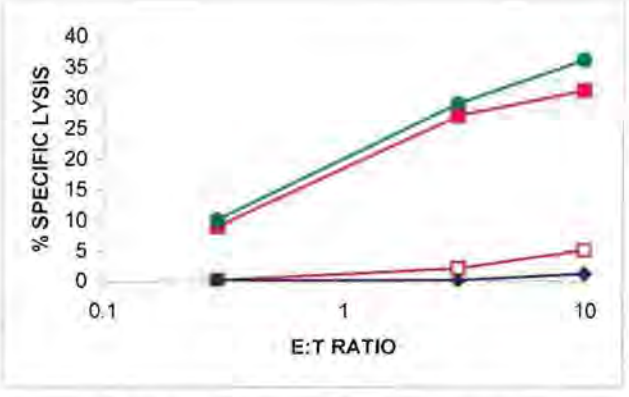


Figure 4.1. The effect of EGTA on CD4+ CTL clone 3.2D-mediated lysis of infected macrophages. H37Rv antigen-specific lysis (■) was strongly inhibited in the presence of EGTA (□). PMA and ionomycin pre-activation did not significantly enhance clone-mediated macrophage lysis (●). Pre-treatment of the clones with EGTA prior to the cytolytic assay led to complete abrogation of killing (◆). Data represent mean values of 3 experiments

Residual killing represented the Fas-FasL pathway (Section 4.3.4.3). EGTA had minimal effect on Fas-mediated killing if added at the beginning of cytolytic assays, but prior incubation for 4 hours resulted in inhibition of this component of CTL cytotoxic activity. This was probably due to the requirement for Ca^{2+} for persistent FasL up-regulation. Removal of Ca^{2+} was therefore unable to affect Fas-FasL-mediated cytolytic potential if CTL were

optimally activated to express FasL. Lack of EGTA impact on Fas-FasL interactions was confirmed by the inability of EGTA to inhibit clone killing of Jurkat cells (discussed in Section 4.3.4.3.). The strong inhibition of CD4+ CTL clone killing by EGTA therefore suggested that the majority of killing was attributable to Ca^{2+}-dependent cytolytic granule release.

4.3.2. CD4+ CTL clones possessed serine protease (esterase) activity

Cytolytic granule activity was functionally demonstrated using an assay for BLT-esterase enzyme activity (Trapani, 1998). Since granzymes (which ar. serine proteases) are the most common constituent of cytolytic granules, assays for their specific activity are commonly performed as evidence for their presence within a particular cell type.

The presence of cytolytic granule serine proteases (BLT-esterases) was determined by enzymatic assay for BLT-esterase activity (Stenger *et al.*, 1997). The absorbance values (relative activity) of 4 clones are presented in Figure 4.2. U937, a non-cytolytic myelomonocytic cell line, was used as an additional negative control. Cytolytic granule activity was present in all the clones, but not in the U937 cells. The clones gave absorbance readings of between 3.6 and 4.9 fold higher than U937 cells, which was indicative of enzymatic activity. The absorbance readings generated by the clones were significantly different to those observed in the U937 cells ($P < 0.01$). The discrepancy between U937 absorbance readings, and those in the absence of any cells (0.07 and 0.01, respectively) may have been due to interference by other enzymatic pathways in the U937 cells. The presence of cytolytic granules is consistent with CTL activity involving granule exocytosis.

Patient-derived CD4+-enriched EMNC and PBMNC lines both possessed serine protease activity. Absorbance readings of 0.32 ± 0.06 and 0.26 ± 0.07 were obtained for the EMNC and PBMNC lines, respectively. These values are comparable to those generated by the clones.

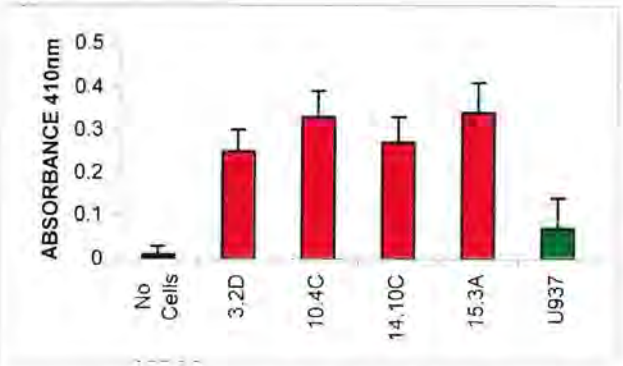


Figure 4.2. Serine protease (BLT-esterase) activity of 4 clones compared to U937. The negative control (no cells) consisted of growth medium treated in the same way as the clones and the U937 cells. Absorbance readings for the clones varied from 0.25 to 0.34. U937 cells gave an absorbance reading of 0.07. Data represent mean values of 3 experiments showing standard deviations

4.3.3. Inhibition of CTL clone granule-mediated cytotoxicity by CsA, CMA and $SrCl_2$

The role of granule exocytosis was investigated using chemical and pharmacological agents previously shown to inhibit this pathway. Three agents, cyclosporin A (CsA), concanamycin A (CMA), and strontium ($SrCl_2$), each of which inhibits cytolytic granule-mediated killing in a different way, were used to assess the involvement of this killing pathway.

Representative profiles of CsA inhibition of cytolysis for 3 clones are illustrated in Figure 4.3. CsA inhibits cytolytic granule release (exocytosis) by blocking granule transport and release through inactivation of calmodulin binding protein (Trenn *et al.*, 1989). Mean levels of inhibition of clone-mediated cytolysis between 51.5% and 74.3% were observed for the 3 clones illustrated at an effector to target ratio of 10:1 (n = 4).

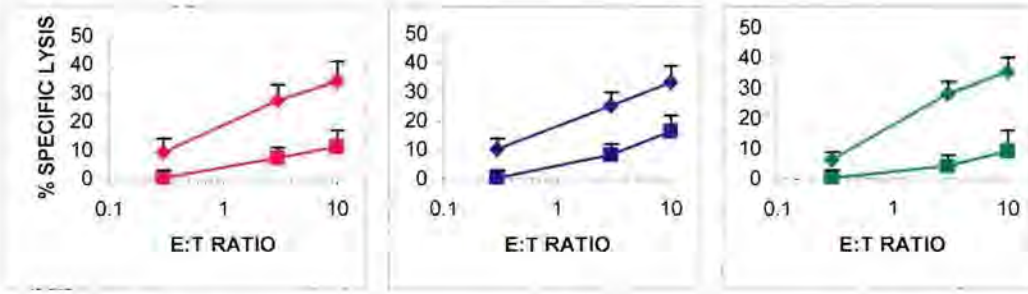


Figure 4.3. CsA-mediated inhibition of macrophage lysis by 3 CD4+ CTL clones. Percent antigen specific (H37Rv) macrophage lysis by the clones following 16 hours of incubation in the presence (■) or absence (◆) of CsA are indicated. Data represent mean values of 4 experiments showing standard deviations

Representative profiles of CMA inhibition of cytolysis for 3 clones are illustrated in Figure 4.4. CMA is an agent which prevents maturation of cytolytic granules by inhibiting vesicle acidification by blocking H^+-ATPase activity (Kataoka *et al*, 1994; 1996). Pre-treatment of

clones with CMA for 4 hours, followed by the continued exposure to CMA for the duration of the cytolytic assay (16 hours), led to mean inhibition of levels of between 39.4% and 54.3% at an effector to target ratio of 10:1 for the 3 clones illustrated. Inhibition of cytolysis by CMA was not as strong as observed with CsA.

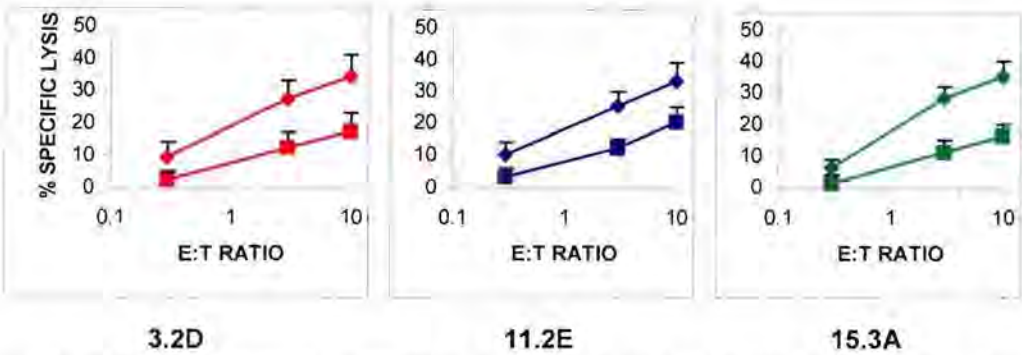


Figure 4.4. CMA inhibition of macrophage lysis mediated by 3 CD4+ CTL clones. Percent specific (H37Rv) macrophage lysis by the clones following 16 hours of incubation in the presence (■) or absence (◆) of CMA are indicated. Data represent mean values of 4 experiments showing standard deviations.

Representative profiles of $SrCl_2$ inhibition of cytolysis for 3 clones are illustrated in Figure 4.5. $SrCl_2$ induces degranulation of cytotoxic cells (Neighbour and Huberman, 1982; Stenger *et al.*, 1997). This agent had the strongest inhibitory effect on clone-mediated macrophage killing. $SrCl_2$-induced degranulation led to mean inhibition of levels of killing between 72.7% and 80.0% at an effector to target ratio of 10:1 for the 3 clones illustrated.

Due to the high concentration $SrCl_2$ required for optimal degranulation, both viability and proliferative responses of the clones were assessed following the overnight treatment. Minimal viability loss was detected by trypan blue staining following overnight degranulation. There was also only minimal interference in clone proliferative responses with and without degranulation treatment. Normal delta cpm values (cpm in the presence of H37Rv minus cpm in the absence of antigen) for clones 3.2D and 15.3A were 13309 ± 2407 and 11947 ± 1694. Following degranulation overnight, the values were 11441 ± 3106 and 10843 ± 2407, respectively. This illustrates that $SrCl_2$ did not affect normal physiological functioning of the clones.

To ensure that $SrCl_2$ treatment resulted in complete removal of cytolytic granules, functional granule activity was determined by serine protease enzymatic activity assays both before and after degranulation. Absorbance readings at 405nm following $SrCl_2$ treatment were consistently below 0.06 (as compared to absorbances above 0.25 prior to treatment). Levels of serine protease activity after degranulation were similar to those detected in the non-cytolytic U937 cell line, which confirmed that cytolytic granules had been removed.

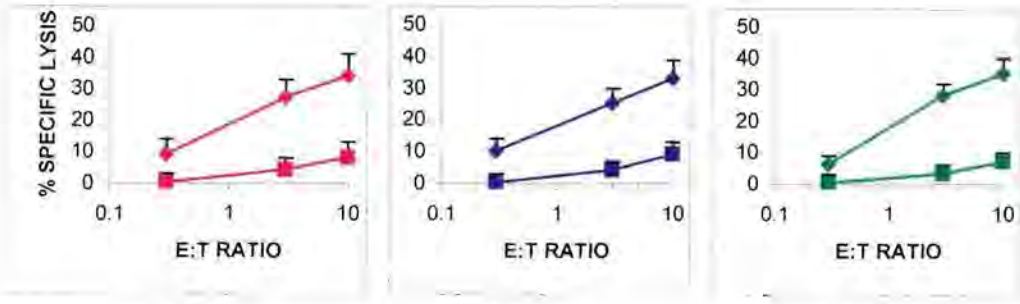


Figure 4.5. $SrCl_2$ inhibition of macrophage lysis mediated by 3 CD4+ CTL clones. Percent specific (H37Rv) macrophage lysis by the clones following 16 hours of incubation in the presence (■) or absence (◆) of $SrCl_2$ are indicated. Data represent mean values of 4 experiments showing standard deviation

The inhibition of the granule-mediated pathway of killing by the agents CsA, CMA and $SrCl_2$ confirmed that this mechanism was predominant in the CD4+ CTL clones. Inhibition by strontium was greater than that observed using either CsA or CMA, but did not reach maximal levels observed with EGTA. A hierarchy of inhibition by various agents was thus established, with EGTA-mediated inhibition being strongest, followed by inhibition mediated by $SrCl_2$, CsA and CMA.

All the agents which inhibit granule exocytosis showed strong inhibition of clone-mediated cytolysis. Although inhibition varied from approximately 40% to 70%, the presence of large granule-mediated component of killing was clearly illustrated. The importance of this component of killing in the mediation of mycobactericidal activity was investigated further (Section 4.3.9). A comparison of the levels of inhibition of CTL clone-mediated macrophage killing by the various agents and the effects on mycobacterial viability is presented in tabular form (Table 4.2).

4.3.4. Assessment of the contribution of Fas-mediated killing

4.3.4.1. Flow cytometric analysis of Fas expression on macrophages

The expression of Fas on the surface of cultured macrophages was assessed by flow cytometry. Levels of expression were measured over several days, and the effect of infection with *M. tuberculosis* H37Rv on Fas expression was also determined. Infection with H37Rv was found to decrease Fas expression at all time points assessed. Optimal expression of Fas was detected on days 5 and 6 of culture. The data are presented in Table 4.1, and indicate that a significant proportion of macrophages expressed the Fas receptor on their surfaces. Levels of Fas expression of >30% were detected after *M. tuberculosis* infection at day 5, the time point at which cytolytic assays were initiated, which indicated that macrophages were susceptible to the Fas-FasL-mediated cytolytic pathway. The role of this pathway in clone cytolytic activity was thus investigated further.

Table 4.1. Expression of Fas (CD95) on adherent macrophages.

Time (days)	Control	H37Rv-infected 24 hours
1	12.2	15.3
2	24.3	22.5
3	33.4	25.8
4	41.2	30.5
5	43.9	32.4
6	43.5	31.2

Data illustrate the percentage of cells expressing Fas as determined by flow cytometry. Macrophages were assessed for Fas expression from 1 day up to 6 days after plating. The effect of infection of macrophages with H37Rv for 24 hours prior to assay time point is also indicated.

4.3.4.2. Functional assessment of FasL expression on CTL clones

FasL expression was demonstrated functionally by assessing the ability of the CTL clones to lyse Jurkat cells (Vergelli *et al.*, 1997). Prior to these functional assays, the level of Fas expression on Jurkat cells was determined by flow cytometry following indirect labelling with anti-Fas. The level of Fas expression was 86 ± 11% (n = 3).

CTL clones showed strong cytolytic activity of 56-62% against the Jurkat target cells (Figure 4.6.). Inhibition of Jurkat killing with anti-Fas blocking antibody (ZB4) was variable, with maximal blocking of lysis of 63% being observed with clone 15.3A at an effector to target ratio of 10:1. The range of inhibition of cytolysis for all 3 clones was 52% to 63%. Pre-treatment of the clones with PMA and ionomycin did not significantly affect the level of lysis observed, which suggests that the clones were optimally expressing FasL at the time of the cytolytic assays.

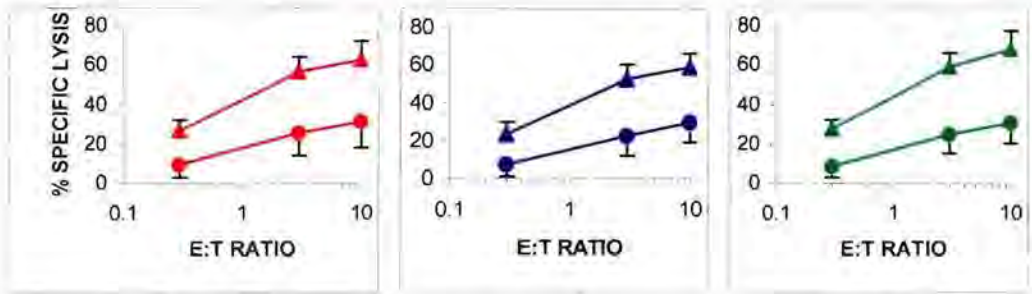


Figure 4.6. Lysis of Jurkat cells by 3 CD4+ CTL clones, and the inhibitory effect of anti-Fas. Percentage specific lysis in the absence (▲) and presence (●) of anti-Fas are illustrated. Data are representative of mean values of 3 experiments showing standard deviations

4.3.4.3. Fas-mediated killing was not affected by EGTA or $SrCl_2$

The effect of EGTA on clone 3.2D-mediated lysis of Jurkat cells was evaluated, and results are presented in Figure 4.7. EGTA did not inhibit killing of the Jurkat cells by the CTL clones.

Although treatment with $SrCl_2$ showed optimal inhibition of lysis of macrophages by the CTL clones (Figure 4.5.), this agent had no effect on Fas-dependent Jurkat cell lysis. Taken together, these findings indicate that Jurkat cell lysis was exclusively dependent on Fas-FasL interactions.

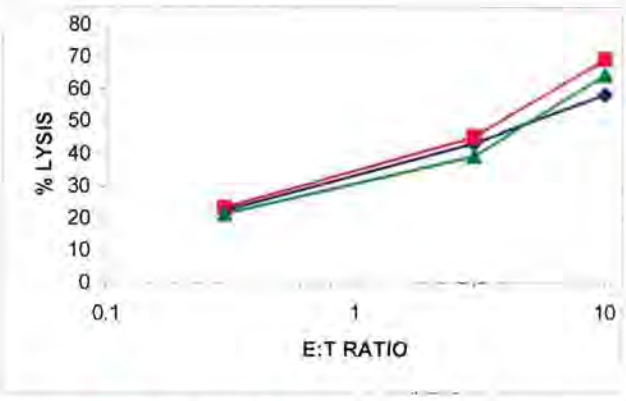


Figure 4.7. Effect of EGTA (■) and $SrCl_2$ (▲) on lysis of Jurkat cells by CD4+ CTL clone 3.2D (◆). Percent lysis was calculated after 16 hours. Spontaneous release of ^{51}Cr was <20% in all experiments. Data represent mean values 3 experiments. (Error bars showing standard deviation omitted for clarity, at 10:1 standard deviation was < 10% of the mean for all data points)

4.3.5. Assessment of the contribution of the Fas pathway in CD4+ CTL clone-mediated lysis of macrophages

Having shown target cell Fas expression and clone FasL expression, the contribution of Fas-FasL interactions in CD4+ CTL killing of infected macrophages was assessed. The inhibitory anti-Fas monoclonal antibody (ZB4) was used to determine the level of Fas-FasL involvement in target cell death. The effect of binding of anti-Fas (ZB4) to macrophage Fas receptors on the cytotoxic activity of the CTL clones is presented in Figure 4.8. The functional activity of ZB4 was verified by examining its ability to block the ability of the pro-apoptotic CH-11 anti-Fas antibody to induce macrophage apoptosis in the presence of CHX (Section 4.3.11). Levels of inhibition of lysis at a 10:1 effector to target ratio varied between and 19% and 24% for the 3 clones illustrated. Maximal inhibition by anti-Fas (clone 10.4C, 24.4%) was significantly lower than inhibition by the various agents which block granule-mediated killing. No inhibition was observed when isotypic IgG1 control antibodies were added to the infected macrophages prior to the cytolytic assays.

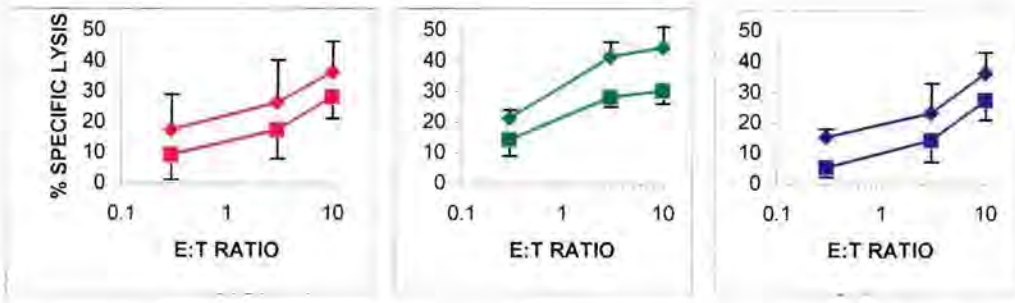


Figure 4.8. Effect of anti-Fas antibody ZB4 (500ng/ml) on CTL clone-mediated lysis of H37Rv-infected macrophages. 3 clones are illustrated at 3 effector to target ratios. Data are representative of mean values of 3 experiments showing standard deviation.

4.3.6. Inhibition of both granule exocytosis- and Fas-mediated pathways led to complete abrogation of macrophage lysis

Detection of both granule- and Fas-mediated killing pathways in the CTL clones prompted an investigation into whether these two pathways accounted for all CTL cytolytic activity. Simultaneous inhibition of granule exocytosis and Fas-mediated killing by a combination of anti-Fas and $SrCl_2$ resulted in almost complete abrogation of clone cytolytic function as shown for clone 15.3A in Figure 4.9. This pattern of abrogation of cytolytic activity in the presence of these two agents was observed in all clones analysed (n = 6), with mean levels of inhibition of cytolysis >93% consistently being detected. These data confirm that these two major mechanisms of lysis accounted for all CTL-mediated lysis observed. TNF-α- and ATP-mediated alternative pathways of cytolysis were also evaluated by the ability of inhibitory agents to affect CTL clone cytolytic activity (Sections 4.3.8.).

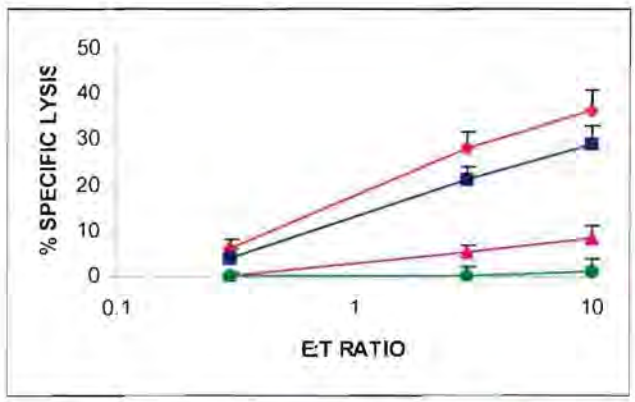


Figure 4.9. Effect of anti-Fas (500ng/ml) (■), $SrCl_2$ (25mM) (▲), and a combination of both (●) on H37Rv-infected macrophage lysis by clone 15.3A (◆). Mean percentage antigen-specific lysis at 3 effector to target ratios is illustrated. Standard deviation values are shown as error bars. Data represent 3 experiments

4.3.7. CD4+ CTL clones were MHC class II- (HLA-DR-) restricted

CD4+ T lymphocytes are classically MHC class II-restricted. However, due to reports of mycobactericidal CD8+ CTL being CD1-restricted (Stenger et al., 1997), the involvement of MHC class II-restricted antigen presentation was investigated. All the cytolytic CD4+ clones examined were MHC class II-, HLA-DR-restricted in their activity. Mouse anti-human HLA-DR antibody (L243) was able to inhibit CTL clone proliferative responses to H37Rv by >96% (n = 6). This complete abrogation of proliferation of the clones in the presence of L243 indicated that the clones were HLA-DR-restricted. No effect on clone proliferation was observed when an isotypic control (mouse IgG1) antibody was used. Since the clones were MHC class II-restricted, a role for CD1 molecules in clone activity was excluded. This was confirmed by an inability to detect CD1 molecules on *M. tuberculosis* H37Rv-infected monocyte derived-macrophages, even after treatment of macrophages with GM-CSF (4mM) and IL-4 (4mM), which has been reported to up-regulate CD1 expression (Porcelli *et al.*, 1992).

4.3.8. ATP and TNF-α were not components of CD4+ CTL clone killing machinery

Both ATP (Zheng *et al.*, 1991; Blanchard *et al.*, 1995) and TNF-α (Laster *et al.*, 1988) have been implicated in the lysis of macrophages via induction of apoptosis. In addition, both these molecules have been implicated in apoptosis of mycobacteria infected macrophages (Lammas *et al.*, 1997; Keane *et al.*, 1997), and in mycobactericidal activity (Kindler *et al.*, 1989; Bermudez and Young, 1992; Molloy *et al.*, 1994). The ability of ATP to induce apoptotic cell death in macrophages and to reduce mycobacterial viability has been confirmed in various experiments in the present study (Sections 3.3.1., 3.3.5., and 3.3.6.). In order to assess the contribution of these molecules in the mycobactericidal killing of the CD4+ CTL clones, inhibitors of both pathways (i.e. hexokinase and anti-TNF-α) were used.

ATP was evaluated for its ability to lyse macrophages at various concentrations. Optimal induction of macrophage lysis (53 ± 5%) at a concentration of 2.5 mM was observed (Figure 4.10). Hexokinase (Sigma) which catalyses the conversion of ATP to ADP was then assessed for its inhibitory effect on ATP-mediated lysis (Blanchard *et al.*, 1995).

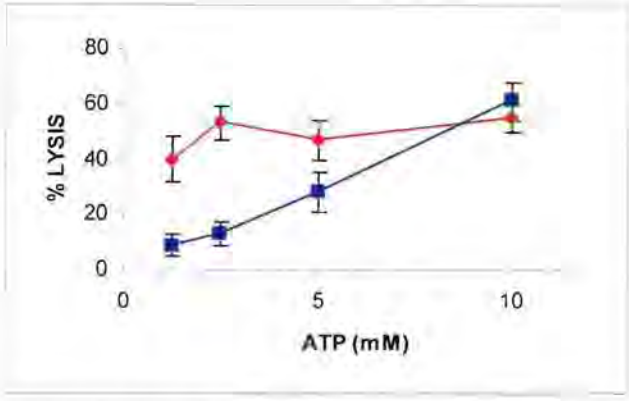


Figure 4.10. ATP-mediated lysis of adherent (day 6) macrophages as determined by ^{51}Cr-release. The level of lysis at various concentrations of ATP is illustrated (◆). The effect of hexokinase (2U/ml) on ATP-mediated lysis at the same concentrations is also indicated (■). Optimal lysis was observed at an ATP concentration of 2.5mM. Data represent mean values of 3 experiments showing standard deviation

Optimal inhibition of lysis (75%) induced by 2.5mM ATP was observed in the presence of 2U/ml of hexokinase (53% lysis in the absence of kexokinase, reduced to 13% lysis). The same concentration of hexokinase was then used to determine whether ATP played any role in CD4+ CTL clone-mediated lysis (Figure 4.11). The figure illustrates the inhibitory effect of hexokinase on clone-mediated lysis of H37Rv-infected macrophages. No inhibition of clone mediated macrophage lysis was observed for any of the clones tested. The addition of hexokinase prior to the addition of clones to the CTL assays ensured inhibition of all ATP-mediated activity. These results therefore exclude a role for ATP in CD4+ clone-mediated cytolysis.

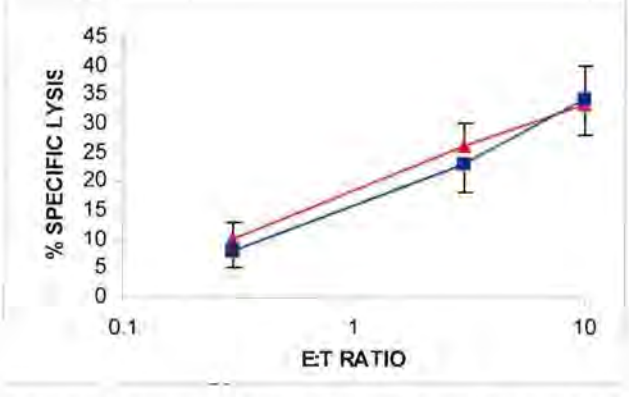


Figure 4.11. Effect of hexokinase on clone 3.2D-mediated lysis of infected macrophages. Lysis in the absence of hexokinase (▲) was not inhibited by the addition of 2U/ml of hexokinase (■). Data are representative of mean values of 3 experiments showing standard deviation.

Induction of macrophage lysis by TNF-α (Genzyme, Cambridge, MA, USA) was initially assessed at various concentrations. Optimal lysis was detected using 100ng/ml of TNF-α. Inhibition of TNF-α-mediated lysis was then observed using 1μg/ml anti-TNF-α monoclonal

antibody (Genzyme, Cambridge, MA, USA). Even though levels of lysis as determined by ^{51}Cr-release were poor (7 ± 4%), anti-TNF-α abrogated this lysis (down to 2 ± 3%, or mean inhibition of 71%). The low levels of induction observed suggested minimal involvement of TNF-α in macrophage lysis. The low level of susceptibility of infected macrophages to TNF-α may have been due to low receptor expression. Alternatively, infected macrophages may have secreted soluble TNF receptors, which would have abrogated any TNF-α-mediated effects. Due to the low level of detection of TNF-α killing, additional experiments were carried out using U937 cells which are susceptible to TNF-α-induced apoptosis. TNF-α was able to cause lysis of 42 ± 6% of these cells. In the presence of anti-TNF-α only 12 ± 5% of cells were lysed, which represents an inhibition of 71%. (Figure 4.12).

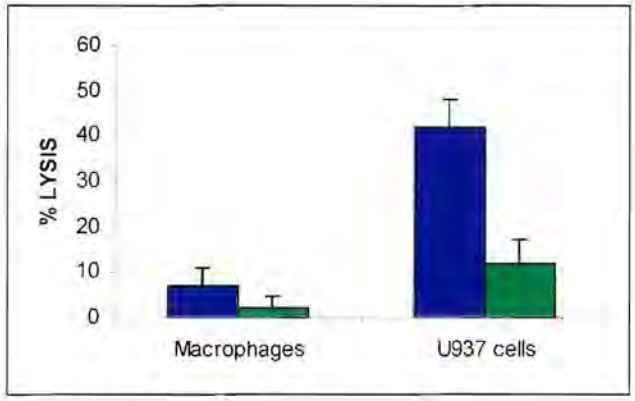


Figure 4.12. TNF-α-mediated lysis of adherent macrophages and U937 cells. Blue bars illustrate the level of ^{51}Cr-release in the presence of 100ng/ml of TNF-α. Green bars illustrate the level of ^{51}Cr-release with the addition of 1μg/ml of anti-TNF-α monoclonal antibody. Data are representative of mean values of 3 experiments showing standard deviations

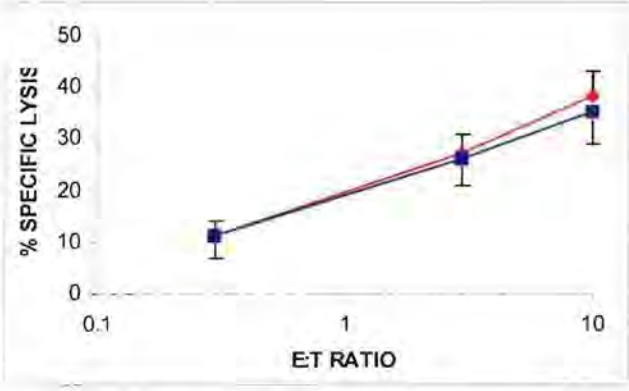


Figure 4.13. The effect of anti-TNF-α monoclonal antibody blocking on the level of macrophage killing mediated by clone 14.10C. Lysis in the absence of anti-TNF-α (◆) was not significantly affected by the addition of 1μg/ml of anti-TNF-α (■). Data represent mean values of 3 experiments showing standard deviation

Confirmation that TNF-α did not play a significant role in clone-mediated killing was obtained by the inability of anti-TNF-α to affect clone-mediated lysis of macrophages. The effect of anti-TNF-α on the cytolytic activity of clone 14.10C is illustrated in Figure 4.13. Maximal inhibition of clone-mediated lysis of 9% at an effector to target ratio of 10:1 was detected. Similar data were generated from all the clones examined (n = 4), with maximal inhibition consistently <10%. The protein synthesis inhibitor, cycloheximide, enhanced the level of cytolysis of infected macrophages induced by ATP, TNF-α, and the CTL clones. These findings are discussed in Section 4.3.11.

4.3.9. Both granule- and Fas-mediated pathways contributed to mycobactericidal activity of the clones

Investigation of the mechanism of killing employed by the CTL clones indicated that both Ca2+-dependent granule exocytosis and Fas-FasL pathways were employed in the lysis of infected macrophages. It was therefore important to establish whether one or both of these pathways was responsible for the reduction in viable mycobacteria. The contribution of each cytolytic pathway in mycobactericidal activity of the clones was examined using the same inhibitory agents described above.

Table 4.2 summarises the effect of inhibition of clone-mediated macrophage lysis by various agents as well as the associated inhibition of mycobactericidal activity of the clones. Four clones are illustrated, data representing mean values of 6 experiments including standard deviation. The data summarised in this table is presented in graphic form in Figure 4.14.

Table 4.2. Levels of lysis of infected macrophages by 4 CD4+ CTL clones (% killing) in the presence or absence of 5 inhibitory agents. The simultaneous reduction in CFU counts (% CFU ↓) is also shown. The level of abrogation of both macrophage lysis and CFU reduction in the presence of the inhibitory agents is indicated by the percentage values in parentheses

		No Inhibitor	CsA (500ng/ml)	CMA (100nM)	$SrCl_2$ (25mM)	αFas (0.5μg/ml)	BFA (10μM)
3.2D	% Killing	34±7	10±6 (70%)	17±6 (50%)	8±5 (76%)	26±6 (24%)	6±3 (82%)
	% CFU ↓	42±7	8±6 (81%)	7±4 (83%)	7±3 (83%)	31±7 (26%)	1±9 (98%)
10.4C	% Killing	44±6	26±9 (41%)	33±5 (25%)	10±4 (77%)	34±7 (23%)	3±2 (93%)
	% CFU ↓	31±6	14±3 (55%)	13±4 (58%)	4±4 (87%)	24±4 (23%)	3±7 (90%)
14.10C	% Killing	39±6	20±7 (49%)	25±6 (36%)	9±4 (77%)	30±5 (23%)	2±2 (95%)
	% CFU ↓	37±7	13±7 (65%)	10±5 (73%)	5±5 (86%)	27±6 (27%)	-3±10 (100%)
15.3A	% Killing	35±8	11±7 (69%)	16±4 (54%)	7±3 (80%)	28±5 (20%)	0±2 (100%)
	% CFU ↓	38±6	7±5 (82%)	9±6 (76%)	5±4 (87%)	25±6 (34%)	-12±14 (100%)

Inhibition of either Fas-, or granule-mediated pathways, led to a reduction in the level of mycobacterial killing. The mean levels of inhibition of CTL clone-mediated lysis of infected macrophages by cyclosporin A, concanamycin A and $SrCl_2$ for the 4 clones illustrated was 56%, 40% and 78%, respectively. This inhibition of macrophage lysis was accompanied by mean levels of abrogation of CFU reduction of 69%, 71%, and 85%, respectively. Mean inhibition of macrophage lysis by anti-Fas was 22%, which was accompanied by a mean abrogation of CFU reduction of 29%. Brefeldin A, which inhibits target cell apoptosis, resulted in mean inhibition of macrophage lysis of 91%. This inhibition resulted i. a mean abrogation of CFU reduction of 95%.

The level of inhibition of macrophage cytolysis correlated positively with the level of inhibition of CFU reduction for all 5 inhibitors ($r = +0.994$ for CsA; $r = +0.925$ for CMA; $r = +0.661$ for $SrCl_2$; $r = +0.169$ for αFas; $r = +0.787$ for BFA). This indicates that levels of CFU reduction were directly linked to levels of macrophage lysis. The hierarchy of inhibition of macrophage cytolysis (BFA > $SrCl_2$ > CsA/CMA > anti-Fas) was mirrored in the degree of abrogation of mycobacterial killing caused by the specific inhibitors. This finding is illustrated in Figure 4.14.

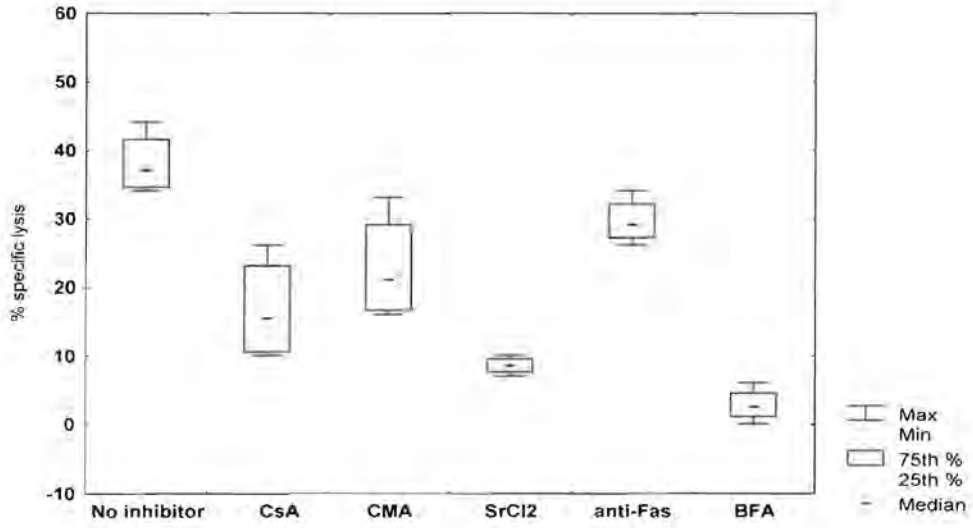
Inhibition of both cytolytic pathways (in clone 15.3A) by co-administration of anti-Fas and $SrCl_2$ led to inhibition of macrophage lysis of 96 ± 3%. This was accompanied by an abrogation of CFU reduction of 97 ± 2%. The two major pathways of cytolysis therefore accounted for all the clone-mediated cytotoxic activity and all the associated mycobactericidal activity. Brefeldin A inhibition of target cell lysis resulted in similar levels of abrogation of both macrophage and mycobacterial death.

Box-and-whisker plots summarising the data generated using the inhibitory agents are presented in Figure 4.14. The median % specific lysis and % CFU reduction values of 6 clones are illustrated. The box indicates values within the 25th and 75th percentage, with minimum and maximum values being covered by the error bars.

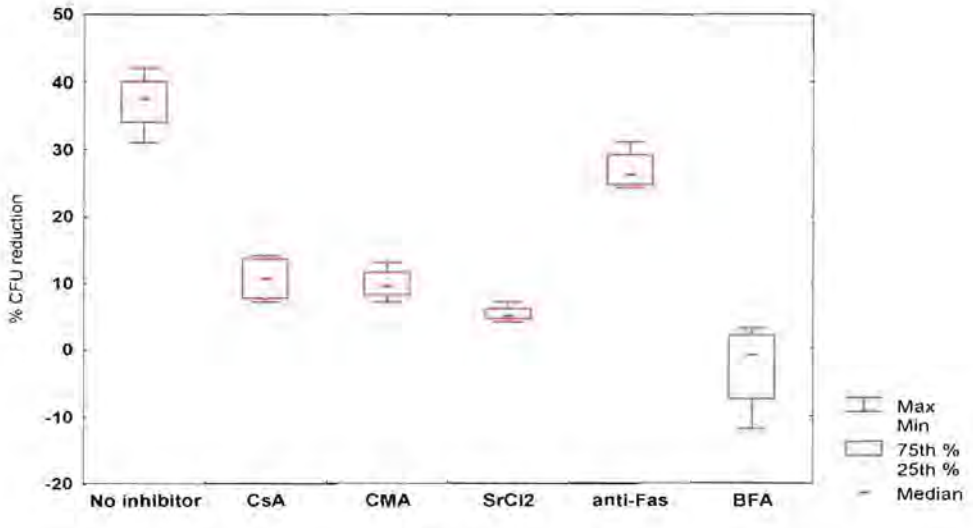
4.3.10. CD4+-enriched EMNC and PBMNC lines also possess dual killing mechanisms

In view of the striking difference in mycobactericidal activity of the cytolytic CD4+-enriched PBMNC line as compared to the EMNC line (chapter 3), the mechanism of killing was compared. CD4+-enriched lines were used to exclude any non-CD4+-mediated cytolytic activity. Table 4.3. summarises the effect of various inhibitory agents on macrophage lysis mediated by both the EMNC- and PBMNC-lines. Granule exocytosis predominated in the EMNC line, and Fas-mediated killing in the PBMNC line, however both lines possessed both mechanisms of killing as observed with the clones. Mean inhibition of EMNC-mediated macrophage lysis by granule exocytosis inhibitors ranged from 56-64%, with mean inhibition by anti-Fas at 40%. The PBMNC possessed a larger Fas component (anti-Fas inhibition =

61%), with the granule exocytosis component accounting for 36-43% of the line killing. Thus, EMNC and PBMNC lines, enriched for CD4+ lymphocytes, possesed dual killing mechanisms. While the EMNC line appeared to posses a larger component of granule-mediated killing, the opposite was true for the PBMNC line. In both cases, inhibition of granule-mediated- and Fas-FasL-killing accounted for all the cytolytic activity of the lines. The difference in mycobactericidal activity of the two lines (EMNC vs. PBMNC) may be related to differences in the components of the granules themselves, or in events linked to apoptosis mediated predominantly by granule exocytosis.



A.



B.

Figure 4.14. Box and whisker plots of effect of inhibitory agents on CTL-mediated lysis of macrophages and on CFU reduction. A. Effect of the inhibitory agents on cytolytic activity of 6 clones. B. Effect of the inhibitory agents on the mycobactericidal activity of the clones, expressed as % CFU reduction

Table 4.3. Levels of antigen-specific lysis of H37Rv-infected macrophages by CD4+-enriched EMNC and PBMNC lines. The effect of inhibition by 4 agents is also indicated. The percentage reduction of lysis in the presence of the inhibitory agents is indicated in parentheses. Data are representative of mean values of 3 experiments showing standard deviations

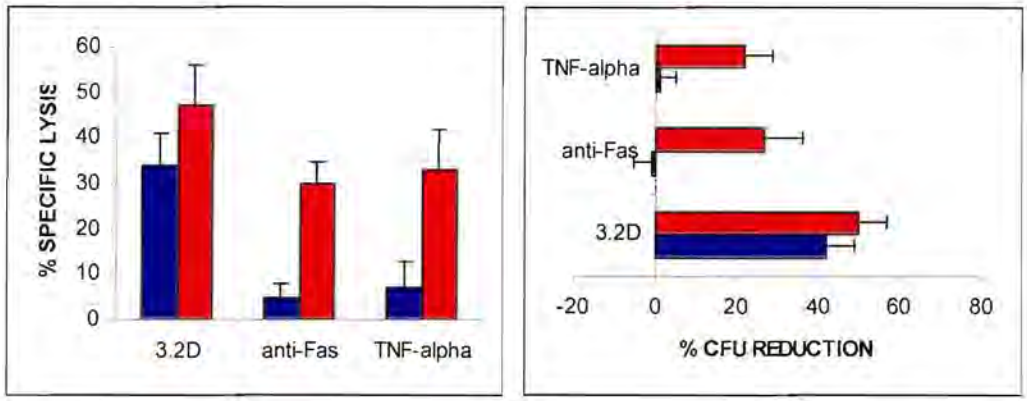
	Cell line-mediated macrophage lysis	
	PBMNC	EMNC
Control	28±3	25±2
Anti-Fas	11±2 (61%)	15±4 (40%)
CsA	18±2 (36%)	11±3 (56%)
CMA	16±4 (43%)	9±4 (64%)
$SrCl_2$	17±3 (39%)	10±5 (60%)

4.3.11. Cycloheximide enhanced killing of macrophages by the CTL clones, TNF-α and anti-Fas

Since cycloheximide has been reported to enhance lysis of macrophages and associated mycobactericidal activity mediated by soluble FasL (Oddo *et al.*, 1997), the effect of CHX on clone-, TNF-α- and anti-Fas-mediated lysis of macrophages was investigated. CHX was found to enhance the level of macrophage killing induced by the clones, as well as lysis mediated by TNF-α, and the anti-Fas pro-apoptotic antibody (CH-11). The effects of CHX on clone macrophage lysis following exposure to CTL clone 3.2D, TNF-α or anti-Fas are presented in Figure 4.15.

1mM CHX was found to enhance lysis following all treatments. For both TNF-α and anti-Fas, CHX treatment enhanced the level of macrophage lysis from <10% to >30%. The enhancement of clone killing was less dramatic, however mean lysis increased from 33% to 46% for clone 3.2D. CHX has been reported to inhibit the anti-apoptotic proteins such as bcl-2 (Weil *et al.*, 1996), and the increased level of killing in the presence of CHX may be attributable to inhibition of such inhibitory influences.

CHX treatment was also found to enhance the mycobactericidal activity of the clones. Figure 4.15.(B) illustrates how CHX treatment resulted in enhancement of both TNF-α- and anti-Fas- mediated mycobactericidal activity. Percentage reduction in CFU counts caused by TNF-α-mediated macrophage lysis increased from 3 ± 5% to 22 ± 9%. The reduction in CFU counts resulting from anti-Fas induced macrophage lysis increased from –3 ± 5% to 25 ± 11%. The mycobactericidal activity of the clone (3.2D) was enhanced moderately in comparison, from 42 ± 9% to 50 ± 10%. The level of enhanced mycobactericidal activity for these three treatments was proportional to the level of increase of macrophage lysis. This finding again confirms that mycobactericidal activity was linked to target cell lysis.



A. B.

Figure 4.15. Effect of cycloheximide on lysis of macrophages by cytolytic clone 3.2D, anti-Fas and TNF-α (A.). Lysis in the absence of CHX, but in the presence of the clone (E:T 10:1), anti-Fas (0.5μg/ml), or TNF-α (100ng/ml) is shown by the blue bars. The addition of CHX (1mM) enhanced the level of macrophage lysis in all three situations (red bars). Enhancement of macrophage killing by CHX also resulted in an increase in CFU reduction (B.). CFU reduction in the absence of CHX (blue bars), was enhanced in all three situations following CHX treatment (red bars). Data represent mean values of 3 experiments and standard deviations are shown

4.4. Discussion

This chapter investigated the mechanism of killing employed by the effusion-derived mycobactericidal CD4+ CTL clones. In addition the killing mechanisms of CD4+-enriched lines derived from peripheral blood and pleural fluid were also investigated to determine whether any particular cytolytic pathway was associated with the reduction in numbers of viable mycobacteria. All cytolytic CD4+ T lymphocyte populations assessed in this study possessed both granule-dependent and Fas-FasL-mediated mechanisms for killing of infected macrophages. An important distinguishing feature of the mycobactericidal clones and line was the predominance of the Ca^{2+}-dependent granule exocytosis cytolytic pathway. This finding suggests that at the site of infection cytolytic CD4+ T lymphocytes acquired a predominant granule-mediated cytolytic pathway which was directly involved in the killing of intracellular mycobacteria.

The importance of CD4+ lymphocytes in control of *M. tuberculosis* infection is undisputed (Kaufmann and Andersen, 1998). These cells are expanded at sites of disease and loss of CD4+ cells in AIDS patients correlates with susceptibility to TB (Barnes *et al.*, 1989; 1991). In addition, the absence of functional CD4+ cells in mice results in rapid disease progression and death (Pedrazzini *et al.*, 1987; Ladel *et al.*, 1995). Protective immunity can be transferred from one animal to another by immunocompetent CD4+ T cells (Orme and Collins, 1984). Functionally, CD4+ T cells are thought to contribute to protection against *M. tuberculosis* by antigen-specific production of cytokines which activate macrophages to kill intracellular mycobacteria (Orme *et al.*, 1993). Of particular importance is the cytokine IFN-γ which appears to be a crucial effector molecule in both mice and humans (Flynn *et al.*, 1993; Newport *et al.*, 1996). Activation of infected macrophages by IFN-γ is thought to be a pivotal step in clearance of infection (Barnes *et al.*, 1994). Although the activation of macrophages is sufficient for infection clearance in mice via the activation of reactive nitrogen intermediates (Rook *et al.*, 1986a; Chan *et al.*, 1992; Sypek *et al.*, 1993), conclusive evidence that this occurs in humans has not been forthcoming (O'Brien *et al.*, 1996). Indeed, a recent report has indicated that both IFN-γ and primed lymphocytes are necessary for induction of macrophage anti-mycobacterial activity (Bonencini-Almeida *et al.*, 1998). In the absence of conclusive proof that CD4+ T cell-derived cytokines such as interferon-γ can activate macrophage mycobactericidal activity, cytolytic activity of CD4+ cells remains a viable alternative mechanism for control of organism replication and spread.

The occurrence of antigen-specific CD4+ cytolytic T lymphocytes in tuberculosis has previously been described (Ab *et al.*, 1990; Ottenhoff and Mutis, 1990; Lorgat *et al.*, 1992a; Tsukaguchi *et al.*, 1995; Oftung *et al.*, 1998). The relevance of the cytolytic effector mechanism *in vivo* has, however, remained elusive. The recent description of MHC class II-restricted CD4+ CTL isolated from BAL fluid may indicate the importance of such cells at the disease site (Tan *et al.*, 1997). Although antimicrobial activity of CD4+ CTL has been

described (Ab *et al.*, 1990), subsequent reports indicated that cytolytic CD4+ T lymphocytes had no effect on viability of intracellular mycobacteria (Fazal *et al.*, 1995; Pithie *et al.*, 1995). Recently, however, it has been demonstrated that co-incubation of CD4+ T cells with infected monocytes led to a reduction in numbers of viable mycobacteria (Silver *et al.*, 1998; Yoneda and Ellner, 1998). Soluble factors could only partially account for this anti-mycobacterial activity, suggesting that contact-dependent processes were important. This contact-dependent activity may involve CD4+ T cell-mediated macrophage lysis These findings taken together implicate cytolytic activity of CD4+ cells in localised containment of infection.

Although Fas-FasL interaction is considered the predominant mechanism of killing employed by CD4+ CTL (Trapani, 1998), recent reports have shown that these cells can kill by either Fas-FasL interaction, or granule exocytosis, or a combination of both pathways (Vergelli *et al.*, 1997; Sevilir-Williams and Engelhard, 1996; Lewinsohn *et al.*, 1998). The cytolytic granule-mediated pathway has been described in an allogeneic-responsive CD4+ CTL, and also in PPD-specific CD4+ CTL (Sevilir-Williams and Engelhard, 1996; Lewinsohn *et al.*, 1998). The description of mycobacterial antigen-specific CD4+ CTL which possess granule-mediated killing mechanisms raises the possibility that this unique pathway may mediate important *in vivo* antimycobacterial activity.

The majority of reports describing CTL which were able to effect a reduction in mycobacterial viability have focused on CD8+ CTL. Although HLA-DR-restricted CD4+ CTL have been shown to reduce viable intracellular mycobacteria (Ab *et al.*, 1990), subsequent reports refuting this finding have resulted in a shift of focus onto CD8+ CTL. Murine CD8+ CTL have been reported to directly reduce infecting organism viability on lysis of bone marrow-derived macrophages. This study did not address the mechanism of killing employed by the CTL (De Libero *et al.*, 1988). Human MHC class I-restricted CD8+ CTL which possess mycobactericidal activity have also been reported, however the mechanism of killing was not characterised in any detail (Lalvani *et al.*, 1998). By far the best characterised mycobactericidal CTL activity has been that of CD1-restricted CD8+ cells (Stenger *et al.*, 1997, 1998). These CTL killed infected macrophages exclusively by cytolytic granule release. The mycobactericidal activity of these cells was linked to a particular granule component - granulysin. Although these findings have important implications regarding CD8+ activity in TB, induction of CD1 expression on macrophages requires *in vitro* stimulation of these cells. Whether this pathway is of major significance *in vivo* has yet to be established.

Not all triggers of cell death result in reduction of organism viability. Neither induction of necrotic cell death by high dose H_2O_2 (Molloy *et al*, 1994), nor osmotic lysis (present study), had any effect on mycobacterial viability. Apoptotic cell death is also not always

accompanied by killing of intracellular organisms. Lysis of infected macrophages by T lymphocytes has been reported to be ineffective in reducing mycobacterial viability. This phenomenon has been observed with both CD4+ and CD8+ CTL (Fazal *et al.*, 1995; Pithie *et al.*, 1995; Stenger *et al.*, 1997). Studies on the effect of CD4+ CTL on mycobacterial viability did not address the mechanism of killing employed by the cells. Non-mycobactericidal CD8+ cells killed infected macrophages by Fas-FasL interaction. These findings indicate that the mechanism of killing employed by CTL may be important in determing mycobactericidal potential.

The kinetics of CTL killing of macrophages (discussed in chapter 3) indicated that secondary necrosis or other late events of macrophage death could be important for maximising the reduction in organism viability (Laochumroonvorapong *et al.*, 1997). However, since all apoptotic cell death results in secondary necrosis, this process itself is unlikely to account for all mycobactericidal activity. Rather, it appears that the efficacy of late apoptotic events may depend on the triggering of certain anti-mycobacterial processes when apoptosis is induced. The strong correlation between level of killing of macrophages and reduction in organism viability by the CTL clones suggests that pathways linked to induction of apoptosis effect killing of mycobacteria.

Although only granule exocytosis killing has been linked to CTL-mediated reduction of organism viability, it has been suggested that Fas-mediated killing may potentially be equally mycobactericidal (Kaufmann, 1999; Oddo *et al.*, 1998). Induction of infected macrophage apoptosis *in vitro* by soluble FasL has been shown to be mycobactericidal. However, this reduction in numbers of viable mycobacteria occurred in the absence of any CTL. In addition optimal reduction in organism viability was only possible in the presence of cycloheximide. This suggests that under normal *in vivo* situations this Fas-pathway may be less important in organism clearance at the disease site than granule exocytosis. Additional pro-apoptotic signals have also been linked to mycobacteridal activity. These include apoptosis induced by ATP, H_2O_2 and TNF-α (Lammas *et al.*, 1997; Laochumroonvorapong *et al.*, 1997; Keane *et al.*, 1997). This suggests heterogeneity in the apoptosis pathway, with mycobactericidal activity being dependent on the type and/or intensity of the apoptotic signal as well as the possible interference by anti-apoptotic proteins. The *in vivo* relevance of mycobactericidal apoptosis induced by agents such as ATP is still being investigated. Levels of ATP produced by T lymphocytes are much lower than doses required for induction of apoptosis (Zheng *et al.*, 1991; Blanchard *et al.*, 1995).

The present study implicates granule-mediated killing of CD4+ T lymphocytes in mycobactericidal activity, and therefore in localised control of infection. Although the importance of CTL in control of viral infections is well established, the activity of these cells in intracellular bacterial infections has only recently begun receiving detailed attention. The

difficulty in conclusively proving that CTL are essential in protective immunity is due to the multiple cell types which mediate cytotoxicity coupled with multiple cytolytic pathways. No conclusive demonstration of the importance of CTL in the control of intracellular infection has been possible since elimination of all cytolytic cells and/or pathways would be catastrophic for the host. Studies with gene knockout mice have indicated that loss of either major cytolytic pathway had no significant effect on disease outcome. These studies have raised important questions as to the importance of cytolytic T cells in protective immunity in TB. Gene knockout mice lacking functional Fas, granzyme B and perforin have been examined (Laochumroonvorapong *et al.*, 1997; Cooper *et al.*, 1997), and in no case was disease susceptibility significantly affected. Although these findings appear to minimise the importance of CTL in tuberculosis, none of these gene deletions could successfully account for all cytolytic activity in the host animal. Loss of Fas-mediated killing (Laochumroonvorapong *et al.*, 1997) does not preclude the activity of granule-exocytosis. Likewise, perforin or granzyme B knockout mice (Cooper *et al.*, 1997; Laochumroonvorapong *et al.*, 1997) still retain functional Fas-mediated activity. In any of these models, cytolytic activity is not completely destroyed. Deletion of both Fas and perforin, results in death from excessive macrophage expansion and pancreatitis (Spielmann *et al.*, 1998). This has prevented detailed assessment of absence of cytolytic activity in tuberculosis. Due to the importance of granule exocytosis in mediating mycobactericidal effects as observed in the present study and others, the deletions of genes coding for perforin and granzyme B are of particular importance. Although the finding that loss of either of these proteins did not affect disease susceptibility appears to minimise a role for granule-mediating killing in disease control, compensatory pathways may have resulted in functional granule-mediated killing being unaffected. Granzyme A, or additional granule constituents such as granulysin, could compensate for loss of granzyme B. Perforin is primarily involved in granzyme release, activation and transport within the target cell (Trapani and Jans, 1999). Its importance for activation of other granule constituents (e.g. granulysin) is not known. In addition, alternative entry and activation pathways for cytolytic granule constituents may exist, minimising the importance of perforin as an essential component of granule-mediated killing.

The importance of the localised disease site in containment of infection has been highlighted by the ability of only effusion-derived cells to mediate mycobactericidal activity. This activity is most dramatically associated with a predominant granule exocytosis mechanism of killing. CD4+ T cells possessing such a predominant granule-mediated killing mechanism may have evolved to control intracellular bacterial infections, similar to the activity of CD8+ CTL in the control of viral infection (Trapani, 1998). The generation of CD4+ CTL in the absence of functional CD8+ CTL which possess cytolytic granule components has been implicated in mediating acquisition of the granule-pathway in CD4+ CTL (Sevilir-Williams and Engelhard, 1997). In tuberculosis, expansion of the CD4+ T cell population in response to mycobacterial

antigen may occur in the absence of any significant expansion of CD8+ CTL. The absence of antigen-specific CD8+ CTL could somehow be envisaged to stimulate production of cytolytic granule components in the CD4+ T cells.

A major debate in TB has revolved around whether lysis of infected macrophages leads to containment or spread of infection. The current study indicates that the former option is likely. Only in the absence of any mycobactericidal activity occurring at the time of host cell death would spread become a problem. The localised disease site appears to prime for the development of granule-mediated cytolytic cells, and the granule constituents themselves may mediate organism killing. Only by killing the host cell, depriving the organisms of their protected niche, and exposing them to potent lytic agents such as granulysin, can the infection be controlled. These findings have important implications for possible future therapies, in that enhancement of the cytolytic activity may lead to rapid and efficient disease clearance. Further characterisation of particular antigenic proteins that stimulate CD4+ CTL is of great importance in the development of DNA-based vaccines. Introduction of immunogenic proteins that induce CD4+ T cells which produce cytokines and mediate mycobactericidal lysis of infected macrophages, is likely to result in protective immunity.

The CD4+ CTL clones in the present study are derived from a disease associated pathological site, and may thus closely approximate CTL activity in-vivo. The clones were generated from an effusion mononuclear cell population using live, virulent *M. tuberculosis* as antigen and autologous macrophages as antigen presenting cells. In addition the cells were MHC class II-restricted and infected macrophages did not require manipulation such as cytokine activation or cycloheximide treatment in order for any mycobactericidal activity to be detected. Reports which indicated that CD4+ CTL were not mycobactericidal used clones which were derived from peripheral blood of healthy donors, *M. bovis* BCG was used as antigen, and HLA-matched donor macrophages were used as antigen presenting cells (Fazal *et al.*, 1995; Pithie *et al.*, 1995). Although the mechanism of killing of the cells in these studies was not addressed, it is likely that Fas-FasL interactions predominated. The current study has also found that peripheral blood-derived CD4+ CTL, which utilised a predominant Fas-FasL pathway, were not mycobactericidal. This contrasts with effusion-derived cells which possessed predominant granule-mediated killing pathways and which were mycobactericidal. The requirement for granule constituents indicates some similarities with mycobactericidal CD8+ CTL. Whether the mycobactericidal activity of the CD4+ CTL was dependent on the occurrence of a predominant granule-mediated pathway, or on the presence of a particular cytolytic granule constituents at an elevated concentration will need to be addressed in subsequent studies.

The findings of the present study have important implications in the understanding of localised immune responses at sites of infection. The presence of large numbers cytolytic

CD4+ T lymphocytes, which can kill infecting mycobacteria, could aid in the rapid clearance of pathogenic organisms. This activity would account for the contact-dependent activity of CD4+ T lymphocytes necessary for antimycobacterial effects.

Chapter 5.

A non-cytolytic CD8+ effusion-derived T lymphocyte clone mediates suppression of autologous T lymphocyte proliferation

5.1. Introduction

Several features of immune suppression or hyporesponsiveness have been observed in patients with active tuberculosis. These features include depressed tuberculin skin test reactivity, decreased proliferative responses to *Mycobacterium tuberculosis* antigens, and suppression of the production of the cytokines IL-2 and interferon-γ (Toossi and Ellner, 1996). The cause of immune suppression in active TB is not known, however several possible mediating factors have been proposed. By far the best characterised has been the production of immunosuppressive cytokines by both macrophages and T lymphocytes. Exposure of mononuclear cells to mycobacterial components results in enhanced production of IL-4 and IL-6, both of which suppress the production of Th1 cytokines (Wadee *et al.*, 1993). Similarly, infection of macrophages with *M. tuberculosis* results in production of IL-6 which suppresses T cell proliferation and IL-2 production (van Heyningen *et al.*, 1997). TGF-β production by macrophages has also been implicated in immunosuppression (Hirsch *at al.*, 1996). TGF-β inhibits lymphocyte proliferation, down-regulates macrophage effector functions and promotes fibrosis (Wahl, 1992). The T lymphocyte-derived cytokine IL-4 and the macrophage-derived cytokine IL-10 have both also been directly implicated in inhibition of T cell responses to mycobacterial antigens (Sieling *et al.*, 1993).

The frequency of the two major components of immunosuppression (decreased tuberculin reactivity and depressed lymphocyte responses) correlates with the spectrum of clinical disease. In pulmonary TB 17-25% of patients are PPD skin test unresponsive (Daniel *at al.*, 1981). Low blastogenic responses are present in 40-60% of patients, which includes almost all skin test negative and some skin test positive patients (Kleinhanz and Ellner, 1987). In disseminated (miliary) TB, 50-75% of patients are skin test non-responsive, and low blastogenic responses are common (Bhatnagar *at al.*, 1977). Both skin test anergy and lymphocyte responses to PPD vary according to mycobacterial load and/or the extent of pathology at the site of disease (Toossi *at al.*, 1986). In pulmonary TB advanced disease is accompanied by higher frequency of skin test negativity as well as very low blastogenic response to PPD.

The fact that depression of *M. tuberculosis*-induced T cell responses is not only immediate (at time of infection), but also protracted (for several months after infection), suggests that cytokine-mediation is not solely responsible for suppression of T cell function (Vanham *at al.*,

1997; Hirsch *at al.*, 1999). Alternative explanations of suppression of immune responsiveness have included enhanced levels of apoptosis in responder T cells (Hirsch *at al.*, 1999), and compartmentalisation of antigen-specific T cells to disease sites (Dieli *at al.*, 1999). In addition, both anergic T cells and antigen-specific suppressor T lymphocytes are able to mediate inhibition of T lymphocyte responses. Although the activity of these cells has been noted in experimental systems and certain other diseases, no detailed characterisation of the activity of either of these cells in TB has been reported. Although the activity of suppressor T lymphocytes in this disease has previously been noted (Kleinhanz and Ellner, 1987), no detailed assessment of functional activity has been possible due to the inability to generate clones of *M. tuberculosis*-specific suppressor T cells. The existence of suppressor cells could provide an alternative explanation for immune suppression in TB. T cell-mediated suppression may involve the production of immunosuppressive cytokines, or direct interference in responder T cell functioning via disturbances to normal signal transduction pathways, resulting in induction of anergy or non-responsiveness.

Studies of effusion mononuclear cell activity in tuberculous pleuritis have shown that protective immunity in TB correlates with CD4+ Th1 memory T cell activity. (Lukey *et al.*, 1998). Severe pulmonary disease resulting in miliary TB, on the other hand, has been found to correlate with depressed responsiveness and possible type-2 cytokine predominance (Dlugovitzky *et al.*, 1999). This dichotomy is more pronounced in leprosy where distinct polarisation of disease outcome is observed. Protective immunity, which is associated with a strong Th1-type cellular immune response, results in the manifestation of the tuberculoid form of leprosy. The lepromatous form of the disease, on the other hand, has been linked to predominance of a type-2 response. Thus, a switch from a type-1 to a type-2 predominance has a profound effect on disease outcome (Bloom *et al.*, 1992). Both CD4+ and CD8+ *M. leprae* antigen-specific suppressor T cell lines and clones have been isolated from the lesions of lepromatous leprosy patients. These cells produce type-2 cytokines, and the CD8+ suppressor T cells are uniquely MHC class II-restricted. These cells are able to inhibit or suppress autologous PBMNC or PBMNC-derived clone proliferation. These findings have implicated the activity of such suppressor cells in the inhibition of protective Th1 responder cell activity (Salgame *et al.*, 1991b). This would allow for the enhancement of a type-2 response as observed in the lepromatous form of the disease.

Extrapulmonary TB resulting in an effusion offers a unique opportunity to obtain a window to the site of active pathology. Compartmentalisation of PPD CD4+ proliferative and cytotoxic responses in the mononuclear population of the effusion has been described (Barnes *et al.*, 1989; Lorgat *et al.*, 1992). Although CD4+ cell numbers, and their functional activity, are increased at sites of disease, evidence exists that other cell types such as CD8+ T cells also play a role (Orme, 1993). The activity of T lymphocytes in tuberculous effusions is in itself complex. Three patterns of responsiveness have been defined. Accelerated responsiveness

of T cells to mycobacterial antigen is a feature of the majority of effusion-derived populations. However, both non-accelerated and "flat" *in vitro* responses have also been observed (Lukey *et al.*, 1996; 1998; Lorgat *et al.*, 1992b). This is suggestive of suppression that may be mediated by the other cell populations within the effusion which are not components of protective immunity.

In this chapter a CD8+ T cell clone derived from a pleural effusion was shown to mediate suppression of T cell responses *in vitro*. The clone produced IL-4, was poorly responsive to mycobacterial antigens and did not display any antigen-specific cytolytic activity. The clone was MHC class II- (DR) –restricted and did not express the CD28 co-receptor. This clone was able to suppress the proliferation of type-1 CD4+ clones, as well as PBMNC cells. This active suppression was mycobacterial antigen-specific, dependent on contact between suppressor and responder cells, and partially mediated by the type-2 cytokines IL-4 and IL-10. The activity of suppressor T cells may help to explain the unusual response kinetics and LDA data of the ex vivo-derived patient cells used in the study. In addition the activity of suppressor T cells may help to explain certain of the immunosuppressive features observed in a wide range of TB patients.

5.2. Materials and methods

5.2.1. Generation and maintenance of a CD8+ T cell clones

CD8+ T lymphocyte clones were generated by limiting dilution as described in Chapter 2. EMNC (10ml of 10^6/ml) were cultured together with *M. bovis* BCG (10^5 CFU/ml) at 37°C for 7 days, and then for an additional 3 days with 100 IU/ml recombinant human IL-2 (Cetus, Emeryville, USA). The CD8+ cells were purified by magnetic bead separation (Minimax, Miltenyi Biotec, Bergisch Gladbach, Germany) as described. Population purity was assessed by flow cytometry prior to cloning. The CD8+ fraction of antigen-primed EMNC cells was then cloned by limiting dilution at ratios of 1 and 0.3 cells per well. Cloning was carried out in RPMI 1640 medium (Flow Laboratories) to which the following were added: 2mM L-glutamine (Highveld Biologicals, Johannesburg, SA), 10mM non-essential amino acids (Highveld Biologicals, Johannesburg, SA), $5x10^{-5}$M 2-mercaptoethanol (BDH Chemicals, England), 1mM Sodium Pyruvate (Highveld Biologicals, Johannesburg, SA). This cloning medium was supplemented with 10% autologous human serum. Autologous feeder cells (PBMNC) were prepared, diluted to a concentration of 10^6/ml and irradiated at 40 Gray. *M. bovis* BCG was added to the feeder cells at a ratio of 10 bacteria per cell (10^7CFU/ml) together with 100 IU/ml recombinant human IL-2 (Cetus). Aliquots (20µl) of feeder cells were plated into Terasaki well plates (Becton-Dickinson, Mountain View, CA) using a multi-channel microdispenser (Titertek). The T-cells (EMNC) were then plated (20µl) into the wells containing feeder cells at ratios of 1 and 0.3 cells per Terasaki well. Each well contained a final volume of 40 µl. Plates were wrapped in foil and sterile distilled water added to each package to ensure minimal evaporation of well contents. Plates were incubated at 37°C in a 5% CO_2 incubator. After 10 days the plates were assessed for growth by examining well contents using an inverted microscope. The contents of the wells showing positive growth were expanded into 96-well round-bottomed plates (Greiner) containing freshly prepared feeder cells (irradiated PBMC), BCG and rIL-2. Following further expansion into 24-well plates (Becton-Dickinson) clones were phenotypically assessed by flow cytometry and they were screened for proliferative responses (^{3}H-thymidine incorporation) and cytotoxic function (^{51}Cr-release). Aliquots (1-5 x 10^6) were cryopreserved in human AB serum containing 10% DMSO (Merck, Darmstadt, Germany) and stored in liquid nitrogen.

The CD8+ clones grew slowly and it was difficult to obtain sufficient cell numbers for detailed characterisation and cryopreservation. Several clones died before characterisation was possible. Due to the slow growth rate of the CD8+ clones and their poor proliferative responses to mycobacterial antigens, cells were passaged by alternate stimulation with the mitogen PHA and *M. tuberculosis* H37Rv. Clones were maintained in culture by weekly restimulation with fresh autologous irradiated APC's (PBMNC), antigen or mitogen (*M. tuberculosis* H37Rv (2 x 10^5 CFU/ml) or PHA (5.75 x 10^{-3} mitogenic units/ml)) and IL-2. After 72 hours, fresh IL-2 (100 IU/ml) was added and the clone cells were resuspended to dislodge clumps.

Five CD8+ clones were generated, however only 2 could be maintained and expanded for further characterisation. These were clones 11.5A and 2.9D. Both these clones were phenotypically characterised by flow cytometry, and they were analysed for their cytokine production profile.

5.2.2. Phenotypic characterisation and cytokine production profile of the CD8+ clones

The 2 CD8+ clones were phenotyped by flow cytometric analysis of monoclonal antibody-labelled cells. Markers used for phenotyping included; CD3, CD4, CD8, and CD56 (see section 4.*) Clone cells (100µl of 10^6/ml) were incubated for 10 minutes with 5µl of FITC-labelled monoclonal antibody (Coulter), or with isotypic controls (FITC-labelled mouse anti-human IgG1)(Coulter, Hileah, CA). Cells were fixed prior to flow cytometry by the addition of 100µl of freshly prepared 1% paraformaldehyde (Merck, Darmstadt, Germany). Flow cytometry was carried out using a Coulter Epics Profile II flow cytometer.

The cytokine production of the clones was assessed by analysis of clone-derived supernatant fluid. Initial characterisation of clone cells into type-1, type-2 or type-0 was based on levels of IFN-γ and IL-4 production. Clones were later also assessed for the production of IL-5, IL-10, and TGF-β. Supernatants were generated following antigen stimulation in the absence of IL-2. Feeder cells (irradiated PBMNC) were prepared and diluted to 10^6/ml. Clone cells (1ml of 10^6/ml) were then added to 500µl of feeder cells and 500µl of antigen (H37Rv at 2 x 10^5 CFU/ml) in 24 well plates (Becton-Dickinson). Supernatants were removed after 48 hours of incubation at 37°C. Supernatants from wells containing feeder cells and antigen but no clone cells were also collected. Supernatants were filtered through 0.2µm syringe filters to remove any mycobacteria. Aliquots (1ml) were frozen at -20°C.

Commercial ELISA kits were used for cytokine detection. These included kits for IFN-γ, IL-4, IL-5, IL-10 (all Pharmingen), and TGF-β (Promega). All ELISA tests were performed according to manufacturer's instruction (see section 2.2.8). For TGF-β, supernatants were acid activated by treatment with 0.1N HCl. TGF-β levels in un-activated and activated samples were compared.

5.2.3. Proliferative and cytotoxic activity of CD8+ clones

CD8+ clones were assessed for their proliferative responses to various mycobacterial antigens (PPD, *M. bovis* BCG, *M. tuberculosis* H37Rv), irrelevant antigen (streptokinase-streptodornase) and mitogen (PHA). Proliferation assays were carried out as described in Chapter 2. Briefly, CD8+ clones (100µl 0f 10^6 cells/ml) were cultured in round-bottomed 96 well microtitre plates (Greiner) in the presence of irradiated feeder cells (50µl of 10^6 PBMNC/ml), with or without antigen or mitogen (50µl). Plates were incubated at 37°C for 2

days. ^{3}H-thymidine was added to the wells the the last 8 hours of culture (44-52 hours). Well contents were harvested onto filter paper using a cell harvester (Titertek). Filter paper discs were placed into scintillation vials and 1ml of scintillation fluid was added. The amount of incorporated label was determined by measuring β-emission.

The cytotoxic activity of the CD8+ clones was evaluated using standard clone cytotoxicity assay techniques (see section 2.2.9). Briefly, day 4 adherent macrophage cultures derived from fresh PBMNC were infected with H37Rv and labelled with ^{51}Cr for 24 hours prior to the assay. On day 5, clone cells were prepared by washing free the growth medium and resuspending in fresh RPMI 1640 supplemented with 10% human AB serum. Clone cells were used 6 days (120 hours) after exposure to feeder cells, antigen and IL-2, 48 hours after re-stimulation with IL-2. Cells were counted using trypan blue exclusion staining, and cell concentration was adjusted to 10^6 cells/ml. Three effector to target ratios were used (i.e. 10:1, 3:1, and 0.3:1). Clone cells were then added to infected ^{51}Cr-labelled macrophages after extensive washing with warm PBS supplemented with 5% FCS to remove unincorporated ^{51}Cr label, non-adherent cells and antigen. Spontaneous release was assessed in wells to which no clone cells were added. The cytotoxicity assay was carried out over 16 hours. The percentage lysis was determined as discussed in Chapter 2.

5.2.4. Assessment of suppressor activity of the CD8+ clones by determining their ability to inhibit proliferation of autologous CD4+ clones and PBMNC

The suppressor activity of the CD8+ clones (11.5A and 2.9D) was assessed by determining the level of inhibition of proliferation mediated by the clones. Suppressor activity against both autologous PBMNC and CD4+ clones was examined. The protocol was an adaption of several published methodologies (Sasazuki *at al.*, 1989; Salgame *at al.*, 1991). Standard proliferation assays of both PBMNC and CD4+ clones were carried out in the presence or absence of CD8+ clone cells. 100μl of 10^6/ml responder cells (PBMNC or CD4+ clone cells) were incubated with 50μl of 2 x 10^5/ml CD8+ clone cells. The final concentration of cells was therefore 10^4 CD8+ clone cells plus 10^5 responder cells. 50μl of antigen was also added to the wells. Various mycobacterial antigens were used including PPD, *M. bovis* BCG, *M. tuberculosis* H37Rv and a sonicated preparation of *M. tuberculosis* H37Rv. The irrelevant antigen streptokinase-streptodornase, and the mitogen PHA, were also included in certain experiments. Optimal concentrations of antigen for inhibition of proliferation were determined by dose response. Optimal suppression was observed at concentrations slighly lower than used to generate peak proliferative responses. Concentrations of antigens used in suppressor assays were as follows: PPD 2μg/ml; *M. bovis* BCG, 2.5 x 10^5 CFU/ml; *M. tuberculosis* H37Rv, 7.5 x 10^4 CFU/ml; sonicated preparation of *M. tuberculosis* H37Rv, 24μl/ml; streptokinase-streptodornase, 200U/ml and 50U/ml respectively; PHA, 4.5 x 10^{-3} mitogenic units/ml. Proliferative responses of PBMNC were assessed after 3 days (PHA) and 6 days (all antigens). The clone proliferative responses were assessed after 44 hours

for both mitogenic and antigenic responses (see section 2.2.9). In PBMNC assays, ^{3}H-thymidine was added for the last 18 hours of culture for both PHA and antigen assays. In CD4+ clone proliferations, ^{3}H-thymidine was added for the final 8 hours of culture. The level of ^{3}H-thymidine incorporation was determined as described in Chapter 2. Briefly, microtitre well contents were harvested onto filter paper, filter paper discs were placed into scintillation vials containing scintillation fluid, and the level of β-emission was determined using a β-counter. Percentage suppression was calculated using the formula: % suppression = [1-(cpm in presence of suppressor clone/cpm in absence of suppressor clone)] x 100%.

To determine whether suppression mediated by clone 11.5A was dependent on cell function, the effect of inhibition of cellular metabolism on suppressor activity of the CD8+ clones was assessed. The ability of clone 11.5A to suppress PBMNC proliferation was determined after irradiation. Clone 11.5A cells were irradiated (50 Gy) and then used in standard assays for inhibition of proliferation (section 5.2.4.). Control, untreated clone cells were also used to compare the effects of irradiation on suppressor activity.

Depletion or inactivation of IL-2 by T lymphocytes mediating suppression has been implicated in affecting normal proliferative responses of responder cells (Li *et al.*, 1990). To assess the importance of IL-2 depletion on activity of responder cells, proliferation inhibition assays were set up in which endogenous IL-2 was added to the microtitre wells. IL-2 (100 IU/ml) was added to wells containing PBMC, clone 11.5A cells, and mycobacterial antigen. In addition the effect of IL-2 addition on normal PBMC proliferation in the absence of clone 11.5A was assessed.

5.2.5. Assessment of the suppressor activity of CD8+ clone-derived supernatant fluid

To determine whether the suppressor activity of the CD8+ clone was mediated by soluble factors, clone-derived supernatants were examined for their ability to suppress proliferation of autologous PBMNC. Supernatants were generated as for cytokine analysis (section 5.2.2.) by incubating resting clone cells (1 week post exposure to antigen presenting cells and antigen) with freshly isolated PBMNC and antigen. Feeder cells (irradiated PBMNC) were prepared and diluted to 10^{6}/ml. Clone cells (1ml of 10^{6}/ml) were then added to 500μl of feeder cells and 500μl of antigen (H37Rv at 1 x 10^{5} CFU/ml) in 24 well plates (Becton-Dickinson). Each well (total volume 1ml) therefore contained 10^{6} clone cells, 5 x 10^{5} feeder cells and antigen. In addition to H37Rv, the mitogen PHA was also used for generation of supernatants. Control wells containing feeder cells and antigen or mitogen, but no clone cells, were also set up. No IL-2 was added to any of the wells. Supernatants (500μl) were removed after 48 hours of incubation at 37°C. The supernatants were filtered using a 0.22μm syringe filter (Sterilin) to remove any cells or mycobacteria.

To assess the ability of the clone supernatants to mediate suppression of freshly isolated PBMNC, 100μl of 10^6/ml responder cells (PBMNC) were incubated with 50μl of clone cell-derived supernatant and 50μl of antigen. The level of proliferative activity was determined after 6 days by adding ^{3}H-thymidine to the cultures for the last 18 hours of culture. Cells were harvested, bottled, and the level of radiolabel incorporation was determined as described above. Parallel wells were set up for standard clone inhibition of proliferation assays (section 5.2.3.). Comparison of the levels of proliferation of the PBMNC to various antigens in the presence of either cloned suppressor T cells, or clone-derived supernatants allowed for an evaluation of the suppressor activity of soluble mediators.

5.2.6. Assessment of the importance of cell-to-cell contact in suppression

The importance of contact between the CD8+ clone cells and the responder cells (PBMNC) in the induction of suppression was determined using transwell plates (Costar). These plates are flat-bottomed 24-well plates which hold between 1 and 2ml of culture fluid. Experiments were initially carried out to determine whether the suppressor activity observed using microtitre plates (total volume 200μl/well) was mirrored when assays were scaled up to 2ml. The ratios of suppressor cells to responder cells (1:10) were maintained, but 10 times the number of cells were added to each well (1 x 10^5 suppressor cells in upper chamber, 1 x 10^6 PBMNC in lower chamber). Increasing the volume and numbers of cells was found not to affect levels of suppression observed.

In experiments utilising transwell plates, clone cells were placed in the upper chamber and responder cells in the lower chamber. Irradiated feeder cells (5 x 10^4 PBMNC) were co-cultured with the suppressor cells to ensure antigen presentation and clone stimulation in the absence of responder PBMNC. In certain wells, feeder cells were excluded. Replicate wells were set up in which suppressor cells were incubated together with PBMNC in normal 24-well plates. The level of proliferation of the PBMNC was determined in both normal wells, and the PBMNC cultured in the lower well of transwell plates. The effect of physical separtion of suppressor cells from reponder cells was then compared in the presence of various mycobacterial antigens, as well as the irrelevant antigen SK-SD.

5.2.7. The role of IL-4, IL-10 and TGF-β in the mediation of suppressor activity

Cytokine analysis of clone 11.5A-generated supernatants indicated that this clone produced the cytokines IL-4, IL-5, IL-10, and TGF-β. The role of two of these cytokines (IL-4 and IL-10) in the suppressor activity of clone 11.5A was determined by adding neutralising antibodies to the cytokines in certain experiments. Anti-human IL-4 (Boehringer Mannheim, Germany), and anti-human IL-10 (Serotec, Oxford, UK) were used at various concentrations (10ng/ml - 10μg/ml) in assays which assessed levels of inhibition of proliferation mediated by clone 11.5A. Additional experiments using neutralising antibody to TGF-β (1μg/ml) (Promega) were also carried out. Isotypic control antibodies (mouse anti-human IgG1 from the

respective commercial companies) were used in control experiments. Antibodies were used singly or in combination with each other.

In addition to blocking cytokine activity, the effect of addition of endogenous recombinant human IL-4 (Boehringer Mannheim) and IL-10 (Genzyme) on suppression of PBMNC proliferation was assessed. A range of concentrations (0.1pg/ml – 10ng/ml) were used for both cytokines. The combined effect of addition of both cytokines was also monitored. For comparative purposes endogenous IFN-γ (Genzyme) was used in certain experiments.

5.2.8. Analysis of the MHC-restriction of the Ts clone

The importance of MHC-restriction in mediation of clone suppressor activity was determined by examining the ability of the clone to suppress PBMNC proliferation of MHC-DR or –DQ matched or mismatched donors. Tissue typing (HLA A, B, C, DR, DQ) of the patient was carried out by the Tissue Immunology Department, University of Cape Town. Donors for experiments were selected from a database of tissue typed individuals who were readily available for blood donation. The ability of the clone to mediated suppression of proliferation of PBMNC from patients who were matched at DR or DQ loci was determined by standard assays as described above.

The role of DR in mediation of suppression was also investigated by examining the ability of L243 ascites to block suppression. Ascites containing anti-human HLA-DR antibodies (IgG1) was collected from mice immunised with the antibody (anti-DR) secreting cell line L243 (Department of Immunology, University of Cape Town). Various dilutions of antibody preparations were used, with optimal inhibition being observed at a dilution of 10^{-6}. Control experiments were carried out using isotypic mouse anti-human IgG1. L243 blocking was also carried out in standard CD4+ clone proliferation assays to determine the efficacy of the chosen concentrations to block proliferation. L243 was added to standard inhibition of proliferation assays in varying concentrations, and the proliferative response of PBMNC was determined as described above.

5.3. Results

5.3.1. Proliferative and cytolytic activity of CD8+ T lymphocyte clones

The EMNC-derived CD8+ clones 2.9D and 11.5A both displayed poor proliferative responses to mycobacterial antigens. The proliferative responses of clone 11.5A are illustrated in Figure 5.1. Clone 2.9D had a similar proliferation profile, with no single antigen response resulting in a mean delta cpm value > 5000. The poor proliferative responses to mycobacterial antigens may have been due to sub-optimal stimulation by the mycobacterial antigens used. These antigens may have been ineffective in stimulating a proliferative response in the CD8+ clones due to the absence of the specific response-inducing mycobacterial epitope. The epitope specificity of HLA-DR-restricted CD4+ suppressor T cell clones has been shown to reside between amino acid residues 439 and 448 of the mycobacterial heat shock protein, hsp65 (Mutis *et al.*, 1994). Additional epitopes may induce proliferative responses in CD8+ T cell clones which mediate suppression, however the use of whole organisms, PPD or sonicated cell material did not appear to provide the correct epitope for succesful proliferation. The nature of the mycobacterial antigen (whole cell versus soluble cell extract) did not appear to play a role in T cell clone response as evidenced by both PPD and H37Rv responses being similar. The absence of the co-receptor CD28 in clone 11.5A may have contributed to the apparent non-responsiveness. However, clone 2.9D, which was equally non-responsive to mycobacterial antigens, did possess CD28. This suggests that absence of this co-receptor was unlikely to account for the non-responsiveness of both the clones. Both clones were, however, able to proliferate more strongly in response to the mitogen PHA. The proliferative responses to this polyclonal activator were similar to mycobacterial antigen-specific responses of CD4+ clones (delta cpm values > 20000).

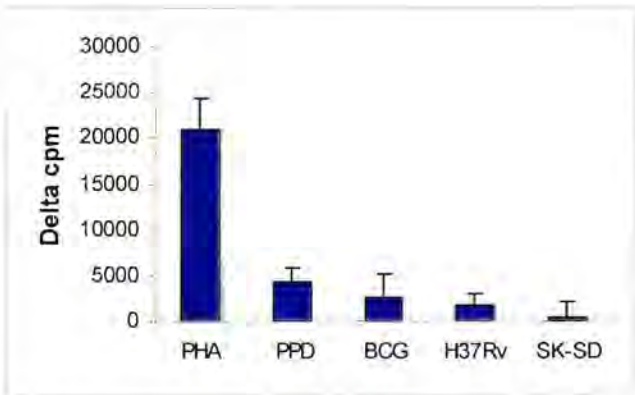


Figure 5.1. Proliferative responses of clone 11.5A to PHA, PPD, BCG, H37Rv and SK-SD. Poor proliferative responses (mean Δcpm < 5000) were observed for all the antigens. Mitogenic (PHA) response is also illustrated. Data are representative of 4 experiments showing standard deviation.

Although proliferative responses were consistently low for all mycobacterial antigens, stimulation indexes of > 3 were observed using PPD. This indicates that the PPD responses were significantly greater than those observed in non-stimulated cells. In the absence of any defined epitope specificity, PPD appeared to be the most successful activator of the CD8+ T cell clones. The broad range of secreted mycobacterial antigens present within PPD may have accounted for this responsiveness.

The cytolytic activity of the CD8+ clones was also examined using a range of mycobacterial antigens. No cytolytic activity was apparent with either of the clones using standard 16 hour ^{51}Cr-release assays, as illustrated for clone 11.5A in Figure 5.2. Standard 16 hour adherent macrophage target cytolytic assays involve labelling of the cells with ^{51}Cr and infection with antigen for 24 hours prior to the addition of the CTL clones. The kinetics of MHC class I antigen presentation differ from MHC class II (Rock and Goldberg, 1999), and the extended incubation prior to assessment of CTL activity may have resulted in sub-optimal antigen presentation. To address this, certain assays were carried out with short 4 hour pulsing with antigen directly before addition of the clones. This variation in antigen challenge did not affect the levels of cytolysis observed for either of the CD8+ clones.

In both the overnight and 4 hour antigen challenge assays, the CD8+ clones were able to lyse target cells in the presence of PHA. This finding confirms that the CD8+ clone cells possessed lytic machinery, but were not successfully stimulated to kill infected target macrophages by the antigens examined. This again may have been linked to the absence of the clone-specific mycobacterial epitope. The absence of any mycobacterial antigen-specific cytolytic activity appears to exclude cytolysis as the primary functional activity of these cells *in vivo*.

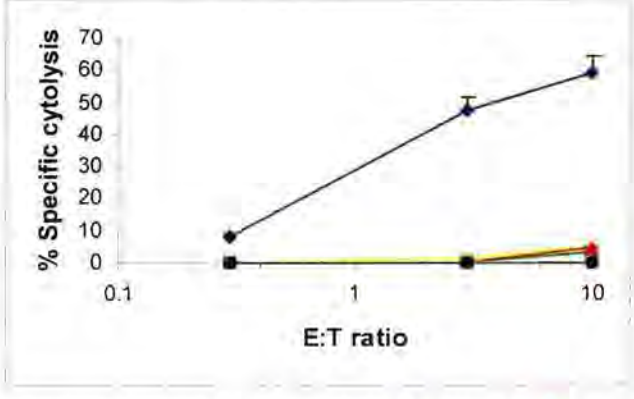


Figure 5.2. Cytolytic activity of clone 11.5A to PHA (♦), H37Rv (▲), PPD (■), BCG (▲) and SK-SD (•). Percentage specific cytolysis of antigen pulsed macrophages by the clone following 16 hours of incubation is illustrated. Poor cytolytic activity (specific cytolysis < 10% at all E:T ratios) was observed with all the antigens. Data represent 3 experiments showing standard deviation

The inability of the CD8+ clones to respond to various mycobacterial antigens in both proliferation and cytotoxicity assays may have been indicative of induction of anergy. Anergy has been shown to be induced by inappropriate or unusual antigen presentation (Allen, 1994, Broeren *et al.*, 1995). The possibility of the CD8+ clones being anergic was investigated further by assessing the ability of exogenous IL-2 to abrogate the anergic status of the clones. Addition of 100 IU/ml of recombinant IL-2 to proliferation assays had no effect on their apparent non-responsiveness. This contrasts with other reports of anergic T cells, where addition of exogenous IL-2 could restore antigen-specific responsiveness (Taams *et al.*, 1998). Additional assessment of the role of IL-2 in CD8+ clone function is addressed in section 5.3.4. Anergy was also excluded due to the ability of the CD8+ clones to produce a range of cytokines (discussed below).

5.3.2. Phenotypic characterisation and cytokine production by CD8+ clones

Phenotyping of both of the CD8+ clones showed that they were CD3+, CD4-, CD8+, CD56-. As mentioned above, clone 11.5A was CD28-, whereas clone 2.9D was CD28+. The status of CD28 expression was examined due to the report that suppressor T lymphocytes lack this co-receptor molecule (Li *et al.*, 1990). These authors also reported that although suppressor clones lacked CD28, they were still able to respond to antigen, a finding not reproduced in the present study.

Initial assessment of cytokine production by the clones has been discussed in Chapter 2. Following the initial characterisation of the clones, production of additional cytokines was examined. These included IL-5, IL-10, and TGF-β. A detailed comparison of cytokine production by the two CD8+ clones as well as several CD4+ clones and PBMNC is illustrated in Table 5.1. Clone 11.5A was novel in that it not only produced the lowest level of IFN-γ (8000 pg/ml) but uniquely produced IL-4 (150 pg/ml) as well, a pattern reminiscent of type-2 clones isolated from lepromatous leprosy as previously described (Modlin *et al.*, 1986; Mutis *et al.*, 1993b). Clone 11.5A also produced high levels of IL-5 (4027 pg/ml), another type-2 cytokine. By contrast, clone 2.9D produced high levels of IFN-γ (22500 pg/ml), no detectable IL-4, and the lowest levels of IL-5 (923 pg/ml), TGF-β (2992 pg/ml) and IL-10 (505 pg/ml). Comparable levels of type-2 cytokines IL-10 and TGF-β were produced by clone 11.5A and all the CD4+ clones. The high levels of production of IL-4 and IL-5 by clone 11.5A were not observed in any of the CD4+ clones or PBMNC.

Could the individual profile of cytokines account for the function of the specific clones? It is important to consider that these cytokines have cross-regulatory, counter balancing activities. Therefore, the possibility of suppressive function for any clone will be more related to the ratio of type-1 to type-2 cytokines than the absolute level of any single cytokine produced. The type-1:type-2 ratio therefore more accurately reflects type-2 suppressive potential, and dictates the nett suppressive effect. These ratios are illustrated in Table 5.2.

As shown in the table, clone 11.5A had the lowest IFN-γ:type-2 cytokine ratio of all the clones, confirming its greater suppressive potential. Most significant is the striking difference in IFN-γ:IL-4 ratio between clone 2.9D and clone 11.5A (>3750 vs 53). Furthermore, clone 11.5A had the lowest IFN-γ:type-2 ratio for IL-5, IL-10, and TGF-β of all the clones and PBMNC, which probably accounts for the functional difference between the two CD8+ clones and the unique suppressive activity of clone 11.5A.

Table 5.1. Cytokine production by CD8+ and CD4+ clones as determined by clone-derived supernatant ELISA. All values expressed in pg/ml. TGF-β values are for acid hydrolysed supernatant fluids

	IFN-γ	IL-4	IL-5	IL-10	TGF-β
CD8+ 11.5A	8000	150	4027	2121	4288
CD8+ 2.9D	22500	<6	923	505	2992
CD4+ 3.2D	56300	<6	<12	2122	8032
CD4+ 11.2E	50105	<6	<12	2579	6700
CD4+ 14.10C	52340	<6	<12	1767	4792
CD4+ 15.3A	42610	<6	<12	2345	6484
PBMNC	24936	<6	<12	509	4192

Table 5.2. Ratios of cytokine production by CD8+ and CD4+ effusion-derived clones and PBMNC. The ratios of IFN-γ to type-2 cytokines (IL-4 and IL-5) as well as IL-10 and TGF-β are indicated.

	IFN-γ:IL-4	IFN-γ:IL-5	IFN-γ:IL-10	IFN-γ:TGF-β
CD8+ 11.5A	53	1.9	3.7	1.8
CD8+ 2.9D	>3750	24	44	7.5
CD4+ 3.2D	>9383	>4691	26	7
CD4+ 11.2E	>8350	>4175	19.4	7.4
CD4+ 14.10C	>8723	>4361	29.1	11
CD4+ 15.3A	>7101	>3550	18	6.5
PBMNC	>4156	>2078	49	6

5.3.3. Inhibition of autologous CD4+ clone and PBMNC proliferation by CD8+ clone 11.5A

Several unique features of the EMNC population including generation of a v-shaped from the LDA data, and the unique proliferation kinetics of the effusion mononuclear cells, suggested possible suppressor activity. In addition, the non-responsiveness of the CD8+ clones, coupled with the unique type-2 cytokine predominance and lack of CD28 (in clone 11.5A) was suggestive of suppressor T cells. The ability of clone 11.5A to mediate suppression was assessed by examining clone inhibition of both autologous CD4+ clone and PBMNC. proliferation.

The levels of suppression of proliferation of CD4+ clones and PBMNC mediated by clone 11.5A varied considerably. Figure 5.3 illustrates the level of suppression detected in response to both PPD and H37Rv. Optimal suppression was observed when PBMNC were

used as responder cells, with mean levels of suppression > 78% for both mycobacterial antigens. The stronger suppression of PBMNC may have been due to less potent production of pro-inflammatory cytokines as compared with the clones. Production of IFN-γ by PBMNC was 24936 pg/ml, whereas all CD4+ clones produced >40000pg/ml. If cytokines were involved in suppression of proliferation, the very high levels of IFN-γ produced by the CD4+ clones may have masked the suppressor effects mediated by the type-2 cytokines. In addition fresh PBMNC are not antigen primed like CD4+ clones, so the kinetics of induction of pro-inflammatory cytokines may differ from those of the clones. The delay in cytokine production by PBMNC may have allowed for early suppression mediated by clone-derived type-2 cytokines. The variability of suppression of CD4+ clone proliferation may also have been related to differing cytokine production patterns. Clone 11.2E produced lower levels of IFN-γ than either clones 3.2D or 10.4C. Clone 3.2D, which was most resistant to suppression, produced the highest level of IFN-γ of all the CD4+ clones. The balance between of clone 11.5A-derived type-2 and CD4+ clone type-1 cytokines may therefore have affected the level of suppression of proliferation observed.

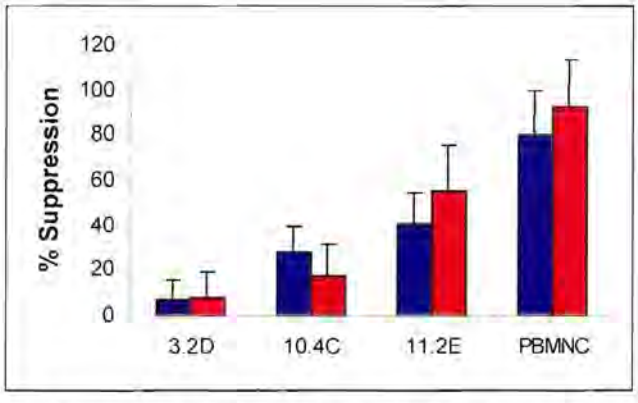


Figure 5.3. Percentage suppression of proliferation of CD4+ clones and PBMC mediated by clone 11.5A. Blue bars represent suppression in the presence of H37Rv, red bars in the presence of PPD. Data are representative of 3 experiments showing standard deviation

Figure 5.4. illustrates the inhibition of proliferation of PBMNC by clone 11.5A in response to various antigens. Clone 11.5A was able to induce high levels of suppression in response to all four mycobacterial antigens, with no suppressor activity being detected in response to SK-SD. Interestingly, suppression of proliferation in the presence of PHA was also detected. This contrasts with earlier reports on leprosy-derived clones in which suppressor activity was restricted to mycobactrial antigens (Modlin *et al.*, 1986; Ottenhoff *et al.*, 1986). A possible explanation for this finding is that the suppression observed was artefactual. PHA (Figure 5.1) optimally stimulated clone 11.5A. Resultant cell replication and activation may have led to exhaustion of IL-2 in the system. The absence of IL-2 may then have prevented any PBMNC proliferation from taking place. In addition, optimal activation of clone 11.5A may

have resulted in high levels of type-2 cytokine production, which would also have inhibited PBMNC proliferation.

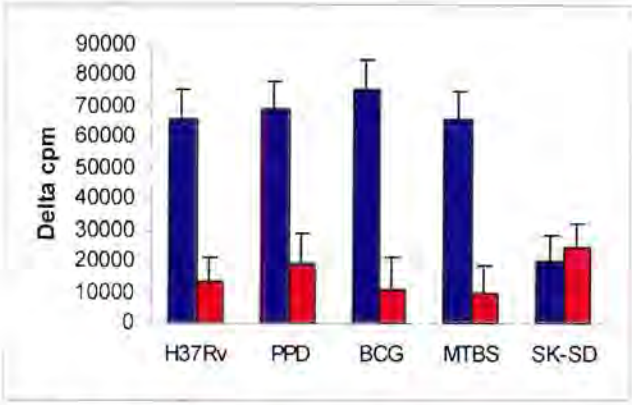


Figure 5.4. Suppression of PBMNC proliferation by Ts clone 11.5A. Blue bars represent proliferation in the absence of Ts, Red bars with Ts added. 4 mycobacterial antigens were used, as well as the mitogen PHA and the irrelevant antigen SK-SD. Data are representative of 4 experiments showing standard deviation

By contrast, the CD8+ clone 2.9D was unable to suppress proliferative responses. The presence of this clone led to an increase in delta cpm values irrespective of antigen used. Suppressor activity was thus unique to the type-0 clone 11.5A. The effect of clones 11.5A and 2.9D on PBMNC proliferation are summarised in Table 5.3. Negative levels of suppression in the presence of clone 2.9D are indicative of enhanced proliferative activity.

Table 5.3. Levels of suppression (%) of PBMNC proliferation by clone 2.9D and 11.5A

Antigen	2.9D	11.5A
H37Rv	-25±9	78±8
PPD	-13±8	92±8
BCG	-21±11	83±11
Rv-sonicate	-9±9	84±10
PHA	-19±10	69±16
SK-SD	-27±13	-26±9

5.3.4. IL-2 depletion could not account for antigen-specific suppression

IL-2 is important for T cell growth and proliferation. Earlier studies have suggested that "suppression" mediated by T cells may simply be a consequence of IL-2 depletion by the suppressor cells (Li *et al.*, 1990). This possibility is unlikely due to the very low number of suppressor cells being added to responder cells in the inhibition assays. Final ratios of 1 suppressor cell to 10 responder cells were used throughout the study. Such low numbers of suppressor T cells are unlikely to have exhausted IL-2 in the system, especially since clone 11.5A proliferated poorly in response to mycobacterial antigens. To ensure that IL-2 depletion was not taking place, certain assays were performed in the presence of exogenous IL-2. The addition of IL-2 (100 IU/ml) at the onset of suppressor assays had no

significant effect on either the level of PBMNC proliferation, or on the ability of clone 11.5A to inhibit proliferation. These data indicate that IL-2 depletion was not a contributory factor in the activity of clone 11.5A. However, as discussed above, IL-2 depletion may indeed have accounted for "suppression" in response to PHA

5.3.5. CD8+ clone-derived supernatants could not mediate suppression

Due to the fact that only clone 11.5A, and not clone 2.9D, was able to mediate suppression, the role of the predominant type-2 cytokines produced by clone 11.5A in this process was investigated further. Initially, the ability of clone 11.5A-derived supernatants to mimic the activity of the cells themselves was assessed. The addition of clone cell-derived supernatant fractions to PBMNC did not result in the high levels of suppression observed in the presence of the clone cells. These findings are illustrated in Figure 5.5. Although the clone 11.5A-derived supernatant fractions could not replicate the suppressive effect of the cells themselves, some inhibition of PBMNC proliferation was still apparent. This suggests that optimal suppression may have been dependent on localised production of type-2 cytokines. In addition, these findings indicate that cytokine activity was not solely responsible for all suppressor activity observed. The role of type-2 cytokines in the mediation of suppression is discussed further in section 5.3.8.

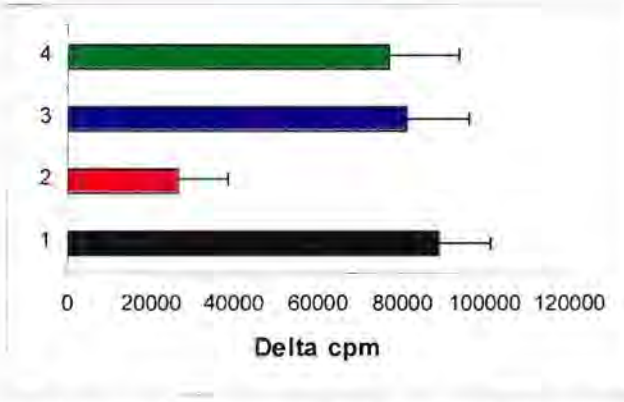


Figure 5.5. Clone 11.5A-derived supernatant and irradiated clone cells could not mediate suppression. Black bar (1) represents proliferation of PBMNC to H37Rv. Red bar (2) illustrates the effect of the presence of clone 11.5A on PBMNC proliferation. Blue bar (3) illustrates the effect of addition of clone 11.5A-derived supernatant on PBMNC proliferation. Green bar (4) illustrates the effect of irradiation of clone 11.5A on its ability to inhibit PBMNC proliferation. Data are representative of 3 experiments ± standard deviation.

5.3.6. Suppression was dependent on functional integrity of the CD8+ clone

Since cytokines or other soluble lymphocyte-derived factors could not solely account for the strong suppressor activity of clone 11.5A, the importance of cellular events was examined by irradiating the clone cells. Irradiation serves to damage the DNA and also to initiate apoptosis. Irradiation would not affect any receptors expressed on the surface of the cells,

but it would inhibit cellular activation and associated processes such as expression of novel receptors and/or the production of novel cytokines.

The effect of irradiating clone cells on their ability to inhibit PBMNC proliferation is presented in Figure 5.5. The addition of irradiated clone cells resulted in potent inhibition of suppression. However, irradiated cells did not completely abrogate inhibition of PBMNC proliferation. The absence of possible activation-dependent processes may have accounted for this finding. Although irradiated cells show diminished levels of cytokine production, it seems unlikely that inhibition of cytokine release could account for the strong inhibition of suppression observed. Interaction of clone cells with antigen-presenting cells may have resulted in the altered expression of cell surface receptors, or the down-regulation of APC-specific receptors such as CD80 or CD86, a phenomenon which has been characterised in xeno-specific suppression (Jiang *et al.*, 1998; Liu, Z. *et al.*, 1999). Alteration in receptor expression may therefore represent an important alternative mechanism of suppression of PBMNC proliferation. The importance of an intimate interaction between the suppressor cells and the resoponder cells for optimal suppression was addressed by physically separating the two populations during the suppressor assays.

5.3.7. Contact between suppressor cells and effector cells was essential for suppression

Dual chamber tissue culture dishes are useful for examining whether biological phemonena are dependent on cell-to-cell contact, or whether soluble mediators are sufficient to elicit a response. This is possible since cell populations remain separated by a semi-permeable membrane between the two chambers but diffusion of soluble mediators is unaffected. In the current study, transwell plates (Costar) were used to determine whether contact between suppressor cells (clone 11.5A) and responder cells (PBMNC) was necessary for inhibition of proliferation.

Standard assays, which assess inhibition of proliferation, were adapted to 24-well plate format. Preliminary experiments illustrated that increasing cell volumes and numbers for the larger wells, but retaining cell ratios, did not affect PBMNC proliferation or the inhibition thereof. Transwell plates were subsequently used to physically separate the two populations of cells. The upper wells contained clone 11.5A cells and irradiated PBMNC as antigen presenting cells. The lower well contained PBMNC alone. Control experiments were carried out in standard 24 well plates (Becton-Dickinson) without separation of the cell populations.

The effect of separating suppressor cells from effector PBMNC is illustrated in Figure 5.6. Although inhibition of proliferation occurred in standard 24-well plates, in the Transwell plates inhibition of proliferation of PBMNC was reduced. The abrogation of suppression was dramatic, irrespective of which mycobacterial antigens were used. Although separation of

the two cell populations greatly inhibited the suppressor activity of clone 11.5A, some residual suppression still occurred, possibly mediated by cytokines. No suppression of PBMNC proliferation was observed in the presence of SK-SD.

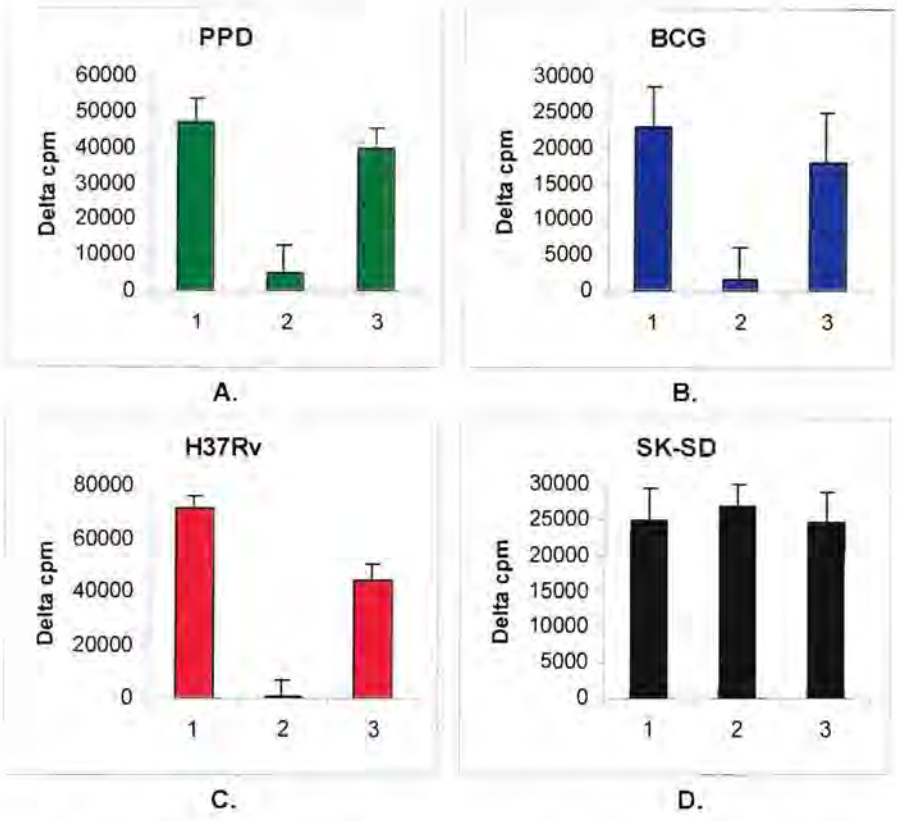


Figure 5.6. The effect of separation of clone 11.5A cells and responder PBMNC. Column 1 illustrates PBMNC proliferation to PPD (A), BCG (B), H37Rv (C), and SK-SD (D). Column 2 illustrates the effect of addition of clone 11.5A to PBMNC. Column 3 indicates the effect of incubating clone 11.5A and responder PBMNC in transwell plates which inhibits cell-to-cell contact. Data are representative of 3 experiments showing standard deviation.

Contact between suppressor and responder cells was necessary for optimal suppression of PBMNC proliferation. These experiments could not conclusively indicate whether direct contact between the suppressor cells and the responder PBMNC, or indirect contact via the antigen presenting cells was the pivotal interaction. Direct interactions between suppressor and responder cells may include MHC-TCR interactions without co-receptor engagement. T lymphocytes which express MHC class II molecules, and which present antigen to helper T cells in the absence of co-stimulation can induce anergy in the responder T cells (Pichler and Wyss-Coray, 1994; Broeren *et al.*, 1995). Alternatively, the suppressor cells may affect expression of certain molecules on the surface of the APC, which in turn could inhibit responder T cell activity. Xenospecific suppressor T cells are able to down-regulate expression of CD80 and CD86 on APC (Jiang *et al.*, 1998). Presentation of antigen to responder to cells in the absence of co-stimulation, or in the presence of altered co-stimulation, could result in non-responsiveness in the responder cell (Liu, L., *et al.*, 1999). In

addition, interaction between suppressor cells and APC may result in induction of immunosuppressive cytokine production and release. Macrophage-derived cytokines such as IL-6 and TGF-β have been implicated in suppressor activity in TB (Wadee *et al.*, 1993; van Heyningen *et al.*, 1997; Rojas *et al.*, 1999). Additional undefined macrophage-derived suppressor factors have been implicated in induction of non-responsiveness or oral tolerance (Channon *et al.*, 1999; Channon and Kasper, 1996). Interaction of all three cell populations (i.e. suppressor T cells, responder T cells, and macrophages) may thus be important for induction of optimal suppression. The interaction of th. se cells at the APC surface may also amplify the suppressive effect of type-2 cytokines produced by the suppressor cells. Release of cytokines such as IL-4 and IL-10 by suppressor cells situated adjacent to responder cells could result in inhibition of responder cell activation. Although the exact nature of the contact-dependent interactions remains unknown, the fact that both suppressor and responder populations were provided with APC in these experiments, suggests that competition for receptor sites on APC was not an important contributory factor in suppression.

5.3.8. IL-4 and IL-10 partially mediated suppression

The role of type-2 cytokines produced by clone 11.5A in the mediation of suppressor activity was investigated further by neutralisation of IL-4, IL-10 and TGF-β. The contribution of each cytokine to the net suppressive effect was assessed by determining the level of abrogation of PBMNC proliferation inhibition (i.e. suppression) in the presence of cytokine-specific neutralising antibodies. The effect of addition of neutralising antibodies to both IL-4 and IL-10 is illustrated in Figure 5.9.

The addition of both anti-IL-4 and anti-IL-10 abrogated the inhibition of proliferation mediated by clone 11.5A. Neither of these antibodies affected antigen-specific PBMNC proliferation in the absence of suppressor T cells. Isotypic control antibodies had no effect on clone 11.5A-mediated suppression. The levels of inhibition of suppression were optimal at 0.5 μg/ml and 1 μg/ml for anti-IL-4 and anti-IL-10, respectively. The addition of anti-IL-4 reduced suppression in the presence of both PPD (65% to 50%), and H37Rv (59% to 44%). Anti-IL-10 reduced suppression from 65% to 46% and from 59% to 34% in the presence of the same antigens. Maximal reduction of suppression was observed when these two antibodies were added simultaneously. Levels of reduction of suppression from 65% to 40% in the presence of PPD, and 59% to 31% in the presence of H37Rv were observed. The enhanced inhibition of suppression in the presence of both anti-IL-4 and anti-IL-10 was not, however, truly synergistic. Partial inhibition of *M. leprae*-specific suppressor cell activity by neutralisation of IL-4 and IL-10 has previously been reported (Mutis *et al.*, 1993b). In that study, variable levels of abrogation of T cell unresponsiveness were observed following inhibition of type-2 cytokine activity. However, Salgame *et al.* (1991b) have reported that the activity of IL-4 secreting suppressor clones could be completely inhibited when anti-IL-4 antibodies were

added. This contrasts with the findings of both the present study and that of Mutis *et al.* (1993b), and suggests that the suppressor activity in the latter two studies was not solely cytokine-mediated.

Anti-TGF-β antibody did not affect clone mediated suppression, nor did the antibody to the type-1 cytokine IFN-γ. These findings exclude a role for these cytokines in the mediation of suppression.

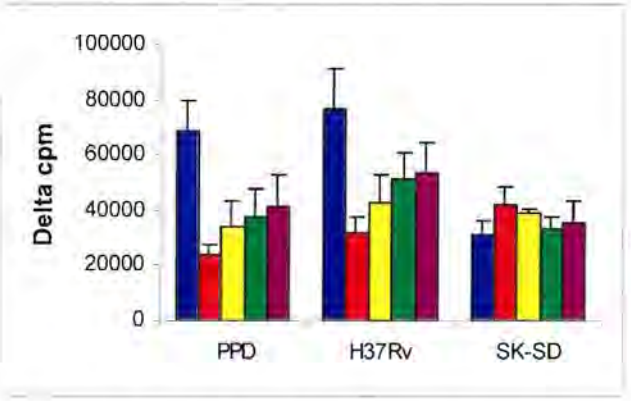


Figure 5.7. Effect of anti-IL-4 (0.5 μg/ml) and anti-IL-10 (1 μg/ml) on suppression of proliferation mediated by clone 11.5A. Experiments were carried out using three antigens: PPD, H37Rv and SK-SD. Standard PBMNC proliferation is indicated by the blue bars. The red bars indicate proliferation in the presence of clone 11.5A. The effect of anti-IL-4, anti-IL-10 and a combination of both are indicated by the yellow, green, and purple bars, respectively. Isoypic control antibodies had no effect on suppressor activity. Data are representative of 3 experiments showing standard deviation

Clone-mediated suppression was therefore at least partly mediated by the cytokines IL-4 and IL-10. To confirm this, the effect of addition of exogenous IL-4 and IL-10 on clone-mediated suppression was examined. These data are illustrated in Figure 5.8. The addition of both these cytokines augmented the inhibition of PBMNC proliferation by clone 11.5A. Maximal inhibition of proliferation (82%) was observed in the presence of a combination of 100 pg/ml of IL-4 and 2000 pg/ml of IL-10. This was equivalent to a 13% increase in suppression over levels observed in the absence of exogenous cytokines, but with the suppressor T cell clone cells present.

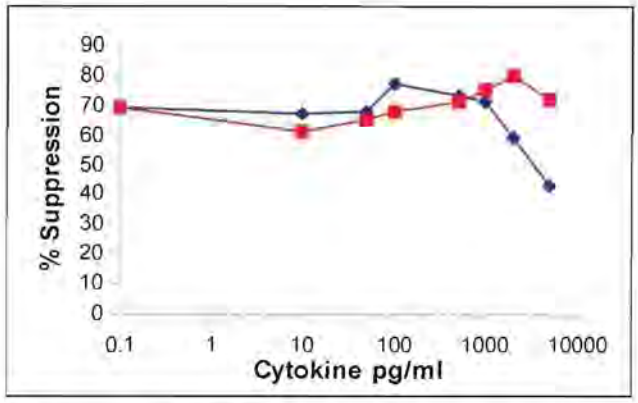


Figure 5.8. Effect of addition of IL-4 and IL-10 at various concentrations on level of suppression mediated by clone 11.5A. Maximal enhancement of suppression was observed at 100pg/ml IL-4 (77% suppression) and 2000pg/ml IL-10 (80% suppression). The increase in suppression was not statistically significant. Data represent mean values of 3 experiments

These results appear to conflict with the finding that clone generated supernatants could not mediate suppression (section 5.3.5). However, addition of IL-4 or IL-10 to PBMNC in the absence of clone 11.5A had no effect on proliferative ability of the PBMNC. Only when IL-4 and IL-10 were added to the suppressor clone cells was suppression enhanced. This suggests that both clonal receptor and cytokine involvement was necessary for suppression to occur. The fact that blocking cytokine activity did not completely abrogate suppressor activity confirmed that cytokines alone were not capable of inducing suppression.

5.3.9. Suppression was MHC class II DR-restricted

Detailed analysis of suppressor T cell activity in lepromatous leprosy has indicated that these cells are MHC class II-restricted. Both HLA-DR-restriction (Ottenhoff *et al.*, 1986; Li *et al.*, 1990), and HLA-DQ-restriction (Modlin *et al.*, 1986) have been reported. The MHC-restriction of suppressor activity mediated by clone 11.5A was investigated on several levels. Initially, the suppressor activity of clone 11.5A in HLA-DR-matched and –mismatched donors was investigated. The patient was tissue typed at the Tissue Typing Laboratory, Groote Schuur Hospital, Cape Town. The tissue typing profile of the patient, as well as other donors used in the study, are illustrated in Table 5.4.

Table 5.4. MHC class I and II (HLA-A, -B, -C, -DR, -DQ) typing of patient AJ and 3 other donors

	MHC class I			MHC class II	
AJ	A2,24	B15,47	Cw-,-	DR12,13	DQ1,7
RG	A2,31	B51,62	Cw3,-	DR4,11	DQ3,-
BR	A2,24	B7,51	Cw3,6	DR2,13	DQ1,-
EC	A3,30	B7,65	Cw7,8	DR2,11	DQ1,7

Using donors matched at DR or DQ, the activity of clone 11.5A in inhibiting proliferation was investigated. PBMNC isolated from individuals matched at both DR and DQ (BR), DQ alone

(EC), or neither DR nor DQ (RG) were used in standard suppressor assays. The ability of clone 11.5A to suppress proliferation of the PBMNC is illustrated in Figure 5.9

Apart from suppression of autologous PBMNC, clone 11.5A was only able to inhibit PBMNC proliferation in donor BR. This donor was matched at both DR and DQ loci. PBMNC from donor EC (matched at DQ alone) were unaffected by clone 11.5A. These findings indicate that matching of the DR13 loci between AJ and BR was sufficient to induce suppression at comparable levels. This finding confirms earlier reports that suppressor clones are DR restricted (Ottenhoff *et al.*, 1986; Li *et al.*, 1990).

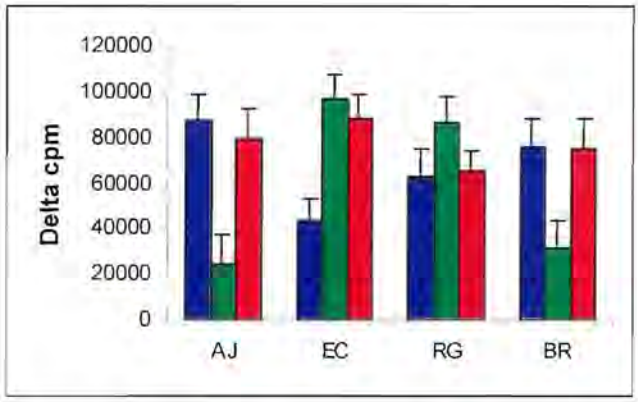


Figure 5.9. The ability of clone 11.5A to mediate suppression of PBMNC in HLA-DR-matched and –mismatched donors. Blue bars represent PBMNC proliferative responses to H37Rv in the 4 donors. AJ is the response of autologous PBMNC. EC and RG are two DR-mismatched donors, and BR is DR-matched to AJ. Green bars represent proliferation in the presence of clone 11.5A. Red bars illustrate the effect of addition of the type-1 CD8+ clone, 2.9D.

The co-culturing of CD8+ clone 11.5A with allogeneic donors resulted in enhanced proliferation of PBMNC cells from donors EC and RG. This enhanced proliferation was probably due to allogeneic stimulation of T cell activity. Co-culturing of clone 11.5A with PBMNC from donor BR did not result in this enhanced proliferative activity. This may have been due to early induction of suppressor activity in the DR-matched cultures. Early suppressor activity may have prevented subsequent expansion of T cells due to any allogeneic interaction. The role of DR-restriction was investigated further in neutralisation assays.

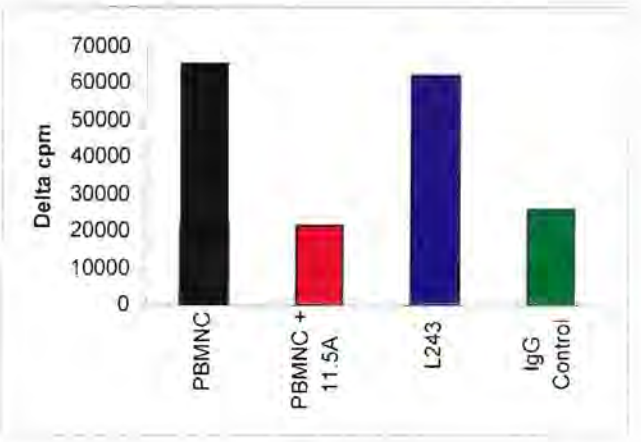


Figure 5.10. Inhibition of suppressor activity mediated by clone 11.5A. Addition of L243 (anti-DR) ascites (10^{-6}) to PBMNC and clone 11.5A completely abrogated inhibition of H37Rv-specific proliferation of PBMNC (column marked L243). Addition of an isotypic control antibody to PBMNC and clone 11.5A had no effect on clone 11.5A-mediated suppression (column marked IgG control)

Mouse anti-human HLA-DR antibody from ascites fluid of animals inoculated with L243-derived HLA-DR molecules was used in neutralisation assays. Anti-HLA-DR antibody was able to completely abrogate suppressor activity of clone 11.5A at a dilution of 10^{-6} (Figure 5.10). This finding appeared to confirm HLA-DR locus involvement in mediation of suppressor activity. Why did neutralisation of HLA-DR not affect PBMNC proliferation? A possible explanation may be that low concentrations of anti-DR antibodies were used. Antigen presenting cells are likely to express higher levels of DR than suppressor T cells. Complete inactivation of the T cell-associated DR molecules may not necessarily have been accompanied by complete APC-associated DR blocking. Residual DR molecules on the APC could then have enabled CD4+ T cell proliferation to occur in the presence of low concentrations of neuralising antibody. The detection of PBMNC proliferation in the presence of anti-DR indicates that complete blocking of DR molecules did not occur. However, abrogation of suppressor cell activity in the presence of neutralising antibody, suggests that suppressor cell–associated DR was important in the mediation of suppressor T cell activity.

5.4. Discussion

In tuberculosis the paradox of simultaneous immune activation and suppression has been well documented (Ellner, 1997; Vanham *et al.*, 1997). Features indicative of immunosuppression include suppressed proliferative responses to mycobacterial antigens and impaired cytokine secretion by T lymphocytes. The relative strength of either activation or suppression may be important in determining the outcome of the disease. The patient in the current study encapsulated both of these phenomena, with the mononuclear cell population derived from the pleural effusion displaying elements of both activation and suppression. Immune activation is advantageous for the host in that a strong pro-inflammatory response generally results in infection clearance and recovery. Immune suppression, on the other hand, is more often associated with systemic spread of infectious organisms and ultimately death. Enhanced antigen-specific immunosuppression therefore bestows a distinct advantage upon the infecting organism (Marrack and Kappler, 1994). Antigen-specific immune suppression is particularly well characterised for viral diseases (Smith, 1994; Ploegh, 1998), however it is also a feature of certain intracellular bacterial diseases, particularly the mycobacterial diseases leprosy and tuberculosis (Bloom *at al.*, 1992).

Induction of suppressor activity in T lymphocytes represents one of several possible responses following exposure to antigen. Generally, antigenic stimulation of T lymphocytes results in one of the following responses: (i) activation, (ii) ignorance, (iii) apoptosis, (iv) anergy, or (v) suppression. Activation encompasses both clonal expansion and enhanced functional activity (e.g. cytokine secretion and cytotoxicity). Activation represents the up-regulatory arm of cellular immunity. Ignorance, also termed non-responsiveness, refers to the decision of a T lymphocytes not to respond to a particular antigen. Apoptosis (or programmed cell death) is the process which results in clonal deletion of T lymphocytes. Apoptosis is an important homeostatic control mechanism which regulates T lymphocyte expansion at disease sites. Anergy (or induced non-responsiveness), unlike ignorance, refers to antigen-specific unresponsiveness in situations which would normally result in activation. Suppression is an antigen-specific T lymphocyte response which results in active inhibition of normal responder T lymphocyte activation. Not all immune suppression can be accounted for by the activity of T lymphocytes, indeed a range of possible mediating factors has been proposed.

The processes of clonal deletion, anergy and suppression all represent the down-regulatory arm of normal cellular immune responses. Anergy and suppression are closely related phenomena, and as such these terms are sometimes used interchangeably. Both suppressor cells and anergic cells are often non-responsive to antigenic stimulation *in vitro*. In addition, both cell types are able to induce suppression (Lombardi *et al.*, 1994; Frasca *et al.*, 1997; Taams *at al.*, 1998). T cell anergy is defined as a state in which the T lymphocyte

is alive but fails to display certain functional responses (e.g. proliferation, IL-2 or other cytokine secretion, cytotoxicity) upon exposure to antigen under otherwise stimulatory conditions (Schwarz, 1996). T cell anergy can be induced by exposure to an antigen stimulus in the absence of co-stimulation (Mueller *at al.*, 1989). In the presence of co-stimulation, the addition of altered peptide ligands can also cause anergy (Allen, 1994). In addition, antigen presenting T cells which express MHC class II molecules can induce anergy in preactivated T cells even when co-stimulation is provided (Pichler and Wyss-Coray, 1994; Broeren *at al.*, 1995). In models of autoimmunity e.g. experimental arthritis and allergic encephalomyelitis, states of immunological unresponsiveness have been induced by T cell clones that following *in vitro* culture have lost their effector capabilities (van Eden *at al.*, 1985; Sun *at al.*, 1988). These findings seem to support the notion of anergic T cells themselves inducing anergy or unresponsiveness in other responder T cells (Frasca *et al.*, 1997; Taams *et al.*, 1998). The suppressor activity of anergic cells is linked to interference in normal T cell activation processes, and excludes a role for cytokines (Lombardi *et al.*, 1994). Anergic cells can often be induced to respond to antigen in the presence of exogenous IL-2, which suggests that inability to produce IL-2 is an important defining feature of these cells (Taams *et al.*, 1998). This represents an important distinction between anergic and suppressor cells, since suppressor cells retain the ability to produce IL-2, and several other cytokines, following antigen stimulation.

Although the two CD8+ clones generated in the current study were non-responsive to antigenic stimulation *in vitro*, their mechanism of action was not linked to their apparent anergic status. Since only one of the non-responsive CD8+ clones could suppress proliferation of lymphocytes, non-responsiveness or anergy did not explain the suppressor activity of clone 11.5A. The inability to induce antigen responsiveness in the presence of exogenous IL-2 coupled with the ability to produce a range of cytokines in response to antigenic stimulation, confirmed that these clones were not anergic (Taams *et al.*, 1998; Schwartz, 1996). The non-responsiveness to mycobacterial antigens *in vitro* was probably due to the absence of specific mycobacteria-derived epitopes to which the clones were able to respond.

Immune suppression in intracellular infections such as *M. tuberculosis* and *M. leprae* generally refers to the inhibition of antigen-responsive helper T cell activity. Suppression in TB may be associated with one or more overlapping phenomena. These include (i) compartmentalisation of antigen-responsive cells to disease sites, (ii) cytokine-mediated induction of T lymphocyte non-responsiveness, or interference in antigen presenting cell function, (iii) enhanced levels of apoptosis of responder lymphocytes, (iv) anergic cell-mediated suppression, and (v) antigen-specific suppressor T cell-mediated inhibition of T lymphocyte activation.

Compartmentalisation of antigen-responsive cells to the disease site is accompanied by a reduction in the number of responder cells in the peripheral blood (Barnes *et al.*, 1989; Dieli *et al.*, 1999). The reduction in responder lymphocytes in the peripheral blood is reflected by an *in vitro* reduction in proliferation and/or cytokine production. Compartmentalisation occurs during active disease and peripheral blood anergy can be reversed following chemotherapy (Dieli *et al.*, 1999). The suppression detected in the present patient was not related to compartmentalisation since peripheral blood responses to mycobacterial antigen were normal.

The predominance of immunosuppressive *cytokines* in TB has been linked with skin test non-responsiveness. The ratio of IL-4/IL-10 to IL-12 seems to be important in determining skin reactivity, and suggests that cytokine ratios may determine disease outcome (Baliko *et al.*, 1998). In addition, a recent study has correlated enhanced patient IL-4 mRNA expression with more extensive disease manifestations (Seah *et al.*, 2000). These patient-based studies seem to indicate that predominance of cytokines such as IL-4 and IL-10, at time of infection, may dictate outcome of disease. T cell unresponsiveness in TB has also been linked to direct inactivation of T lymphocytes by the macrophage-derived cytokines TGF-β and IL-10 (Vanham *et al.*, 1997; Hirsch *et al.*, 1996). The synergistic effects of these cytokines have recently been shown to inhibit T cell production of interferon-γ (Othieno *et al.*, 1999), suggesting that cytokines may actively inhibit important T cell functions. In addition, inhibition of APC function (i.e. antigen presentation and co-stimulatory molecule expression) by cytokines such as TGF-β and IL-10 may result in immune suppression (Wahl, 1992). *M. tuberculosis* infection of macrophage APC itself can results in enhanced production of inhibitory cytokines such as IL-6 (Van Heyningen *et al.*, 1997), and decreased production of important pro-inflammatory cytokines such as IL-12 (Ellner, 1997). In the present study, the CD8+ clone, which was able to inhibit T cell proliferation, produced type-2 cytokines, and had the lowest ratio of type-1 to type-2 cytokines of all the clones and PBMNC. The activity of type-2 cytokines in the mediation of suppression was also demonstrated by the partial abrogation of suppression in the presence of neutralising antibodies to IL-4 and IL-10. However, cytokines alone could not account for all suppressor activity, suggesting that additional components were involved.

Enhanced *apoptosis*, possibly related to T cell overstimulation, may account for much of the immune suppression observed in TB (Hirsch *et al.*, 1999). Increased numbers of apoptotic cells in responder Th1 populations have been reported in TB. Although levels of apoptosis were not examined in the present study, viability of both effusion- and peripheral blood-derived mononuclear populations was high. This does not, however, exclude a possible role for clonal deletion in effecting certain immunosuppressive features of the patient. The activity of CD8+ clone 11.5A could not be explained by induction of enhanced T cell apoptosis. The CD8+ clone was not cytolytic towards infected macrophages, and is unlikely to have induced

death of other responder cells. If the suppressor clone cells induced apoptosis of lymphocytes, neutralising cytokine antibodies would have been unable to affect levels of suppression. However, these findings do not exclude the possibility of more than one mechanism of suppression induction.

Suppressor T cell activity has been defined in two circumstances. Firstly, in situations where there is a failure to respond to a particular antigen in the presence of certain T cells and where responsiveness is restored by removing the T cells (Gershon and Kondo, 1971). Secondly, in situations where unresponsiveness can be induced in individuals otherwise capable of responding. This normally occurs when low dose soluble antigen is introduced, or where antigen is chemically modified or coupled to self antigens (Mitchison, 1964; Mueller *at al.*, 1989; Claman *at al.*, 1980). Of particular importance in the present study has been the description of antigen-specific suppressor T cells in leprosy.

Mediation of suppression in TB by *anergic and/or suppressor T lymphocytes* has not been documented. Suppressor T cells have, however, been isolated from the lesions of lepromatous leprosy patients. In lepromatous disease there is an increase in the number of CD8+ T cells in lesions (Modlin *et al.*, 1983). In addition lepromatous lesions have been found to contain an abundance of the type-2 cytokines IL-4, IL-5 and IL-10 (Yamamura *et al.*, 1991). T lymphocytes isolated from such lesions display antigen unresponsiveness and removal of the CD8+ cells resulted in restoration of antigen responsiveness in certain cases (Modlin *et al.*, 1986). Lines and clones of lepromatous lesion-derived T cells (both CD4+ and CD8+) have been generated and characterised (Modlin *et al.*, 1986; Ottenhoff *et al.*, 1986). At the clonal level these cells produce high levels of IL-4 and IL-5, but little IFN-γ (Salgame *et al.*, 1991a). In several studies, suppressor T lymphocytes have been shown to lack CD28 (Li *et al.*, 1990). The activity of these cells has been found to be MHC class II-restricted, either at the HLA-DQ locus (Modlin *et al.*, 1986), or the HLA-DR locus (Ottenhoff *et al.*, 1986). However, exceptions to this pattern have been noted (Mutis *et al.*, 1994). Although the exact mechanism whereby such cells induce immune suppression remains unknown, the outcome of their activity is the induction of clonal anergy or apoptosis (Salgame *et al.*, 1991b; Sambhara and Miller, 1991).

The mechanisms by which antigen-specific suppressor and/or anergic T lymphocytes effect inhibition of proliferation of responder cells are not clearly understood. Several possible scenarios have been suggested focusing on either the APC or the responder T lymphocyte as the prime target. At the level of the APC, suppressor cells may directly lyse the APC, induce suppressor factor/cytokine production by the APC, compete for MHC molecules and/or co-receptors on the APC, mediate alteration in expression of receptors (MHC, co-receptors) on the APC, or directly inhibit APC function via cytokine activity. At the level of the responder cell, suppressor cells may directly lyse lymphocytes, dysregulate normal IL-2

activity (i.e. exhaust IL-2 supplies, inhibit IL-2 production by T cells, or down-regulation IL-2 receptor expression), directly inhibit T cell responses via inhibitory cytokine activity, or provide inappropriate signalling to responder cells (e.g. direct antigen presentation without co-receptor involvement), resulting in non-responsiveness.

CD8+ suppressor clones in leprosy appear to induce unresponsiveness by interference with normal antigen recognition by the responder T cell (Salgame *et al.*, 1989). This may involve direct antigen presentation by the suppressor T cell to responder cells, or blocking of antigen recognition by the responder T cell. The activity of suppressor T cells is dependent on CD8 and TCR expression, as well as MHC class II expression (Salgame *et al.*, 1991b). Monoclonal antibodies to all these molecules abrogated suppression. Other potential mechanisms proposed include release of suppressor factors which inhibit responder cell activity, release of cytokines such as IL-4 and IL-5 which directly inhibit responder cells, competition for antigen or MHC receptors, and deactivation or blocking of IL-2 resulting in responder cell anergy (Bloom *et al.*, 1992).

Competition for MHC molecules or co-receptors on the surface of antigen presenting cells by the suppressor T cells (Webb *et al.*, 1994), was unlikely to have played a role in the present study since there were far fewer CD8+ clone cells than PBMNC present in suppressor assays. Direct lysis of APC by suppressor cells (Bloom *et al*, 1992; Taams *et al.*, 1998), was excluded on the basis that the clone showed minimal cytolytic ability when exposed to macrophages pulsed with mycobacterial antigen. Dysregulation of IL-2 activity by suppressor cells (Li *et al.*, 1990), was excluded due to the finding that addition of exogenous IL-2 did not affect suppressor activity. Although IL-2 depletion was unlikely to have accounted for antigen-specific suppression, it may have been a contributory factor in PHA-mediated suppression due to optimal activation of suppressor cells.

Direct inhibitory cytokine effects are linked to a predominance of type-2 cytokines in generalised immunosuppression. Suppressor T cell-derived cytokines such as IL-4, IL-5, IL-10, and TGF-β have been associated with immune suppression. The activity of immunosuppressive cytokines was confirmed in the present study. Both IL-4 and IL-10 were directly implicated in T lymphocyte-mediated suppressor activity. Macrophages can also produce immunosuppressive cytokines such as IL-6, IL-10 and TGF-β, as well as other mediators such as arachodonic acid metabolites (prostaglandins and leukotrienes). The induction of cytokine release from macrophages by the suppressor cells may involve a suppressor cell cytokine mediator. In addition, alteration of macrophage receptor expression may also be linked to the activity of a suppressor cell-derived cytokine. This latter phenomenon is important since xenospecific suppressor T cells are able to affect expression of B7.1 and B7.2 molecules (Lederman and Sucia-Foca, 1999). These molecules are important in the activation of lymphocytes, and interference in expression could result in

induction of anergy in responder cells. Enhancement of B7.2 expression, and engagement with the CTLA-4 receptor would result in non-responsiveness. Similarly, reduced expression of B7.1 would reduce CD28 engagement, also resulting in non-responsiveness. Direct activity between suppressor and responder cells may be linked to cytokine effects, but also to direct signalling. Suppressor cells may express MHC class II, and as such may be capable of presenting antigen to T cells (Schwarz, 1996). If this occurs in the absence of appropriate co-stimulation, anergy may be induced.

How are suppressor T cells generated? There is no clear indication of how cells develop a suppressor phenotype, however several studies have suggested that antigen-primed CD4+ Th cells when exposed to CD8+ cells in the absence of specific antigen, induce a suppressor phenotype. When the suppressor T cells are subsequently incubated with responder cells in the presence of antigen, suppression occurs (Koide and Engleman, 1990; Damle and Engleman, 1989; Damle *et al.*, 1984). These findings suggested that suppressor cells respond to antigen recognition molecules on Th cells. In studies using xenospecific suppressor cells, it has been demonstrated that suppressor cells inhibit CD4+ Th1 responder cell activity via modulation of macrophage receptor expression, most importantly B7.1 and B7.2. These studies implicate cross-talk between three cell types as pivotal in determining response (Lederman and Suciu-Foca, 1999; Jiang *et al.*, 1998). A similar scenario has been proposed in anergic cell induced suppression (Taams *et al.*, 1998). At least two functional scenarios have thus been demonstrated *in vitro*. This, in combination with suppressor cells in leprosy that have been shown to mediate effects at least partially via cytokine inactivation, suggests complex interactive pathways in the mediation of active suppression.

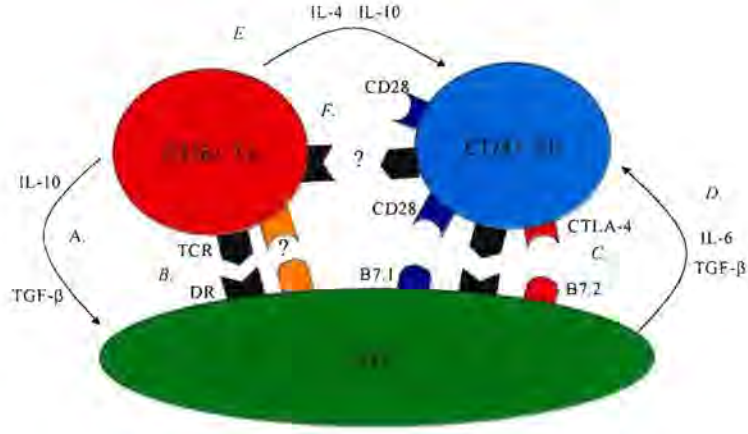


Figure 5.11. Schematic illustration of possible suppressor activity mediated by CD8+ clone 11.5A.

Possible mechanisms of CD8+ T lymphocyte-mediated suppression are illustrated in Figure 5.11. The CD8+ T cell clone 11.5A, which produced both type-2 and type-1 cytokines, was able to inhibit responder PBMNC and clone proliferation. The clone lacked CD28, was HLA-DR-restricted, was non-responsive to mycobacterial antigen, was non-cytolytic and was not anergic. Suppression was partially mediated by T cell-derived cytokines IL-4 and IL-10 (A. and E., Figure 5.11), but cytokine activity was apparent only in the presence of the cloned suppressor T cell. Cell-to-cell contact was required for suppression to occur (B., C., and F., Figure 5.11). This contact dependency has been observed in both lepramin-specific and anergic suppressor clones as well (Li *et al*, 1990; Taams *et al*, 1998). It seems possible that the suppressor cell on recognising antigen, was activated and produced a range of cytokines. These cytokines may have induced macrophages to alter receptor expression, preventing optimal signalling to responder cells (C., Figure 5.11). Macrophages may also have been stimulated to produce immunosuppressive cytokines (D., Figure 5.11). Alternatively, interaction between responder cells and suppressor cells (F., Figure 5.11) – possibly via inappropriate MHC-TCR signalling – may have induced non-responsiveness.

The activity of suppressor T cells, similar to that described in the present study, may help to explain immunosuppressive phenomena at disease sites. The occurrence of cells that can mediate immune suppression would help to explain the divergent manifestations of infection with *M. tuberculosis*. An increase in the number of suppressor cells and/or in their activity could account for rapid organism spread observed in certain patients. An increase in mycobacterial numbers is disadvantageous to the host, therefore inappropriate stimulation of suppressor cells activity needs to be counteracted. It is possible that infectious mycobacteria have exploited the activity of these cells. Manipulation of antigen presentation, co-stimulation, cytokine secretion or other mechanisms would enable enhanced replication and spread. A more detailed assessment of the universality of such suppressor cells and their role in the pathogenesis of TB is required, however it appears that the activity of such cells may enhance certain features of immune suppression, and therefore they represent a possible target for therapy.

Peripheral blood-derived CD4+ CTL possessed a predominant Fas-mediated killing component, whereas CTL derived from the effusion had a predominant granule exocytosis killing mechanism. What causes this discrepancy? It seems that the unique localised environment of the effusion is able to initiate an enhanced granule-dependent mode of killing in TB antigen-responsive cells. This may be due to the cytokine milieu, or to the recognition of unique pathogenic epitopes derived from *M. tuberculosis*. The predominant granule-mediated component of killing may also be accompanied by an increased concentration of particular cytolytic granule components. These components may themselves be pivotal in the clearance of infection following lysis of infected macrophages by CD4+ CTL.

CD8+ CTL have been reported to be mycobactericidal if they kill macrophages by granule exocytosis (Stenger *et al.*, 1997). Although CD8+ lymphocytes are now accepted as playing some role in immunity to TB, the fact that they are not expanded at disease sites, and that elimination of CD8+ cells does not cause death of mice, or exacerbation of disease in humans, suggests their role is not pivotal (Schluger and Rom, 1998). This is probably due to the nature of the infection, with mycobacteria remaining within the phagosome in host cells. Access to the MHC class I pathway is in this way prevented, and subsequent stimulation of antigen-specific CD8+ cells is sub-optimal. For this reason MHC class II-responsive CD4+ cells may have assumed the role traditionally played by CD8+ cells in intracellular viral infections.

Generation of cytolytic activity *in vitro* in the absence of CD8+ cells has been shown to result in CD4+ CTL which possess a granule exocytosis mechanism of killing (Sevilir-Williams and Engelhard, 1997). In TB, CD4+ cells would be expanded (possibly in the lymph nodes) and then recruited to the disease site, in the absence of extensive CD8+ expansion. This may result in generation of granule mediated killing, which in itself is mycobactericidal.

Mycobactericidal killing is important and suggests that the cytolytic activity of CD4+ T cells, together with cytokine activation of macrophages, may be essential in clearance of infected organisms from the disease site. This has major implications in the development of possible immunotherapeutic control measures. Determination of the factors unique to the disease site, which may play a role in the generation of mycobactericidal CD4+ cells, could be incorporated into possible vaccine strategies. Promoting CD4+ CTL activity could enhance the efficacy of novel antibiotic treatments, or other anti-microbial measures. A clearer understanding of the mycobacterial peptides that stimulate cytolytic CD4+ T cells, and of the cytokine milieu of the disease-associated site, would aid in development of possible future therapeutic procedures. In addition, an investigation of the cytolytic granule components of the CD4+ CTL, and how particular granule constituents are able to kill mycobacteria, is another major thrust of future research.

The other important finding in this study has been that a CD8+ clone derived from the effusion could suppress proliferation of autologous lymphocytes. This suggests that CD8+ T cells may play a regulatory role in TB. Switching off the expansion of Th1 cells at disease sites may be important to prevent tissue damage caused by certain cytokines. Without regulation of the localised inflammatory response, necrosis and disseminated spread of organisms may occur. The activity of any population of cells, which can down-regulate the inflammatory response, would thus normally be beneficial to the host. The activity of suppressor T lymphocytes becomes problematic if they are over-active or stimulated before adequate clearance of infecting organisms has occurred. In the present study suppressor cells may have accounted for an inhibition of the pattern of accelerated *in vitro* responsiveness to mycobacterial antigens observed in over two thirds of patients with TB pleuritis (Lukey *et al.*, 1996, Lorgat *et al.*, 1992b). The generalised non-responsive immune reactions to TB of a sub-population of patients may be due to expansion and stimulation of suppressor T cells. A more detailed understanding of the mechanisms by which these cells operate would aid in treating such patients. Switching off active suppression may result in the development of a normal Th1 dominant response.

Although this study has highlighted important new functional roles for CD4+ and CD8+ lymphocytes, certain limitations are apparent. Due to continuous use of T cell clones, this study concentrated on a single patient. A cross-sectional study would aid in determining the universality of these findings. If mycobactericidal cytolytic CD4+ cells and/or suppressor CD8+ cells can be demonstrated in a broad cross-section of TB patients, the findings of the present study would have served as the basis for novel immunotherapeutic approaches in tuberculosis research.

Mycobactericidal activity may be the result of late events associated with apoptosis, or novel components of the cytolytic granules. Future experiments need to address involvement of apoptosis-associated caspases and compare PBMNC and effusion lines, in order to identify any mediators selectively involved in effusion induced apoptosis. An understanding of the components of the cytolytic T cell granules - what initiates their production, and of how they kill bacteria when the cell is undergoing apoptosis – would aid in the development of future treatment strategies. If novel mycobacterial epitopes can be shown to be involved in development of predominant granule-dependent CTL, then a vaccine incorporating these mycobacterial determinants that stimulate mycobactericidal CD4+ T cell activity would ensure a rapid and efficient clearance of organisms. Similarly, an understanding of the antigenic determinants which stimulate suppressor cells, definition of unique markers expressed by these cells, and an understanding of the environment which favours their expansion, could help in the design of anti-tuberculosis therapies. Exclusion of any mycobacterial components that could trigger suppressor activity, would ensure greater vaccine efficacy. Vaccine design needs to incorporate an understanding of localised immune

responses. Enhancing the protective host response will result in more rapid organism clearance, allowing for shorter treatment time, and thus aid in the future combating of this disease.

In conclusion, the current study describes novel functional activities of both CD4+ and CD8+ T lymphocytes in the host immune response to *M. tuberculosis* infection. This study represents the first report of both TB patient-derived mycobactericidal cytolytic CD4+ T cells, and a CD8+ suppressor T cell clone. The description of the novel activities of these two cell populations may be important for a better understanding of protective immune responses at the localised disease site, and may ultimately aid in the development of a TB vaccine and/or novel immunotherapeutic treatments.

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