The role of the cytolytic mediators, Granulysin and Perforin, in Tuberculosis

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DEDICATION

I dedicate this thesis to my daughter Tarryn and to Almighty God for entrusting her in our love and care

DECLARATION

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ABBREVIATIONS

7AAD	7-aminoactinomycin D
AFB	acid fast bacilli
Ag	antigen
Ag85	antigen 85
a-IFN-γ	aerosolized interferon gamma
APC	antigen presenting cell
-APC	allophycocyanin
BAL	bronchoalveolar lavage
BCG	Bacillus Calmette Guérin
Ca^{2+}	calcium cation
CB	cord blood
CBA	Cytokine Bead Array
CBMC	cord blood mononuclear cell/s
CCR	chemokine receptor
CD	cluster of differentiation
cDNA	copy deoxyribonucleic acid
CFP 10	culture filtrate protein 10
CFU	colony forming units
CMI	cell mediated immunity
CN	*Cryptococcus neoformans*
cpm	counts per minute
CTL	cytotoxic lymphocyte/s
CXCR	chemokine CXC motif receptor, where C is a cysteine and X is any other residue
DC	dendritic cell
DID	Danish intradermal
DMSO	dimethylsulphoxide
DOTS	directly observed treatment shortcourse
ELISA	enzyme-linked immunosorbent assay
ESAT 6	early secreted antigenic target 6kDa protein
FACS	fluorescence activated cell sorter

Fas/L	Fas ligand
FCS	foetal calf serum
FITC	fluorescein
Fc	fragment crystallizable (region)
FcR	fragment crystalizable (region) receptor
g	gravitational force
γδ	gamma delta
HBHA	heparin-binding haemagglutinin
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HPCSA	Health Professional Council of South Africa
ICC	intracellular cytokine
Ig	immunoglobulin
IFN-γ	interferon gamma
IFN-γ R	interferon gamma receptor
IL-	interleukin
iNOS	inducible nitric oxide synthase
IP-10	interferon inducible protein 10
IRF	interferon regulatory factor
IU	international units
JID	Japanese intradermal
JPC	Japanese percutaneous
kDa	kiloDalton
LAMP	lysosomal-associated membrane protein
M.avium	*Mycobacterium avium*
Mb	mega bases
M.bovis	*Mycobacterium bovis*
MCP-1	monocyte chemoattractant protein 1
MDR-TB	multi-drug resistant tuberculosis
Mg^{2+}	magnesium cation
MHC	major histocompatibility complex
MIP-1β	macrophage inflammatory protein-1 beta
MIG	mitogen inducible gene

MMP	matrix metalloproteinase
MMR	macrophage mannose receptor
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
M.tb	*Mycobacterium tuberculosis*
MTOC	microtubule-organising centre
ND	healthy PPD+ adult volunteers
NEB	nebulizer
NK	natural killer
NKT	natural killer T-cell
NO	nitric oxide
NOS-2	nitric oxide synthase-2
PB	peripheral blood
PBMC	peripheral blood mononuclear cell/s
PBS	phosphate buffered saline
PE	phycoerythrin
PEL	pleural effector lymphocyte/s
PerCP	peridin chlorophyll protein
PF	pleural fluid
PPD	purified protein derivative
PRR	pattern-recognition receptor
PS	phosphatidyl serine
PTB	pulmonary tuberculosis
RCT	randomized clinical trial
rIFN-γ	recombinant interferon gamma
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SAPLIP	saposin-like family of proteins
SCID	severe combined immunodeficiency
SDS	sodium dodecylsulphate

SNF	supernatant fluid
STAT	signal transducer and activator of transcription
SUB	subcutaneous
TB	tuberculosis
TCR	T-cell receptor
TGF-β	T-cell growth factor-beta
Th	T-helper
TLR	toll like receptor
TNF-α	tumour necrosis factor-alpha
T-reg	regulatory T-cell/s
UCB	umbilical cord blood
UCT	University of Cape Town
uNK	uterine natural killer
XDR-TB	extensively drug-resistant tuberculosis
WHO	World Health Organisation

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ABSTRACT

Protective immunity against mycobacterial infection requires an effective cytolytic response, in addition to an intact Type 1 (Th1) cytokine pathway. Natural killer (NK) cells and cytolytic T-cells (CTL) are essential components of protective immunity against tuberculosis (TB) and mediate granule-dependent killing of infected cells. Granulysin, an antimicrobial protein, and perforin, a pore-forming molecule, have been found to co-localise in the granules of these two cell types. Granulysin has been shown to be directly cytotoxic to extracellular *Mycobacterium tuberculosis* (M.tb) and, together with perforin, is cytolytic against intracellular mycobacteria. This project evaluated the role of these two cytolytic mediators in TB.

Firstly, a flow cytometric assay was validated for detection of granulysin and perforin within CTL and NK cells, and the number and phenotype of cells expressing both cytolytic mediators was determined in a cohort of healthy PPD+ adult volunteers. A novel finding was the detection of granulysin, but not perforin, on the surface of the cells. Secondly, cord blood cells expressing granulysin and perforin were evaluated. Both cytolytic markers were found to be constitutively expressed in NK cells in cord blood, but neither granulysin nor perforin was found in PPD-stimulated T-cells from cord blood. BCG vaccination at birth resulted in significant cytolytic mediator expression in T-cells of 10 week-old infants, which did not differ with variation in vaccine strain or route of vaccination. Thirdly, expression of these cytolytic mediators was evaluated in a large clinical trial with recombinant interferon gamma (rIFN-γ) treatment of patients with active TB. Interferon gamma (IFN-γ) therapy administered by subcutaneous injection in addition to standard anti-TB therapy (DOTS) showed a significant increase in granulysin expressing cells compared to DOTS alone or DOTS and IFN-γ administered via a nebulizer, in a cross-sectional analysis. No difference in the percentage of T-cells or NK cells expressing either marker was found in a smaller paired group evaluation in the longitudinal study. Fourthly, the impact of HIV was evaluated in a small number of patients. TB and HIV co-infection resulted in a reduced number of CD4+ T-cells expressing granulysin and perforin, but no difference in the percentage of cells expressing either marker was seen in patients with HIV who had been successfully treated for PTB.

Finally, expression of cytolytic mediators was evaluated in tuberculous pleuritis. Effective host immunity against intracellular pathogens requires antigen-specific T-cell activity at the site of infection. Granulysin and perforin were found to be expressed in many more pleural effusion effector cells than effectors from the peripheral blood (PB) and this correlated both with measurement of cytolysis in a chromium release assay, and results of CFU assay which documented the survival of mycobacteria in infected macrophages following exposure to CTL.

In summary, granulysin and perforin were constitutively expressed in NK cells in cord blood and in peripheral blood from BCG-vaccinated babies and individuals with latent and active TB. These cytolytic markers were not expressed in T-cells from cord blood but were upregulated in T-cells from 10 week-old babies having been vaccinated at birth with BCG. Patients receiving IFN-γ in addition to standard anti-TB therapy (DOTS) had an increase in granulysin expressing cells compared to DOTS alone. Cells obtained from the site of infection also expressed more granulysin and perforin than cells from PB in patients with TB and mycobacterial survival was reduced in infected macrophages when exposed to effector PEL compared to effector cells from PB. Therefore it is possible that granulysin and perforin may contribute to the immune response to TB and may indeed be correlates of protection against M.tb.

CHAPTER 1

1.1 INTRODUCTION TO TUBERCULOSIS

1.1.1 Epidemiology of Tuberculosis

The World Health Organisation (WHO) declared tuberculosis (TB) a global health emergency in 1993. The latest WHO report claims that there are approximately 8.8 million new cases of TB annually (WHO, 2007) which translates to a newly diagnosed case every 3.6 seconds. One third of the world's population is infected with TB and with more than 2 million deaths per year, TB is second only to the human immunodeficiency virus (HIV) as a cause of death arising from a single infectious agent (WHO, 2006). TB has been endemic in Europe since the early 1600s but was virtually unknown in Sub-Saharan Africa and the India subcontinent till the second half of the 1900s (Daniel *et al.*, 1994). The rates of TB in Europe have been increasing steadily in the last decade but there are alarming disparities in TB rates in countries in Western Europe compared to Eastern Europe. This is due to socio-economic differences between the more affluent Western Europe and the poorer Eastern European countries which are plagued with overcrowding, malnutrition and other impoverished situations that favour disease conditions. Africa, South East Asia and the Western pacific region accounts for 83% of all TB cases worldwide. Amongst the fifteen countries with the highest TB incidence rates, twelve are in Africa with 23% of cases worldwide being on the African continent (WHO, 2007). This has been attributed to the high number of individuals infected with HIV where the likelihood of developing active TB is 10% per annum as opposed to 10% per lifetime in non-HIV infected individuals (Rook *et al.*, 2005). South Africa (SA) is ranked 7th in the world for TB incidence, with the number of cases estimated at 600/100 000 and although it has only 0.7% of the worlds population, 19% of all cases of TB in adult HIV positive people in 2005 were found in SA (WHO, 2007).

1.1.2 History of Tuberculosis

Evidence of pulmonary TB dates back as far as 1000-400 BC (Zimmerman, 1979), but it was only in 1881 that Robert Koch, using special staining techniques, demonstrated slender rods which he called tubercle bacilli. Koch later produced a glycerine extract of dead tubercle bacilli which was named tuberculin, out of which the tuberculin skin test for the identification of TB arose. Only a few members of the genus mycobacterium have evolved to a pathogenic lifestyle. These include amongst others *Mycobacterium tuberculosis* (M.tb), the causative agent of tuberculosis in humans, *Mycobacterium bovis* (M.bovis) which causes TB in mammals including cattle and humans, and *Mycobacterium avium* (M.avium) which can occur as opportunistic pathogens in immunosuppressed individuals (Cosma *et al.*, 2003). The genomes of several members of pathogenic mycobacteria have been sequenced. M.tb was one of the first organisms whose complete genome sequence was known; the genome size of M.tb is 4.4Mb and encodes 4000 genes (Cole *et al.*, 1998).

1.1.3 Diagnosis and Treatment of Tuberculosis

Exposure to M.tb results in a 10-30% infection rate but only 5-10% will develop active disease. The most common symptom of pulmonary TB at the onset of disease is a non-productive cough. If left unchecked the infection causes inflammation and necrosis of the lung tissue, which induces productive sputum. A definitive diagnosis of M.tb can only be made by culturing samples for the causative agent taken from specimens, usually sputum, from infected patients. Diagnosis of TB is often made by identification of acid fast bacilli (AFB) using fluorescent microscopy. A disadvantage of the test is the number of false negative results obtained due to the need for a minimum of 5000 bacilli/ml of sputum (Teixeira *et al.*, 2007). Extra-pulmonary forms of TB are more difficult to diagnose and invasive procedures are frequently required in order to make a diagnosis.

Two factors, persistence – the survival of TB despite the use of antibiotics, and resistance – the loss of susceptibility to antibiotics, have made the treatment of M.tb difficult (Sacchettini *et al.*, 2008). Anti-TB therapy involves the use of a combination

of different drugs for a six month period and is usually administered by the directly observed treatment short course (DOTS) program, which ensures that patients comply with the treatment regimen. The anti-TB drugs include isoniazid, rifampicin, pyrazinamide and ethambutol for two months, then isoniazid and rifampicin alone for a further four months. The rationale for using multiple drugs to treat TB are firstly to combat resistance to one particular drug and secondly to utilize the different mode of action of drugs e.g. isoniazid is bactericidal against replicating bacteria while pyrazinamide is effective against bacteria located in acidic environments, inside macrophages or in areas of acute inflammation. In most instances, treatment with properly implemented DOTS has a success rate exceeding 95% and prevents the emergence of further multi-drug resistant strains of tuberculosis (MDR-TB). In untreated M.tb, fatality rates exceed 50% (Onyebujoh and Rook, 2004).

1.1.4 Multi drug resistant TB (MDR-TB)

Drug resistant strains have been documented in every country surveyed. MDR-TB is defined as TB that is resistant at least to isoniazid and rifampicin, which are considered the most powerful anti-TB drugs (Rook *et al.*, 2004). Isolates which are multiply-resistant to any other combination of anti-TB drugs but not to isoniazid and rifampicin are not classed as MDR-TB although resistance to all major anti-TB drugs has emerged. Recently, in South Africa, a more serious form of drug resistant TB has emerged and has been classified as "extensively drug-resistant tuberculosis" (XDR-TB). This is defined as MDR-TB that is resistant to quinolones and also to any one of kanamycin, capreomycin, or amikacin (WHO, 2006). XDR-TB does not transmit easily in healthy populations, but is capable of causing epidemics in populations already stricken by HIV and therefore, more susceptible to TB infection (Gandhi *et al.*, 2006). This strain of TB does not respond to any of the drugs currently available in South Africa for first- or second-line treatment.

1.1.5 Pathogenesis of Tuberculosis

M.tb is a highly pathogenic bacterium which is spread by inhalation of droplets, usually by a cough, from infectious tuberculous patients. It has been estimated that a droplet containing 1-10 bacteria is sufficient to initiate infection (Sundaramurthy and Pieters, 2007). Disease in adults is almost exclusively of the lungs. Most people exposed to M.tb will not become infected and over 90% who are infected remain asymptomatic throughout their lives, as the mycobacteria can remain dormant giving rise to latent TB. Although M.tb can cause primary disease, the most common manifestation in adults is reactivation of pre-existing latent infection, which is usually triggered by conditions of immunosuppression such as malnutrition and HIV infection (Russell, 2007).

For the minority who develop active TB, interaction of M.tb with the host begins with droplet nuclei measuring 1-2μm, containing micro-organisms, reaching the distal airways where they encounter alveolar macrophages and dendritic cells (DC) and are phagocytosed (Meya and McAdam, 2007). This is mediated by various host receptors including toll-like receptors (TLR), macrophage mannose receptor (MMR) and complement receptor 3 (Ernst, 1998). Ten functional human TLR have been cloned and the natural ligands for these receptors are mainly microbial components (Salaun *et al.*, 2007). Inside the phagocyte, mycobacteria interfere with the host trafficking pathways by modulating events in the endosomal/phagosomal maturation pathway and preventing lysosomal fusion. In this way, mycobacteria prevent their destruction by reactive nitrogen and oxygen intermediates, and degredation by lysosomal enzymes (Boshoff and Barry, 2005; Hart and Young, 1991). Thus, mycobacteria circumvent immediate eradication, enabling them to form a niche inside the macrophage where they can survive and even replicate (Houben *et al.*, 2006).

During early stages of infection, M.tb occupies one or more compartments of the lung that do not promote antigen presentation to naïve CD4 T-cells and this allows for the bacterial population to expand in the lungs and resist effector function of the adaptive immune response (Wolf *et al.*, 2008). At this stage M.tb-infected macrophages can be lysed and the bacilli taken up by other macrophages and DC, recruited to the site of

infection by chemotaxis. This usually occurs without associated pathology. Although both DC and macrophages engulf the bacteria, only DC carry M.tb from the lungs to the draining lymph nodes where the initiation of the adaptive immune response occurs (Salgame, 2005). Infected DC upregulate MHC class II and co-stimulatory molecules, secrete IL-12 and pro-inflammatory cytokines, and present captured antigens to naïve T-cells in the lymph nodes (Alaniz *et al.*, 2004). Approximately 3-4 weeks afterwards primed T-cells, mediated by chemokines and adhesion molecules migrate to the sites of inflammation and activate macrophages which may or may not destroy the bacilli (von Andrian and Mackay, 2000). The dynamic interaction of T-lymphocytes and macrophages leads to chronic antigenic stimulation and T-cell accumulation around macrophages resulting in the remodelling of the site of infection, forming a cellular mass called a tubercle or granuloma, hence the name of the disease (Russell, 2007).

1.2 THE INNATE IMMUNE RESPONSE TO TB INFECTION

1.2.1 The innate immune response to Tuberculosis

Cells of the innate immune system, which represents resistant mechansims non-specific to a particular pathogen, are the hosts' first response to invading pathogens (Andersen *et al.*, 2006). The innate immune response comprises several different cell types including macrophages, DC, NK cells, NKT cells, neutrophils and γδ cells, and has its own receptor system to recognize the presence of pathogens. Reciprocal activation interactions occur between NK and DC via mechanisms dependent on cell-cell contact and soluble factors (Gerosa *et al.*, 2002). NK cells do not require prior activation and are available for early microbicidal activity (Ma *et al.*, 2004). Using an *in vitro* model, unstimulated NK cells incubated with intracellular H37Rv, could mediate killing of mycobacteria within 24 hours (Brill *et al.*, 2001). NK cells are recruited to the lungs early during M.tb infection where they expand and provide resistance by the secretion of interferon gamma (IFN-γ). NK cells are known to cause lysis of infected macrophages by utilizing NK cell receptors in a TLR-dependent manner (Vankayalapati *et al.*, 2005). However, their role in protection still remains to be elucidated, as although NK cells were expanded in the lungs of mice in response to aerosol infection with M.tb, and produced IFN-γ, no alteration in the bacterial load within the lungs was demonstrated after the depletion of NK cells (Junqueira-Kipnis *et al.*, 2003).

1.2.2 NKT cells in innate immunity to TB

NKT cells are TCR-expressing T cells and have been implicated as a link between innate and acquired immune responses (Dalbeth *et al.*, 2004). A high percentage of NKT-cells were demonstrated in patients with M.tb before treatment but this was significantly reduced after treatment (Al Majid and Abba, 2008; Barcelos *et al.*, 2006). NKT cells promote protection by the rapid production of IFN-γ and IL-4 (Godfrey *et al.*, 2000). It has been shown that NKT cells induce a granulomatous

response to a glycolipid fraction in the cells wall of M.tb cell in murine models (Apostolou *et al.*, 1999).

1.2.3 Gamma delta (γδ) T-cells in innate immunity to TB

γδ T-cells contribute to the innate immune response against M.tb by recognising antigens without any requirement of antigen processing and presentation on MHC molecules (Chien and Konigshofer, 2007). They respond to microbial infection by recognising small non-peptide molecules. Recently, it has been shown that γδ T-cells secrete IL-17 in response to IL-23 secreted by DC, thereby implicating them as a main player in the resistance against M.tb infection at the initial stage (Lockhart *et al.*, 2006). Upon contact with M.tb, γδ T-cells have been shown to secrete IL-2 and exhibit cytolytic function (Munk *et al.*, 1990). In addition, mycobacteria-specific γδ T-cells are potent inhibitors of BCG growth (Worku and Hoft, 2003). Once activated, γδ T-cells secrete IFN-γ and TNF-α leading to the induction of nitric oxide synthase 2 (NOS-2) which strengthens the bactericidal capacity of macrophages (Bhatt and Salgame, 2007). T-regulatory cells inhibit the secretion of IFN-γ from γδ cells showing that T-regulatory cells play an important role in the regulation of immune responses of antigen-specific human memory γδ cells (Li and Wu, 2008).

1.2.4 Neutrophils in innate immunity in TB

The role of neutrophils in M.tb infection is less specific. Neutrophils are found at the site of infection at the onset of mycobacterial infection, and depletion of neutrophils before infection enhances mycobacterial growth in lungs of infected mice (Fulton *et al.*, 2002). Macrophages acquire antimicrobial activity against intracellular pathogens through the uptake of neutrophil granules which results in killing of the intracellular pathogens (Tan *et al.*, 2006).

1.3 THE ADAPTIVE IMMUNE RESPONSE TO TB INFECTION

Adaptive immunity involves the response of antigen specific cells to antigen, and includes the development of immunological memory. Recognition of M.tb enlists the participation of many cells and molecules and DC play a pivotal role in the initiation and modulation of the adaptive immune response. This is achieved by processing and presenting antigens to T-lymphocytes including CD4+ T-cells, CD8+ T-cells and CD1-restricted T-cells. CD4+ T-cells recognise large peptides presented by MHC class II molecules, and CD8+ T-cells recognize small amino acid peptide antigens presented by MHC class I molecules. CD1-restricted T-cells are activated directly by microbial lipid antigens. In comparison to the MHC class II restricted T-cell repertoire, CD1-restricted T-cells are limited to recognition of cross-reactive antigens imparting a distinct role in the host response to immunologically related pathogens (Sieling *et al.*, 2005). However, although immunological memory induced by M.tb provides short-term protection, it does not translate into long-term protection as Kamath *et al.* (2006) reported that memory T-cells generated following BCG infection in mice, fail to protect the host against disease.

1.3.1 CD4+ T-cells in the adaptive immune response to TB

Activated macrophages and DC produce IL-12, polarizing cells to a Th1 type of response. CD4+ T-cells are essential for host defense against mycobacteria as M.tb infection induces a potent Th1 type CD4+ T-cell response. Although the decision to differentiate a naïve CD4+ T-cell into a Th1 or Th2 cells is made at the level of translation, extracellular cytokines are still required to differentiate the cells (Yates *et al.*, 2004). Activated CD4+ T-cells secrete IFN-γ and TNF-α which induces antimicrobial mechanisms in macrophages by the generation of reactive nitrogen and oxygen intermediates (Winkler *et al.*, 2005). IFN-γ knock-out mice rapidly succumbed to M.tb infection (Flynn *et al.*, 1993), and humans deficient in the genes encoding IFN-γ or IL-12, or their receptors, are susceptible to M.tb infection (Newport *et al.*, 1996; Picard *et al.*, 2002). In active TB in humans, M.tb-specific stimulation of IFN- γ production and IFN-γ R signaling are significantly depressed

correlating with disease severity and activity and normalizing after successful anti-TB therapy (Sahiratmadja *et al.*, 2007b). In contrast, Flynn (2004) reported that the amount of IFN-γ production does not appear to be a reliable predictive marker for disease status.

The role of CD4+ T-cells in TB is more than just for the secretion of IFN-γ and macrophage activation, as normal levels of IFN-γ and NO2 production in CD4+ depleted mice did not prevent reactivation of TB, implicating another role for CD4+ T-cells in immunity against TB (Scanga *et al.*, 2000). Accumulation of terminally differentiated CD4+ T-cells in the lungs of M.tb-infected mice correlated with protection against M.tb (Kapina *et al.*, 2007), and Sud *et al* (2006) reported that the depletion of CD4+ T-cells in active disease is more detrimental than a loss of IFN-γ. This is supported by the increased susceptibility of HIV infected people to TB where these individuals often have an increased production of IFN-γ.

IL-17 and IL-23 are central to lymphocyte migration to the lungs (Kolls and Linden, 2004). Recently, the presence of two mycobacteria-specific CD4+ T-cell populations in the peripheral blood (PB) of M.tb exposed, or diseased people, was described (Scriba *et al.*, 2008). IL-17 or IL-22 producing CD4+ T-cells constituted >20% of the specific cytokine-producing CD4+ T-cells and were shown to contribute to the adaptive immune response to mycobacteria. In addition, these cells were detectable in the PB of 10 week-old infants after vaccination with BCG at birth (Scriba *et al.*, 2008).

A more recently described subset of CD4+ T cells called regulatory T-cells (T-reg) has been implicated in adaptive immunity against M.tb. These CD4+/CD25^{high} cells have been shown to be expanded in patients with TB and to inhibit BCG-specific induction of IFN-γ by CD4+ T-cells (Guyot-Revol *et al.*, 2006; Li and Wu, 2008). Th1-secreting CD4+ T-cells were capable of controlling M.tb in the absence of T-reg as shown in an adoptive cell transfer model in mice (Kursar *et al.*, 2007). It has not been established whether the presence of T-regs in patients with TB is beneficial or detrimental. FoxP3+ T-regs have been shown to depress T-cell mediated immune

responses to the protective mycobacterial antibody, anti-HBHA, during active TB (Hougardy *et al.*, 2007) yet have been reported to function effectively in an inflammatory milieu by resisting the pro-inflammatory effects of IL-6 (Zheng *et al.*, 2008b).

1.3.2 CD8+ T-cells in the adaptive immune response to TB

The role of HLA class I-restricted CD8+ T-cells in host defense against M.tb is not as well defined as that of CD4+ T-cells. Both CD4+ and CD8+ T-cells specific for M.tb protein antigens are primed, and expand and accumulate at the site of disease. CD8+ T-cells are induced early in infection with M.tb, but appear to play a major role in the latter phases of infection (van Pinxteren *et al.*, 2000). In murine models, CD8+ T-cells are essential for protection against M.tb as β_2-microglobulin-deficient mice are unable to develop MHC class I-restricted CTL, as they lack functional MHC class I molecules, and readily succumb to M.tb infection (Flynn *et al.*, 1992). This was supported in other murine studies which indicated CD8+ CTL have a critical role in protective immunity against TB by the release of IFN-γ, lysis of target cells, and antimicrobial activity (Cho *et al.*, 2000), and where mice lacking in functional CD8+ T-cells were more susceptible to mycobacterial infection (Ladel *et al.*, 1995). Using a mathematical model of the immune response to M.tb in the lung, Sud *et al* (2006) showed that there were two subsets of CD8+ T-cells, the cytotoxic and the IFN-γ producing cells, and the removal of both of these subsets always resulted in the development of active disease in mice. This supports the notion that contribution of either subset is necessary to control disease in murine models.

Human studies have also shown that MHC class I-restricted CD8+ T-cells recognise M.tb infected cells (Tan *et al.*, 1997) and are activated by microbial antigens present in the cytoplasm of APC. Effector functions of CD8+ T-cells depend on the stage of the disease (Lazarevic *et al.*, 2005). CD8+ T-cells are present within the granuloma where they have access to infected cells and prevent bacillary dissemination (Caccamo *et al.*, 2006). Like CD4+ T-cells, CD8+ T-cells secrete IFN-γ and TNF-α

on recognition of mycobacterial antigens (Caccamo *et al.*, 2002). Human CD8+ CTL specific for Ag85A were demonstrated by Smith *et al* (2000b) but before 2000 only three human CD8+ T-cell reactive epitopes to M.tb had been described. Antigen 85A epitope-specific memory and effector CD8+ T-cells are reduced in the blood of children with TB compared to healthy PPD+ children providing evidence that these cells play a role in defense against TB (Caccamo *et al.*, 2006). More recently, Weichold *et al* (2007) measured Ag85B-specific CD8+ T-cell responses across the different MHC class I molecules in patients with active PTB. Other CD8+ epitopes identified in M.tb proteins include ESAT 6-related proteins TB10.3 and TB10.4 (Kamath *et al.*, 2006; Majlessi *et al.*, 2003), M.TB32A (Irwin *et al.*, 2005) and CFP10 (Kamath *et al.*, 2004). CFP10 is recognized by T-cells in most people with latent and active TB and Shams *et al* (2004) showed that a peptide from CFP10, namely $CFP10_{71\text{-}85}$, stimulated IFN-γ production and CTL activity by CD4+ and CD8+ T-cells from people with latent TB. In addition, CFP10-specific CD8+ T-cells have been shown to mediate protection in challenge models of TB (Wu *et al.*, 2008). The recognition of these M.tb-specific protein epitopes by CD8+ T-cells further demonstrates that infection with M.tb does induces a strong CD8+ response. Although the role of CD4+ T-cells in TB infection cannot be understated, CD8+ T-cells were shown to protect M.tb-infected mice lacking CD4+ T-cells (Wang *et al.*, 2004).

1.3.3 CD1-restricted cells in the adaptive immune response to TB

Some CD8+ T-cells have a critical role of recognizing cells infected with viruses, bacteria and parasites as they recognize non-peptide antigens in the context of CD1 molecules. CD1 molecules transport microbial lipid antigens through the endosomal network to the cell surface so that CD8+ T-cells can detect infected cells whether the pathogen is in the cytoplasm or in an intracellular vacuole (Stenger and Modlin, 1999). Evidence for the supportive role of CD1-restricted cells in M.tb infection includes the production of the protective cytokine IFN-γ and the detection and lysis of mycobacteria infected cells (Stenger *et al.*, 1997). CD1-restricted T-cell responses were detected in patients with latent TB but not naïve healthy individuals indicating that these cells are activated following infection with mycobacteria (Moody *et al.*,

2004). Conflicting reports for their role are provided by studies showing that anti CD1 monoclonal antibodies impaired early immunity to M.tb infection (Szalay *et al.*, 1999), yet mice deficient in CD1-restricted cells were able to fight M.tb infection (Behar *et al.*, 1999; Sousa *et al.*, 2000). These murine studies differ from those of human mycobacteria models in leprosy, where CD1-restricted T-cells lysed mycobacteria-infected cells, produced macrophage-activating cytokines, and released cytolytic granule proteins that directly inhibit mycobacterial growth (Sieling *et al.*, 2005). This illustrates that immunity to M.tb in humans and mice display definite differences which could be due to the cytotoxic granule content in human cells compared to that of mice.

1.4 THE ROLE OF CYTOLYTIC MEDIATORS IN CYTOTOXIC GRANULES OF CTL

1.4.1 Cytotoxic granules of T-cells and NK cells

Human CTL and NK cells package secretory and lysosomal proteins in a specialized, cytotoxic organelle or lytic granule, which serves as a secretory lysosome (Smyth *et al.*, 2001). These organelles have an acidic pH and perform both hydrolytic and degradative functions of CTL and NK cells (Burkhardt *et al.*, 1990). The granules have a distinctive dense core which contains most of the soluble lytic proteins including granulysin, perforin and granzymes. In addition, the granules contain lysosomal-associated membrane proteins (LAMP) including LAMP 1 and LAMP 2, soluble proteins and chemokines (Clark and Griffiths, 2003; Stegelmann *et al.*, 2005). Similar to other secretory lysosomes, lytic granule mobilisation and secretion requires activation which occurs through the TCR in CTL (Takayama *et al.*, 1987). Upon TCR recognition of MCH class II peptides on target cells, a rapid reorganisation of the secretory machinery within the CTL occurs, which results in a reorientation of the Golgi apparatus and the microtubule-organising centre (MTOC) inside the effector cells (Kupfer *et al.*, 1985; Stinchcombe *et al.*, 2001). This ensures that secretory vesicles containing cytotoxic granules are directed to the area of target cell binding. On reaching the contact site, lytic granules dock with the plasma membrane and release their cytolytic proteins into the intracellular space where the proteins trigger rapid death of the target cells (Stinchcombe and Griffiths, 2007).

1.4.2 Perforin

Perforin was first described in 1985 and characterized as a calcium (Ca^{2+}) dependent, pore-forming cytolytic protein found in the cytotoxic granules of T-cells and NK cells (Masson and Tschopp, 1985; Podack *et al.*, 1985). Perforin is synthesized as a 70kDa inactive precursor which is cleaved at the C-terminus to yield the 60kDa active form (Smyth *et al.*, 2001). Perforin shares homology with the C9 component of

complement and is able to insert into the lipid bilayer of target membranes and polymerize, forming pores in the target membrane (Stinchcombe and Griffiths, 2007).

In humans, almost 95% of uterine NK cells (uNK) have large granules containing perforin and granzymes which are thought to play a role in the protection of the mother and foetus against a microbial attack (Crncic *et al.*, 2007). Perforin was found to be constitutively expressed in NK cells in cord blood (CB) and could efficiently lyse NK-sensitive target cells, but the protein is not expressed in cord blood T-cells (Berthou *et al.*, 1995). Naïve CD8+ T-cells do not express mRNA for perforin, (Grayson *et al.*, 2001) but perforin mRNA was documented seven hours after *in vitro* stimulation of Ag-specific CD8+ T-cells (Veiga-Fernandes *et al.*, 2000), and perforin was shown to influence the expansion of Ag-specific CD8+ T-cells *in vivo* (Harty and Badovinac, 2002).

Perforin is considered to be responsible for the release of granzymes as, in the absence of perforin, granzymes remain sequestered in endocytic vesicles and are not associated with apoptosis (Froelich *et al.*, 1996). The development of perforin knock-out mice demonstrated the critical role for perforin in cytotoxicity *in vitro*, and the role it plays in the immunological resistance to some intracellular bacteria infections *in vivo* (Catalfamo and Henkart, 2003; Kagi *et al.*, 1994). T-cells in the lungs of M.tb-infected mice were found to express perforin *in vivo* and lyse M.tb-infected macrophages (Serbina *et al.*, 2000). NK cells were shown to expand in the lungs of mice challenged with aerosolised exposure to M.tb which was associated with IFN-γ and perforin production, although *in vivo* depletion of NK cells did not influence bacterial load in the lungs (Junqueira-Kipnis *et al.*, 2003). However, it has also been reported that in murine models, perforin is ineffective in the initial stages of M.tb infection (Laochumroonvorapong *et al.*, 1997) but is critical for the containment of mycobacteria in the chronic stage of disease (Sousa *et al.*, 2000).

The role of perforin in the immune response to M.tb in humans is still under debate. Perforin was shown to promote enhanced bacteriolysis by another granule protein, granulysin, by an increase in endosome-phagosome fusion, triggered by an increase in

intracellular Ca+. Inhibition of Ca^{2+} signalling by M.tb is associated with reduced phagosome-lysosme fusion and is critical for prolonged survival of M.tb within phagosomes of macrophages (Malik *et al.*, 2003). Perforin has been shown to kill M.tb-infected macrophages but Stenger *et al.* demonstrated that although perforin did not reduce the viability of M.tb, it was essential for intracellular killing of M.tb, as perforin formed pores in the target membranes allowing for the entry of cytolytic granules into the target cell (Stenger *et al.*, 1998). In addition, γδ T-cells, known to be effector cells against M.tb, require the presence of perforin for the killing of intracellular M.tb (Dieli *et al.*, 2001).

Other studies have refuted the necessity of perforin in the hosts' defense against M.tb. Canaday *et al.* (2001) reported that the inhibition of perforin and Fas/FasL activity did not have an effect on CD4+ or CD8+ T-cell mediated restriction of M.tb growth. However, more recently, evidence supporting the contribution of perforin was provided, as the cytolytic and mycobactericidal potential of a perforin expressing CD4+ T-cell clone was inhibited by the addition of concanamycin A (Klucar *et al.*, 2008), which prevents perforin from introducing pores into the target membrane (Kataoka *et al.*, 1994). This was further supported by a study using *Listeria innocua,* where there was a significant enhancement of granulysin-mediated lysis of the intracellular organism in human DC in the presence of perforin (Walch *et al.*, 2007).

Reports of the number of perforin expressing cells in M.tb infection are conflicting. A significantly increased percentage of total perforin positive cells and CD8+ perforin-expressing cells has been documented in patients with PTB compared to healthy controls, in a study conducted in a high tuberculous burden area in India (Rajeswari *et al.*, 2007). In addition, CD8+/CD57+ T-cells from patients with TB, but not healthy controls, in Mexico expressed high levels of perforin and were able to exhibit spontaneous cytotoxicity against autologous monocytes in the absence of antigenic stimulus, suggesting that the cells were primed (Sada-Ovalle *et al.*, 2006). CD8+ T-cells were shown to contain perforin and were cytolytic in the initial phase of TB infection, but exhibited minimal cytotoxicity in chronic infection although their ability to synthesize perforin was not impaired (Lazarevic *et al.*, 2005). In contrast, low numbers of perforin positive cells have been associated with TB. The frequency

of M.tb-specific CD8+ T-cells was reduced in children with active TB compared to PPD+ healthy controls but was recovered to that of healthy controls after anti-TB therapy (Caccamo *et al.*, 2006). Perforin HLA-DR2 antigens regulate immunity by facilitating the secretion of various cytokines including IL2, IL-6 and IL-12, which can rapidly induce perforin in CTL, but HLA-DR2 was associated with a downregulation of perforin in NK cells and CTL in patients with pulmonary TB compared to healthy controls (Rajeswari *et al.*, 2007). The contradictory levels of the number of perforin expressing cells in active TB may be explained by compartimentalization at the site of disease, as these cells were found in a high concentration in the cerebrospinal fluid of a child with tuberculous meningitis (Caccamo *et al.*, 2006).

In the bovine model of M.tb, expression of perforin in CD4+ T-cells in BCG-vaccinated cattle, was confined to memory M.bovis-specific cells, and the expression of perforin and IFN-γ correlated with the reduction of BCG colony forming units (CFU) in infected macrophages (Endsley *et al.*, 2007). Unvaccinated cattle had less than 1% of perforin expressing CD4+ T-cells. CTL memory cells, specific for BCG, have been generated and maintained for long periods of time in BCG-vaccinated hosts and these cells mature into effector cells upon restimulation, produce perforin, and kill BCG- infected DC. However, perforin expressing CD8+ T-cells were not observed in all PPD+ individuals after reactivation, suggesting that there must be some variability in the maintenance of functional memory T-cells responsible for delayed type hypersensitivity reaction and CTL in BCG-vaccinated individuals (Tsunetsugu-Yokota *et al.*, 2002).

1.4.3 Granulysin

Granulysin, previously known as 519, is a human T and NK cell-specific protein and was discovered by a subtractive hybridization procedure of late activated T-cells (Pena *et al.*, 1997). The gene for granulysin shows extensive alternative splicing unlike perforin and granzymes, which are encoded by only one transcript (Latinovic-Golic *et al.*, 2007; Zhou and Shi, 2002). A similar gene product, NKG5, was found in

the NK cell line as well as T-cell clones (Houchins *et al.*, 1990). At mRNA level, all transcripts except NKG5 are upregulated between day 0 and day 6 after stimulation. Granulysin belongs to the saposin-like family of proteins (SAPLIP) and shares a particular polypeptide motive and affinity to a variety of lipids, especially shingolipids, and cholesterol. Granulysin kills bacteria, fungi, parasites and tumour cells and is similar to NK-lysin, a porcine T and NK cell granule protein. Both granulysin and NK lysin consist of a precise fold comprising of five α-helices spaced by three loops (figure 1.1). The basic residues in granulysin are arginine which were shown to be critical for cytotoxicity but not for antimicrobial activity, whilst the basic residues in helix 2-3 appear to have significant antimicrobial activity (Linde *et al.*, 2005).

Figure 1.1: Ribbon representative of Granulysin structure.
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Granulysin is found in cytoplasmic granules of NK cells and CTL, and translocates to the region of the CTL/target contact where it is exocytosed upon stimulation of the TCR (Pena *et al.*, 1997). It is synthesized as a 15kDa precursor form which is then sorted to the cytolytic granules and processed to a 9kDa effector form. Stimulation of CTL with anti-CD3 antibodies induced exocytosis of granulysin where approximately 60% of the 9kDa protein was secreted constitutively, whereas the remainder appears to accumulate in granules (Pena *et al.*, 1997). Both CD4+ and CD8+ primed T-cells express granulysin and there is no preferential presentation in MHC class 1 or class II molecules (Latinovic-Golic *et al.*, 2007). In unstimulated cells, only the 15kDa

protein is found, but after 4 days of IL-2 stimulation, the 9 kDa granulysin can also be detected. After 7 days of stimulation, the 15kDa protein diminishes and the 9kDa form is more strongly expressed. The 15kDa form is present in human serum and its levels have been shown to correlate with NK and/or CTL activity in patients with acute viral infections, and in patients with immunodeficiencies (Ogawa *et al.*, 2003). This form was believed to be derived by both non-exocytotic spontaneous release and stimulated granule exocytosis.

Granulysin frequently co-localizes with other proteins although it can be found on its own. There is no homolog in mice, which has prevented studies aimed at identifying the relevance of the protein *in vivo*, as it has been shown that perforin knock-out mice either do not, or have reduced, ability to kill certain target cells (Catalfamo and Henkart, 2003). Peptides derived from granulysin and NK lysin have similar activities against M.tb (Andreu *et al.*, 1999). The fate of M.tb in human infection appears to rely on a co-operation of granulysin and perforin through the granule exocytosis pathway which involves direct and regulated secretion of lytic granule contents including perforin, granzymes and granulysin. Antimicrobial activity of granulysin-like molecules is particularly important against M.tb as the killing of infected cells does not necessarily result in the killing of the mycobacteria. The alternative Fas/FasL pathway can lyse M.tb-infected cells, however, this may result in the release of mycobacteria which may infect other neighbouring cells, thereby perpetuating the containment of the disease.

The dependence of perforin on granulysin killing has been extensively studied but no concrete conclusions have been drawn. Early studies describe a perforin-independent mechanism of granulysin-induced death of Jurkat cells, where recombinant granulysin caused typical apoptotic features including cell shrinkage, chromatin condensation, nuclear fragmentation, and PS translocation, by interacting with lipids in the plasma membrane and forming pores (Gamen *et al.*, 1998). Others reported that granulysin kills microbial targets by altering membrane permeability thereby leading to osmotic lysis (Ernst *et al.*, 2000). Kaspar *et al* (2001) proposed that positively charged granulysin lyses negatively charged liposomes causing the disruption of cell membranes, resulting in a calcium influx, mitochondrial damage and eventual cell death. Perforin was shown to be necessary for granulysin-mediated killing of

intracellular M.tb but not extracellular M.tb, suggesting that perforin is required to permit granulysin to enter and destroy M.tb-infected macrophages and DC (Stenger *et al.*, 1997). In contrast, NKT cells were able to control mycobacterial infection using granulysin but not perforin (Gansert *et al.*, 2003), and Canaday *et al* (2001) reported that CTL killed intracellular M.tb by a perforin-independent and Fas/FasL independent mechanism, implicating only granulysin in the killing process. Recently, using an intracelluar *listeria* model, it was shown that granulysin binds to infected cells via lipid rafts, which consist of membrane microdomains containing phospholipids and cholesterol in the inner membrane and shingomylin and cholesterol in the outer membrane (Walch *et al.*, 2005). It was also suggested that simultaneous secretion of IFN-γ at the site of infection by CTL would enhance granulysin targeting in this model (Walch et *al.*, 2005). Barman reported that the lipid-raft bound granulysin was endocytosed, transferred via membrane vesicles to early endosomes, and then to phagosomes where the killing of the bacteria takes place (Barman and Nayak, 2007). However, this may not be the case in M.tb infection, as M.tb has evolved mechanisms to prevent phagosome maturation by interfering with the endocytic machinery of the host cell. This could explain why granulysin is incapable of gaining access to M.tb-containing phagosomes without the assistance of perforin (Walch *et al.*, 2007)

The importance of CD4+ T-cells in protective immunity to mycobacterial infection is highlighted by the susceptibility of CD4 gene-depleted mice, and HIV-infected individuals to TB infection (Flynn, 2004). Inhibition of mycobacterial growth by CD4+ T-cells has been shown to be mediated by supernatants from stimulated T-cells (Yoneda and Ellner, 1998) or by granulysin (Ochoa *et al.*, 2001). Using a CD4+ T-cell clone generated against a single epitope on CFP10, a specific mycobacterial peptide encoded on the RD1 region present in M.tb but not M.bovis BCG, granulysin and perforin were shown to contribute to the lysis of M.tb-infected target cells and to inhibit intracellular mycobacterial growth (Klucar *et al.*, 2008). Human and bovine TB have similar patterns of transmission, clinical presentation and tissue pathology (Buddle *et al.*, 2005) and bovine homologues of granulysin and perforin were induced upon T-cell activation and expressed in the granuloma cells of M.bovis-infected cattle (Endsley *et al.*, 2004). In addition, memory T-cells expressing elevated levels of

perforin and granulysin strongly lysed BCG-infected macrophages in the bovine model (Endsley *et al.*, 2007).

A critical function of CD8+ T-cells in the hosts' response to M.tb is the lysis of infected targets cells, and the maintenance of effective CD8+ T-cells is believed to depend, in part, on CD4+ T-cells (Bennett *et al.*, 1997). Indeed CD4+ T-cells have been reported as being essential for the development of CD8+ T-cells during M.tb infection in the murine model (Serbina and Flynn, 2001). Samten *et al* (2003) proposed that CD4+ T-cells enhanced M.tb reactive CD8+ T-cells through CD40/CD40L interactions and showed that a CD40 ligand trimer enhanced the responses of CD8+ T-cells to M.tb by greatly increasing the expression of granulysin and perforin in these cells. Others have suggested that NKT cells participate in the host defense against M.tb infection, as degranulation of NKT cells resulted in an abrogation of antimycobacterial activity (Gansert *et al.*, 2003). In the bovine model, a subset of NK cells were shown to constitutively express perforin and granulysin and these cells dramatically reduced CFU in M.bovis BCG-infected alveolar and blood derived macrophages (Endsley *et al.*, 2006).

Although a protective role for granulysin and perforin in M.tb has been suggested *in vitro*, evidence of granulysin and perforin expressing cells *in vivo* has been less explored. Effective host immunity against intracellular pathogens requires antigen-specific T cells at the site of infection. The number of granulysin positive T-cells in cutaneous leprosy lesions were found to be six-fold greater in patients with a localized tuberculoid form of leprosy compared to the disseminated lepromatous form, and the frequency of granulysin positive cells correlated with the clinical form of the disease (Ochoa *et al.*, 2001). Perforin positive cells were distributed equally in both forms of leprosy indicating that granulysin, but not perforin, was a relevant marker for effective host defense in leprosy. Interestingly, granulysin was expressed in many more CD4+ T cells and perforin was expressed by more CD8+ T-cells whilst co-localization of granulysin and perforin in cytolytic granules was seen in a small percentage of cells. Expression of the bovine gene for granulysin was also detected in

lymph node lesions from an M.bovis BCG-infected animal, adding evidence to local expression of this marker at diseased sites (Endsley *et al.*, 2004).

More recently, granulysin and perforin expressing T cells were found to be expressed at ratios two to three-fold lower in tuberculous lesions compared to distal lung parenchyma from patients who did not respond to anti-TB therapy (Andersson *et al.*, 2007). Although there was a general increase in CD3+ T cells within the granulomas of these chronically infected patients, CD8+ granulysin and perforin positive cells were scarce suggesting that there was an insufficient upregulation of these cytolytic molecules in CD8+ T-cells at the site of infection (Andersson *et al.*, 2007). Granzyme A expressing T-cells were expressed at a similar ratio in uninfected and infected lesions of the lung tissue in patients with TB. However, although the expression of granzyme A and B is enhanced following M.tb activation of peripheral blood mononuclear cells (PBMC) from vaccinated individuals, neither granzyme appears to have a protective role (Toossi *et al.*, 2004). The lack of granulysin and perforin expressing cells in the infected lesions of lungs from patients who were resistant to anti-TB drugs could implicate a protective role for these cytolytic mediators. Deng *et al* (2005) reported that granulysin is a potent chemo-attractant of monocytes, CD4+ and CD8+ memory T cells, NK cells, and mature monocyte-derived DC. Using a micro-chemotaxis chamber, recombinant granulysin caused a 2- to 7-fold increase in chemotaxis of these cells but had no effect on naïve T-cells and immature DC. In addition, granulysin released from CTL and NK cells in the local inflammatory environment caused lysis of microbes but further from the site of granule release, granulysin functioned as a chemo-attractant for additional immune cells (Deng *et al.*, 2005). Taken together, these data indicate that high concentrations of granulysin at the site of infection contribute to cytotoxicity, but diluted concentrations further from the site of release act as a chemo-attractant for the recruitment of immune cells to sites of inflammation (Deng *et al.*, 2005).

No data is currently available on granulysin expression in CD4+ and CD8+ T-cells of patients with TB before and after treatment. However, perforin expressing antigen-specific CD8+ T-cells were found to be low in patients with active TB compared to

healthy PPD+ controls which normalized after anti-TB treatment (Caccamo *et al.*, 2006). M.tb-specific γδ T cells have been shown to be potent inhibitors of intracellular BCG growth, an observation which correlated with an upregulation of perforin, granzymes and granulysin mRNA and IFN-γ (Worku and Hoft, 2003). Vγ9/Vδ2 T-cells from children with different clinical forms of TB had decreased IFN-γ production and granulysin expression which normalized after successful anti-TB treatment suggesting that both granulysin and IFN-γ are involved in curative responses (Dieli *et al.*, 2002). In addition, serum levels of granulysin were significantly lower in children with active PTB compared to healthy controls but no difference was observed between the levels in successfully treated children and healthy controls (Di Liberto *et al.*, 2007). Similar results were reported in adults from the same geographical area where patients with active PTB had significantly lower plasma granulysin levels compared to healthy controls, which increased to the levels of controls after two months of chemotherapy, and surpassed the levels of the controls after completion of successful anti-TB treatment (Sahiratmadja *et al.*, 2007a).

Although both CD8+ T-cells and NK cells were shown to express granulysin in transgenic mice, only CD8+ T-cells were responsible for the elimination of tumours in these mice (Huang *et al.*, 2007). This has been shown in *Cryptococcus neoformans* (CN), a microbial pathogen commonly found in patients with HIV, where granulysin is the major effector molecule against CN and is required for CD8+ T cell-mediated antifungal activity (Ma *et al.*, 2002). In contrast, NK cells used perforin for the anticryptococcal activity (Ma *et al.*, 2002). More recently, granulysin expressing CD4+ T-cells were implicated in the demise of this fungus and this was shown to be defective in patients with HIV (Zheng *et al.*, 2007). Patients with chronic progressive HIV have also been shown to have deficient perforin production (Andersson *et al.*, 2002). An explanation for defective granulysin expression in CD4+ T-cells of HIV-infected patients was recently given by Zheng *et al* (2008a) who reported that CD4+ T-cells from HIV-infected patients can be deficient in P13K and STAT2, and IL-2 signals via P13K and STAT2 to increase expression of IL-2Rβ, which in turn, is required for the production of granulysin. The IL-2Rβ gene is expressed constitutively in NK cells and in low levels in CD8+ T-cells but is induced in activated CD4+ T-cells.

1.5 IFN-γ AS A CORRELATE OF PROTECTION AND AS AN ADJUVANT IN THE TREATMENT OF TB

The critical role of IFN-γ in the fight against M.tb was firstly shown by the inability of mice, with a disrupted IFN-γ gene, to control TB infection (Cooper *et al.*, 1993; Flynn *et al.*, 1993) and secondly, by the enhancement of host defense by an IFN-γ gene transfer in SCID mice (Xing *et al.*, 2001). Inherited deficiencies in IFN-γ signaling render humans susceptible to mycobacterial infections, and the absence of IFN-γ signaling abrogates the ability of effector cells to kill mycobacterial-infected macrophages (Boselli *et al.*, 2007). IFN-γ signaling is involved in the generation of mycobacterial-specific cytotoxic CD4+ T cells. In M.tb infection, CD4+ T-cells mount a much stronger IFN-γ response than CD8+ T cells due, not only to an increase in the number of the antigen-specific CD4+ T cells, but also because of a greater capacity of IFN-γ secretion by these cells (Ngai *et al.*, 2007). Although controversial, IFN-γ, not cytotoxicity, has been attributed to antimycobacterial protection mediated by CD8+ T cells (Lazarevic *et al.*, 2005; Tascon *et al.*, 1998).

IFN-γ has many important activities such as activation of macrophages, stimulation of antigen presentation, regulation of cellular functions, including apoptosis, proliferation and cell adhesion (Reljic, 2007). In addition, IFN-γ controls FasL dependent cytotoxicity of CD4+ T cells and may control the export of cytotoxic effectors like perforin and granulysin (Boselli *et al.*, 2007). The percentage of IFN-γ producing CD4+ and CD8+ T-cells in patients with active TB were reported to be significantly higher than healthy controls but normalized during treatment (Veenstra *et al.*, 2007). IFN-γ has been successfully used as an adjuvant in the treatment of human mycobacterial infections. Raad *et al* (1996) reported that a patient with refractory MDR-TB of the brain showed substantial neurological and radiologic improvement after 5 month of treatment with subcutaneous IFN-γ, and complete eradication of lesions in the brain and spinal cord after 12 months. Successful immunomodulatory treatment with subcutaneous recombinant IFN-γ was seen in a child with disseminated BCG infection (Ulrichs *et al.*, 2005). However, subcutaneous administration of IFN-γ in two patients co-infected with HIV and *M. avium* resulted in

transient clinical improvement but no long-term beneficial effect (Lauw *et al.*, 2001) and eight patients with MDR-TB had no improvement in clinical, radiological, microbiological or immunological parameters (Park *et al.*, 2007). Intramuscular administration of IFN-γ has also had mixed successes. Six months of intramuscular IFN-γ as adjuvant to chemotherapy in patients with MDR-TB in Cuba, resulted in clinical improvement and sputum negative AFB (Suarez-Mendez *et al.*, 2004) but the treatment showed no significant improvement in bacillary clearance in lepromatous leprocy patients compared to chemotherapy alone (Barral-Netto *et al.*, 1999).

Drugs administered topically to the lungs via aerosols are attractive as they may achieve higher levels in the lungs with fewer systemic side effects (Hubbard and Tattersfield, 2004). Aerosol therapy with IFN-γ (a-IFN-γ) in patients with PTB is widely distributed and results in enhancement of IFN-γ levels in the lower respiratory tract (Condos *et al.*, 2004). MDR-TB patients receiving a-IFN-γ reverted from smear positive to smear negative and there was cavitary reduction within 1 month of treatment (Condos *et al.*, 1997). IP10 recruits T-cells to site of inflammation and IP-10 mRNA was increased in bronchoalveolar lavage (BAL) cells from patients with PTB which was augmented after treatment with a-IFN-γ (Raju *et al.*, 2004). No activation of macrophages was seen by systemic administration of IFN-γ but a-IFN-γ resulted in expression of activation markers in alveolar macrophages (Condos *et al.*, 2003). IFN-γ activation of human alveolar macrophages *in vitro* is mediated by STAT1 signaling and requires IRF-1 and IRF-9 (Condos *et al.*, 2003). Normal levels of expression of these markers by macrophages of TB patients is low (Reljic, 2007) and a-IFN-γ caused a marked increase in the expression of these markers in ten patients with MDR-TB (Condos *et al.*, 2003). Partial success of a-IFN-γ was also reported in MDR-TB patients in Korea (Koh *et al.*, 2004).

NK cells are a major source of IFN-γ early after M.tb infection and help to maintain a Th1 cytokine profile (Schierloh *et al.*, 2007). A study in Germany reported a cure of four patients with MDR-TB due to the admission of a-IFN-γ and anti-microbial treatment (Grahmann and Braun, 2008). Interestingly, an increase of NK cells and γδ T-cells during and after treatment was observed in these patients. NK cells and γδ T-

cells are a rich source of granulysin and perforin and it is possible that, beside the addition of IFN-γ to the treatment regimen, these two cytolytic mediators may have contributed to the successful cure of MDR-TB.

1.6 BCG AND VACCINE CANDIDATES

The vaccine against TB, an attenuated *Mycobacterium bovis* strain, was developed by Albert Calmette and Camille Guérin and used for the first time in 1921 as an oral vaccine but later intradermal and percutaneous administration was favoured. Bacillus Calmette Guérin (BCG) vaccine is the most widely used vaccine worldwide with over three billion individuals having been immunized since its inception. Although the efficacy of BCG is variable, ranging from 0-80%, the mortality rates of vaccinated infants are much lower than those who remain unvaccinated against TB (Hussey *et al.*, 2007). In Eastern Germany, vaccination of newborns caused a lower TB incidence in children under the age of 15 years but no decline in the number of adults contracting the disease was observed (Nasser Eddine and Kaufmann, 2005). BCG offers good protection against childhood miliary disease and TB meningitis but its protection against pulmonary TB and other manifestation of adult disease is poor.

The limited protection of this vaccine in adult TB has been blamed on contact with atypical environmental bacteria which stimulate an immune response that eradicates BCG prematurely thus, curtailing vaccine-produced protection (Andersen and Doherty, 2005). However, there appear to be many reasons for the wide variability of the success of BCG including genetic variability, immunological different BCG strains, over-attenuation of parent vaccine strain, different ages of vaccination recipients, different routes of administration and genetic variability of BCG to the human strain of M.tb (Nasser Eddine and Kaufmann, 2005; Sable *et al.*, 2007). In addition, BCG is a potent inducer of CD4+ T-cells but an insufficient stimulator of CD8+ cells that are required for long-term control of the pathogen (Kaufmann and McMichael, 2005).

It is generally considered that T cell-mediated immunity is not transferred to offspring (Marchant and Goldman, 2005) but antigen-specific cells can be primed *in utero* as shown by the proliferation of cord blood mononuclear cells (CBMC) in response to PPD stimulation (Devereux *et al.*, 2001). Memory cells, as defined by the phenotype CD45RO, comprise of approximately 10% of CBMC (Cossarizza *et al.*, 1996). CD4+ effector cells generated from naïve cord blood have been reported to be intrinsically

as competent as naïve cells generated from adult PB, in response to TCR-mediated stimulation (Canto *et al.*, 2005). However, neonatal T lymphocytes are fundamentally different from naïve adult cells and CD4+ T lymphocytes from newborns produce lower levels of IFN-γ than naïve adult cells in vitro (White *et al.*, 2002).

M.tb is a common cause of severe infection in children, especially in developing countries, and the T-cell mediated immune responses are different in young infants, limiting the efficacy of vaccines against intracellular pathogens (Marchant and Goldman, 2005; Marchant and Newport, 2000; Siegrist, 2001). Mature cellular immune responses however, can be developed in early life and stimulation of neonatal T lymphocytes can be instructed to fight intracellular pathogens. Infants immunized at birth with BCG develop mature Th1 responses (Hussey *et al.*, 2002; Marchant *et al.*, 1999; Vekemans *et al.*, 2001). Nevertheless, the capacity for CD4+ T-cells to produce IFN-γ and of DC to promote Th1 responses is lower in infants than adults (Marchant and Goldman, 2005). A Th1 response is critical for the control of mycobacterial infections and the extent of the response is different in developing countries as described by Weir *et al* (2006) who reported that BCG vaccination administered to Malawians induced a lower type 1 immunity than vaccination of British citizens. This could be attributed to exposure to environmental mycobacteria prior to vaccination. However, a study in South Africa showed that BCG induced a potent T cell response including, cytokine-producing or cytotoxic CD8+ T-cells and regulatory CD4+ T-cells in ten week-old babies having been vaccinated with BCG at birth (Hanekom, 2005).

As discussed previously, granulysin and perforin are constitutively expressed in innate γδ T-cells and NK cells. In comparison to maternal PBMC, a lower number of γδ and CD3+ T-cells from cord blood expressed perforin and granzyme B after non-specific *in vitro* stimulation, but comparable numbers of NK cells expressed perforin and granzyme B (Engelmann *et al.*, 2006). A marked upregulation of perforin, granzyme A and granzyme B was shown in CD8+ T-cells of 10 week-old infants having received BCG at birth (Murray *et al.*, 2006). Evidence of the functionality of these cytotoxic cells was provided by the presence of CD107 on the surface of the

cells indicating that degranulation had occurred. Similar levels of IFN-γ were found in CD4+ and CD8+ T-cells of infants after BCG vaccination, but Soares *et al* (2008) reported that many mycobacteria-specific CD4+ and CD8+ T cells did not produce IFN-γ. The route and strain of BCG vaccination has also induced different immune responses with regards to IFN-γ, as it was reported that percutaneous Japanese BCG induced significantly higher frequencies of BCG-specific IFN-γ producing CD4+ and CD8+ cells than intradermal Danish BCG (Davids *et al.*, 2006). The variability of these responses suggests that IFN-γ may not be an optimal readout in studies of the host cytokine response to BCG vaccination.

Waning of BCG induced protection through childhood and young adult life coincides with a gradual increase in TB incidence which, in highly TB endemic countries, reaches a peak in early adulthood (Andersen and Doherty, 2005). A 15 year follow-up of patients receiving BCG or placebo revealed that BCG was of little value in preventing sputum positive cases of PTB (Tuberculosis Research Centre, India, 2006). In fact, BCG vaccine has been exposed as a risk factor rather than a preventative factor against TB in people infected with Beijing strains (Abebe and Bjune, 2006). The poor protective response of BCG, and the increase in the prevalence of MDR-TB, has sparked renewed interest in TB, especially with regard to the development of a proficient vaccine. Many vaccine candidates have been identified including ESAT 6 and Ag85 (Agger *et al.*, 2006; McShane *et al.*, 2005; Sable *et al.*, 2005). Although no murine homologue of granulysin has been discovered, a recombinant plasmid containing full length cDNA of granulysin injected into muscles of M.tb-infected mice, significantly reduced the number of viable bacteria in the lungs and spleen of the mice and resulted in fewer granulomatous lesions (Liu *et al.*, 2006). A viable therapeutic vaccine of recombinant *M.smegmatis* containing IL-12 and granulysin genes, was also shown to induce proficient protective immune responses against M.tb (Yi *et al.*, 2007). In addition, leucocytes from transgenic mice, containing the human granulysin gene and its 5′ and 3′ flanking regions, showed significant protection against a lethal dose of a cancer T-cell line compared to the non-transgenic littermates, implicating a protective role of granulysin in malignacy (Huang *et al.*, 2007). This was supported by Sekiya *et al* (2002) who showed that a gene transfer of 9kDa granulysin was therapeutic *in vivo* in

a murine lung cancer model. Six vaccine candidates have entered human clinical trials and over 2000 candidates have been identified (Hussey, 2007). As granulysin has been shown to be efficient in the eradication of many pathogens, and a progressive loss of granulysin has been shown to correlate with the progression of a number of cancers (Kishi *et al.*, 2002; Nagasawa *et al.*, 2006; Pages *et al.*, 2005), the use of granulysin as a candidate for a vaccine against TB should not be overlooked.

1.7 AIMS OF THE STUDY

Tuberculosis claims the lives of more than two million individuals globally per annum, and is especially endemic in developing countries where HIV is prevalent. BCG is the only vaccine available for TB but is ineffective in adult disease and childhood pulmonary TB. Protective immunity against TB requires an effective cytolytic response and an intact Th1 cytokine pathway. Granulysin and perforin are cytolytic mediators present in NK cells and cytolytic T-cells, and granulysin has been shown to kill both intracellular and extracellular mycobacteria. As the efficacy of BCG is poor, it is imperative that a new, more effective vaccine against TB is produced. In order to do this, correlates of protection against TB need to be defined.

The aims of this study were;

i) to document kinetics of granulysin and perforin expression in NK, CD4+ and CD8+ PBMC subsets from healthy PPD+ donors,

ii) to determine the expression of these markers in naïve lymphocytes isolated from cord blood,

iii) to establish if granulysin and perforin are upregulated in peripheral blood lymphocytes from 10 week-old infants having been vaccinated at birth with BCG,

iv) to determine granulysin and perforin expression in individuals with TB or TB/HIV coinfection, at baseline and after anti-TB treatment, with or without rIFN-γ supplementation,

v) to determine if the "protective" Th1 cytokine profile correlates with an increase in either or both of these effector molecules,

vi) to determine if granulysin and perforin expressing CTL are detectable at the site of infection in TB pleuritis, and to determine their cytotoxic potential and

vii) to establish whether there is evidence for granulysin or perforin surface expression on the membranes of CTL.

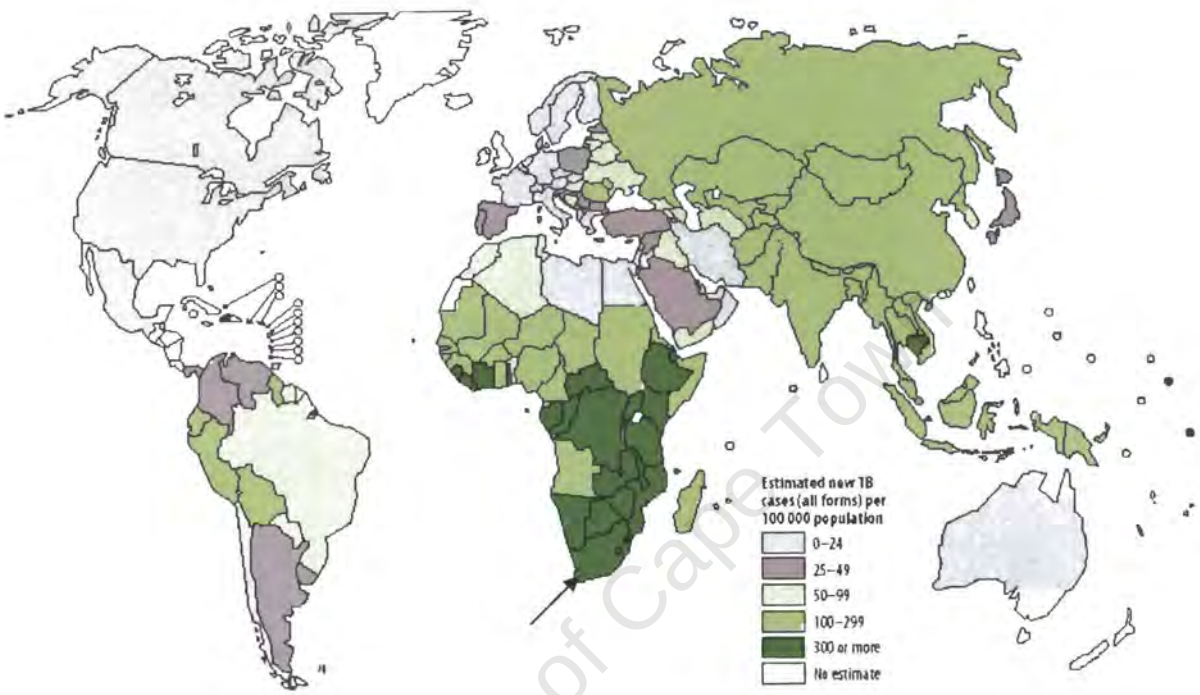


Figure 1.2: Incidence of TB worldwide. The arrow shows the region where the experiments for this thesis were performed.

CHAPTER 2

OPTIMISATION OF ASSAY AND DETERMINATION OF A "NORMAL RANGE" OF GRANULYSIN AND PERFORIN EXPRESSING CELLS IN HEALTHY PPD+ ADULT VOLUNTEERS

2.1 INTRODUCTION

2.2 MATERIAL AND METHODS

2.2.1 Human participation and ethical approval

2.2.2 Peripheral blood mononuclear cell (PBMC) isolation from healthy PPD+ adult volunteers (ND)

2.2.3 Stimulation of PBMC from ND

2.2.4 Staining of stimulated PBMC with granulysin polyclonal antiserum

2.2.5 Granulysin and perforin expression in ND to establish a "normal range"

2.3 RESULTS

2.3.1 Titration of granulysin antisera

2.3.2 Kinetics of granulysin and perforin expression in T-cells and NK cells

2.3.3 Granulysin and perforin expression in T-cells and NK cells of ten ND

2.4 DISCUSSION

2.1 INTRODUCTION

Tuberculosis, caused by the intracellular bacterium *Mycobacterium tuberculosis* (M.tb) is a major cause of mortality world-wide. Mechanisms involved in the protective immune response against M.tb are not fully understood but cytolytic T-lymphocytes (CTL) are known to be key players in the host defense against intracellular pathogens (Kaufmann, 1993). CD4+ T-cells produce IFN-γ, which activates macrophages to kill intracellular mycobacteria, and possess lytic granules which are able to carry out granule-mediated cytotxicity in the same way as CD8+ cytotoxic cells and NK cells (Appay and Rowland-Jones, 2002).

CTL lyse M.tb-infected cells by the release of effector molecules that have the capacity to kill intracellular and/or extracellular mycobacteria (Stenger *et al.*, 1998). These molecules include perforin, which is not directly cytotoxic but assists by "punching" holes in the membranes of the infected cells, and cytotoxic molecules granzymes and granulysin, which enter and destroy the cell, releasing the invading pathogen. No gene for granulysin has been detected in mice but T-cells from granzyme B or perforin knock-out mice do not, or have reduced ability to, kill certain infected target cells (Heusel *et al.*, 1994; Kagi *et al.*, 1994; Lowin *et al.*, 1994) suggesting that these molecules may have an important role in protective immunity.

The aims of this thesis was to establish the status of granulysin and perforin in CTL of infants vaccinated at birth with BCG, and in diseased states such as TB and TB/HIV co-infection, and to determine whether these cytolytic mediators have a protective role in tuberculosis. The normal range of serum granulysin levels has been established by ELISA in infants and adults; serum levels were shown to be elevated in acute viral infections and to correlate with immune reconstitution in immune deficient infants (Ogawa *et al.*, 2003). No antibody for the detection of granulysin in CTL was commercially available at the start of the study but a polyclonal antibody to granulysin was provided as a gift by Professor Alan Krensky from Stanford University in USA. The antibody has previously been used in other studies (Canaday *et al.*, 2001) but optimization of the flow cytometric assay was still required for this study, to ensure optimal kinetics and veracity of the findings. This chapter describes

optimization of an assay for granulysin expression and also determines a "normal range" of granulysin and perforin in healthy PPD+ adult volunteers (ND).

2.2 MATERIAL AND METHODS

2.2.1 Human participation and ethical approval

Approval for this study was obtained from the Human Ethics Committee at the University of Cape Town (UCT) in South Africa. Written, informed consent was obtained from all healthy adult volunteers prior to the collection of peripheral blood. Only individuals over the age of 21 were recruited and the blood was taken by qualified professional nursing staff registered with the Health Professionals Council of South Africa (HPCSA).

2.2.2 PBMC isolation from healthy PPD+ adult volunteers (ND)

Peripheral blood was collected into Na-heparin tubes according to manufacturer's instructions and diluted with an equal volume of Ca^{+} and Mg^{+} free phosphate buffered saline (PBS, Bio Whittkaker, Walkersville, MD, USA). Peripheral blood mononuclear cells (PBMC) were isolated by density sedimentation using Ficoll-Hypaque (Sigma-Aldrich, Steinham, Germany). The gradient was centrifuged at 400g for 30 minutes at room temperature. The resultant interface was removed and washed twice with PBS and centrifuged at 300g for 10 minutes. After the final wash the PBMC were adjusted to $1x10^{6}$ per milliliter (ml) in RPMI (Bio Whittkaker) supplemented with 10% human AB serum (Western Province Blood Transfusion Service, South Africa).

2.2.3 Stimulation of PBMC from ND

CTL were generated from PBMC by stimulation with 6µl/ml purified protein derivative (PPD, Statin's Serum Institut, Copenhagen, Denmark), *Mycobacterium Bovis* Bacillus Calmette Guérin (BCG, Statins Serum Institut) at a multiplicity of infection (MOI) of 5:1 (5:1 has been validated in our laboratory as the optimal MOI in adult PBMC), 100 IU/ml interleukin 2 (IL-2, Aldesleukin, Chiron) or 25ug/ml interleukin 15 (Recombinant human IL15, BD Biosciences Pharminogen, San Jose, CA) for 6 days at 37°C in a 5% CO_2 humidified chamber. IL-2 has been shown to upregulate granulysin expression (Houchins *et al.*, 1990) and was therefore used as a positive control. Cells were cultured in RPMI supplemented with 10% human AB serum. Unstimulated cells served as a background control. Subjects that did not proliferate to PPD in culture were excluded from this group.

2.2.4 Staining of stimulated PBMC with granulysin polyclonal antiserum

At the end of the stimulation period, cells were washed in FACs buffer which comprises of PBS containing 1% human AB serum and 0.1% sodium azide (Sigma-Aldrich), and resuspended in the same buffer. Cell viability was determined by trypan blue dye exclusion (Sigma Aldrich). Approximately 100µl of $1x10^6$ cells were stained with 10µl CD4-APC (Caltag, Burlingame, CA) for 15 minutes at room temperature. After staining the cells were washed then fixed and permeabilised with FACs™ Permeabilising Solution (BD Biosciences) according to manufacturer's instructions. Intracellular granulysin staining was performed by adding 10ul of neat granulysin antiserum or 10ul of the antiserum diluted 1/10, 1/100 and 1/1000 in FACs buffer to 100ul of cells. As a negative control the antiserum was replaced with undiluted normal rabbit serum (Sigma Aldrich) or at the same dilutions as the granulysin antiserum. The cells were incubated for 30 minutes at 4°C then washed and 1µg/ml (recommended by manufacturer) of $F(ab)_2$ goat anti-rabbit IgG (H+L)-PE conjugated secondary antibody (Caltag) was added for 30 minutes at 4°C. After another wash the cells were resuspended in FACs buffer and analysed on a FACsCalibur using *Cell Quest* software.

2.2.5 Granulysin and perforin expression in ND to establish a "normal range"

PBMC from ND were isolated and stimulated as above and the cells were surface stained with 10µl CD3 FITC for granulysin expression or 10µl CD3 PerCP (BD Biosciences) for perforin expression, and 10µl CD4 APC or CD8 APC or CD56 APC. After fixation and permeabilisation, intracellular granulysin and perforin was determined by the addition of 1/100 dilution of granulysin antiserum or 20ul of perforin FITC as per manufacturers' instructions (BD Biosciences). The method was performed as above. Isotypic matched controls were used for all monoclonal antibodies while normal rabbit serum (Sigma Aldrich) served as a control for the granulysin polyclonal antibody.

2.3 RESULTS

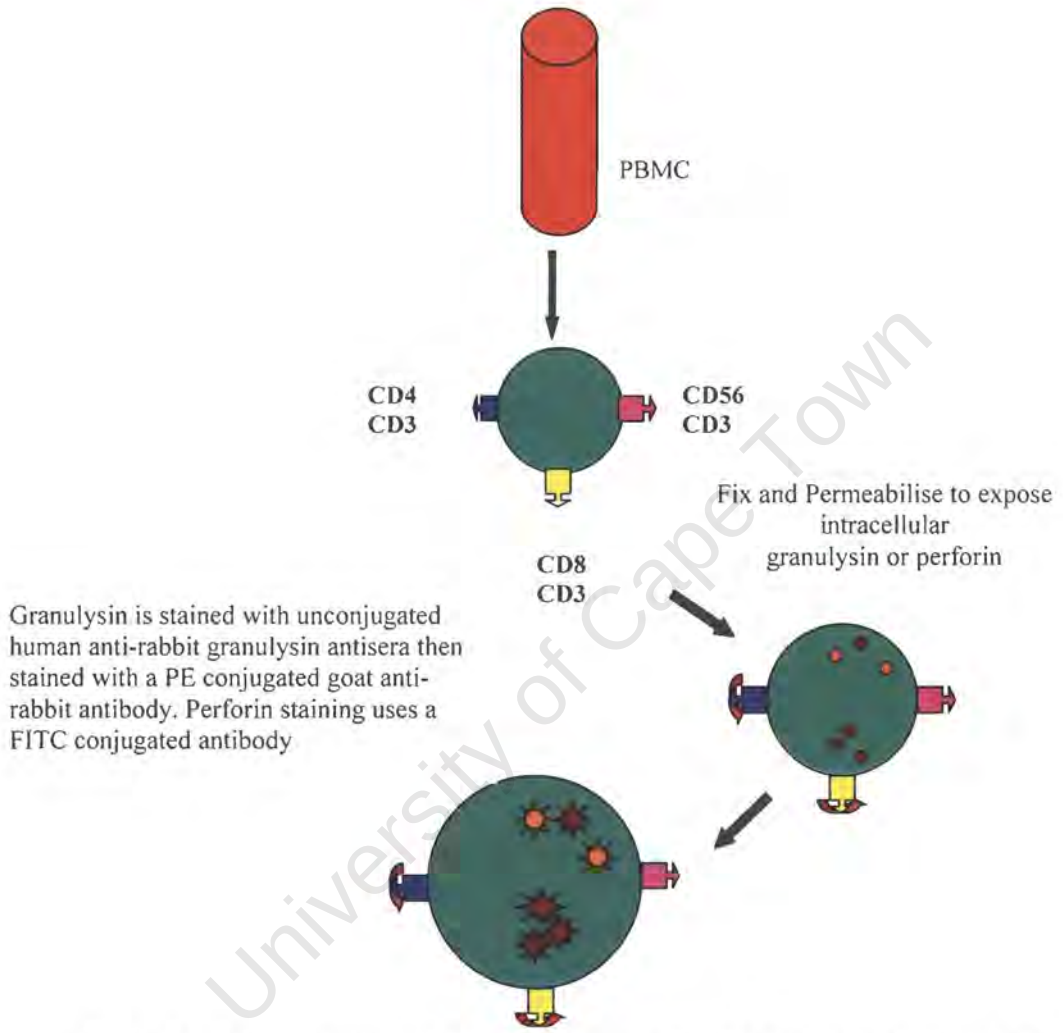


Figure 2.1: Schematic diagram of the staining method used for intracellular expression of granulysin and perforin within T-cells and NK cells

2.3.1 Titration of granulysin antisera

To determine the optimal concentration of the polyclonal antibody to human granulysin, PBMC from two ND were isolated and stimulated with 6µl/ml PPD for six days. The strategy adopted in order to evaluate granulysin or perforin expression in three reciprocal lymphocyte subsets is outlined in figure 2.1. Flow cytometric analysis was carried out by evaluating granulysin expression in CD4+ cells using granulysin antisera at different dilutions. Normal rabbit serum was used as a negative control. Figure 2.2 shows that at a dilution of 1/10 and 1/100 there was a mean of 19.5% and 24% granulysin expressing CD4+ cells respectively. Concentrated antisera and a dilution of 1/1000 resulted in a mean of 6% and 2% respectively of CD4+ granulysin expressing cells. It was therefore decided to use the antiserum at 1/100 giving a final concentration of 1/1000 for granulysin detection and neat normal rabbit serum as a negative control (10µl of antisera to 100µl of cells).

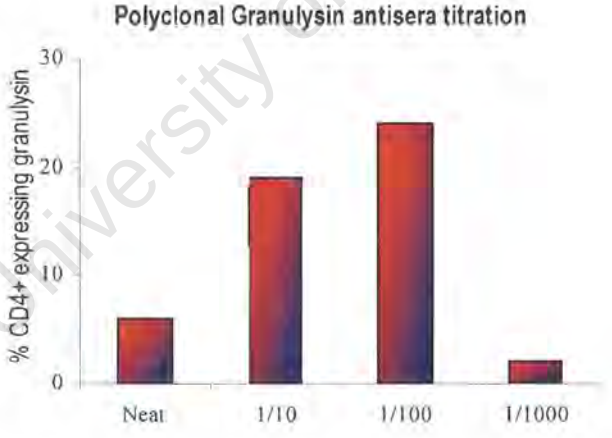


Figure 2.2: Comparison of granulysin expression in CD4+ cells in two donors using granulysin antisera at different dilutions.

2.3.2 Kinetics of granulysin and perforin expression in T-cells and NK cells

Granulysin in T-cells has been reported to be expressed 3-5 days after stimulation (Pena *et al.*, 1997) but has also been shown to be constitutively expressed in natural killer (NK) cells (Clayberger and Krensky, 2003). To determine at what time-point granulysin and perforin were optimally expressed in T-cells and NK cells, PBMC from three ND were stimulated with PPD and expression of both cytolytic mediators was determined in T-cells and NK cells on day 1, 4 and 7 of stimulation. Unstimulated cells served as a background control. Figure 2.3 illustrates the gating strategy employed. A lymphocyte gate was defined by forward and side scatter cell characteristics (R1) and within this gate CD3 cells were divided into CD3+ (R2) and CD3- (R3). Granulysin and perforin was detected in T-cells which were CD4+/3+ or CD8+3+ and in NK cells which were CD56+3-. Percentage expression was calculated by dividing the value of the second quadrant (A) by the sum of the lymphocytes in the second and fourth quadrant (A+B).

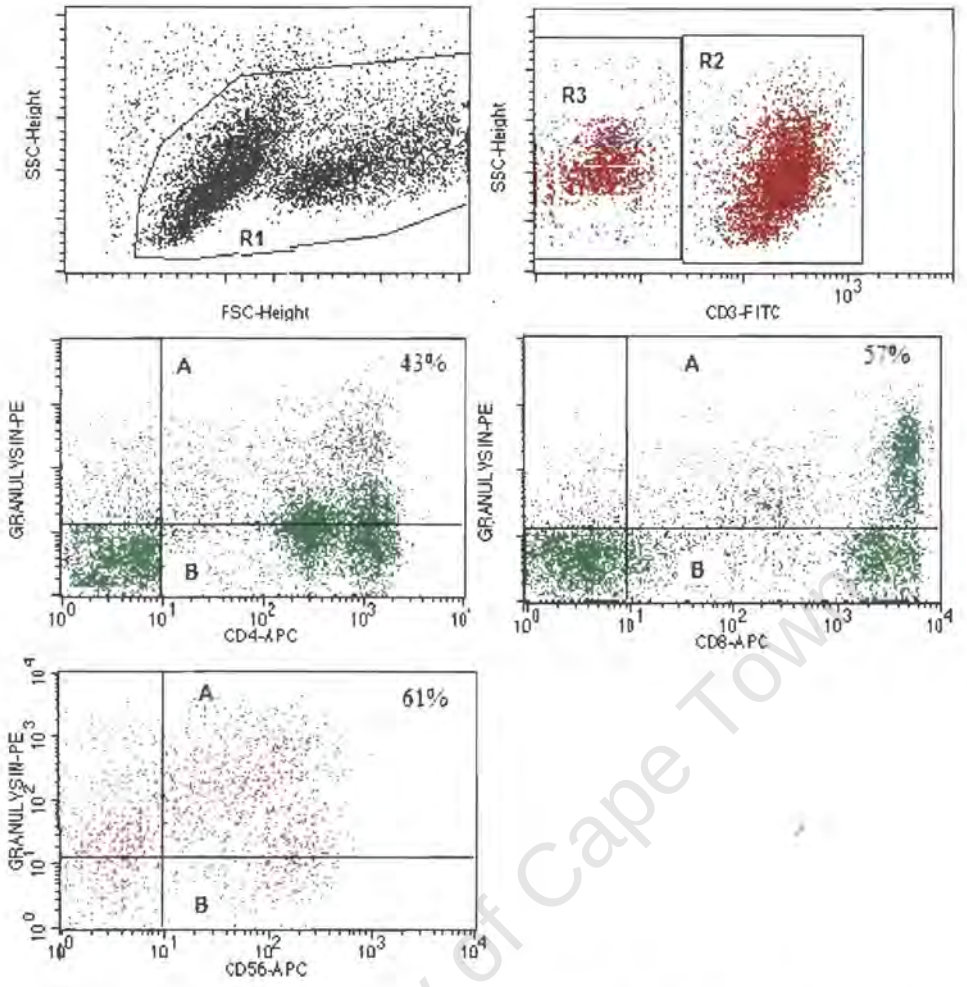


Figure 2.3: A representative flow cytometric diagram showing the FACS gating strategy employed in order to define three distinct peripheral blood lymphocyte subsets for quantification of granulysin and perforin expression. The lymphocyte gate was defined by forward and side scatter cell characteristics (R1). Within this population $CD3^+$ T-cells were split into $CD3^+4^+$ helper or $CD3^+8^+$ cytotoxic subsets (R1 and R2), while NK cells were operationally defined as CD3-56+ (R1 and R3).

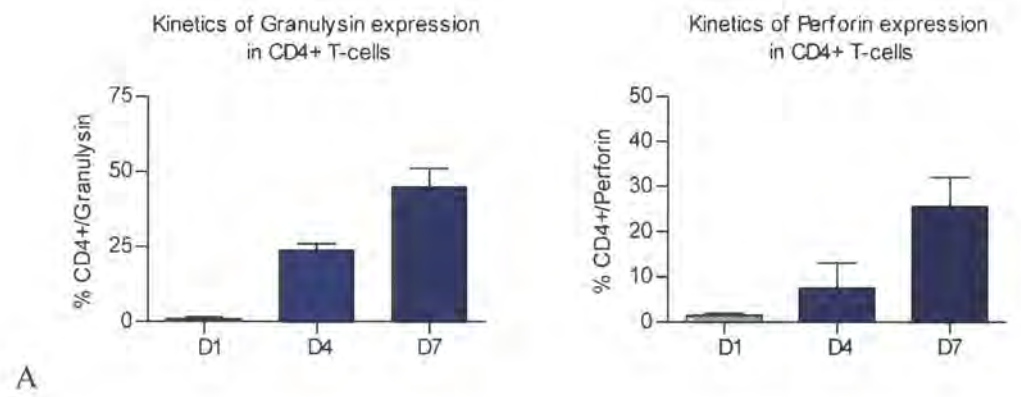
After one day of PPD stimulation, a mean of 1% of CD4+ T-cells, 6.7% of CD8+ T-cells and 61% of NK cells expressed granulysin (figure 2.4). There was a large increase of granulysin expressing T-cells on day four of PPD stimulation where a mean of 23.7% of CD4+ and 29% of CD8+ T-cells expressed this cytolytic marker. In contrast, the percentage of NK cells expressing granulysin had reduced to 37.5% after PPD stimulation and the constitutive expression had been reduced to a similar percentage which may suggest that these cells had spontaneously degranulated. By day 7 granulysin was detected in a mean of 44.7% of CD4+ and 45.3% of CD8+ T-

cells. There was an increase of granulysin expressing NK cells from day 4 to day 7 in both unstimulated (64.5%) and PPD stimulated cells (69.1%).

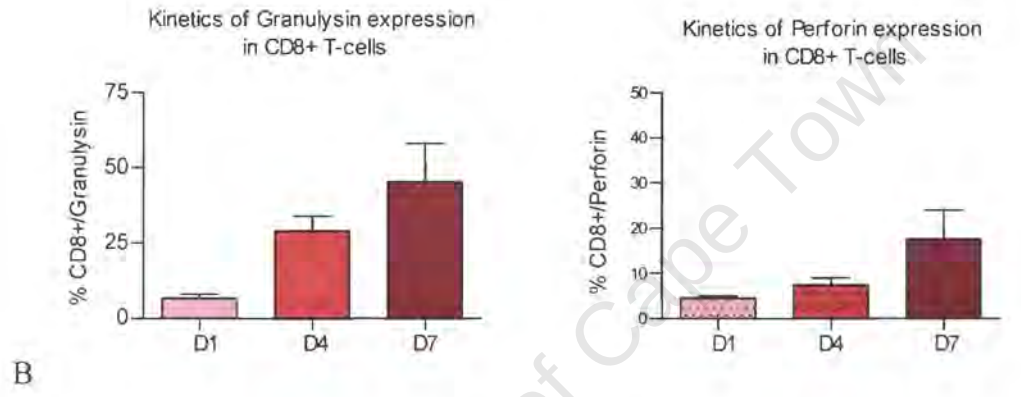
Although the mean percentage of perforin expressing T-cells was lower than the mean percentage of granulysin expressing T-cells, a similar pattern emerged in response to PPD stimulation (figure 2.4). CD4+ perforin expressing cells increased from an initial mean percentage of 1.5% to 25.5% seven days after PPD stimulation. There was also a small number of CD8+ T-cells expressing perforin one day after PPD stimulation (4.4%) and this figure increased to 19±1% on day 7. As seen in granulysin expressing cells, there were a high number of NK cells constitutively expressing perforin (87.1%), which was lower on day 4 after PPD stimulation (28.5%) and then increased again on day 7 (56.3%). Figure 2.5 is a histogram illustrating granulysin and perforin expression on day 7 following PPD stimulation. The rabbit serum negative control for granulysin, the isotypic control for perforin, and unstimulated and PPD stimulated expression of granulysin and perforin are shown.

As there was a continual increase from day 4 to day 7 in the number of T-cells expressing both cytolytic makers, granulysin expression was evaluated in T-cells and NK cells in four adult volunteers six, seven and eight days after PPD stimulation to validate if day seven was the optimal time-point. As the antibody for perforin is commercially available and the assay is well established, only granulysin expression was determined within these volunteers.

The number of T-cells expressing granulysin was highest on day 7 in both CD4+ and CD8+ T-cells but there was a gradual reduction in NK cells expressing granulysin from day 6 to day 8 (Figure 2.6). As the difference was not significant, the optimal time-point was set at day seven for all subsequent experiments in this thesis.



A



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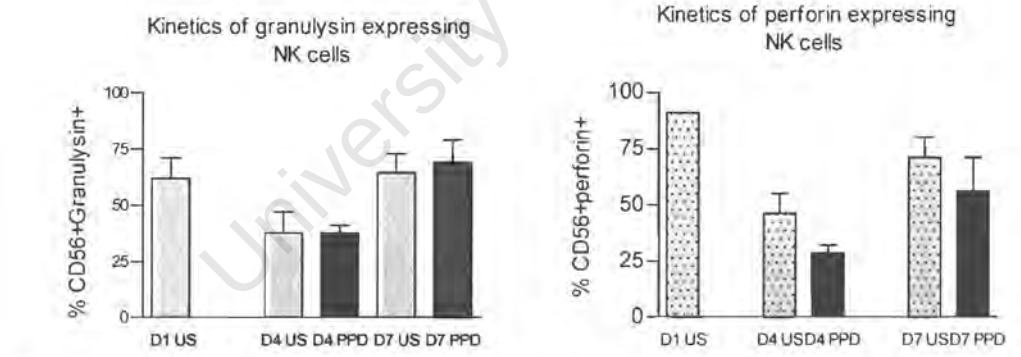


C

Figure 2.4: Percentage of granulysin and perforin expressing CD4+ (A) and CD8+ (B) T-cells and NK cells (C). Expression was evaluated on day 1, 4 and 7 after PPD stimulation. Data of unstimulated NK cells (C) is shown as there was constitutive expression of granulysin and perforin in NK cells. The results are shown as means (n=3) and error bars represent standard deviations.

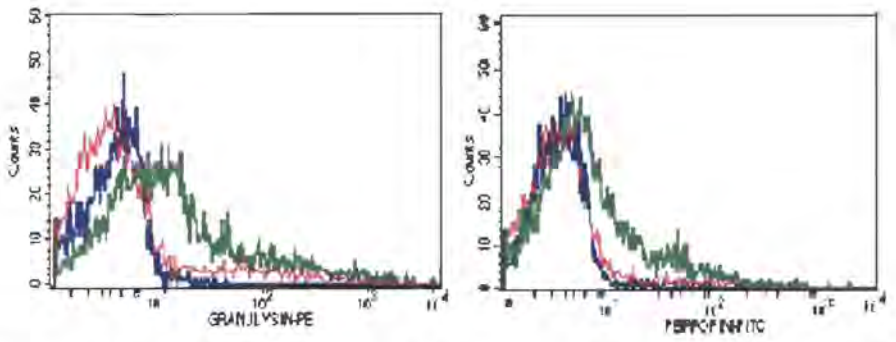


Figure 2.5: Flow cytometric histogram of granulysin and perforin expression in PBMC on day 7 following PPD stimulation. The negative control is shown in pink, unstimulated cellsin blue and PPD stimulated cells in green.

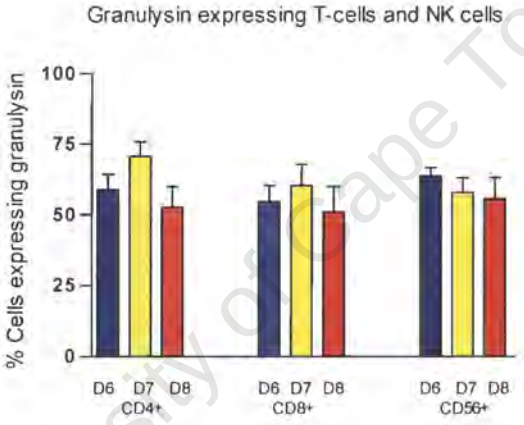


Figure 2.6: Granulysin expression in CD4+ and CD8+ T-cells and NK cells 6, 7 and 8 days after PPD stimulation. The results are shown as means (n=4) and error bars represent standard deviations.

2.3.3 Granulysin and perforin expression in T-cells and NK cells from ten ND

Granulysin and perforin was evaluated in T-cells and NK cells in PBMC from ten ND including 5 males and 5 females with a mean age of 36 years (24-55 years). PBMC were stimulated with PPD and BCG to determine which mycobacterial antigen gave the best expression of these two cytolytic markers. In addition, the cells were stimulated with IL-2 as a positive control. As IL-2 did not result in an increase of CD4+ granulysin or perforin expressing cells in the first five patients, IL-15 was employed as an extra positive control in the next 5 patients as it has been shown to

activate memory cells (Weng *et al.*, 2002). Unstimulated cells served as a negative control.

In PBMC, unstimulated CD4+ T-cells had very little or no granulysin expressing cells but constitutive granulysin expression was seen in a mean of 14.4±4% of $CD8^+$ T-cells and 51.4±7% of NK cells. After 7 days of PPD or BCG stimulation, a mean of 35.5±5% and 30.2±6% of CD4+ T-cells respectively expressed granulysin. The mean percentage of granulysin expressing $CD8^+$ T-cells after PPD or BCG stimulation was 39.1±4% and 44.9±6% respectively. There was very little difference in the percentage of NK cells expressing granulysin after PPD or BCG stimulation compared to unstimulated cells (55.6±4.4% for PPD and 59.4±3.7% for BCG). IL-2 did not result in an increase of granulysin expressing T-cells or NK cells to the same extent as was found when PPD or BCG stimuli were used. Stimulation with IL-15 resulted in an increase in the mean percentage of CD4+ granulysin expressing T-cells (48.4±3%) but did not increase the percentage of CD8+ T-cells or NK cells expressing granulysin more than PPD and BCG stimulation (figure 2.7).

A similar pattern of results was observed in perforin expressing cells (figure 2.8). Perforin was constitutively expressed in a mean of 8.4±3% of CD8+3+ cells and 71±5% of NK cells. No constitutive expression of perforin in CD4+ T-cells was detected but PPD and BCG induced a mean of 13.6±1.8% and 12.4±1% of CD4+ perforin expressing cells respectively. There were very similar percentages of CD8+ T-cells expressing perforin after PPD or BCG stimulation (22.4% and 24.8% respectively) but PPD and BCG stimulation resulted in reduction of perforin expressing NK cells (58±5.3% and 66.8±3.9% respectively). Again, IL-2 and IL-15 stimulation did not induce an increase of perforin expressing cells over and above PPD or BCG stimulation.

Figure 2.9 is a representative flow cytometric dotplot showing forward (size) and side scatter (granularity) of unstimulated and stimulated PBMC after 7 days of PPD, BCG, IL-2 or IL-15 stimulation, showing the ability of the cells to be activated.

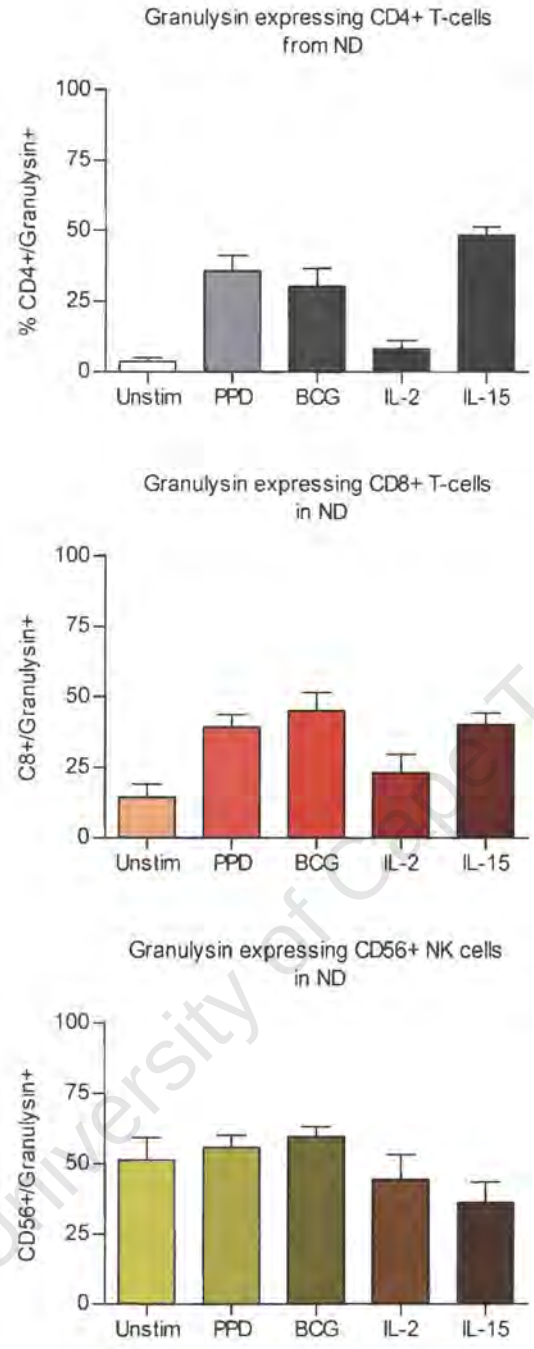


Figure 2.7: Granulysin expressing T-cells and NK cells in unstimulated (unstim) and stimulated PBMC from ten ND. Mann Whitney stats were performed and the results are shown as means and error bars represent standard deviations.

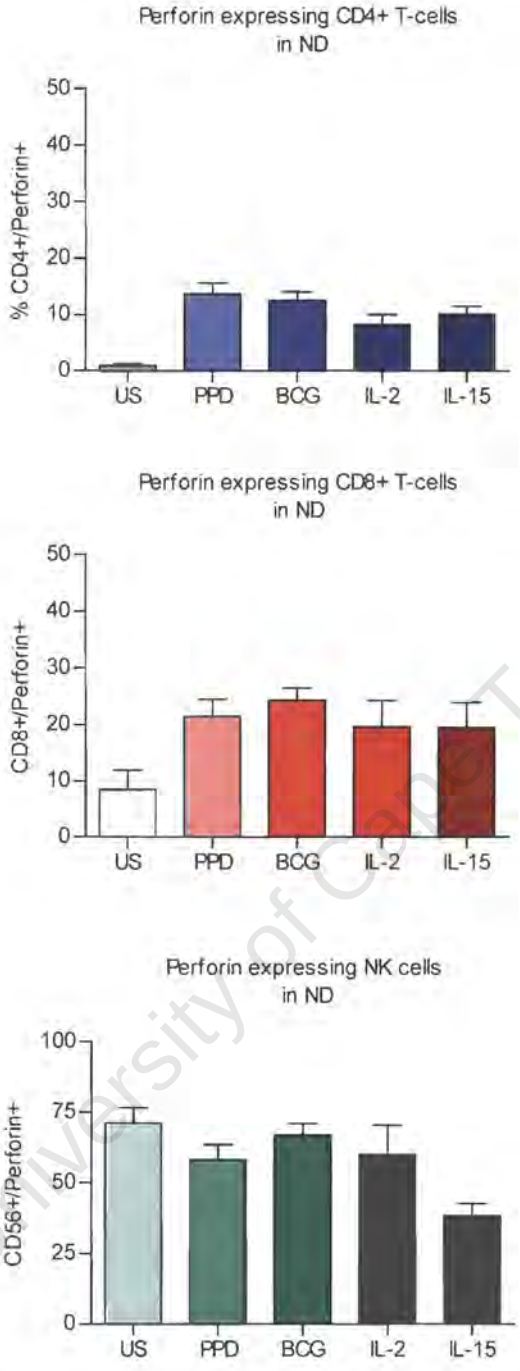


Figure 2.8: Perforin expressing T-cells and NK cells in unstimulated (unstim) and stimulated PBMC from ten ND. The results are shown as means and error bars represent standard deviations.

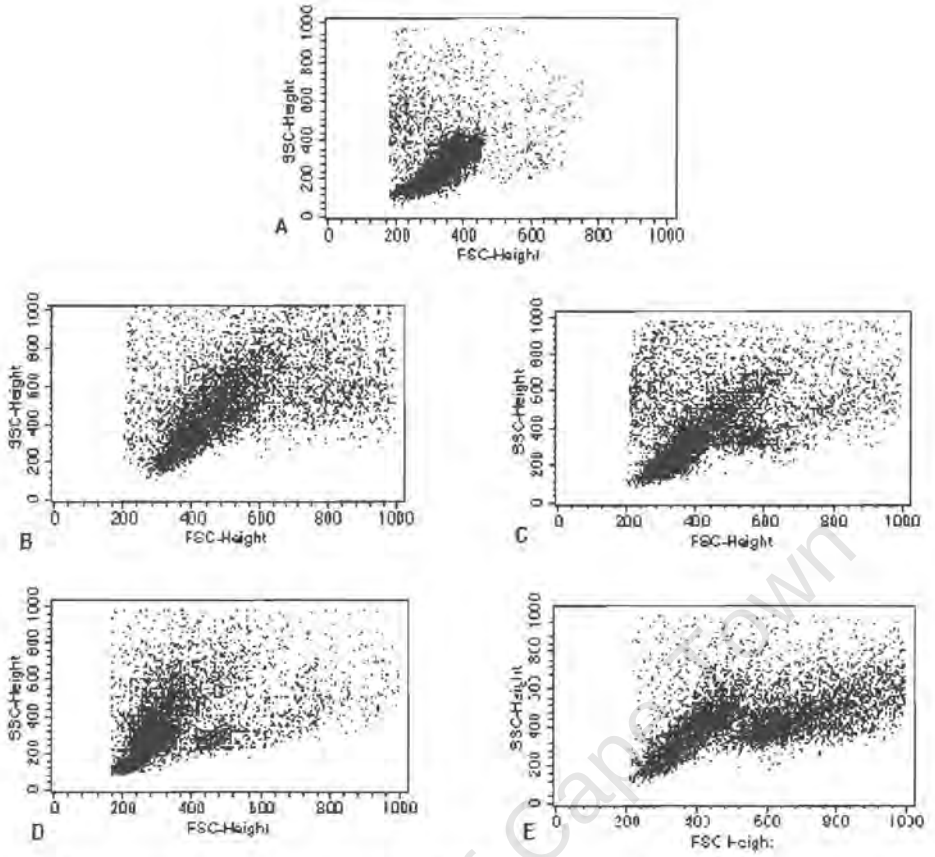


Figure 2.9: Representative flow cytometric dotplots showing forward and side characteristics of unstimulated (A) and PBMC stimulated with PPD (B), BCG (C), IL-2 (D) and IL-15 (E) from a healthy PPD+ adult volunteer. PBMC were stimulated for 7 days with PPD, BCG, IL-2 and IL-15.

2.5 DISCUSSION

Granulysin and perforin were constitutively expressed in more than 50% of NK cells and in approximately 10-15% of CD8+ T-cells in healthy PPD+ adult volunteers. CD4+ T-cells did not constitutively express either marker. Four days after PPD, BCG, IL-2 or IL-15 stimulation both granulysin and perforin could be detected in stimulated PBMC from ND, and maximal numbers of cells expressing granulysin and perforin were seen seven days after stimulation. IL-15 appeared to be a more potent inducer of granulysin in T-cells than IL-2, but the opposite was true for NK cells. The percentage of T-cells and NK cells in the lymphocyte population expressing granulysin and perforin were similar whether PPD or BCG was used as a stimulant to induce those expressing cells.

Granulysin expressing CD4+ and CD8+ T-cells have been detected using the same polyclonal antisera as used in this study, in purified cells using H37RA as a stimulant (Canaday *et al.*, 2001). IL-2 has also been reported as a potent inducer of granulysin and perforin (Zheng *et al.*, 2008a). For the optimization of the indirect fluorescent assay for granulysin expression in experiments included in this thesis, considerable effort was taken to ensure that the positive fluorescence observed represented specific binding of the granulysin antibody. Rabbit serum was utilised as a negative control in the absence of an isotypic control. Measures taken to avoid non specific binding included firstly, incubation of cells with the primary antibody was limited to 30 minutes, secondly, Fc receptor (FcR) binding was minimized by the addition of human serum to all the washes and dilutions to ensure FcR blocking and thirdly, a F(ab)′ secondary antibody was used to prevent FcR binding. Finally the veracity of the data was supported by the pattern of fluorescence observed on a range of target cells. These included negative staining with the Jurkat cell line as expected, negative staining with naïve T cells from cord blood (see chapter 3), and negative staining in unstimulated CD4+ T-cells. On the other hand there was positive staining in NK cells and to a lesser degree in CD8+ T-cells due to constitutive granulysin expression, which served as an internal positive control for the assay.

For all subsequent experiments in this thesis, granulysin expression will be detected after PPD and BCG stimulation whenever cell numbers are sufficient, but only PPD

stimulation when cell numbers are low. As positive controls IL-2 and IL-15 will be included but only IL-2 if cell numbers are low. Stimulation will be for seven days and unstimulated cells will serve as a background control. Isotypic controls will be used as negative controls except for granulysin where rabbit serum will be used. Granulysin antisera will be used at a final dilution of 1:1000. Granulysin and perforin expression will be evaluated on all samples except where cell concentrations are low and only granulysin will be tested in those subjects.

CHAPTER 3

GRANULYSIN AND PERFORIN EXPRESSING CELLS IN CORD BLOOD AND IN PERIPHERAL BLOOD OF 10 WEEK-OLD INFANTS VACCINATED WITH BCG AT BIRTH.

3.1 INTRODUCTION

3.2 MATERIAL AND METHODS

3.1.1 Human subjects and vaccination.

3.2.2 Isolation of cord blood mononuclear cells (CBMC)

3.2.3 Isolation of PBMC from 10 week-old vaccinated infants

3.2.4 Stimulation of CBMC and PBMC

3.2.5 Flow cytometric analysis

3.2.6 Statistical analysis

3.3 RESULTS

3.3.1 Granulysin expressing cells in CBMC and PBMC from vaccinated infants in response to PPD stimulation

3.3.2 Perforin expressing cells in CBMC and PBMC from vaccinated infants in response to PPD stimulation

3.3.3 Granulysin and perforin expression in $CD4^+$ and $CD8^+$ T-cells from CBMC in response to BCG stimulation, and comparison to ND

3.3.4 Effect of the route and strain of the BCG vaccine on granulysin and perforin expression in T-cells and NK cells from PBMCs from 10 week-old vaccinated infants

3.3.5 Lymphocyte counts pre and post stimulation

3.4 DISCUSSION

3.1 INTRODUCTION

It is estimated that approximately one third of the worlds population is infected with *Mycobacterium tuberculosis* (M.tb) resulting in about 2 million deaths from tuberculosis (TB) annually (WHO, 2007). *Mycobacterium bovis* Bacillus Calmette Guérin (BCG), the most commonly used vaccine worldwide, is the only vaccine used for the prevention of TB but the efficacy of this vaccine is both variable and poor. BCG offers good protection against childhood miliary disease and TB meningitis (Colditz *et al.*, 1995; Fine *et al.*, 2007) and has been associated with a reduced risk of acute respiratory tract infections in neonates (Stensballe *et al.*, 2005). However, it affords very little protection against pulmonary TB and other manifestations of adult disease. Furthermore, the strain of BCG and the route of administration may affect the efficacy of the vaccine (Al Jarad *et al.*, 1999; Hart, 1967; Kemp *et al.*, 1996; Ormerod and Palmer, 1993) and may differ in the extent of antigen-specific T-cell immunity induced (Davids *et al.*, 2006). Kemp *et al* (1996) proposed that intradermal BCG administration induced greater Th1 cytokine responses and lymphoproliferation in response to M.tb whole-cell lysates, than percutaneous administration of same strain. In contrast, a study in South Africa found that Japanese BCG administered percutaneously induced greater specific Th1 immunity than the same strain or the Danish strain administered intradermally (Davids *et al.*, 2006). Recent reports have also suggested that BCG induces different immune responses in Malawi and in the United Kingdom (Black *et al.*, 2002).

Despite having a full repertoire of helper and cytotoxic T-cells, B-cells and dendritic cells (DC), the neonatal immune system is immature (Adkins *et al.*, 2004). Circulating neonatal lymphocytes are different from naïve adult T-cells as there is a functional impairment of T-cells in infants resulting in intrinsic deficiencies that prevent them from becoming maximally activated (Harris *et al.*, 1992). In the first two years of life the infant is unable to mount a T cell-independent B cell response to polysaccharides, resulting in a higher susceptibility to infections by bacterial pathogens (Adderson, 2001). Innate and adaptive cellular immune responses are required for an effective host defense against M.tb, and individuals with defective cell mediated immunity

(CMI) have a predisposition towards developing TB or more severe disease (Barnes, 1994).

T-cell derived interferon gamma (IFN-γ) is widely recognized as being important in antimicrobial protection. The cytokine is essential for the activation of macrophages, a process required to limit the replication of the bacilli in these cells (Kaufmann and Andersen, 1998; Stenger and Modlin, 1999) and it is clear that a Th1 cytokine response is imperative for optimal protection against M.tb (Marchant *et al.*, 1999). However, IFN-γ production in response to mycobacterial antigens is significantly lower in neonates as compared to adults, providing an explanation for the extremely high incidence of TB in babies during the first 2 years of life (Suen *et al.*, 1998; Trivedi *et al.*, 1997). However, the value of IFN-γ as the best correlate of protection has recently been challenged (Elias *et al.*, 2005; Kaufmann, 2006). T-cell derived cytotoxic molecules found in cytotoxic lymphocytes (CTL) and natural killer (NK) cells are also important for protective immunity against intracellular pathogens. These cells contain cytolytic granules rich in granulysin, perforin and granzyme molecules that contribute to lysis of infected cells. Granulysin, a member of the saponin-like family of lipid binding proteins has been shown to directly kill extracellular bacilli and, together with perforin, was able to substantially reduce the viability of intracellular M.tb (Stenger *et al.*, 1998).

The aim of this chapter was (i) to evaluate intrinsic granulysin and perforin expression in cord blood lymphoid cells and document response to *in vitro* PPD and BCG stimulation and (ii) to ascertain the effect of BCG vaccination on cytolytic mediators in peripheral blood lymphoid cells, obtained from ten-week old infants, vaccinated at birth with either Danish BCG (administered intradermally) or Japanese BCG, (administered either intradermally or percutaneously)

3.2 MATERIAL AND METHODS

3.2.1 Human subjects and vaccination

Human participation was according to the United States Department of Health and Human Services and good clinical practice guidelines. This included protocol approval by the University of Cape Town Research Ethics Committee and by the University of Medicine and Dentistry of New Jersey Newark, and informed written consent from a parent or gaurdian of the neonate.

Healthy pregnant females scheduled to undergo elective Caesarian section were enrolled for umbilical cord blood (UCB) collection. The donors comprised 10 full term pregnant subjects (38-40 weeks) with a mean age of 26 years (19-34) who had chosen to have Caesarian sections. Healthy 10-week old infants were enrolled at primary care clinics in a region in the Western Cape in South Africa where the incidence of TB is 2/100 under 2 years of age. At birth the babies had received either Japanese BCG (strain 172: Japan BCG Laboratory) or Danish BCG (strain 1331: Statens Serum Institute). The Japanese vaccine was administered either intradermally (JID) or percutaneously (JPC), while the Danish vaccine was given intradermally. Infants with any acute or chronic disease, born to a HIV^+ mothers, or living with a person with active tuberculosis, were excluded. A positive ELISA test for HIV, performed on all infants, also resulted in exclusion.

3.2.2 Isolation of cord blood mononuclear cells (CBMC)

UCB was collected in the maternity theatre from elective caesarean donors in order to prevent any possible effects of labour. After puncturing the umbilical vein of the placenta, the blood was allowed to flow by gravity into a standard blood donation bag (Sabax, Johannesburg, South Africa) containing 2000 units of sodium heparin (Sigma- Aldrich, Steinham, Germany). In the laboratory, the UCB was diluted with equal volumes of Ca^+ and Mg^+ free phosphate buffered saline (PBS, Bio Whittkaker, Walkersville, MD, USA) and mononuclear cells were isolated by density gradient

sedimentation using Ficoll-Hypaque. The gradient was centrifuged at 1200g (Yang *et al.,* 2001) for 30 minutes at room temperature and the resultant interface containing the mononuclear cells was removed and washed twice with PBS by centrifugation at 300g for 10 minutes. Cells were resuspended in PBS to the original volume of cord blood and subjected to a second density separation to remove the large number of erythroid cells in UCB (Yang *et al.,* 2001). The cells were collected at the interface and washed as before. The CBMC were adjusted to $1x10^6$ per ml in RPMI supplemented with 10% human AB serum (Western Province blood Transfusion Service, South Africa).

3.2.3 Isolation of PBMC from 10 week-old vaccinated infants

PBMC were isolated as described in chapter 2 from forty six babies vaccinated at birth as follows; 15 babies had JID, 15 babies had Danish and 16 had JPC. One infant from the JID group was later discovered as HIV^+ and was excluded from the study.

3.2.4 Stimulation of CBMC and PBMC

$1x10^6$cells/ml of CBMC were stimulated with PPD, BCG, IL-2 and/or IL-15 as described in the previous chapter. Unstimulated cells served as a background control. BCG was optimized at a MOI of 1:1, which induced the stimulation of CBMC without causing cell death, as determined by the viability exclusion dye, 7AAD. In one donor, the CBMC were stained for an activation marker CD25 after seven days, in order to ascertain whether this marker was expressed in response to stimulants. Flow cytometric analysis was carried out at the end of the stimulation period.

Due to the small volume of blood collected from the 10 week-old infants (1-2mls) only PPD was used as a stimulant for the isolated PBMC, and for most infants, only granulysin expression was studied in these cells. In those infants where more PBMC were available, IL-2 stimulation and perforin expression was also evaluated (n=14). Unstimulated cells served as a background control. After 7 days of stimulation PBMC were washed and resuspended in 90% foetal calf serum (FCS Delta, Kempton Park,

South Africa) in RPMI. Equal volumes of cells were mixed with 20% dimethylsulphoxide (DMSO, Merck, Darmstadt Germany) in RPMI to give a final concentration of 10% DMSO. The cells were frozen at 1˚C per minute ("Mr Frosty") and maintained at -70˚C until all samples had been collected. On the day of the assay, the cells were thawed rapidly at 37˚C then washed twice in 10% AB serum in RPMI and resuspended in FACs buffer for flow cytometric analysis. Validation of the cryopreservation process was obtained by lack of significant differences in cytolytic mediator expression between fresh blood and cryopreserved cells as shown in our laboratory and as other studies have shown (Murray *et al.*, 2006).

3.2.5 Flow cytometric analysis

Flow cytometric analysis was carried out as detailed in chapter 2.

3.2.6 Statistical analysis

The Mann-Whitney two-tailed test for non-parametric data or Kruskal Wallis test was used for group comparison. All statistical analysis was carried out using *GraphPad Instat* software (version 3.06).

3.3 RESULTS

3.3.1 Granulysin expressing cells in CBMC and PBMC from vaccinated infants in response to PPD stimulation

Granulysin expression in T-cells and NK cells was determined in unstimulated and PPD stimulated CBMC. A mean of 0.1±0.1% CD4+ granulysin expressing cells were found in unstimulated CBMC and this increased minimally to a mean of 1.4±0.4% after PPD stimulation. A similar result was found in CD8+ T-cells where 1.3±0.3% of cells constitutively expressed granulysin and this was increased to 3.5±0.8% after PPD stimulation. IL-2 stimulation induced an increase in the mean percentage of granulysin expressing CD4+ and CD8+ T-cells to 27.±9.9% and 29.0±9.6% respectively. In contrast, there were a high percentage of granulysin expressing NK cells in the absence of *in vitro* stimulation and the percentage of positive cells decreased from 51±5.3% to 35.2±6.3% following PPD stimulation. IL-2 stimulation resulted in a decrese in granulysin expressing NK cells compared to unstimulated cells (21.1±6.4%).

When infants were vaccinated with BCG at birth and their PBMC evaluated for granulysin expression 10 weeks post vaccination, a significantly different pattern was seen. Granulysin was expressed in a mean of 0.2±0.1% and 2.5±0.4% of unstimulated CD4+ and CD8+ T-cells respectively, which was similar to CBMC, but after *in vitro* PPD stimulation 58.2±3.6% of CD4+ and 58.3±3.5% of CD8+ T-cells expressed granulysin ($p=0.0001$ and $p=<0.0001$ respectively). IL-2 stimulation induced comparable expression with means of 46±8.6% and 58.3±7.6% for CD4+ and CD8+ T-cells respectively. Thus, in contrast to CBMC, vaccinated babies had significantly higher levels of granulysin expressing T lymphocytes after *in vitro* PPD stimulation. Constitutive expression of granulysin was seen in 39.6±4.8% of unstimulated NK cells of vaccinated infants and this expression was greatly enhanced by PPD stimulation to 85.9±2.4% of NK cells expressing granulysin ($p=<0.0001$), a phenomenon not seen in CBMC (figure 3.1). Representative flow cytometric analyses of granulysin expression in PPD stimulated T-cells and NK cells are shown in figure 3.2.

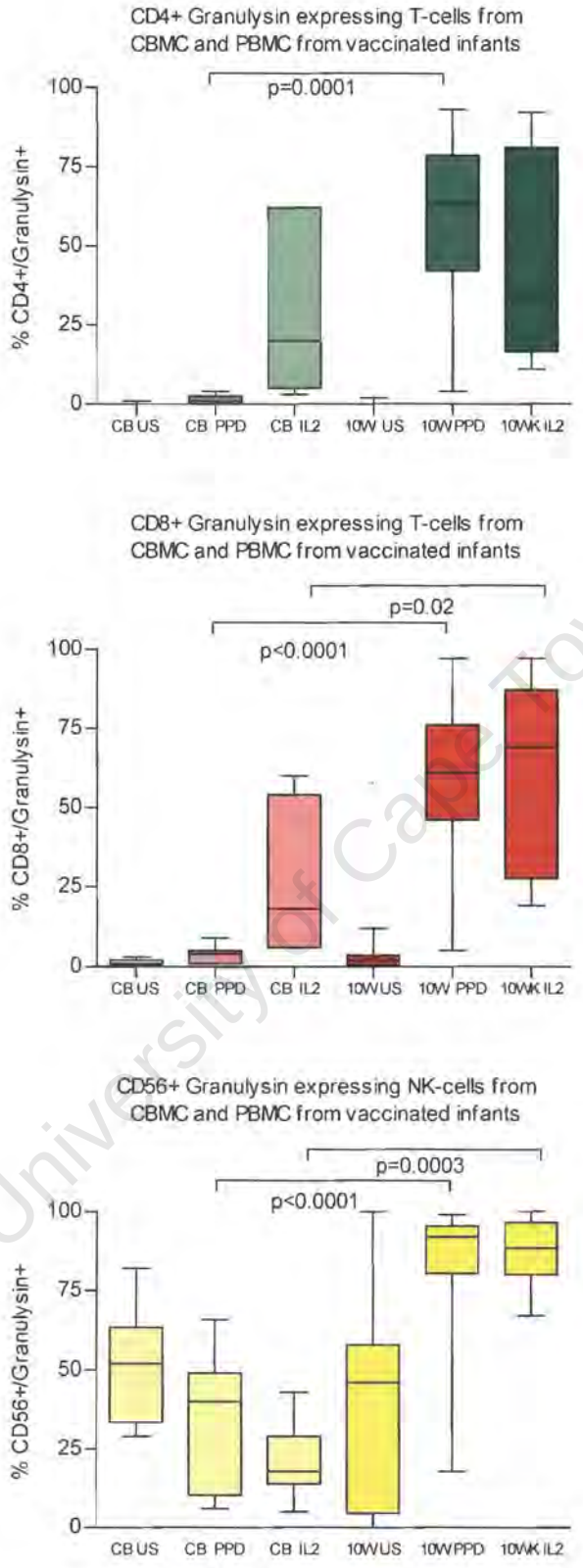


Figure 3.1: Granulysin expressing cells in CBMC (n=10) and PBMC from vaccinated infants (n=45). Granulysin was not expressed in PPD stimulated T-cells from CBMC but vaccinated infants had a significant percentage of granulysin positive T-cells. NK cells constitutively expressed granulysin. The box extends from the 25th-75th percentile, the line represents the median, and the whiskers represent the minimum and maximum values.

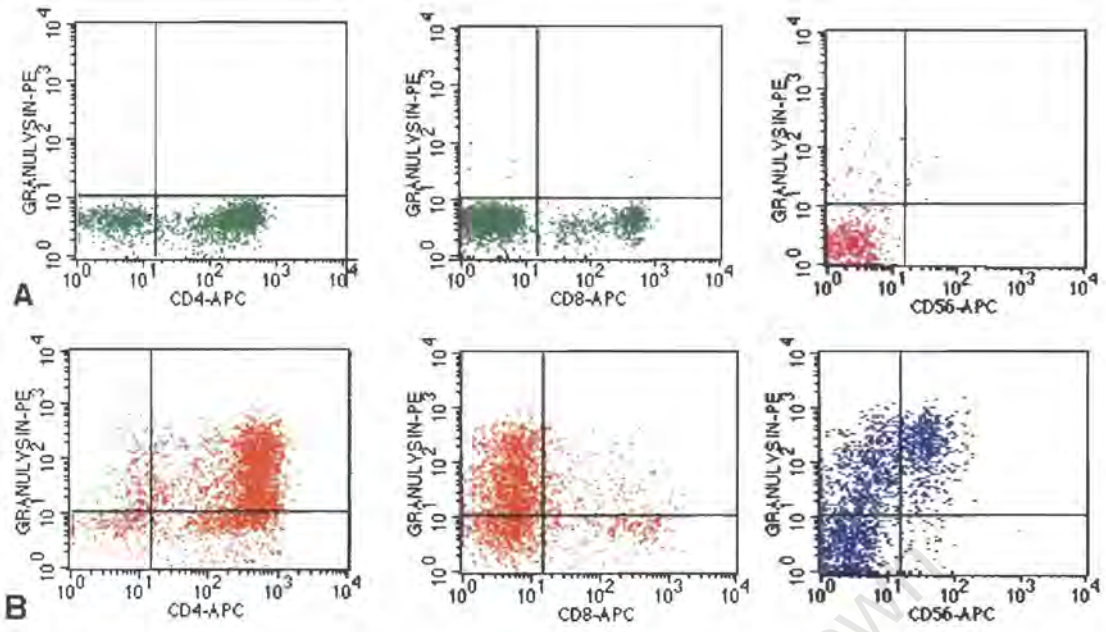


Figure 3.2: A representative flow cytometric analysis of granulysin expression in T-cells and NK cells from CBMC (A) and PBMC from 10 week-old vaccinated infants (B) analysed after 7 days of *in vitro* PPD stimulation.

3.3.2 Perforin expressing cells in CBMC and PBMC from vaccinated infants in response to PPD stimulation

A similar analysis was carried out to determine the lymphocyte subsets expressing perforin in CBMC and 14 infants vaccinated with BCG at birth. Neither T-cells from cord blood nor T-cells from PBMC from 10 week-old vaccinated infants expressed perforin in the absence of stimulation, but means of 38.7±6.8 and 20.1±6.6% of NK cells from CBMC and PBMC from vaccinated infants respectively, constitutively expressed the cytolytic marker. As seen with granulysin, PPD stimulation did not induce expression of perforin in CD4+ (0.4±0.2%) or $CD8^+$ (1.6±0.5%) T-cells from cord blood, and the mean percentage of NK cells expressing perforin decreased after PPD stimulation to a mean of 21.9±5.1%. IL-2 stimulation resulted in an increase in the percentage of CD4+ (5.1±2.5%) and CD8+ T-cells (10±3.9) expressing perforin but no difference in the percentage of NK cells expressing perforin (figure 3.3).

In contrast, PPD stimulation of PBMC from vaccinated infants resulted in a large increase in the percentage of perforin expressing CD4+ and CD8+ T-cells to a mean of 28.2±4.4% and 35.4±5.1% respectively, and in NK cells to a mean of 76.6±6.7%. There was a significant difference in the percentage of T-cell and NK cells expressing perforin compared to CMBC ($p=<0.0001$ for CD4+ and CD8+ T-cells and $p=0.003$ in NK cells). After IL-2 stimulation a mean of 20.7±2.9% of CD4+ T-cells, 33.1±4.2% of CD8+ T-cells, and 65.2±8.4% of NK cells expressed perforin. Therefore, IL-2 stimulation induced a statistically significant difference in perforin expressing T-cells and NK cells in PBMC from vaccinated infants compared to CBMC (figure 3.3).

In order to establish if T-cells from CBMC were being stimulated by PPD despite the lack of granulysin or perforin expression, CD3+ cells were stained for the activation marker CD25 in one donor. Figure 3.4 shows that PPD, BCG and IL-2 stimulation resulted in CD25 surface expression on CD3+ cells of CBMC. Trypan blue exclusion dye and CD25+ surface expression established that the cells were viable and were responsive to PPD stimulation.

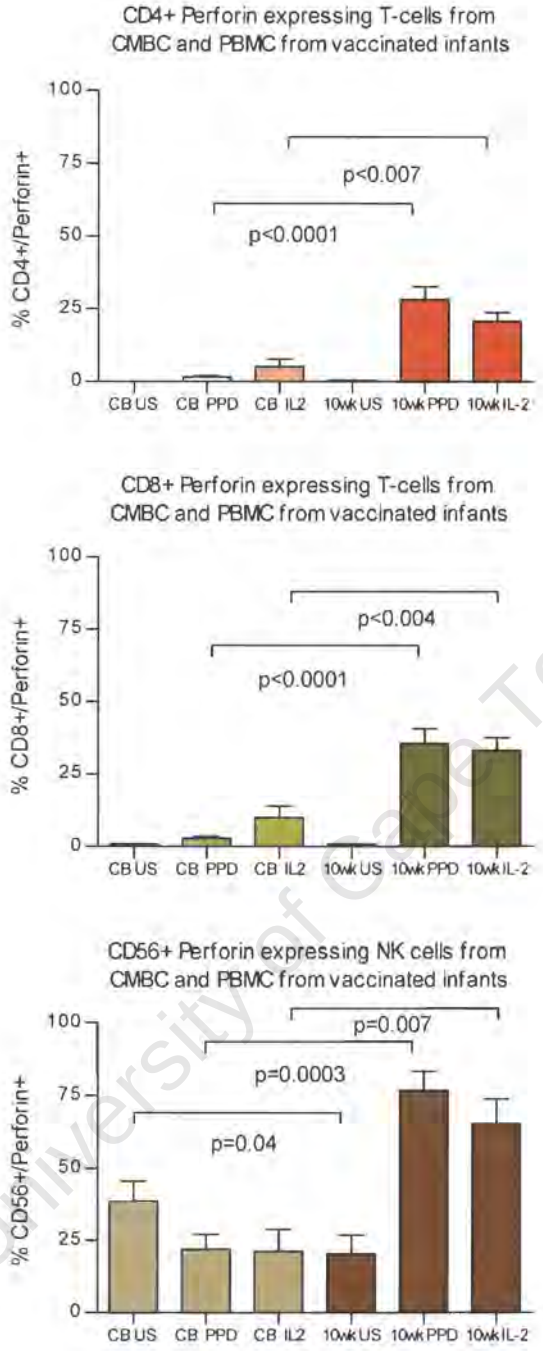


Figure 3.3: Perforin expressing cells in CBMC (n=10) and PBMC from vaccinated infants (n=14). Perforin was not expressed in PPD stimulated T-cells from CBMC but vaccinated infants had a significant percentage of granulysin positive T-cells compared to CBMC. NK cells constitutively expressed perforin. IL-2 stimulation resulted in significant difference in the mean percentage of T-cells and NK cells expressing perforin in vaccinated infants compared to CBMC. The results are shown as means and error bars represent standard deviations.

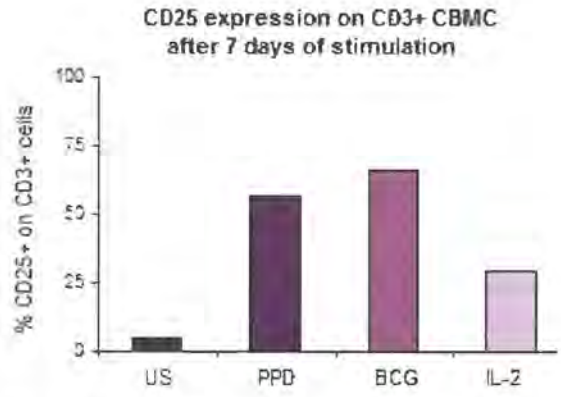


Figure 3.4: Surface expression of CD25 on CD3+cells from CBMC from one donor in the presence and absence of stimulation

3.3.3 Granulysin and perforin expression in CD4+ and CD8+ T-cells from CBMC in response to BCG stimulation, and comparison to ND

CBMC and PBMC from ND were stimulated with Danish BCG for 7 days and granulysin and perforin expression was determined within $CD3^+$ T-cells. Unlike PPD stimulated CBMC, BCG at an MOI of 1:1 induced an increase in both granulysin and perforin expressing T-cells from cord blood. 10.9±2.1% of CD4+ T-cells and 13±2.5% of CD8+ T-cells expressed granulysin after BCG stimulation (figure 3.5). Perforin expression was seen in 7.2±1.4% and 9.3±1.5% of $CD4^+$and $CD8^+$ T-cells respectively (figure 3.6). Thus, although the percentage of cells expressing granulysin and perforin was considerably lower than in ND, significant expression was induced in both lymphocyte subsets compared to PPD stimulation when BCG was used as a stimulant. In contrast, in ND there was no significant difference in the percentage of granulysin or perforin expressing T-cells between PPD and BCG stimulation.

In order to assess that the MOI of BCG did not result in cell death, 7AAD was used to assess the viability of CBMC after BCG stimulation in three donors. The mean percentage of viable cells in unstimulated cells was 99%. Stimulation with PPD and IL-2 resulted in approximately 5% and 2% dead cells respectively. BCG infection at an MOI of 1:1 had the same number of dead cells as PPD stimulation (5%). In

contrast, an MOI of 3:1 and 5:1 resulted in the death of 11% and 17% of CBMC respectively (figure 3.7). Therefore an MOI of 1:1 was optimal.

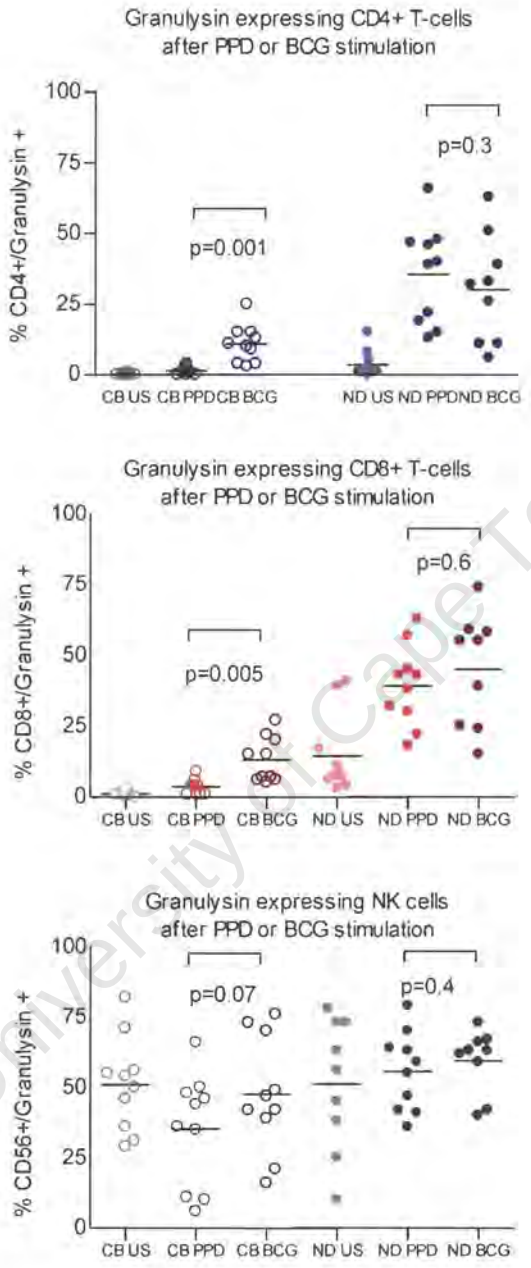


Figure 3.5: Percentage of granulysin expressing T-cells and NK cells in CBMC (CB n=10) and PBMC from ND (n=10) after PPD and BCG stimulation. There was a statistical difference in the mean percentage of T-cells expressing granulysin in CBMC after BCG stimulation compared with PPD stimulation. This was not observed in T-cells from ND. There was no difference in granulysin expression in NK cells from CBMC or ND after PPD and BCG stimulation. The results are shown as mean percentages.

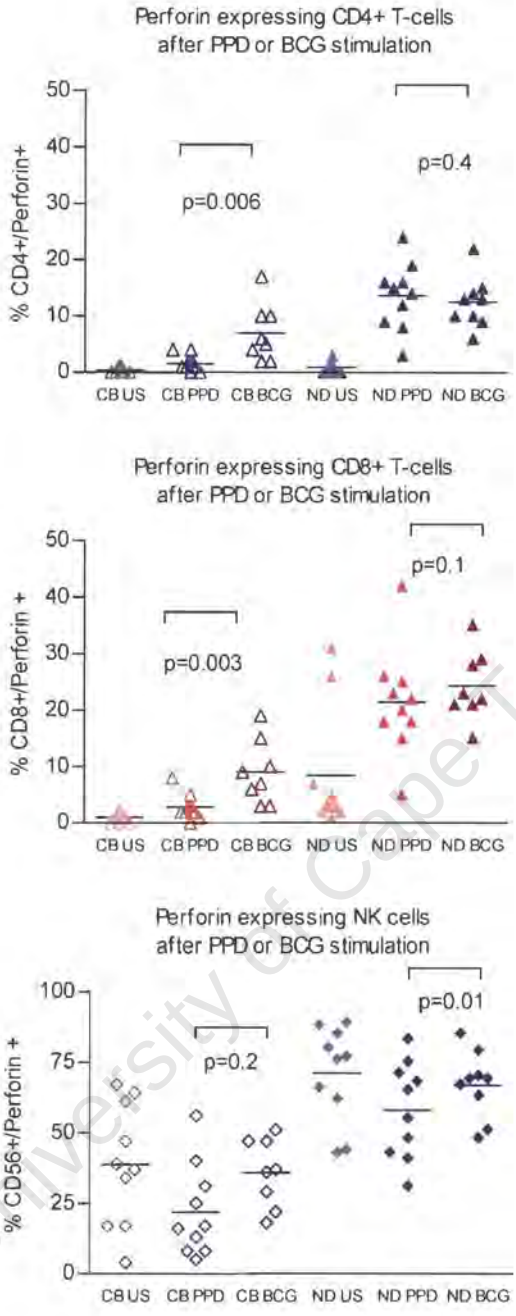


Figure 3.6: Percentage of perforin expressing T-cells and NK cells in CBMC (CB n=10) and PBMC from ND after PPD and BCG stimulation. There was a statistical difference in the mean percentage of T-cells expressing perforin in CBMC after BCG stimulation compared with PPD stimulation. This was not observed in T-cells from ND. There was no difference in perforin expressing NK cells in CBMC but significantly more NK cells expressed perforin in PBMC from ND after BCG stimulation than PPD stimulation. The results are shown as mean percentages.

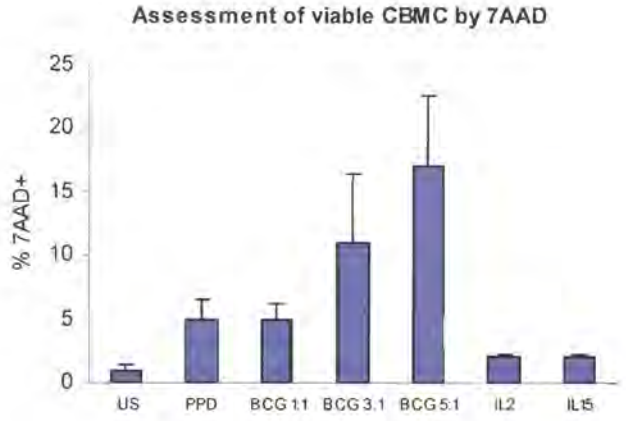


Figure 3.7: 7AAD assessment of cell viability of CBMC stimulated with PPD, IL-2 and BCG at different MOI. The results are shown as means and error bars represent standard deviations.

3.3.4 Effect of the route and strain of the BCG vaccine on granulysin and perforin expression in T-cells and NK cells from PBMC from 10 week-old vaccinated infants

To evaluate whether the strain of BCG, or the route of administration of the vaccine affected the extent of cytolytic mediator expression, PBMC from 10 week-old infants vaccinated with either Danish (n=14 granulysin and n=3 for perforin) or JID administered either intradermally (n=15 for granulysin and n=4 for perforin) or JPC (n=16 granulysin and n=7 for perforin) were evaluated for granulysin and perforin expression. No significant differences were found in granulysin or perforin (figure 3.8) expression between Japanese or Danish BCG. In addition, the route of administration of the vaccine had no effect on expression of these markers. Thus, in contrast to other immunologic assays (Davids *et al.*, 2006) evaluation of cytolytic mediator expression did not identify any differences in immunogenicity of the three vaccination protocols.

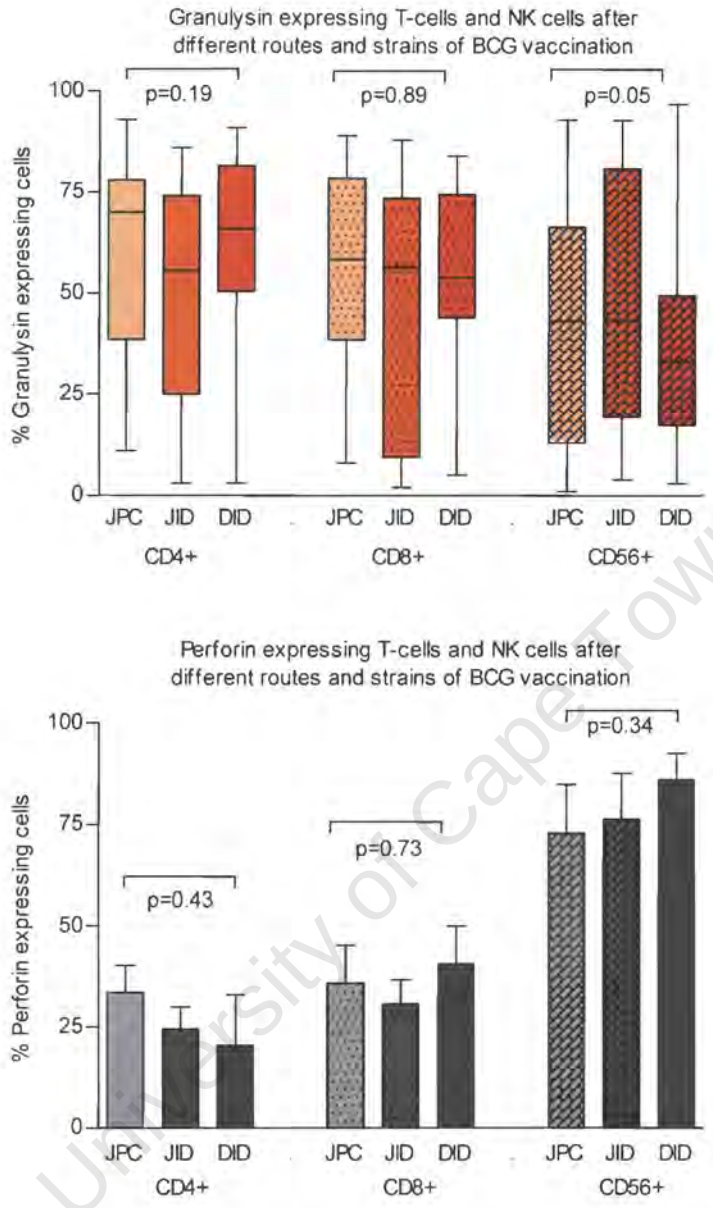


Figure 3.8: Granulysin and peforin expression in PPD stimulated T-cells and NK cells of 10 week-old infants after BCG vaccination with JID (n=14 for granulysin and n=4 for perforin), JPC (n=16 for granulysin and n=7 for perforin) or Danish (DID, n=15 for granulysin and n=3 for perforin). There was no statistical difference in the mean percentage of granulysin or perforin expressing cells between the three groups. For granulysin analysis the box represents the 25^{th}-75^{th} quartile and the horizontal line is the median whilst the whiskers are the maximum and minimum values. For perforin analysis the results are shown as means and error bars represent standard deviations.

3.3.5 Lymphocyte counts pre and post stimulation

Absolute cell counts were not established after stimulation with PPD in PBMC or CBMC. However, although there was expansion of the cells after stimulation as seen both microscopically and by flow cytometry forward and side scatter characteristics, the percentage of CD4+ and CD8+ T-cells were very similar before and after PPD stimulation. In contrast, the percentage of NK cells in the population increased after PPD stimulation of PBMC although this was only minimal in CBMC. In the vaccinated infants the mean NK cells constituted approximately 1% of the lymphocyte population which increased to 12% after PPD stimulation. Unstimulated NK cells from ND constituted a mean of about 8% which was increased to a mean of 14% (figure 3.9).

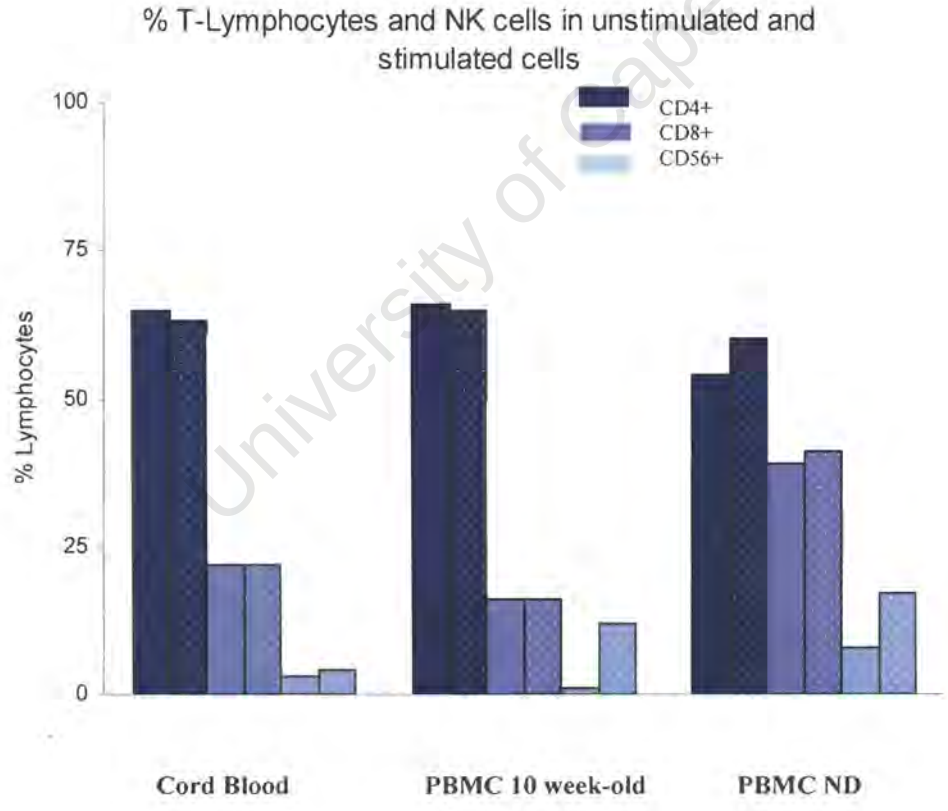


Figure 3.9: Percentage of lymphocyte phenotypes in lymphocyte populations in CBMC and PBMC from vaccinated infants (10wk) and ND, with and without PPD stimulation. PPD stimulated cells are represented by dot filled bars.

3.4 DISCUSSION

Considerable evidence supports a role for cytotoxic T-cell activity in protective immunity in TB (Cho *et al.*, 2000; Sable *et al.*, 2005; Santosuosso *et al.*, 2005). CD4+ cytotoxic activity in BCG vaccinated neonates using a chromium release assay has been reported (Hussey *et al.*, 2002), and flow cytometric assays have been used to measure cytolytic mediators and obtain evidence of degranulation of cytotoxic cells *ex-vivo* (Betts *et al.*, 2003; Chan and Kaur, 2007; Weren *et al.*, 2004). Other studies have shown that the level of expression of cytotoxic mediators is directly related to functional cytotoxic activity (Wolint *et al.*, 2004). Evidence of $CD8^+$ cytotoxic molecule expression in infants who had received BCG vaccination at birth, has also been shown (Murray *et al.*, 2006).

Protective immunity against infection with *M. tuberculosis* requires control of the intracellular growth of the bacilli. Innate immune responses contribute towards restriction of mycobacterial growth through the production of nitric oxide (NO), TNF-α, IFN-gamma and other pro-inflammatory cytokines that drive Th1 immunity. However, mycobacteria appear to be able to evade these effector pathways, and therefore killing of infected target cells by NK and CTL may be required. Perforin and granulysin are important cytolytic effector molecules involved in lysis of infected macrophages. Granulysin, found in the granules of cytotoxic T-lymphocytes and NK cells, has a broad spectrum of antimicrobial activity and has been shown to kill extracellular M.tb and, together with perforin, is bactericidal to intracellular organisms (Stenger *et al.*, 1998). It has been suggested that granulysin and perforin may have important utility as biomarkers of vaccine induced TB immunity (Endsley *et al.*, 2007). This highlighted the need to characterize cord blood and early neonatal cytolytic T-cell mediator expression in response to BCG vaccination.

In this chapter, the expression of the cytolytic mediators, perforin and granulysin, in both CD56+ NK cells and in CD4+ and CD8+ T-cell subsets in cord blood cells was evaluated. It was observed that (i) intrinsic expression of cytolytic mediators was limited to CD56+ cells and not found in CD4+ and CD8+ T-cells from cord blood; (ii) induction of perforin and granulysin expression in CBMC occurred selectively in

response to BCG, but not PPD. In contrast, in 10 week old BCG-vaccinated infants, upregulation of perforin and granulysin expressing CD4+ and CD8+ T-cells was demonstrable in response to both BCG and PPD stimulation. This response was independent of variation in vaccine strain or route of vaccine delivery. Granulysin has not been investigated in CBMC before but previous studies have shown that cord blood CD4+ lymphocytes lack constitutive expression of perforin (Berthou *et al.*, 1995) but most CD8+ T-cells of newborns were reported to contain perforin and granzymes (Murray *et al.*, 2006). In contrast, no granulysin or perforin was expressed by CD8+ T-cells or CD4+ T-cells isolated from cord blood in this study. This difference may be explained by the fact that, in the present study, the CBMC were from mothers undergoing cesarean section. During normal delivery, cytokines including IL-15 may be induced, which could activate CD8+ T-cells to acquire cytotoxic potential (Agarwal *et al.*, 2001).

IFN-γ production by NK cells represents the most obvious contribution of innate immune cells to protection against intracellular pathogens (Marodi, 2006). This mechanism is most needed after birth at a time when CD4+ CD45RO- T-cells have down-regulated Th1 function by transcriptional regulation of the IFN-γ promoter gene due to hypermethylation (White *et al.*, 2002). Intrinsic cytolytic mediator expression provides a second mechanism for NK cell mediated protective immunity during this critical period of heightened susceptibility to mycobacterial infection. The observation that BCG upregulates cytotoxic molecules in cord blood CD4+ and CD8+ T-cells may also be secondary to activation of cells of the innate immune system. Since IL-2 treatment of T-cells of cord blood directly upregulated perforin and granulysin expression, the effect of BCG could be via activation of NK cells to produce IL-2 which, in turn, could affect CD4+ and CD8+ T-cells activation. Support for a central role of NK cells in the host response against TB infection comes from the bovine model where activated NK cells were shown to have increased granulysin and perforin expression and to lyse *Mycobacterium bovis* BCG-infected alveolar and monocyte-derived macrophages (Endsley *et al.*, 2007).

The role of IFN-γ producing CD4+ T-cells in protection against TB is well described (Dieli *et al.*, 2002; Sahiratmadja *et al.*, 2007c; Van Rie *et al.*, 2006). Support for a role

for cytotoxic CD4+ effectors in protective immunity against TB comes from studies carried out in BCG-vaccinated cattle. In the bovine model, memory CD4+ T-cells expressing elevated levels of perforin and granulysin strongly lysed BCG-infected macrophages (Endley *et al.*, 2007). Cytotoxic CD4+ T-cells have also been shown to use granulysin to kill *Cryptococcus neoformans* (Zheng *et al.*, 2007) and cytotoxic granulysin expressing CD4+ T-cells have been isolated from skin lesions of tuberculoid leprosy patients (Ochoa *et al.*, 2001).

In the present study there was no statistical difference in granulysin or perforin expression in cells obtained from neonates vaccinated at birth with either Japanese or Danish BCG, and no difference between vaccinations via the two routes (intradermal versus percutaneous) of administration. This finding is in contrast to another study using the same infants, where Japanese BCG given percutaneously induced significantly more BCG-specific IFN-γ producing CD4+ and CD8+ T-cells and greater Th1-specific immunity, than Japanese BCG given intradermally or Danish BCG given by the same route (Davids *et al.*, 2006). In addition, Japanese BCG percutaneously induced greater CD4+ and CD8+ T-cell proliferation. Taken together, the data suggest that distinct factors are involved in promoting the development of the two pathways, i.e. Th1 responsiveness, and expression of granulysin and perforin by CTL.

In support of this dicotomy, it has been shown that BCG-infected immature DCs selectively expanded perforin-positive CD8+ T-cells with little contribution from cytokines, including IFN-γ, TNF-α, or IL-12 (Tsunetsugu-Yokota *et al.*, 2002). Furthermore, IL-15 promotes granulysin expression (Endsley *et al.*, 2006; Ma *et al.*, 2002) while IL-21 enhances lytic activity of cytotoxic T-cells and NK cells (Leonard and Spolski, 2005). These findings may have implications for immunotherapeutic boosting of cytotoxic CD8+ activity in tuberculosis and BCG vaccination protocols. In addition to therapeutic strategies aimed at inhibition of IL-4 and TGF-β (Rook *et al.*, 2007), molecular engineering of BCG to incorporate IL-15 or IL-21 could result in enhanced cytotoxic activity of both NK cells and $CD8^+$ cytotoxic T-cells. Several new TB vaccine candidates are undergoing clinical trials at present (Kaufmann, 2006). While no currently available immunology test can predict vaccine efficacy, most studies measure type 1 cytokine production, especially IFN-γ, as a measure of

protection in response to vaccination. However, BCG readily elicits a type 1 cytokine response (Marchant *et al.*, 1999) and it has been proposed that additional factors may be important for vaccine efficacy, including IL-4 and cytokine balance (Rook *et al.*, 2004), and induction of cytotoxic activity and memory T-cells (Whelan *et al.*, 2008). Further research is needed to establish if BCG-induced cytolytic mediator expression is sustained in memory CTL, as it has been postulated that protection due to BCG vaccination wanes with time (Sterne *et al.*, 1998).

CHAPTER 4

EXPRESSION OF GRANULYSIN AND PERFORIN IN PATIENTS WITH PULMONARY TUBERCULOSIS (PTB) AND THE EFFECT OF STANDARD THERAPY, WITH AND WITHOUT IFN-γ, ON CYTOLYTIC MEDIATOR EXPRESSION.

4.1 INTRODUCTION

4.2 MATERIAL AND METHODS

4.2.1 Human subjects and study design.

4.2.2 Kinetics of granulysin and perforin expression in PBMC from patients with active PTB

4.2.3 Stimulation of PBMC

4.2.4 Stimulation of PBMC with a TB-antigen specific marker in active and treated PTB

4.2.5 Ex vivo, and post in vitro stimulation, activation status of PBMC in treated PTB

4.2.6 Cytometric Bead Array analyses of Th1 and Th2 cytokines in active and treated PTB

4.2.7 Statistical analysis

4.3 RESULTS

4.3.1 Detection of granulysin and perforin expressing cells in PBMC from patients with PTB

4.3.2 Comparison of granulysin and perforin expressing cells in response to PPD stimulation in ND and in patients with active PTB or treated PTB

4.3.3 Comparison of granulysin and perforin expressing cells in response to PPD stimulation in PBMC from ND and from patients treated with DOTS or DOTS and rIFN-γ

4.3.4 Comparison of granulysin and perforin expressing cells in active PTB and in patients after 16 weeks of anti-TB therapy with or without IFN-γ

4.3.5 CD25 expression on lymphocytes from active and treated PTB patients

4.3.6 Analysis of ex vivo granulysin, perforin and CD25 expression in PBMC from patients with active and treated PTB

4.3.7 CD8+ T-cells respond to PPD by an indirect mechanism involving IL-15. CD4+ T-cells require IL-15 for granulysin expression

4.3.8 PPD stimulation induces more granulysin and perforin expressing T-cells in PTB and treated PTB than Ag85 stimulation

4.3.9 Cytokine profiles in active and treated PTB

4.3.10 TB/HIV co-infection results in a low granulysin and perforin expression in CD4+ T-cells

4.4 DISCUSSION

4.1 INTRODUCTION

An intact IFN-γ pathway is essential for the control of mycobacteria in murine models of infection (Flynn *et al.*, 1993; Orme, 2003). The development of TB toward latency or infection is governed in part by cytokine signaling as cytokines are responsible for the expansion and differentiation of antigen-specific T-cells (Ottenhoff *et al.*, 1997). Humans with mutations in IL-12 or IFN-γ receptors are susceptible to mycobacterial infections (Altare *et al.*, 1998; de Jong *et al.*, 1998) while active TB is associated with reduced *in-vitro* levels of IFN-γ production (Hirsch *et al.*, 1999; Sahiratmadja *et al.*, 2007b). M.tb replicates within the host macrophages thus, effective immunity to intracellular bacterial infection not only requires the lysis of infected cells but also the killing of the invading pathogen. Activated macrophages are essential to limit the infection (Boom *et al.*, 2003) and this requires the presence of IFN-γ. However, *M.tb* may evade nitric oxide (NO) and reactive oxygen species (ROS) and continue to replicate within host macrophages; thus effective immunity against intracellular mycobacterial infection not only requires the lysis of infected cells by CTL, but also the killing of the invading pathogen.

It has been proposed that aerosol IFN-γ is a potent immunomodulator in the treatment of PTB (pulmonary tuberculosis) and may be used as an adjuvant to enhance local immune responses (Condos *et al.*, 2004). Aerosolized IFN-γ in conjunction with anti-TB therapy significantly enhanced IFN-γ levels in the lower respiratory tract which may be important in the treatment of chronic disease (Condos *et al.*, 2004). Alveolar macrophages treated *in vitro* with recombinant IFN-γ (rIFN-γ) showed upregulation of STAT-1 which binds to regulatory regions of genes containing IFN-γ activation sites. This binding activates the transcription factor interferon regulatory factor 1 (IRF-1) which can activate a large number of genes (Condos *et al.*, 2003). Patients with multi-drug resistant TB (MDR-TB) who received 500μg of rIFN via nebulizer three times a week for one month, had negative sputum for acid-fast bacilli (AFB) and reduced cavitary lesions, which persisted two months after the cessation of treatment (Condos *et al.*, 1997). A trial of long-term therapy (6 months) with aerosolized IFN-γ in patients with MDR-TB in Korea concurs with the previous study that aerosolised IFN-γ was effective in some patients with refractory MDR-TB who were not

responding to conventional therapy (Koh *et al.*, 2004). In addition, Giosue *et al* (2000) reported that aerosolised IFN-alpha may be a promising as an adjuvant for MDR-TB.

IFN-γ leads to differentiation of both natural killer (NK) and CD8+ cytotoxic T-cells (Trinchieri *et al.*, 1996). CTL lyse infected target cells via a granule dependent mechanism and are key players in the hosts defence against TB. Both CD4+ and CD8+ effector T-cells contain the cytolytic mediators including granzymes, granulysin and perforin. Several reports have implicated granulysin and perforin as effector molecules directly contributing to host protection against TB (Dieli *et al.*, 2002; Sahiratmadja *et al.*, 2007a; Stegelmann *et al.*, 2005; Stenger *et al.*, 1998). Recombinant granulysin killed more than 90% of extracellular M.tb and together with perforin was able to kill intracellular organisms (Stenger *et al.*, 1998). Synthetic peptides derived from granulysin inhibited *in vitro* growth of clinical isolates of multidrug resistant and drug susceptible strains of M.tb (Toro *et al.*, 2006). NK cells constitutively express these cytolytic mediators and were shown to reduce M.bovis BCG viability within *in-vitro* infected macrophages in cattle (Endsley *et al.*, 2006). The degranulation of NKT-cells resulted in granulysin depletion and the consequent abrogation of antimycobacterial activity that these cells possessed (Endsley *et al.*, 2006; Gansert *et al.*, 2003).

Compared to healthy donors, patients with active TB showed marked reduction in perforin positive CD8+ T-cells which correlated with a reduction in CTL activity (Smith *et al.*, 2000b). Adults with active TB had lower plasma granulysin than community controls but after anti-TB therapy the levels increased to that of normal healthy controls (Sahiratmadja *et al.*, 2007). Although IFN-γ production correlated with activity and severity of TB the granulysin levels inversely correlated with TB disease activity but not with severity. At a cellular level, granulysin and IFN-γ expression correlated inversely with disease activity in TB (Sahiratmadja *et al.*, 2007). As is the case with adults, children with active TB had depressed levels of IFN-γ and granulysin, which increased after successful chemotherapy (Dieli *et al.*, 2002, Sahiratmadja *et al.*, 2007).

Taken together, these data suggested that rIFN-γ used as an adjuvant together with standard chemotherapy for treatment of pulmonary TB, may have a beneficial effect

by augmenting granulysin and/or perforin expression. The aim of experiments in this chapter, therefore was i) to evaluate the granulysin and perforin expression in T-cells and NK cells in patients with active TB at baseline and after treatment, and to compare this with healthy PPD+ controls, ii) to establish if rIFN-γ supplementation of supervised tuberculosis chemotherapy (DOTS) had an effect on the expression of these cytolytic mediators, iii) to determine if HIV or HIV/TB co-infection had an effect on granulysin and perforin expression in T-cells and NK cells and iv) to establish if the "protective" Th1 cytokine profile correlated with an increase in either or both of these effector molecules.

4.2 MATERIAL AND METHODS

4.2.1 Human subjects and study design

Human participation was approved by the University of Cape Town Research Ethics Committee and informed written consent was obtained from all participants. Patients with M.tb were enrolled in the study after the diagnosis had been confirmed by sputum smear positive for acid fast mycobacteria, culture positivity for M.tb and a lung X-ray showing evidence of bilateral pulmonary TB (PTB) with cavitation. Granulysin and perforin expression in PBMC were evaluated at baseline and four months after treatment. The study consisted of a randomized controlled trial of supervised tuberculosis chemotherapy (DOTS) with or without the addition of 200μg IFN-γ administered by either aerosol (via a nebulizer) or subcutaneous injection, three times weekly for four months. Patients who were not sensitive to first line drugs were excluded from the study.

4.2.2 Kinetics of granulysin and perforin expression in PBMC from patients with active PTB

Kinetics of granulysin and perforin expression in PBMC was evaluated in three patients with active PTB as these markers were often expressed *ex vivo* and the optimal time for expression could not be assumed to be the same as ND (Chapter 2). Granulysin and perforin expression by PBMC was performed as previously described using $1x10^6$/ml PBMCs immediately after isolating the cells and repeated on day 4 and day 7 using PBMC that had been left unstimulated or stimulated with PPD.

4.2.3 Stimulation of PBMC

PBMC from 59 patients with active pulmonary tuberculosis (PTB) and 57 patients who had received four months of anti-tuberculosis therapy were stimulated with PPD, or left unstimulated as a control, and granulysin and perforin expression was evaluated on day 7. In forty-one patients, granulysin and perforin expression was

determined on pre-treatment and post-treatment matched samples from the same patients, and paired statistical analysis was performed. IL-2 stimulation served as a positive control. IL-15 stimulation was also performed in some patients in order to ascertain if IL-15 upregulated granulysin and perforin expression in tuberculosis.

Ex vivo granulysin and perforin expression was evaluated in patients with active PTB and in patients after treatment to determine whether these cytolytic mediators were expressed without requirement for *in vitro* stimulation. The results were compared to samples stimulated by PPD *in vitro* for 7 days.

4.2.4 Stimulation of PBMC with a TB-antigen specific marker in active and treated PTB

Whenever an adequate number of PBMC were isolated to permit additional experiments, cells were stimulated with 10μg/ml Ag85 complex (A, B, and C mix purified from BCG culture filtrate; a gift from Dr Kris Huygen). Granulysin and perforin expression was evaluated in Ag85-stimulated T-cells and NK cells in ten patients with active PTB, and 18 patients post treatment, and results compared to the expression of these markers in PPD stimulated cells.

4.2.5 Ex vivo, and post in vitro stimulation, activation status of PBMC in treated PTB

In order to assess whether lymphocytes from patients who had been on anti-TB therapy were activated *in vivo,* PBMC from 24 patients were analysed directly for *ex vivo* IL-2 receptor (CD25) surface expression on T-cells and NK cells after 16 weeks of treatment. $1x10^6$/ml PBMCs were stained with anti-CD25 PE and anti-CD3 FITC. Cells stained with the isotypic controls to the relevant antibodies served as negative controls. PBMCs from the same patients were stimulated with PPD, IL-15 and IL-2 for seven days (as previously described) and CD25 expression was evaluated in stimulated and unstimulated cells within a general lymphocyte gate.

4.2.6 Cytometric Bead Array (CBA) analyses of Th1 and Th2 cytokines in active and treated PTB

PBMC were stimulated with PPD for seven days or left unstimulated as a control and the concentration of Th1 and Th2 cytokines in cultured supernatants was simultaneously quantified by CBA assay according to the manufacturers' instructions. The cytokines included IFN-γ, TNF-α, IL-2, IL-10, IL-4 and IL-5.

4.2.7 Statistical analysis

The Mann-Whitney two-tailed test for non-parametric data or Kruskal Wallis test was used for group comparison. For paired data, paired t-test was used to compare baseline and 16 week data within study arms, while the 2-sample t-test was used to compare differences between the 3 treatment arms. PBMC could not be obtained from each patient at all time points, therefore, for comparison of unpaired data the linear mixed effects model was used, which takes into account randomly missing data. P-values <0.05 were considered significant.

4.3 RESULTS

4.3.1 Detection of granulysin and perforin expressing cells in PBMC from patients with PTB

Initial experiments evaluated kinetics of granulysin expressing cells in three patients with active PTB, to determine the optimal time-point of expression, and to compare this with those cells in ND. There was a possibility that PBMC from PTB patients with active disease were activated *in vivo* and that, following PPD stimulation of patients' cells *in vitro,* hyper activation and subsequent cell death could occur. Figure 4.1 shows that after PPD stimulation, the percentage T-cells expressing granulysin and perforin was low *ex vivo* but increased with time over a 7 day period. NK cells expressing granulysin were detected *ex vivo* but reduced expression was documented over the seven day culture period. However, in contrast to PBMC from ND, granulysin was expressed *ex vivo* in T-cells of patients with active PTB (figure 4.1 A). A mean of 1% of unstimulated CD4+ T-cells from ND expressed granulysin compared to 9% of those cells from active PTB patients. This was more apparent in CD8+ T-cells where a mean of 6.7% of cells from ND had granulysin compared to a mean of 25.6% from active PTB patients. NK cells had similar constitutive expression with 62% expressing granulysin in ND and 79% of in active TB.

Ex vivo perforin expression was also seen in T-cells and NK cells in patients with active PTB. A mean of 2.7% of CD4+ and 20.3% of CD8+ T-cells expressed perforin *ex vivo*. Constitutive expression of perforin in NK cells was very high in these three patients with active PTB, with 90.3% of NK cell expressing this cytolytic mediator (figure 4.1 B).

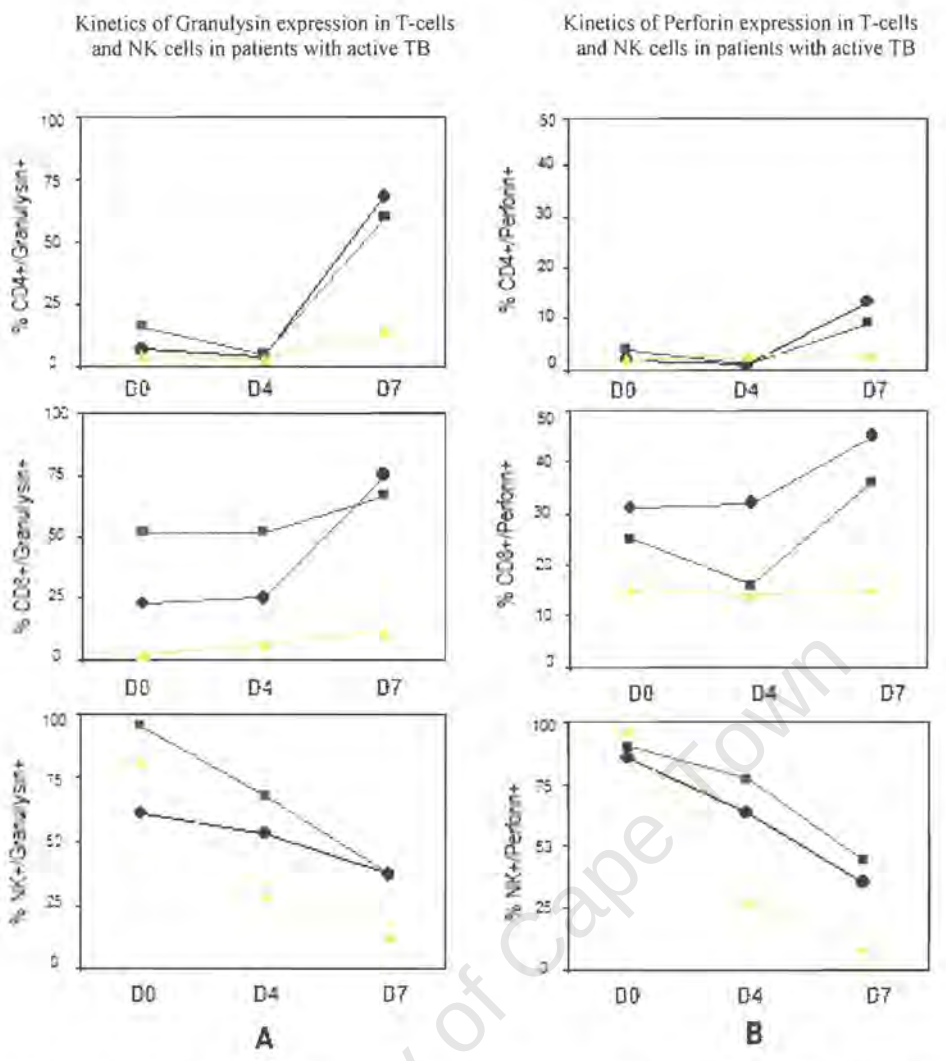


Figure 4.1: Kinetics of Granulysin (A) and Perforin (B) expression in T-cells and NK cells of three patients with active PTB. Kinetics were performed ex vivo (D0) and after stimulation with PPD for 4 days (D4) and 7 days (D7).

Due to the finding of *ex vivo* expression of granulysin and perforin in PBMC from patients with active PTB, *ex* vivo granulysin expression was determined in unstimulated PBMC from five patients with PTB, and compared with results obtained from cultured unstimulated cells on day 7. This was done to investigate whether there was spontaneous degranulation during the culture period as the expression of these markers would be determined in both unstimulated and stimulated cells after a 7 day culture period. Figure 4.2 shows that the percentage of unstimulated T-cells expressing granulysin was similar *ex vivo* and on D7 and, although more unstimulated NK cells expressed granulysin after 7 days in culture, this was not significantly different ($p=0.08$). Therefore, in subsequent experiments, granulysin and perforin

expression in unstimulated and antigen stimulated cells was evaluated on day 7 as for the previous chapters. Figure 4.3 represents a flow cytometric dotplot of *in vitro* unstimulated CD8+ T-cells expressing granulysin on day 7 and demonstrates that the percentage of granulysin-expressing CD8+ T-cells increases after 7 days of PPD stimulation. Thus, despite *ex vivo* expression of granulysin within CD8+ T-cells, day 7 was also optimal for evaluation of granulysin expressing CD8+ T-cells following PPD stimulation.

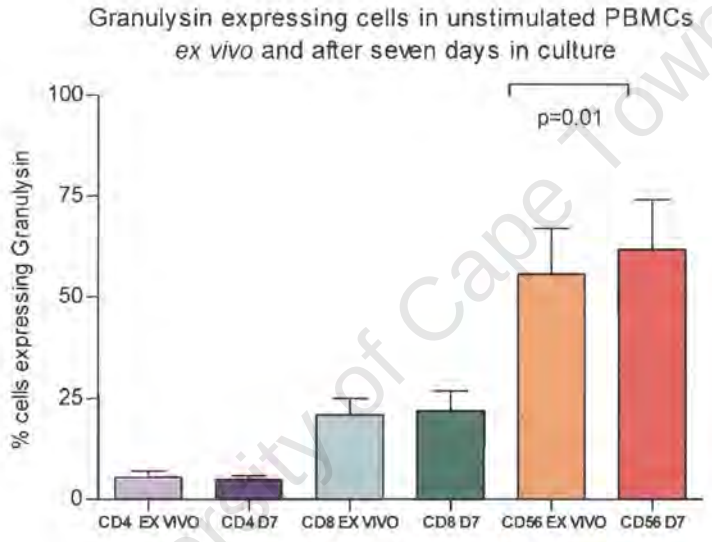


Figure 4.2: Granulysin expression in T-cells and NK cells ex vivo and in unstimulated cells in five patients with active PTB on day 7 (D7). There was no significant difference between granulysin expression in cells *ex vivo* and in unstimulated cells after a seven day culture period. The results are shown as means and error bars represent standard deviations.

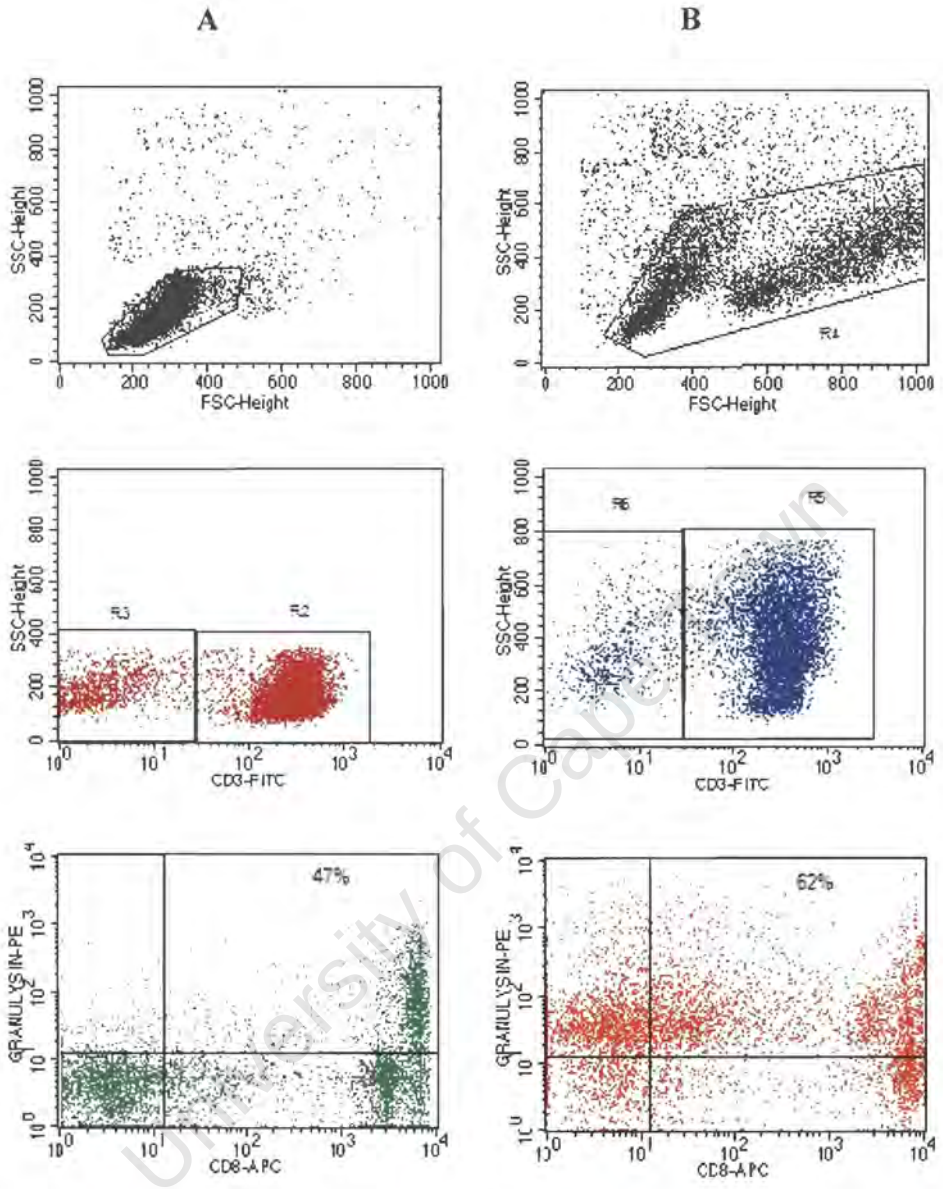


Figure 4.3: A representative flow cytometric dotplot showing that unstimulated CD8+ T-cells (A) from a patient with PTB express granulysin on day 7, and the increase in percentage of granulysin expressing cells after 7 days of PPD stimulation (B).

4.3.2 Comparison of granulysin and perforin expressing cells in response to PPD stimulation in ND and in patients with active PTB or treated PTB

Granulysin and perforin expression in PBMC was evaluated in fifty nine patients with active PTB and fifty seven patients at week 16 of anti-TB chemotherapy, and compared to ten ND (figure 4.4). There was no significant difference in granulysin expression in T-cells of ND and those that had active disease. 35.5±1.5% of CD4+ T-cells from ND and 46.2±3.6% of those from patients with active disease expressed granulysin after seven days of PPD stimulation. Similarly 39.1±4.5% and 45.3±3.1% of CD8+ T-cells from ND and infected patients respectively expressed granulysin after PPD stimulation. While there was no significant difference in granulysin expression between T-cells of ND and those that had active disease, significantly more NK cells from ND (55.6±4.4%) expressed granulysin compared to patients with active disease (41.9±42.9%) after PPD stimulation (p=0.04, figure 4.4). Constitutive granulysin expression by NK cells was similar in ND and patients with active disease (51.4±7% and 51.1±3.6% respectively). There was no significant difference between the percentage of unstimulated T-cells expressing granulysin from ND or from active PTB.

By 16 weeks of therapy all patients were smear and culture negative although the time of conversion from culture positive to negative was variable (6 weeks to 15 weeks; one patient remained culture positive after 4 months of treatment but could not be evaluated due to insufficient PBMC recovery). There was a significant difference in the percentage of granulysin expressing unstimulated CD4+ T-cells and PPD stimulated CD4+ T-cells from treated patients compared to the respective CD4+ T-cells from ND (figure 4.4). 35.5±1.5% of CD4+ T-cells from ND and 55.1±3.6% of those from treated patients expressed granulysin after seven days of PPD stimulation (p=0.02). Similarly, PPD stimulation of PBMC from these patients induced significantly more CD8+ T-cells to express granulysin than CD8+ T-cells from ND (54.7±3.2% and 39.13±4.5 respectively, p=0.04). There was a greater percentage of unstimulated CD8+ T-cells expressing granulysin in patients after anti-TB-therapy than ND but this was not significant (p=0.05). No difference in the percentage of

granulysin expressing NK cells in unstimulated or PPD stimulated cells from ND or patients treated for TB was seen.

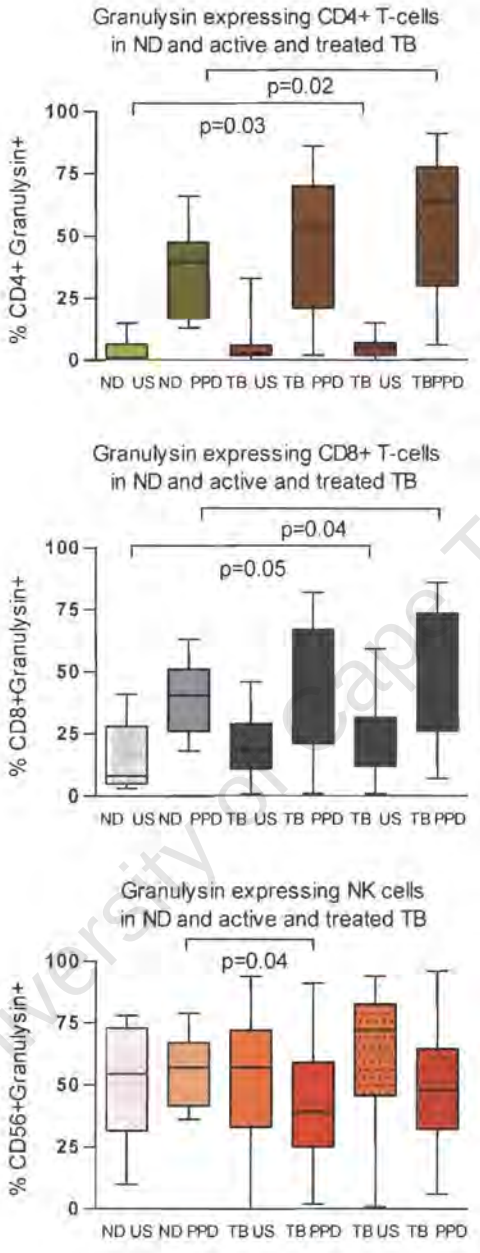


Figure 4.4: Granulysin expressing T-cells and NK cells from ND (n=10), from patients with active PTB (n=59) and from patients at week 16 of anti-TB therapy (n=57, dotted boxes). There was a significantly greater percentage of PPD stimulated T-cells and unstimulated CD4 T-cells from PBMC in treated PTB that expressed granulysin than from T-cells from ND. The percentage of PPD stimulated NK cells expressing granulysin was significantly greater in PBMC from ND than from PBMC from patients with active TB. The box extends from the 25th to 75th percentile, the line represents the median and the whiskers represent the maximum and minimum values.

In contrast to granulysin, significantly more PPD stimulated CD4+ T-cells from ND expressed perforin than these cells from active PTB (figure 4.5). 13.6±1.9% of PPD stimulated CD4+ T-cells from PBMC of ND expressed perforin compared to 6.9±0.7% of T-cells from patients with active PTB (p=0.01). Although there was a larger percentage of perforin expressing CD8+ T-cells from PBMC from ND than from active PTB this was not significant (p=0.06). No difference in the percentages of unstimulated T-cells expressing perforin was seen. The percentage of perforin expressing NK cells in unstimulated and PPD stimulated PBMC from ND was significantly greater than in patients with active PTB. There were 71±5.3% of unstimulated NK cells expressing perforin in ND compared to 49.9±3.4% of the same cells in active TB and 58±5.4% of PPD stimulated NK cells expressing perforin in ND, compared to 36.2±2.5% of the same cells in active TB (p=0.01 and p=0.02 respectively).

In contrast to active TB, there was no significant difference in the percentages of T-cells expressing perforin in PBMC from ND and patients treated with anti-TB drugs. Interestingly, the percentage of unstimulated and PPD stimulated NK cells expressing perforin in ND was significantly higher than in treated patients. There were 71±5.3% of unstimulated NK cells expressing perforin in ND compared to 54.4±3.3% of the same cells in 16 week treated TB patients, and 58±5.4% of PPD stimulated NK cells expressing perforin in ND compared to 36.1±2.5% of the same cells in patients after anti-TB therapy (p=0.03 and p=0.04 respectively).

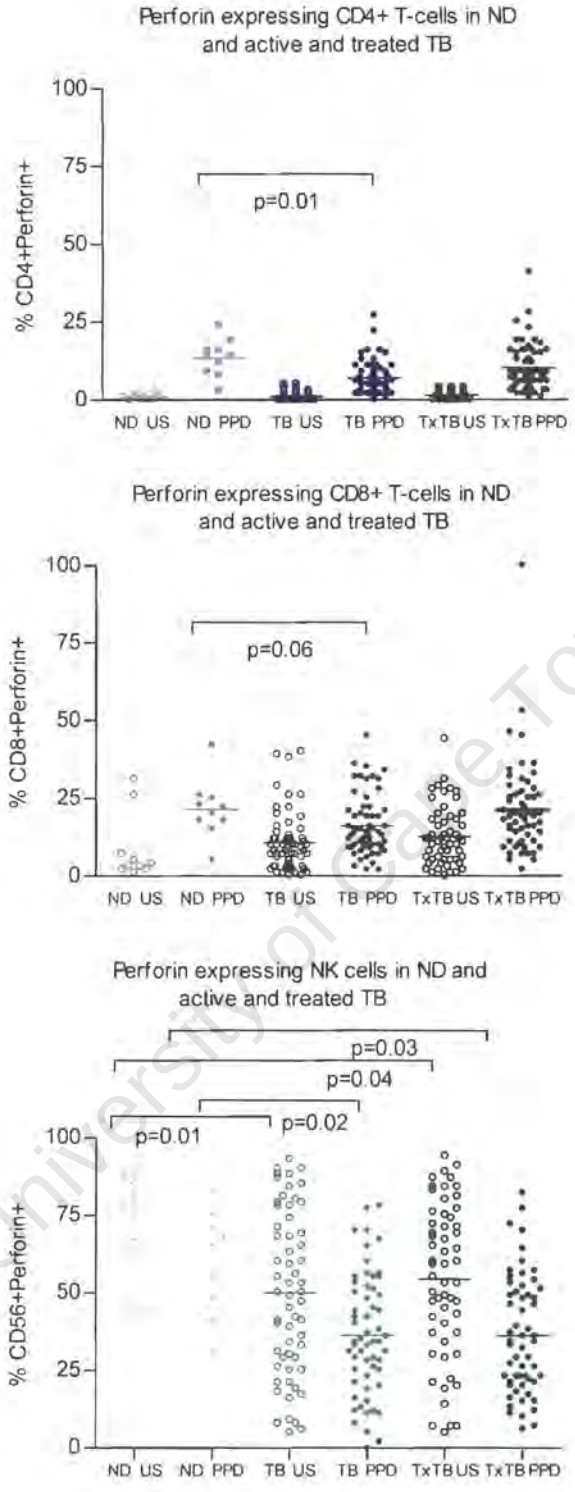


Figure 4.5: Perforin expressing T-cells and NK cells from ND (n=10), and from patients with active PTB (n=59) or after anti-TB therapy (TX TB, n=57). After PPD stimulation there were significantly more CD4+ T-cells and NK cells expressing perforin from ND than from patients with active PTB. The percentage of unstimulated NK cells expressing perforin was also significantly greater in PBMC from ND compared to active and treated PTB. The open circles represent unstimulated cells (US) and the closed cirles are PPD stimulated cells. The horizontal lines represent the medians.

4.3.3 Comparison of granulysin and perforin expressing cells in response to PPD stimulation in PBMC from ND and from patients treated with DOTS or DOTS and rIFN-γ

Granulysin expression in PBMC from ND was compared to that of patients who had received 16 weeks of anti-TB therapy according to the treatment arm of the clinical study. The treatment involved the administration of supervised tuberculosis chemotherapy alone (DOTS), or DOTS and rIFN-γ administered by aerosol (NEB) or by subcutaneous injection (SUB). Results tabulated in table 4.1 show that there was a significant difference in granulysin expressing unstimulated CD8+ T-cells from ND (14.4±4.4%) compared to unstimulated CD8+ T-cells (21.7±3.1%) from patients who had received aerosolised IFN-γ (NEB p=0.0001). The increase in granulysin expressing CD8+ T-cells was not due to TB therapy alone, since DOTS group did not have a similar statistically significant increase in unstimulated CD8+ T-cells over ND (p = 0.06). After PPD stimulation the percentage of granulysin expressing T-cells was significantly higher in patients receiving SUB than ND. 35.5±5.5% of CD4+ T-cells and 39.1±4.5% of CD8+ T-cells from ND expressed granulysin compared to 60.2±6.2% of CD4+ (p=0.01) and 59.4±6.1% (p=0.03) of CD8+ T-cells from SUB treated patients. There was no significant difference in the percentage of NK cells expressing granulysin between ND and the treatment groups. Although there was a general increase of granulysin expressing T-cells from treated patients in all treatment groups compared to ND, no other significant differences between treated groups were observed.

The percentage of perforin expressing T-cells was similar in ND and the different treatment groups, but patients treated with DOTS and NEB had significantly less NK cells expressing perforin than ND after PPD stimulation (p=0.008 and p=0.001 respectively, figure 4.6).

	ND	DOTS	SUB	NEB
CD4+ US	3.5±1.5% (0-15)	5.2±0.9% (0-15) **P=0.09**	5.1±0.7% (0-11) **P=0.05**	5.8±0.95 (1-13) **P=0.07**
CD4+ PPD	35.5±5.5% (13-66)	54.5±1.9% (9-89) **P=0.06**	60.2±6.2% (6-87) ***P=0.01**	50.7±6.9% (6-91) **P=0.1**
CD8+ US	14.4±4.4% (3-41)	21.6±3 % (3-54) **P=0.06**	23.1±3% (1-43) **P=0.1**	21.7±3.1% (2-59) ***P=0.0001**
CD8+ PPD	39.1±4.5% (18-63)	51.2±5.1% (12-84) **P=0.1**	59.4±6% (7-86) ***P=0.03**	50.8±6.1% (7-85) **P=0.4**
CD56 US	51.4±7% (10-78)	65±5.6% (8-94) **P=0.09**	62.3±6.2% (8-94) **P=0.1**	62.8±5.9% (1-88) **P=0.1**
CD56 PPD	55.6±4.4% (36-79)	45.4±4.8% (6-77) **P=0.2**	56.9±5% (20-96) **P=0.8**	45.9±5.4% (7-92) **P=0.2**

Table 4.1: Percentage of granulysin expressing T-cells and NK cells in ND and patients treated with DOTS (n=21) or DOTS + rIFN-γ administered by subcutaneous injection (SUB, n=18) or DOTS + rIFN-γ administered by a nebuliser (NEB, n=18). P-values represent a comparison of granulysin expressing cells between treatments groups and ND only. Data is expressed as a mean and SE with the minimum and maximum values in brackets. Statistical analysis was done using Mann Whitney.

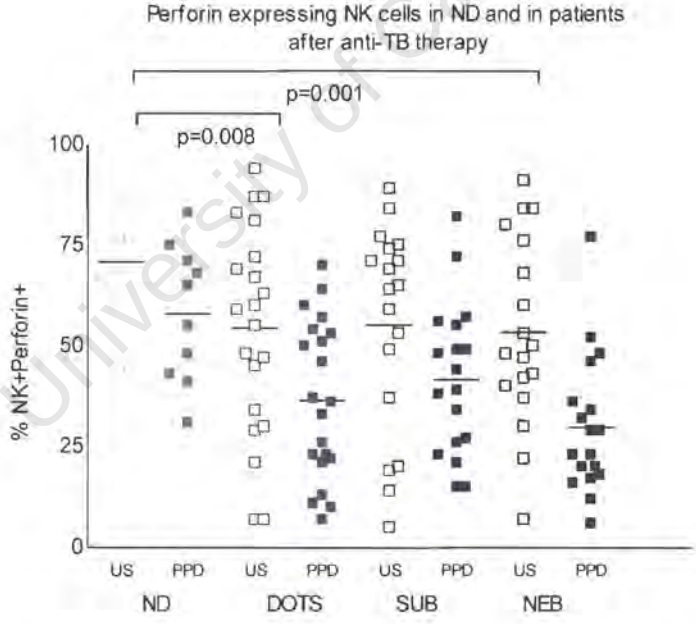


Figure 4.6: Percentage NK cells expressing perforin in PBMC from ND and patients after 16 weeks of anti-TB therapy (n=57). There is a statistical difference between the percentages of NK cells expressing perforin after PPD stimulation from ND and from NK cells from patients after 16weeks of DOTS. Statistical significance was also observed between stimulated NK cells expressing perforin from ND and in patients after NEB. The open squares represent unstimulated cells and the closed squares are PPD stimulated cells. The horizontal lines represent the medians.

4.3.4 Comparison of granulysin and perforin expressing cells in active PTB and in patients after 16 weeks of anti-TB therapy with or without IFN-γ

Granulysin and perforin expression was determined in PBMC from 59 patients with active TB and 57 patients immediately after receiving anti-TB chemotherapy for 16 weeks. Forty one of the patients who enrolled in the trial were studied at baseline (untreated) and after 16 weeks of treatment. The paired data comprised of 14 patients on DOTS alone, 11 patients received SUB and 16 patients had NEB. Granulysin and perforin expression was determined only at baseline in eighteen patients and only after chemotherapy in 16 patients due to non-compliance or insufficient cell numbers. Table 4.2 shows that there was no statistical difference in the percentage of T-cells and NK cells expressing granulysin or perforin at baseline between the different treatment groups indicating that there was no bias in the randomised drug selection.

	DOTS	SUB	NEB	P value
CD4+ US	6±1.9%	4.6±3.5%	5.5±1.3%	p=0.8
CD4+ PPD	50.6±.7%	44.3±6.1%	44.2±5.9%	p=0.8
CD8+ US	21.4±3.6%	21.2±3.2%	19.7±1.8%	p=0.9
CD8+ PPD	48.7±5.5%	41.3±5.6%	46±5.4%	p=0.7
CD56+ US	49.3±7.4%	52.9±6%	51±5.6%	p=0.9
CD56+ PPD	59.8±4.7%	38.2±5.9%	46.7±4.3%	p=0.3

A

	DOTS	SUB	NEB	P value
CD4+ US	1.5±0.4%	1±0.3%	1±0.3%	p=0.6
CD4+ PPD	6.3±1%	6.8±1.4%	11.5±4%	p=0.3
CD8+ US	10.8±1.9%	11.1±2.7%	10.4±1.9%	p=0.9
CD8+ PPD	14.8±2.5%	16.5±2.4%	16.9±1.8%	p=0.5
CD56+ US	48.1±7%	48.2±6.1%	52.6±5%	p=0.9
CD56+ PPD	36±5.3%	34.7±3.7%	37.7±4.1%	p=0.8

B

Table 4.2: Percentage of granulysin (A) and perforin (B) expressing T-cells and NK cells in active TB before the commencement of treatment (n=59). The randomized selection of treatment groups shows no skewing. The data is shown as mean percentages and SE. Statistical analysis was done using Kruskal-Wallis non parametric ANOVA.

There was no statistical difference in the percentage of granulysin or perforin expressing T-cells or NK cells at baseline or after treatment in the longitudinal study as shown in figure 4.7 and 4.8 respectively. Similarly, table 4.3 shows that there was no statistical difference between the granulysin (A) and perforin (B) expressing T-cells and NK cells in the different treatment groups either.

	DOTS (n=14)	SUB (n=11)	NEB (n=16)
CD4+ T-cells	p=0.16	p=0.31	p=0.51
CD8+ T-cells	p=0.56	p=0.31	p=0.33
NK cells	p=0.32	p=0.42	p=0.09

Table 4.3 A: Statistical significance between granulysin expressing T-cells and NK cells before and after treatment with DOTS, SUB or NEB. The paired t-test was used to compare baseline and 16 week data within study arms. No statistical differences in the percentages of T-cells and NK cells expressing granulysin were found at baseline and after 16 weeks of treatment in any of the treatment groups.

	DOTS (n=14)	SUB (n=11)	NEB (n=16)
CD4+ T-cells	p=0.18	p=0.33	p=0.84
CD8+ T-cells	p=0.10	p=0.69	p=0.75
NK cells	p=0.76	p=0.99	p=0.84

Table 4.3 B: Statistical significance between perforin expressing T-cells and NK cells before and after treatment with DOTS, SUB or NEB. The paired t-test was used to compare baseline and 16 week data within study arms. No statistical differences in the percentages of T-cells and NK cells expressing granulysin were found at baseline and after 16 weeks of treatment in any of the treatment groups.

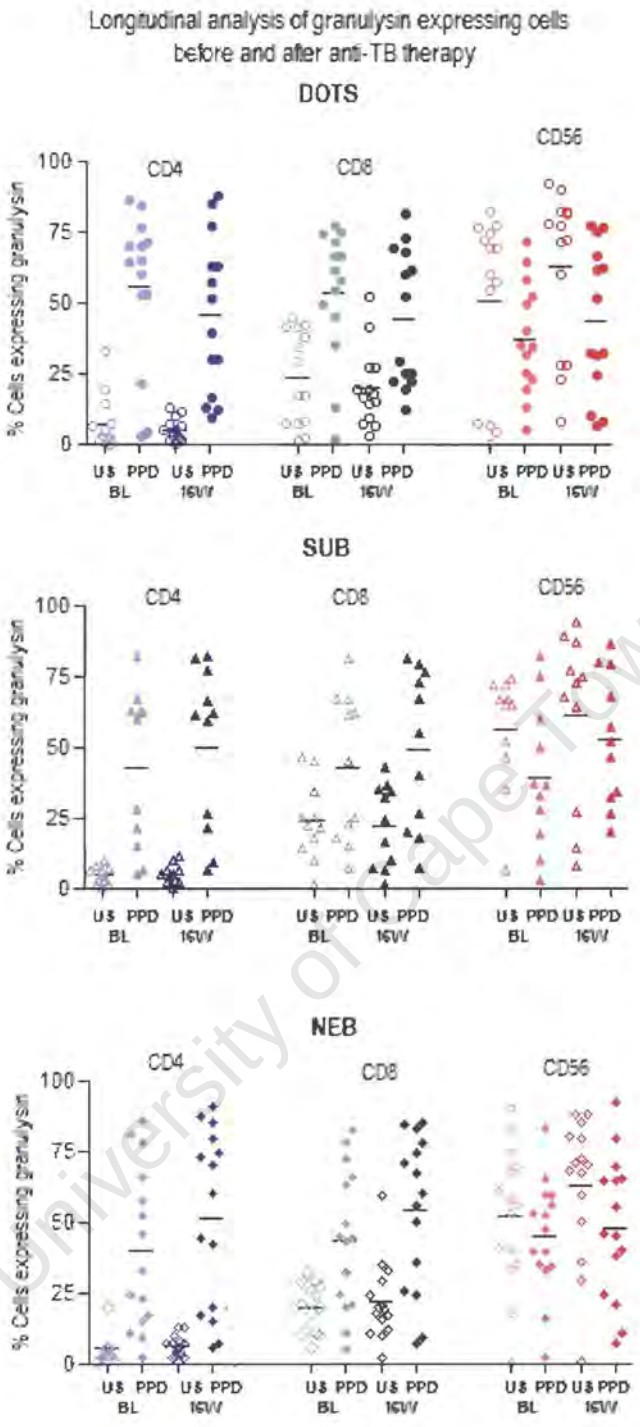


Figure 4.7: Longitudinal analysis of the percentage of T-cells and NK cells expressing granulysin in PBMC from 41 patients at baseline (BL) and after treatment (16W) with anti-TB chemotherapy. Treatment consisted of DOTS alone or DOTS and rIFN-γ administered by subcutaneous injection (SUB) or DOTS and rIFN-γ given via a nebulizer (NEB). There was no statistically significant difference in the percentages of T-cells or NK cells expressing granulysin in active PTB and after treatment. The open circles, triangles and diamonds represent unstimulated cells and the closed ones represent PPD stimulated cells. The horizontal lines represent the medians.

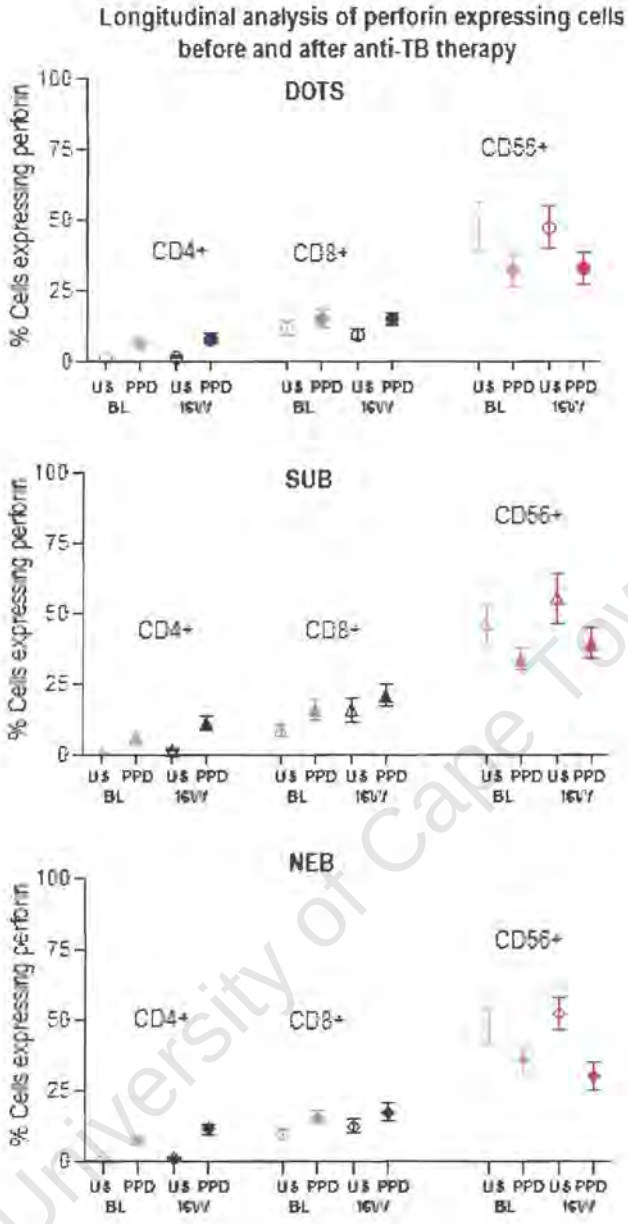


Figure 4.8: Longitudinal analysis of the percentage of T-cells and NK cells expressing perforin from PBMC from 41 patients at baseline (BL) and after treatment (16W) with anti-TB chemotherapy. Treatment consisted of DOTS alone or DOTS and rIFN-γ administered by subcutaneous injection (SUB) or DOTS and rIFN-γ given via a nebulizer (NEB). There was no statistically significant difference in the percentages of T-cells or NK cells expressing perforin in active PTB and after treatment. The open circles, triangles and diamonds represent unstimulated cells and the closed ones represent PPD stimulated cells. The horizontal lines represent the medians.

Because PBMC could not be obtained from each patient at all time points, for comparison of unpaired data the linear mixed effects model was used, which takes into account randomly missing data. P-values <0.05 were considered significant. Patients who had been randomised at baseline to receive SUB had a significantly higher percentage of CD8+ T-cells expressing granulysin after they had received treatment (p=0.04). 41.3±5.6% of CD8+ T-cells expressed granulysin at baseline after PPD stimulation compared with 59.4±6% of CD8+ T-cells after treatment with TB chemotherapy and subcutaneous rIFNγ (SUB; figure 4.9). Although not significant (p=0.08), there was a considerable increase in the percentage of CD4+ granulysin expressing T-cells after treatment in SUB group, compared to baseline. There were no significant differences from baseline to post treatment in the percentages of granulysin or perforin expressing T-cells or NK cells in the other treatment groups, or in the comparison of treatment groups amongst each other.

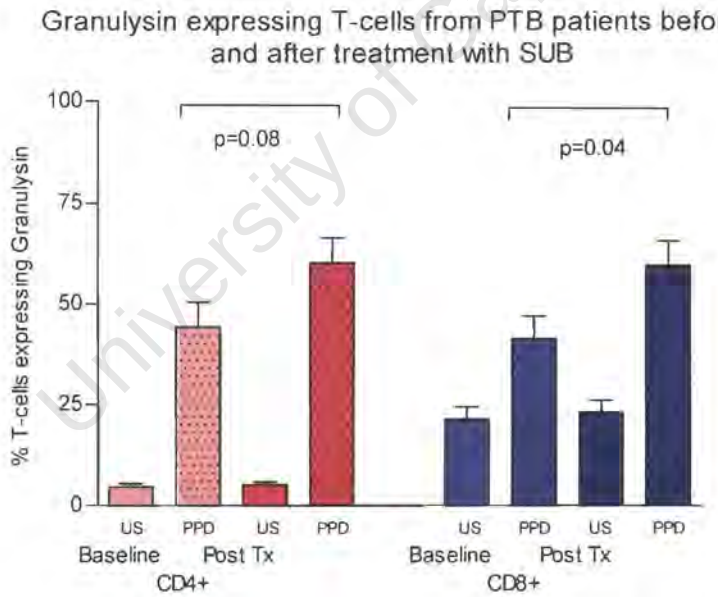


Figure 4.9: Percentage of granulysin expressing T-cells in PBMC from patients at baseline (BL) and after treatment (Post Tx) in the cohort of patients selected for SUB treatment. There was a statistical difference in the percentage of granulysin expressing CD8+ T-cells after patients had received TB chemotherapy plus subcutaneous IFN-γ (SUB n=18) compared to baseline expression (n=19) after PPD stimulation. Although not significant the percentage of CD4+ T-cells expressing granulysin after SUB treatment was markedly more compared to those at baseline. The results are shown as means and error bars represent standard deviations and the mixed effects model was used.

4.3.5 CD25 expression on lymphocytes from active and treated PTB patients

Flow cytometric characteristics of forward and side scatter in unstimulated cells was different in active and treated PTB compared to that of ND. In the absence of antigenic stimulation, two lymphocyte populations were observed, indicative of blasts, suggesting possible *in vivo* activation (figure 4.10A). The "blastic" population comprised of CD4+ and CD8+ T-cells, and NK cells. The cells were nevertheless responsive to PPD stimulation (figure 4.10 B). The expression of CD25 as a measure of cellular activation was investigated in unstimulated and PPD stimulated PBMC after 7 days in culture, in 6 patients with active PTB and in 18 patients after treatment. IL-2 and IL-15 stimulation served as positive controls. In all samples over 95% of cells were viable as determined by exclusion of Trypan blue dye after 7 days of culture. Figure 4.11 shows that there is a statistically significant difference in the percentage of cells expressing CD25 after *in vitro* stimulation compared to unstimulated cells. 18.2±2.6% of unstimulated lymphocytes expressed CD25 compared to 60.6±3.6% after PPD stimulation, 40.4±5% after IL-2 stimulation and 59.5±5.96% after IL-15 stimulation. There was no significant difference in CD25 expression on lymphocytes from patients with active PTB compared to treated PTB and no significant differences between the different treatment groups. Interestingly, CD25 expression was found in cells in both of the populations seen in figure 4.10.

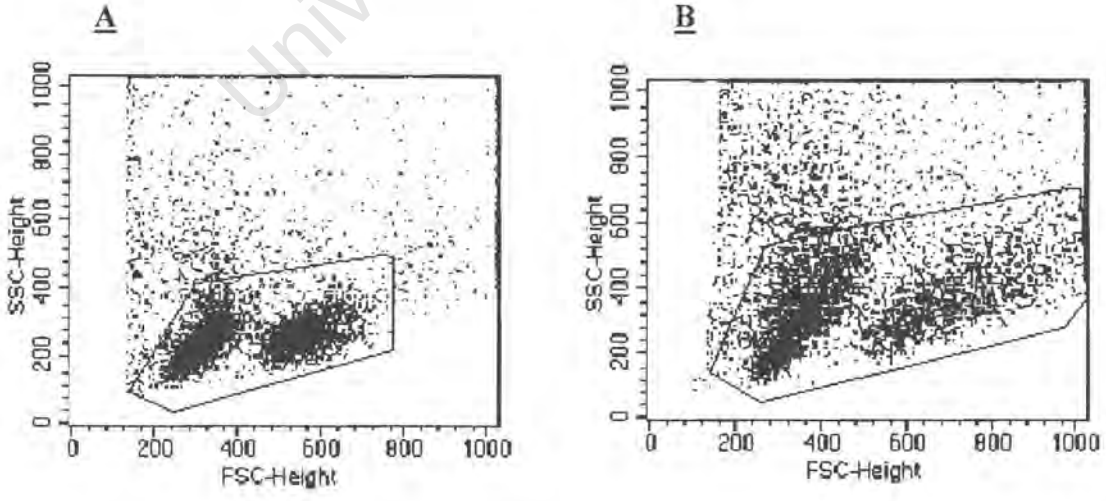


Figure 4.10: A representative flow cytometric dotplot of unstimulated cells (A) and PPD stimulated cells (B) from a patient with active PTB showing two populations of cells, which is usually indicative of stimulated cells.

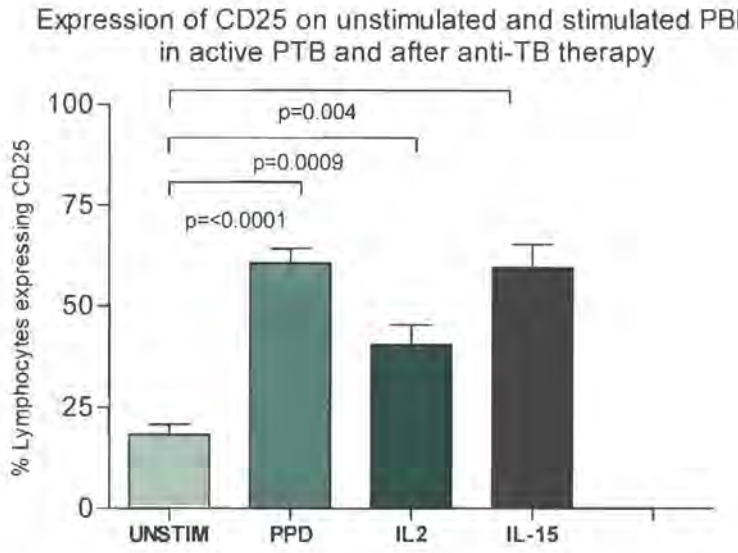


Figure 4.11: CD25 expression on lymphocytes in unstimulated and stimulated cells after 7 days in culture from patients with active PTB and from patients after anti-TB therapy (n=24). Although 18.2% of unstimulated cells expressed CD25, this percentage increased with stimulation. The results are shown as means and error bars represent standard deviations.

4.3.6 Analysis of ex vivo granulysin, perforin and CD25 expression in PBMC from patients with active and treated PTB

The preceding experiments had shown that, in the absence of a stimulus, many lymphocytes expressed CD25 after 7 days of culture. The following experiments were designed to establish whether active TB resulted in *in vivo* T-cell and NK cell activation **and** cytolytic mediator expression, and to compare with results after treatment. Figure 4.12 shows that CD4+ T-cells were activated *ex vivo* but that CD8+ T-cells and NK cells were not. In contrast, *ex vivo* CD4+ T-cells did not express granulysin or perforin but CD8+ T-cells and NK cells did. Previously it was shown that CD8+ T-cells and NK cells constitutively express granulysin and perforin and the percentage cells expressing this marker was considerably higher in active disease than ND. However, expression of the surface activation marker CD25 appeared to be inversely correlated with the expression of granulysin and perforin (figure 4.13). Thus, NK and CD8+ cells constitutively expressed granulysin and perforin without evidence of activation, whereas CD4+ were activated but did not express these

mediators. Since CD4+ CTL are known to play a key role in the immune response to TB, it is possible that they had degranulated *in-vivo* and that stimulation with PPD *in vitro* was needed to induce further cytolytic mediator expression. CD25 was expressed comparably on CD4+ T-cells from active and treated TB. There was no statistical difference between *ex vivo* CD25 expression for the three treatment groups as shown in the box insert of figure 4.12.

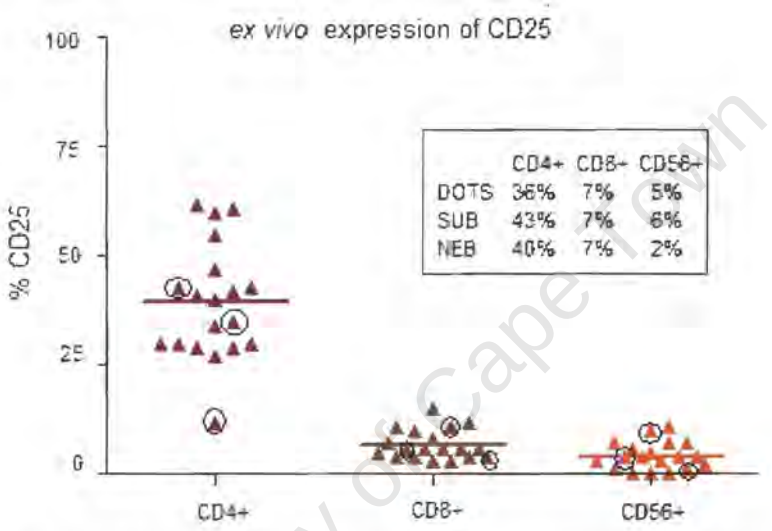


Figure 4.12: T-cells and NK cells from active PTB (n=3; encircled triangles) and treated patients (n=16) were stained with the surface marker CD25 in order to ascertain whether they were activated *ex vivo*. The horizontal lines represent the medians. The box inserted gives the mean percentage CD25 expressing T-cells and NK cells of patients who received the different treatment regimens.

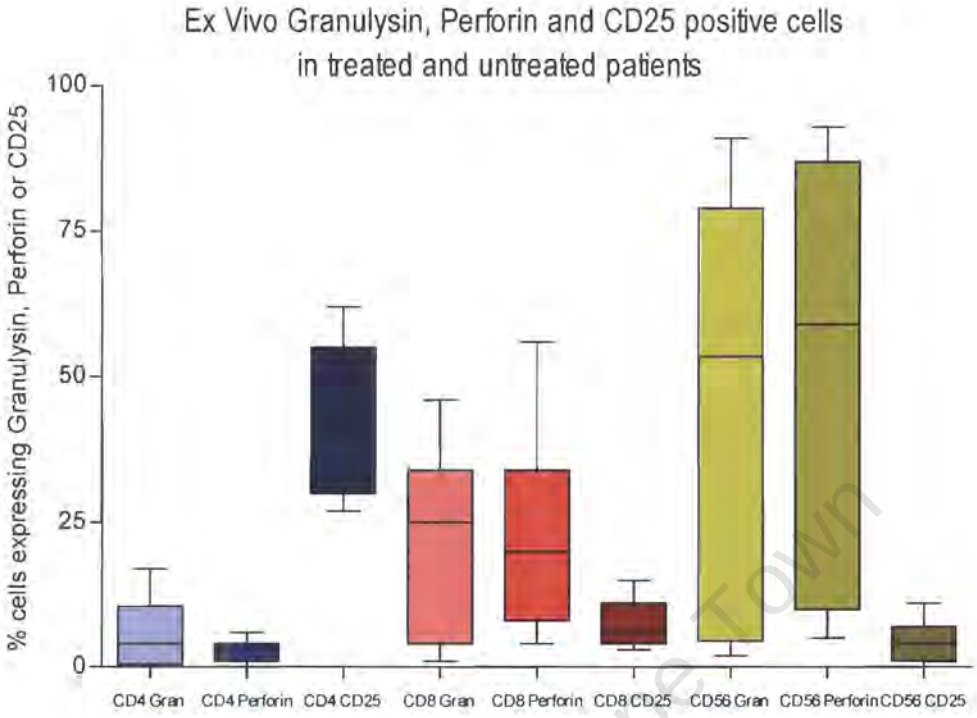


Figure 4.13: *Ex vivo* T-cell and NK cell expression of granulysin, perforin and surface CD25 showing a high percentage of granulysin and perforin expressing CD8+ T-cells and NK cells and a low percentage of CD4+ T-cells expressing these markers (n=19). In contrast, there was a high percentage of CD4+ T-cells expressing the surface marker CD25 *ex vivo* but a low percentage of CD8+ T-cells and NK cells expressing CD25. The box extends from the 25th to 75th percentile, the line represents the median and the whiskers represent the maximum and minimum values.

In fifteen of the above patients who had received anti-TB therapy, surface expression of CD25 was assessed on unstimulated and PPD stimulated CD8+ T-cells after 7 days in culture. In six of these patients CD25 and granulysin was determined within CD8+ T-cells *ex vivo* and again on day seven of culture. Figure 4.14 shows that CD8+ T-cells stimulated with PPD express CD25 and that there is a significant difference between CD25 expression *ex vivo* and on day 7 (p=0.0005). There was also a significant difference in the percentage of CD8+ T-cells expressing granulysin at the same time-points (p=0.04, figure 4.15). This data suggests that CD8+ T-cells expressing granulysin and perforin after a 7 day stimulation with PPD are indeed actived. The mechanism whereby CD8+ T-cells are activated in response to PPD stimulation was then next addressed.

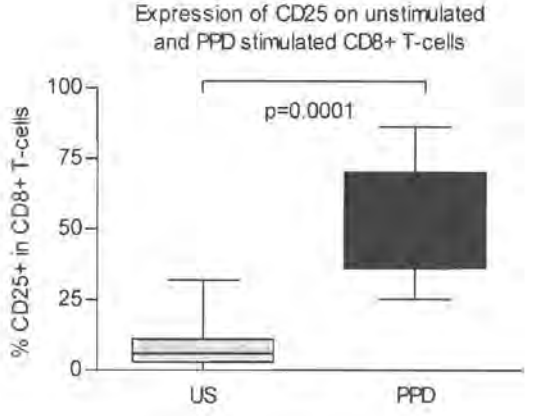


Figure 4.14: Surface CD25 expression on CD8+ T-cells *ex vivo* (US) and after 7 days of PPD stimulation. The box extends from the 25th-75th percentile, the line represents the median, and the whiskers are the maximum and minimum values (n=15)

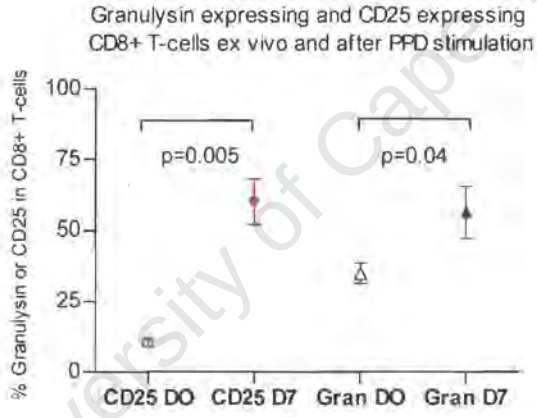


Figure 4.15: Granulysin and CD25 expressing CD8+T-cells *ex vivo* (D0) and after 7 days of PPD stimulation (D7). The results are shown as a mean and standard error (n=6).

4.3.7 CD8+ T-cells respond to PPD by an indirect mechanism involving IL-15; CD4+ T-cells require IL-15 for granulysin expression

It is well documented that CD8+ T-cells recognise peptide antigens of 9-10 amino acids in length presented by MHC class I molecules, and that CD4+ cells recognise peptide antigens 15 amino acids in length presented by MHC class II molecules (Stenger, 2001). As PPD is a highly degraded preparation of secreted proteins, stimulation of CD8+ T-cells by PPD is an interesting observation and the mechanism

was consequently addressed in the following set of experiments. Figure 4.16 shows that in three ND, PPD stimulation resulted in 33±6.1% of CD4+ and 23.6±6.9% of CD8+ T-cells expressing granulysin. The addition of IL-15 to the cultures containing PPD resulted in 54±6.6 % of CD4+ and 38.6±12.1% of CD8+ T-cells expressing this marker. Anti-IL-15 added to the PPD stimulated cultures caused an 80% and 76% decrease in the percentage of CD4+ and CD8+ T-cells expressing granulysin respectively. When mouse immunoglobulin (isotype for the IL-15 and anti-IL-15) or IL-4 (irrelevant antigen) was added to the cultures containing PPD there was no difference in granulysin expression compared to PPD stimulation alone. Therefore, IL-15 was necessary for the expression of granulysin by both CD4+ and CD8+ T-cells. It is probable that PPD stimulation results in macrophage activation which acts in a paracrine way to induce granulysin expression in CD8+ T-cells, which could be a mechanism of CD8+ T-cell stimulation by PPD.

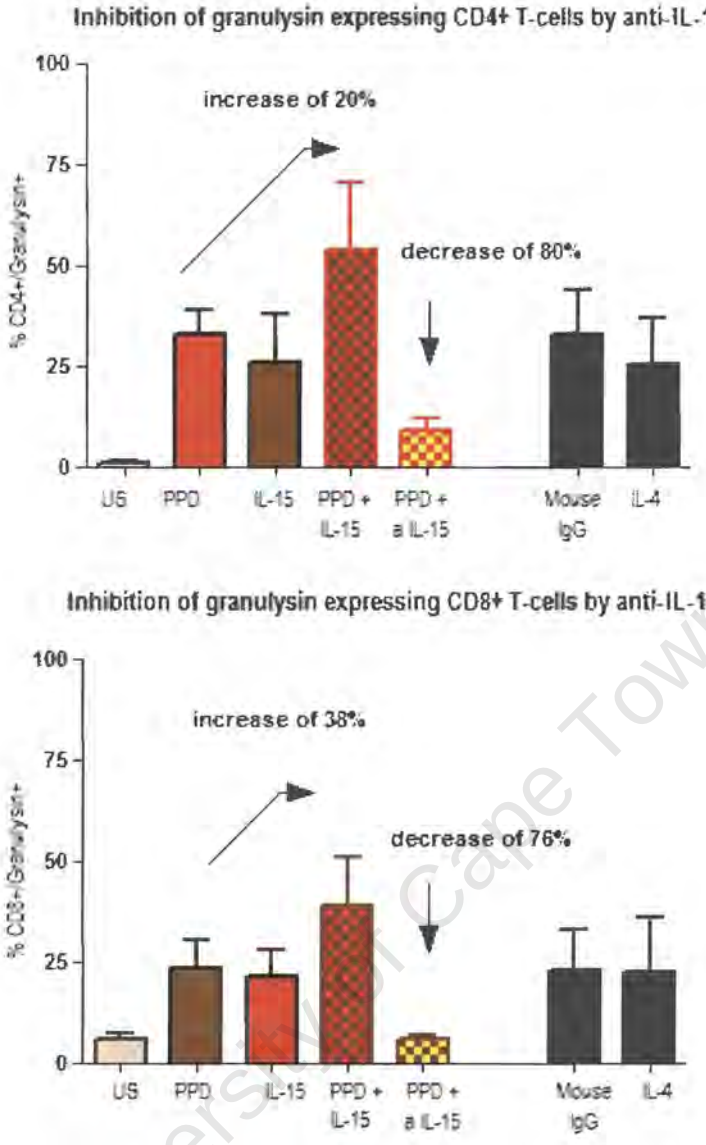
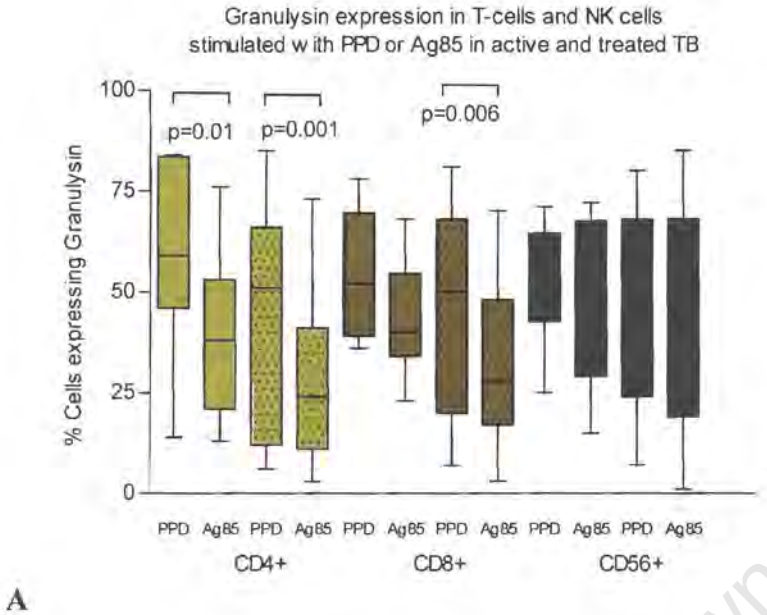


Figure 4.16: Abrogation of PPD stimulated granulysin expressing T-cells by anti IL-15. PPD + IL-15 resulted in more granulysin expressing T-cells whereas PPD + anti-IL-15 resulted in an 80% and 76% reduction of CD4+ and CD8+ granulysin expressing T-cells respectively. The results are shown as means and error bars represent standard deviations.

4.3.8 PPD stimulation induces more granulysin and perforin expressing T-cells in PTB and treated PTB than Ag85 stimulation

Ag85 complex consisting of A, B and C is a highly cross-reactive antigen found in all mycobacterial species. Ag85A is a major component of secreted proteins in the culture filtrate of M.tb (Smith *et al.*, 2000a). The following experiment was designed

to determine the ability of putative M.tb antigens involved in protective immunity and/or vaccine candidates to induce granulysin and perforin expression. Granulysin and perforin expression was evaluated in PBMC in response to Ag85 (A+B+C) stimulation from ten patients with active PTB and from 18 patients after anti-TB therapy for 16 weeks, and compared to stimulation with PPD. The percentage of CD4+ T-cells expressing granulysin and perforin in response to PPD stimulation was significantly greater than Ag85 stimulation in both active TB ($p=0.01$ and $p=0.03$ respectively) and treated TB ($p=0.001$ and $p=0.001$ respectively, figure 4.17). PPD also induced more CD8+ T-cells expressing granulysin in treated PTB than Ag85 ($p=0.006$). There was no difference in granulysin or perforin expression in NK cells stimulated with PPD or Ag85.



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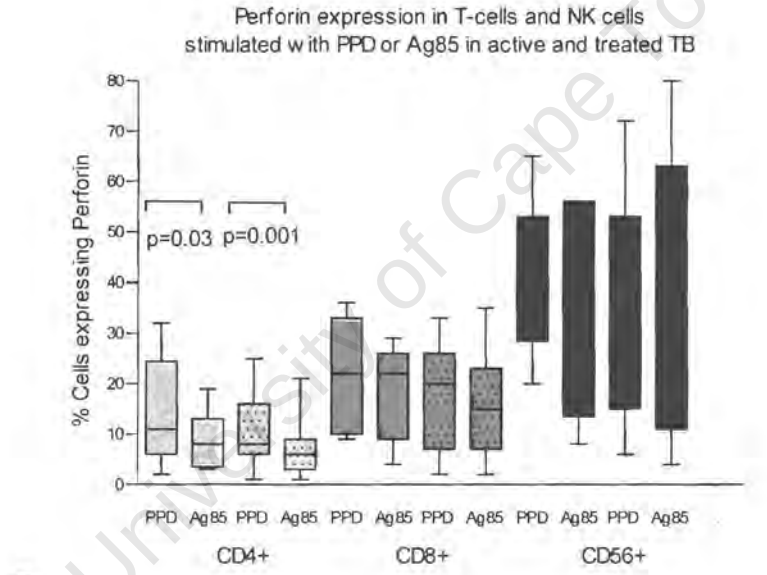

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Figure 4.17: Granulysin (A) and perforin (B) expression in PPD and Ag85 stimulated T-cells and NK cells in active TB (plain boxes) and after anti-TB therapy (dotted boxes). PPD stimulation resulted in significantly more CD4+ T-cells expressing both granulysin and perforin in active and treated TB than cells stimulated with Ag85. Although PPD induced more granulysin and perforin expressing CD8+ T-cells and NK cells than Ag85, only PPD stimulated CD8+ T-cells in treated PTB had significantly more granulysin expressing cells. The box extends from the 25th to 75th percentile, the line represents the median and the whiskers represent the maximum and minimum values.

4.3.9 Cytokine profiles in active and treated PTB

An efficient immune response to M.tb is associated with a Th1 cytokine profile (Flynn and Chan, 2001). Therefore, the following experiments addressed three questions: i) in response to PPD stimulation, what is the cytokine profile in patients before and after anti-TB treatment, ii) in response to PPD stimulation, what is the cytokine profile in rapid and slow responders to anti-TB treatment and iii) in response to PPD stimulation, is there a correlation between granulysin and perforin expression and the cytokine profile? The supernatants of unstimulated and PPD stimulated PBMC were evaluated for Th1 and Th2 cytokines using the cytokine bead array assay.

The cytokine profile of supernatants collected from 7 day PPD stimulated PBMC or unstimulated PBMC, was ascertained in the same seven patients before and after treatment. IFN-γ levels were significantly lower in active TB ($p=0.01$) but there was no statistical difference in the levels of TNF-α, IL-2, IL-10, IL-4 or IL-5 before and after treatment in these patients (Figure 4.18).

Th1 and Th2 cytokines in five patients who had converted from culture positive to culture negative after eight weeks of anti-TB drugs were compared to five patients who were culture positive after 12 weeks of treatment although, culture negative after 16 weeks. Although the numbers were small Table 4.4 demonstrates that there were no significant differences in the cytokine profiles of these two groups. As the exact level of IFN-γ was too high to report (>5000pg/ml) no Th1/Th2 ratios could be done.

	IFNγ	**TNF-α**	**IL-2**	**IL-10**	**IL-4**	**IL-5**
Culture negative	4677±322 (3067->5000)	521±233 (4-1275)	10.5±6 (0-38)	113±56 (20-382)	4±1 (0-6)	77±18 (13-143)
Culture Positive	>5000	493±257 (72-1728)	4±2 (0-11)	90±36 (18-241)	4±2 (-2-9)	164±46 (70-316)

Table 4.4: Cytokine analysis of supernatants from PBMC stimulated with PPD. Five patients from patients who has converted to culture negative after 8 weeks of treatment were compared to five patients who were still culture positive after 12 weeks of treatment. Data is shown as PPD stimulated minus unstimulated background and are expressed as pg/ml. The results are expressed as a mean with a standard error and minimum and maximum values are indicated in brackets.

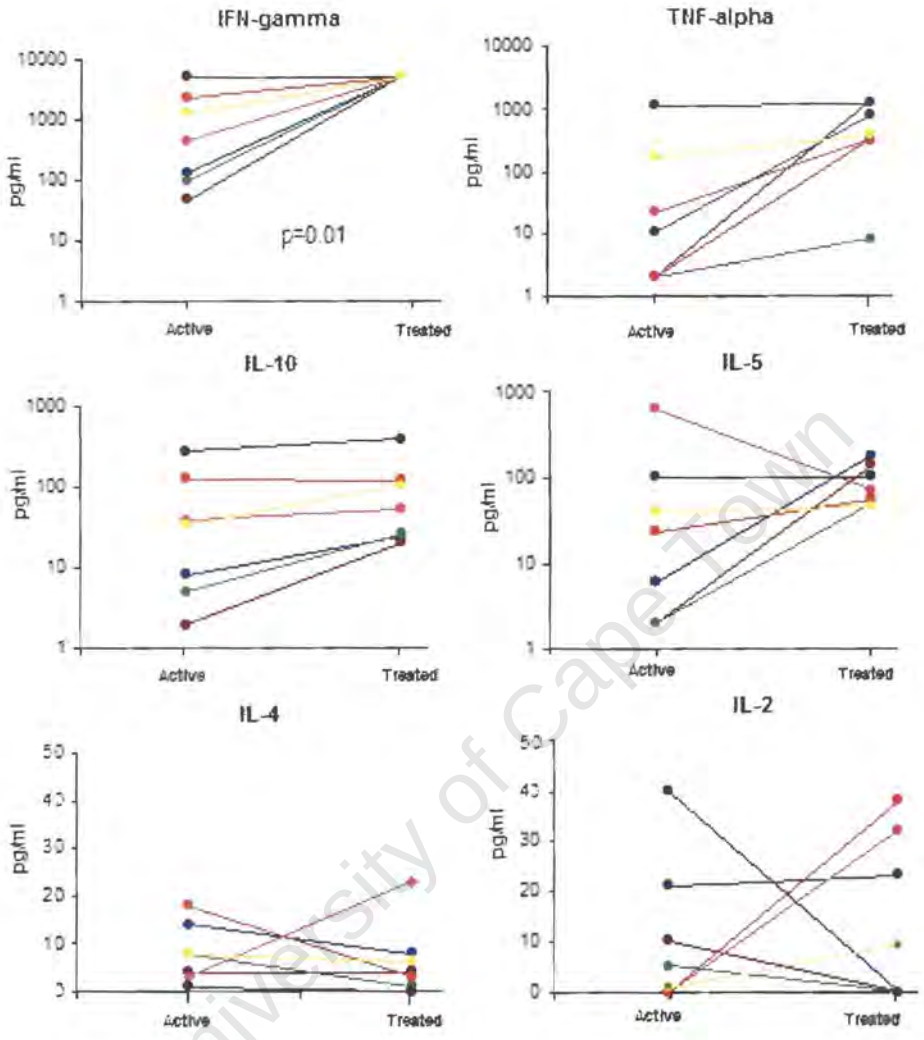
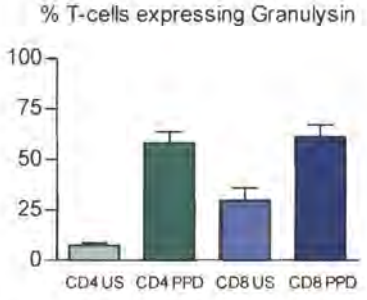


Figure 4.18: Cytokine profile in active and treated TB (n=7). Th1 (IFN-γ, TNF-α, IL-2) and Th2 (IL-10, IL-4 and IL-5) cytokines were measured in supernatants from PPD stimulated PBMC in the same patients before and after treatment. The background consisted of unstimulated cells which were subtracted from the stimulated levels. Only IFN-γ showed any significant difference before and after treatment.

The cytokine profile in PB of five patients who had a low percentage of granulysin expressing T-cells after 16 weeks of chemotherapy was evaluated to investigate if there is a correlation between low cytolytic mediator expression and a possible Th2 immune response. These values were compared to 12 patients who had a high percentage of granulysin expressing T-cells in their PB after anti-TB therapy. There was a significantly reduced level of TNFα ($p=0.009$) in the supernatants of PPD stimulated PBMC from five patients with low granulysin expression compared to 12 patients who had a high percentage of granulysin expressing T-cells (figure 4.19). There was no statistical differences between the levels of the other cytokines evaluated (Table 4.5). In Africa, TB is associated with a low Th1 and high Th2 profile (Rook *et al.*, 2005). While low levels of TNF-α may be consistent with this finding in patients with advanced TB, there was however no increase in IL-4, IL-5 or IL-10. It is also possible that high granulysin expression is accompanied by greater cytolysis and mycobacterial death, which could lead to greater stimulation of TNF-α production.

	IFN (pg/ml)	IL-2 (pg/ml)	IL-10 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)
Low granulysin	4807±193	4±2.5	70.3±31	5±1.5	88.9±26
High granulysin	4679±321	6±2.6	40±8.1	5.5±1.9	95.8±45

Table 4.5: Expression of Th1 and Th2 cytokines in supernatants of PPD stimulated cells from treated patients with high (n=12) or low (n=5) percentages of granulysin expressing T-cells. There was no significant difference in any of the above cytokines between the two groups.



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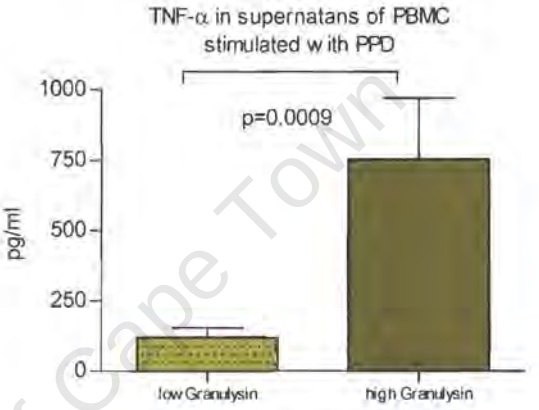

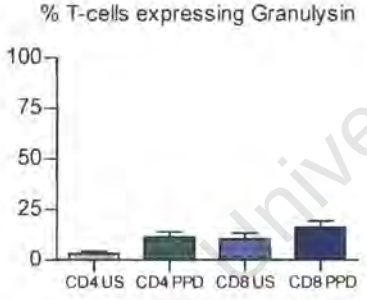

B

Figure 4.19: TNF-α levels in supernatants of PBMC from treated patients with a high (A, n=12) and low (B, n=5) percentage of granulysin expressing T-cells. There was a significant difference in TNF-α levels in patients who had a low percentage of granulysin expressing T-cells compared to those who had a high percentage of granulysin expressing T-cells. The results are shown as means and error bars represent standard deviations.

4.3.10 TB/HIV co-infection results in low granulysin and perforin expression in CD4+ T-cells

Perforin and granulysin have been reported to be low in HIV infection (Sun *et al.*, 2002; Zheng *et al.*, 2007). Granulysin and perforin was determined in T-cells and NK cells of ten patients with PTB and HIV co-infection and in another 5 HIV-infected patients who had been successfully treated for TB. No longitudinal analyses were possible as no cells were available from the five patients prior to treatment. CD4+ T-cells from patients who had HIV/TB co-infection had significantly lower granulysin and perforin expressing cells compared to co-infected patients who had been successfully treated for TB ($p=0.002$ and $p=0.008$ respectively, figure 4.20). In contrast, there was no difference in the percentage of CD8+ T-cells and NK cells expressing granulysin or perforin. This is unlike previous reports as granulysin and perforin was expressed in T-cells and NK cells in patients who were HIV positive. In addition, there was no difference in the percentage of granulysin expressing T-cells or NK cells of these patients compared to normal donors. There was a significant difference in perforin expression in PPD stimulated NK cells of normal donors compared to patients with HIV ($p=0.001$, figure 4.21).

All the patients who had been treated for TB had CD4 counts greater than 200 whilst eight of the ten patients with TB/HIV co-infection had CD4 counts lower than 35 (6-33 with a mean of 21.1). None of the patients were on retroviral treatment. Two patients with TB/HIV co-infection had CD4 counts of 713 and 846 cells/cm^3 but the CD4+ T-cells did not express granulysin or perforin, and therefore the low CD4 counts could not be responsible for the low cytolytic mediator expression. Although the proliferation was not quantified, the forward and side scatter characteristics indicated that the patients with HIV/TB co-infection did not respond to PPD stimulation as strongly as the patients that had been treated for TB. This could, therefore, account for the low granulysin and perforin found in TB/HIV co-infection. It does however appear to be TB infection that caused the low response in addition to HIV, rather than HIV itself.

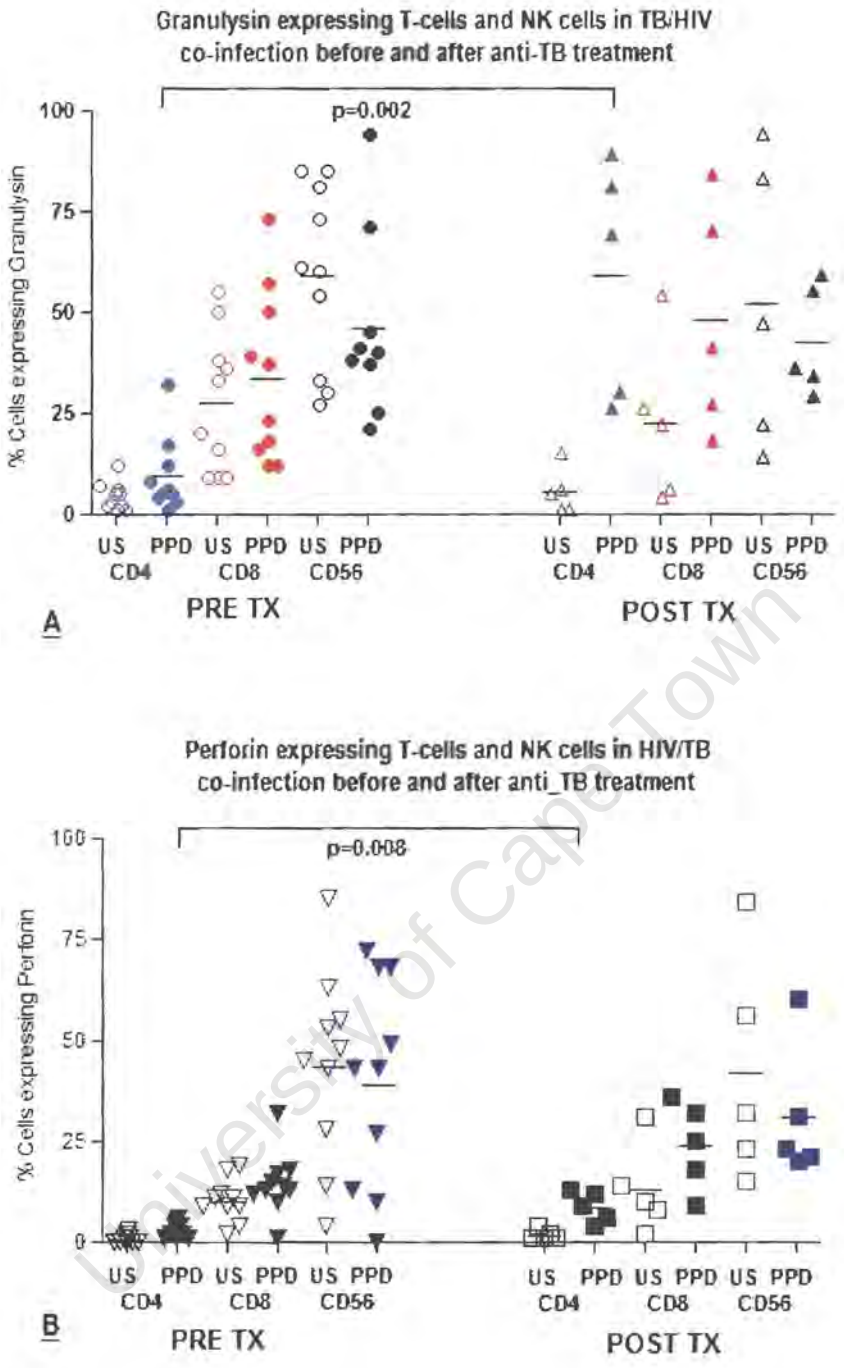

Figure 4.20: Percentage of granulysin (A) and perforin (B) expressing T-cells and NK cells from patients with HIV/TB co-infection before (Pre Tx, n=10) and after successful anti-TB therapy (Post Tx, n=5). There was a significant difference in the percentage of both granulysin and perforin expressing CD4+ T-cells from patients with HIV/TB co-infection compared to HIV only. The open circles or triangles represent unstimulated cells and the closed circles or triangles are PPD stimulated cells. The horizontal lines represent the medians.

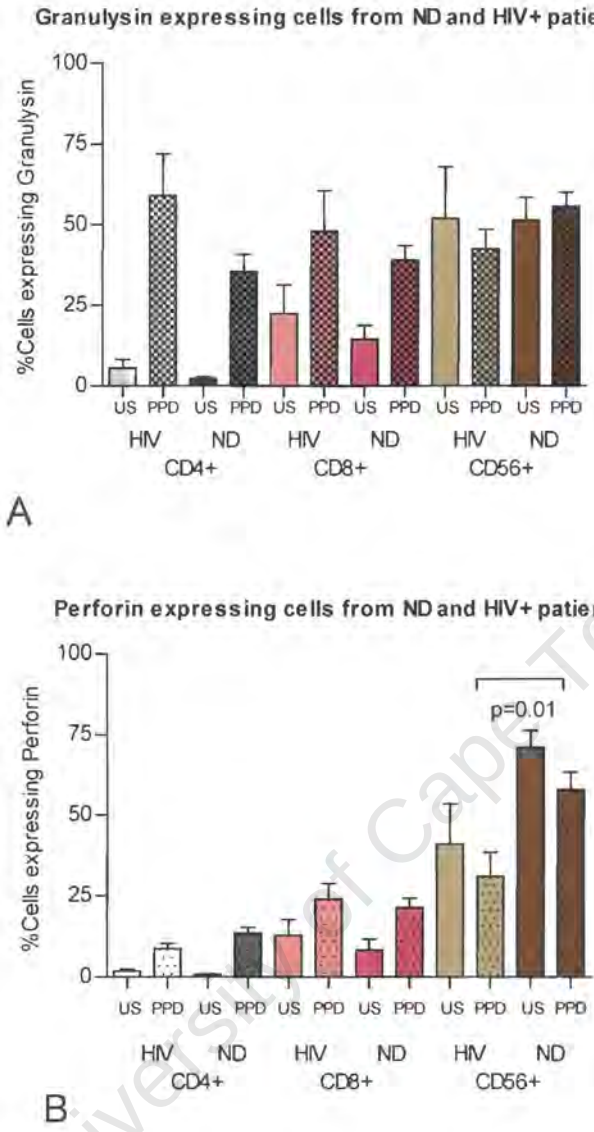

Figure 4.21: Granulysin (A) and perforin (B) positive T-cells and NK cells from patients with HIV after successful treatment for TB (n=5) and in ND (n=10). There were no significant differences in both granulysin and perforin expressing T-cells from patients with HIV compared to ND. There was a significant difference in perforin expressing PPD stimulated NK cells. The results are shown as means and error bars represent standard deviations.

4.4 DISCUSSION

Granulysin expression in T-cells and NK cells in active pulmonary tuberculosis, and the response to treatment, has not previously been described. Reduced expression of perforin has however, been shown in CD8+ T-cells in children with active TB compared to healthy controls, which recovered after chemotherapy (Caccamo *et* al., 2006). Serum or plasma granulysin levels were significantly lower in children with active TB compared to healthy controls (Liberto *et al.*, 2007) as were plasma granulysin levels in adults with active TB in a high burden tuberculous endemic country (Sahiratmadja *et al.*, 2007). After successful treatment the serum granulysin levels in children did not differ from the controls but plasma levels of granulysin in treated adults were significantly higher in the patients than the controls. Thus, granulysin is upregulated in patients with tuberculosis after successful anti-TB therapy.

The results presented in this chapter show that there was no significant difference in the percentage of PPD stimulated granulysin expressing T-cells of patients with active PTB compared to that of healthy PPD+ controls (ND). In contrast, significantly more stimulated CD4+ T-cells and especially CD8+ T-cells from ND expressed perforin than the respective subsets in patients with active PTB. After treatment the percentage of granulysin expressing T-cells was significantly higher in treated patients than controls, which would be consistant with the data of Sahiratmadja *et al* (2007a) who reported upregulation of serum granulysin. Perforin expression in T-cells in treated TB and ND was similar which is in agreement with the finding in children where treatment normalised perforin expression in CD8+ T-cells (Caccamo *et al*,. 2006). The percentage of stimulated NK cells expressing either marker was significantly reduced in active TB, and although the percentage of granulysin expressing NK cells was similar in treated TB and ND, perforin expressing NK cells remained significantly lower after treatment. HLA-DR antigens are HLA class II immune response gene products which contribute to regulation of immunity in part by facilitating the secretion of various cytokines. HLA-DR2 was reported to be associated with down regulation of perforin in NK cells in TB (Rajeswari *et al.*, 2007). The biological significance of this finding is uncertain. NK cells do not require prior activation and are therefore available for microbiocidal activity (Ma *et al.*,

2004). Interestingly, NK cells used perforin for anticryptococcal activity (Ma *et al.*, 2004) and impaired granulysin expression in NK cells in tumour patients correlated with the progression of cancer (Kishi *et al.*, 2002).

The induction of IFN-γ by M.tb is severely reduced during in PB in active TB although levels of IFN-γ do not correlate with *in vitro* growth containment of the mycobacteria (Toossi *et al.*, 2004). Aerosolised IFN-γ in conjunction with standard anti-TB drugs has been reported to be effective in MDR-TB (Condos *et al.*, 1997). Aerosol therapy with IFN-gamma in patients with pulmonary TB resulted in significant enhancement of IFN-gamma levels in the lower respiratory tract (Condos *et al.*, 2004). The data presented in this chapter showed that patients who had been randomised at baseline to receive standard anti-TB drugs and recombinant IFN-γ administered via subcutaneous injection (SUB) had significantly higher percentages of CD8+ T-cells expressing granulysin after they had received treatment, and although it did not reach statistical significance, there was an similar trend with CD4+ granulysin expressing T-cells. In addition, patients in the SUB group had significantly more granulysin expressing T-cells after PPD stimulation compared to those of ND. These results could support a role for the addition of IFN-γ in the treatment regimen of drug sensitive TB. Although the same treatment did not improve clinical, radiological or immunological parameters in MDR-TB (Park *et al.*, 2007), aerosolised IFN-γ has been well tolerated and has been proposed as a useful adjuvant in MDR-TB (Condos *et al.*, 2004).

Reports of cytokine profiles in active and treated TB are conflicting with some studies reporting higher Th1 levels in active TB (Bai *et al.*, 2004) and others proposing a Th2 dominance (Emori, 2004; Selvaraj *et al.*, 2007). In the experiments in this chapter IFN-γ levels in supernatants of stimulated PBMC were significantly lower in active PTB compared to after treatment in the same patients but there was no statistical difference in the levels of TNF-α, IL-2, IL-10, IL4 and IL-5. In addition, there was no statistical difference in cytokine levels in patients who converted to culture negative early in treatment compared to those who responded later. Cozmei *et al* (2007) found that after anti-TB therapy the levels of IFN-γ, TNF-α, IL4 and IL-5 in supernatants of stimulated PBMC from patients with moderate PTB decreased compared to the levels

before treatment while they increased in patients with severe PTB. In other studies patients with moderate and advanced TB had depressed M.tb stimulated IFN-γ in whole blood (Hirsch *et al.*, 1999), and it was recently reported that plasma IFN-γ was strongly depressed during active TB, correlated inversely with TB disease severity and increased during therapy (Sahiratmadja *et al.*, 2007a). This dichotomy proves that although the role of IFN-γ as a protective cytokine cannot be denied, the use of IFN-γ as a surrogate marker of protection is controversial. Interestingly, the data presented in this chapter shows there was significantly reduced levels of TNF-α in the supernatants of PPD stimulated PBMC from patients with a low percentage of granulysin-expressing cells as compared to those with a high percentage of granulysin-expressing cells, although there was no significant difference in *in vitro* IFN-γ levels in the same patients. This may be in agreement with a study performed on patients with PTB in Indonesia, where granulysin was expressed predominantly by IFN-γ negative T-cells but plasma granulysin levels and cellular IFN-γ correlated with curative host responses (Sahiratmadja *et al.*, 2007a).

CD4+ T-cells in active and treated TB in the patients studied in this chapter were activated *ex vivo* as shown by the expression of the surface activation marker CD25. CD8+ T-cells and NK cells did not express this marker *ex vivo* but there was strong expression of CD25 on CD8+ T-cells after seven days of PPD stimulation. In contrast, granulysin and perforin was not expressed in CD4+ T-cells *ex vivo* but there were CD8+ and NK granulysin and perforin expressing cells *ex vivo.* This confirms the finding that granulysin and perforin are constitutively expressed in CD8+ T-cells and NK cells, and activation is not a prerequisite for this expression. NK cells do not require prior activation for microbiocidal activity. In fact, granulysin and perforin expressing NK cells were reduced after PPD stimulation in contrast to the increase in the percentage of granulysin and perforin expressing CD8+ T-cells after PPD stimulation. The data showed that IL-15 was necessary for the expression of granulysin by both CD4+ and CD8+ T-cells and that anti-IL-15 abrogated PPD stimulated granulysin and perforin expression in T-cells.

Ag85 complex is found in all mycobacterial species and has been shown to induce good proliferative and cytolytic responses in BCG vaccinated and M.tb-infected

individuals (Smith *et al.*, 2000). Ag85A-epitope specific CD8+ T-cells were reduced in children with TB compared to those in healthy PPD+ children and there was a partial recovery of these cells after four months of chemotherapy (Caccamo *et al.*, 2006). In the same patients, perforin-expressing CD8+ T-cells specific for Ag85A were decreased during active disease but recovered after therapy (Caccamo *et al.*, 2006). The experiments in this chapter showed that granulysin and perforin expression was induced in T-cells in response to Ag85 and this is encouraging as this antigen is a potent inducer of CD8 T-cells (Smith *et al.*, 2000) and has been suggested as a vaccine candidate for tuberculosis. A promising vaccine for tuberculosis could be one containing granulysin and Ag85, as the delivery of a recombinant plasmid containing full-length cDNA of granulysin significantly reduced the number of viable bacilli in lung lesions in murine studies (Liu *et al.*, 2006) and a viable therapeutic vaccine containing IL-12 and the granulysin genes induced efficient protective immune responses (Yi *et al.*, 2007).

CD4+ T-cells can be classified into three subsets by the expression of receptors for two T-cell-tropic cytokines, IL-2 (CD25) and IL-7 (CD127); the CD127(-)CD25(-) subset includes mainly effector T-cells expressing perforin and IFN-gamma (Dunham *et al.*, 2008). HIV-infected patients showed a relative increase in this subset of T-cells that was related to an absolute decline of CD4+/CD127+/CD25low T-cells, but granulysin-mediated killing of *Cryptococcus neoformans* (CN) by CD4(+) T-cells was defective during HIV infection (Zheng *et al.*, 2007). Although no functional evaluation of granulysin mediated killing was performed, patients with HIV and PTB co-infection had significantly less granulysin and perforin expressing CD4+ T-cells than patients with HIV only, in the experiments described in this chapter. This was not seen in CD8+ T-cells and NK cells. There was also no difference in granulysin and perforin expression in healthy controls compared to HIV only. This is in agreement with Day *et al* (2008) who reported that polyfunctional M.tb-specific CD4+ and CD8+ T-cell responses are maintained in the PB of HIV positive individuals in the absence of active disease, and their function is not affected by the disease. It is possible that the low percentage of granulysin and perforin expressing CD4+ T-cells in TB/HIV co-infection might not result in severely defective killing in PTB as, unlike CN where the major subset of cells responsible for killing the fungus are CD4+ T-cells (Zheng *et al.*, 2007), CD8+ T-cells play a key role in the hosts

defence mechanisms in M.tb (Lalvani *et al.*, 1998; Smith *et al.*, 1999; Turner and Dockrell, 1996).

CHAPTER 5

COMPARISON OF GRANULYSIN AND PERFORIN IN PB AND PLEURAL FLUID LYMPHOCYTES; EVALUATION OF FUNCTION AND CELL SURFACE EXPRESSION

5.1 INTRODUCTION

5.2 MATERIAL AND METHODS

5.2.1 Human subjects

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5.2.3 Isolation of PBMC and Pleural Effusion lymphocytes (PEL)

5.2.4 Assessment of cytotoxic activity of PBMC and PEL

5.2.5 Effect of CTL-mediated lysis of infected macrophages on BCG viability

5.2.6 Assessment of granulysin and perforin expression in PBMC and PEL and the determination of mycobacterial survival after the addition of effector PBMC and PEL to BCG-infected macrophages.

5.2.7 Expression of granulysin and perforin on the surface of lymphocytes and the effect of surface granulysin on BCG viability

5.3 RESULTS

5.3.1 CTL from pleural fluid (PF) are more cytotoxic to BCG-infected macrophages than CTL from PB

5.3.2 Lysis of BCG-infected macrophages by effector PEL results in lower mycobacteria survival than CTL from PB

5.3.3 Granulysin expression correlates with cytoxicity of CTL and with a reduction in mycobacterial survival

5.3.4 Granulysin is expressed on the surface of PPD stimulated CD3+ and CD3- lymphocytes; perforin is not expressed on the surface of cells

5.3.5 Kinetics of surface and intracellular granulysin expression within T-cells and NK cells

5.3.6 The Golgi blocker, Colchicine, inhibits granulysin from being expressed on the surface of cells

5.3.7 Surface granulysin does not result in direct killing of mycobacteria

5.4 DISCUSSION

5.1 INTRODUCTION

Effective host immunity against intracellular pathogens requires the effector activity of antigen-specific T-cells at the site of infection (Ochoa *et al.,* 2001). The previous chapter showed that in PBMC, there was no striking difference in the percentage of PPD stimulated T-cells expressing granulysin in ND compared to patients with active PTB. Although there were significantly more perforin positive T-cells and NK cells in healthy controls after PPD stimulation, no statistical difference in expression of either cytolytic marker was seen in active TB in the longitudinal study before and after treatment.

Many studies have compared the immune response at the site of infection and in peripheral blood. Lorgat *et al* (1992) reported that both proliferation and cytotoxicity of pleural effusion CTL was augmented and accelerated compared to that of effector PBMC from the same patients. CD4+ T-cells were implicated in these differences. The accelerated proliferation kinetics of PPD stimulated lymphocytes from tuberculous pleural effusions suggested reactivation of CTL *in vivo (*Lorgat *et al.,* 1992*)*. Antigen-specific T-cells have also been demonstrated in pleural fluid (PF) and Barnes *et al* reported that the "protective" Th1 cytokines IFN-γ, TNF-α and IL-2 were expressed at greater concentrations in pleural fluid than PB, suggesting that they play an important role at the site of infection (Barnes *et al.*, 1990; Barnes *et al.*, 1993). IL-4, a known antagonist to IFN-γ, was decreased in PF in patients with TB whilst IL-12 was increased (Jalapathy *et al.*, 2004). Pleural and PB lymphocytes from patients with tuberculous pleuritis have been shown to differ in phenotype and specificity for micobacterial antigens (Wilkinson *et al.*, 2005) and unlike other forms of TB, pleuritis usually resolves without chemotherapy (Manca *et al.*, 1991). ESAT-6 specific effector memory CD4+ T-cells were highly concentrated in tuberculous pleural fluid and these cells recognised a broader repertoire of ESAT-6 derived peptides than T-cells from the PB (Wilkinson *et al.,* 2005). Mononuclear cells from PF were reported to have increased proliferative response to M.tb antigens and mitogens than PBMC in TB patients (Jalapathy *et al.,* 2004), although Sable *et al* (2005) found that most polypeptides purified from low molecular-mass secretory proteome of M.tb, were recognised by both PF and PB lymphocytes.

Granulysin expressing-T-cells at the site of infection provide a mechanism of host defence in bacterial infections. This was demonstrated in humans using leprosy as a model. Granulysin-expressing T-cells were detected in cutaneous leprosy lesions at a six-fold greater frequency in patients with localised tuberculoid form as compared with the lepromatous form of the disease (Ochoa *et al.*, 2001). Frequency of perforin expressing cells, however, was at similar levels across the spectrum of the disease. Recently, chronic TB was associated with low perforin and granulysin co-expression in CD8+ cells at the site of infection (Andersson *et al.*, 2007). Cryopreserved lung biopsies from those patients who repeatedly failed to respond to anti-TB therapy, had two to three fold lower perforin and granulysin-expressing CD3+ T-cells in TB lesions than in distal lung parenchyma and uninfected control lung samples (Andersson *et al.*, 2007). This phenomenon was observed at the protein and mRNA levels. Similarly, CD8+ cells expressing perforin and granulysin were scarce in the granulomas of TB lesions although there was an upregulation of granzyme A in the lesions in all patients. Oono *et al* (2004) indicated a role for granulysin in cutaneous defense mechanisms by demonstrating granulysin producing CD4+ T-cells in affected follicles and peri-lesional dermis in superficial microbial folliculitis, but not in sterile pustular lesions.

In the mouse model, intranasal inoculation of a vaccine containing human recombinant granulysin resulted in specific anti-PPD sIgA in bronchoalveolar fluid and systemic protective immune responses in BALB/c mice (Yi *et al.*, 2006). Liu *et al* (2006) injected a eukaryotic granulysin expression plasmid into mice infected with M.tb and found reduced numbers of viable bacteria in the lungs and spleen of these mice compared to control mice. In addition, the lungs had less alveolitis, necrosis and fewer granulomatous lesions. Taken together, these murine studies provide strong support for an *in vivo* role of granulysin in protective immunity in TB.

Several cytokines have been detected both within intracellular compartments, as well as on the cell surface. This includes monocyte surface expression of both IL-1 and TNF-α (Kriegler *et al.*, 1988; Kurt-Jones *et al.*, 1985). Indeed, it has been argued that the membrane-bound form of these two cytokines actually mediate their main biological function. Cytotoxic T-cells contain lytic granules which are membrane-bound secretory lysosomes where lysosomal-associated glycoproteins (LAMPs) such

as CD 107a (LAMP-1) and CD 107b (LAMP-2) are contained in the membranes (Peters *et al.*, 1991). Degranulation of CD8+ T-cells occurs rapidly after TCR stimulation, and once the granules reach the plasma membrane of the CTL, the membranes fuse releasing the granules into the immunological synapse resulting in the lysis of the target cells (Peters *et al.*, 1991). Following degranulation, these glycoproteins have been detected on the cell surface, and flow cytometric detection of CD8+ cell surface CD107a and CD 107b has become a standard assay for detection of degranulation, providing prima facie evidence of cytolytic activity (Betts *et al.*, 2003).

Given the precedent that granulysin has been implicated in a protective role at the site of infection, and that expression of molecules that are normally present within the cytoplasm have been demonstrated on the surface of cells, the following important questions were addressed; i) are CTL in PF more cytotoxic than in the PB in TB pleuritis? ii) are granulysin and perforin expressing cells detectable at the site of infection? iii) if so, do these markers contribute to the cytotoxic potential of effector cells in the pleural fluid? iv) is there evidence for perforin or granulysin expression on the membrane of CTL? v) if so what is the biological relevance of cell membrane expressed granulysin or perforin?

5.2 MATERIAL AND METHODS

5.2.1 Human subjects

Human participation was approved by the University of Cape Town Research Ethics Committee, and informed written consent was obtained from all participants. All patients were proven TB by culture positivity and had not commenced treatment at the time of the blood and pleural fluid collection.

5.2.2 Growth of BCG

M. bovis BCG obtained from State Vaccine Institute, Cape Town, South Africa was grown in Middlebrook 7H9 broth (Difco, Detroit, Michigan) supplemented with 10% OADC enrichment media (State Vaccine Institute, Cape Tow, South Africa) and 0.5% glycerol (Merck, Darmstadt, Germany). The cultures were grown at 37˚C in a 5% CO_2 humidified chamber, shaken daily, and growth was monitored by assessment of culture absorbance readings at 450nm. At mid-log phase 5% glycerol was added to the cultures and they were aliquoted and frozen in liquid nitrogen.

To determine the number of colony forming units (CFU), three individual aliquots were separately passed through an insulin syringe numerous times, and serial dilutions of 10^{-1} to 10^{-6} were made in 7H9 broth. 100µl of each dilution was plated out on 7H11 agar plates (Difco, Detroit, Michigan) supplemented with 10% OADC and 0.5% glycerol. The cultures were incubated at 37ºC and counted approximately 21 days later, and the CFU were determined.

5.2.3 Isolation of PBMC and pleural effusion lymphocytes (PEL)

PBMC were isolated as previously described. Pleural effusion lymphocytes (PEL) were isolated from PF collected from TB patients admitted to Groote Schuur Hospital, and presenting with a pleural effusion. The fluid was collected into 50ml tubes

containing 2.5ml of heparin (final concentration 5 IU/ml of fluid) and mononuclear cells were isolated by density gradient centrifugation using Hypaque-Ficoll as described for PBMC.

5.2.4 Assessment of cytotoxic activity of PBMC and PEL

PBMC and PEL were assessed for cytotoxic potential by determining their ability to lyse macrophages which had been exposed to mycobacterial antigens or an irrelevant antigen. Effector cells were generated from PBMC and PEL by stimulation for 6 days at 37°C with 3µl PPD or BCG at a MOI of 10:1. Simultaneously, adherent macrophages from PBMC were cultured by adding 100ul per well of 10^6/ml PBMC to round-bottomed microtitre plates, to be used as antigen presenting target cells for PBMC and PEL. After 5 days, the cells were removed from the wells and the adherent macrophages were labelled with 6µCi per well of ^{51}Cr (Sodium chromate, Amersham, Aylesbury, UK) in the presence or absence of the antigens BCG (MOI of 5:1) and PPD. (The macrophages were presumed to be at $1x10^5$/ml as monocytes comprise of approximately 10% of mononuclear cells). After the 24 hour incubation, the labelled target macrophages were washed 3x in warm PBS containing 5% foetal calf serum (FCS, Gibco) to remove any excess label. PPD or BCG effector PBMC or PEL (day 6) were added to the labelled target macrophages at effector to target (E:T) ratios of 10:1, 3:1 and 0.3:1 in triplicate wells. Spontaneous ^{51}Cr release was measured in wells containing target cells only. After 16 hours, the supernatants were harvested and placed in Durham tubes (Greiner). Maximum ^{51}Cr release was assessed by lysis of target cells with 5% triton-X (Merck, Damstadt, Germany) and lysates were collected in Durham tubes. Radioactivity was determined using a γ-counter (Tricarb, Packard) and the following formula was used to determine the percentage of kill:

> % specific lysis = [mean test cpm/(mean test cpm+mean cpm after triton-X in same wells)] x 100% - % spontaneous release.
>
> The % spontaneous release was calculated by: mean cpm in spontaneous release wells/(mean cpm in spontaneous release wells + mean cpm after triton-X treatment of same wells) x 100%.

5.2.5 Effect of CTL-mediated lysis of infected macrophages on BCG viability

Mycobacterial survival was determined using the same PBMC and PEL as the cytoxicity assay above. 100µl/well of $1x10^6$/ml PBMC were seeded in 96 well plates and incubated at 37C for 5 days so as to ensure that the monocytes had adhered and had acquired a macrophage phenotype. Non-adherent cells were removed and the macrophages were infected with BCG at a MOI of 5:1 for 24 hours. (The macrophages were presumed to be at $1x10^5$/ml as monocytes comprise of approximately 10% of mononuclear cells). For infection of adherent macrophages, aliquots were thawed and clumps disrupted by passing the bacteria through an insulin syringe several times. Mycobacteria were diluted in growth medium prior to addition to the macrophages. Macrophage infection was confirmed by ZN staining of BCG-infected macrophages grown on chamber well slides (Nunc, Denmark). After 24 hours, the cells were washed with warm PBS containing 10% FCS to remove any bacteria that did not gain access into the macrophages. Effector PBMC and PEL and media alone were added to the infected macrophages at a E:T ratio of 10:1 in triplicate wells for 16 hours. The supernatants of the wells were then removed, triplicate wells pooled, and 0.25% sodium dodecyl sulphate (SDS, Merck, Darmstadt, Germany) in PBS was added to each well to dissolve any residual macrophages. The resultant well contents were added to the original pool of well contents. The pooled lysates were diluted in 7H9 broth (neat, 10^{-1} to 10^{-6}) and 100µl was plated onto 7H11 plates as described above. Colony counts were carried out after three weeks of incubation at 37°C. In later experiments, this method was modified and lysates were plated onto Middlebrook 7H10 agar plates (Falcon, BD Microbiology Systems, Maryland, USA, as shown below) for determination of CFU by counting the colonies using an Olympus S751 microscope (Olympus Corporation, Hamburg, Germany). Cultures were set up in triplicate and plated onto a plate with 6 grids (as shown below), so that the average of 6 replicates was determined in each of the triplicate cultures. The CFU were enumerated between day 10 and day 14.

7H10 microgrid agar plate : Cultures were set up in triplicates using plates with grids as shown by the figure on the left. Each grid comprised of 6x6 squares so for each triplicate there were 6 squares each giving a total of 18 squares for each result.

5.2.6 *Assessment of granulysin and perforin expression in PBMC and PEL and, the determination of mycobacterial survival after the addition of effector PBMC and PEL to BCG-infected macrophages*

Granulysin and perforin was determined in T-cells and NK cells of PBMC and PEL from 4 patients with active TB. As PEL have been shown to respond to PPD stimulation earlier than lymphocytes from the PB (Lorgat *et.al.*, 1992) the expression of these cytolytic markers was determined *ex vivo* and after 7 days of PPD stimulation. Unstimulated cells served as a background control.

In addition, macrophages were generated from the PBMC of two of the above patients and PPD stimulated PBMC and PEL were added to BCG infected macrophages. The modified method of CFU as discussed in *material and methods* (page 122) was carried out, in order to assess if patients with a high percentage of granulysin and perforin expressing PBMC and/or PEL had a greater capacity to reduce mycobacteria survival *in vitro*.

5.2.7 *Expression of granulysin and perforin on the surface of lymphocytes and the effect of surface granulysin on BCG survival*

PBMC, isolated from six adult volunteers were stimulated with PPD for seven days or left unstimulated. The cells were surface stained for CD3 and either permeabilised or left unpermeabilised, in order to determine if granulysin and perforin was found on the surface of the cells. PBMC from a healthy PPD+ adult volunteer were isolated, stimulated with PPD for seven days then surface and intracellular granulysin expression was determined in T-cells and NK cells *ex vivo* and on day 4, 5, 6 and 7 to determine at which time-point granulysin was expressed on the surface of these cells. Unstimulated cells served as a background control. Normal rabbit serum was used as a negative control.

To exclude the possibility that the surface expression was an artefact, PBMC from two healthy PPD+ adult volunteers were stimulated for 7 days with PPD in three separate flasks. Overnight on day 6, 10μM Colchicine, a Golgi blocker which inhibits

cell surface expression, was added to one of the PPD stimulated cultures for 12 hours prior to granulysin assessment. Both surface and intracellular granulysin expression within T-cells and NK cells was determined on day 7. In the third culture flask 10μM colchicine was added daily from day 4 to day 7 to verify if colchicine was toxic to the cells if added to cultures for an extended period of time. Trypan blue exclusion dye was carried out on all cells prior to staining to ensure that the cells were viable. Determination of surface markers CD107a and CD107b (BD Biosciences) expression was performed to determine whether degranulation had taken place.

In one of the above patients, unstimulated PBMC and PPD stimulated CTL with and without 10μM colchicine were added to $3x10^4$ CFU/ml BCG at an MOI of 50:1 for 24 hours. The modified CFU counting method was performed as previously described (page 122).

5.3 RESULTS

5.3.1 CTL derived from pleural fluid are more cytotoxic to BCG-infected macrophages than CTL from peripheral blood

The cytotoxic potential of PPD-stimulated lymphocytes from PB and PF of four patients was assessed by the ability of these cells to lyse BCG infected macrophages. In two patients, the stimulated effector PEL were more cytotoxic than CTL generated from PB. The percentage of cytolysis by PPD specific effector cells at a E:T ratio of 10:1 from PEL was 25% and 49%, compared to 2% and 13% respectively of cytolysis by effector cells generated from PBMC. In the other two patients, the percentage of cell lysis by effector PEL was comparable to that obtained by effector PBMC (26% and 49% lysis by PEL and 22% and 52% CTL from the PB respectively, figure 5.1). These results suggest that in some patients PPD-specific effector cells generated by stimulation of PEL with PPD are more efficient at killing mycobacterial infected macrophages than effector cells from PPD stimulated PBMC.

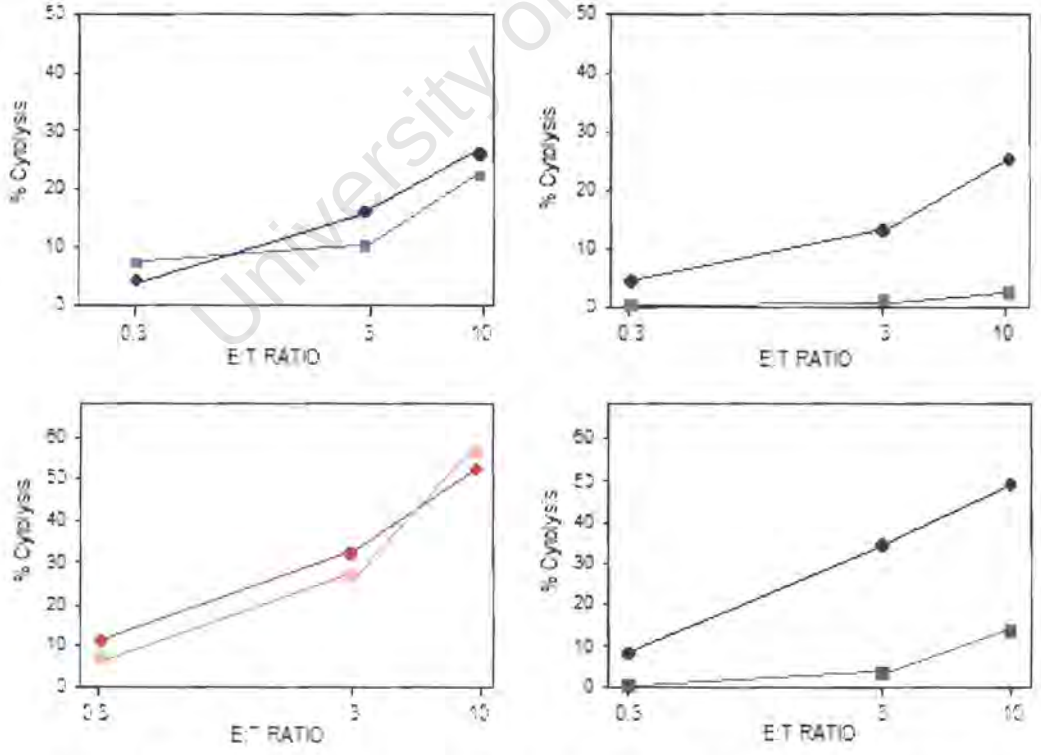

Figure 5.1: Cytolysis of BCG infected macrophages (target cells) by effector primed PBMC (squares) and PEL (circles) at an E:T ratios of 0.3:1, 3:1 and 10:1. Each set of data is from a single patient and data points indicate the percentage of cytolysis of BCG-infected macrophages.

5.3.2 Lysis of BCG-infected macrophages by effector PEL results in a lower mycobacteria survival than CTL derived from PB

In the above four patients, viable bacilli were enumerated following exposure of BCG infected macrophages to CTL derived from PPD or BCG stimulated PBMC and PEL. For the negative control the effector cells were replaced with medium alone (10% human AB serum in RPMI). Figure 5.2 indicates that for all four patients there was a significant reduction in mycobacterial survival after effector PEL were added to the infected macrophages compared to mycobacterial survival after effector PBMC were added to the infected macrophages. CFU recovery was lower in the negative control than the PB in three patients (one was omitted due to technical problem) which suggest a stimulating effect on CFU growth by PBMC. This has been shown before in a PhD thesis in our department (R. Glashoff, unpublished data). In the chromium release assay, effector cells from PEL and PBMC from two of the four patients had comparable killing potential, but in the CFU assay there was a reduction in mycobacterial survival in all four patients by effector PEL compared to effector PBMC. As granulysin has been shown to be directly cytotoxic to mycobacteria, it was possible that the CTL from the pleural fluid expressed greater levels of granulysin which directly killed mycobacteria from lysed macrophages, and this was investigated in the next experiment.

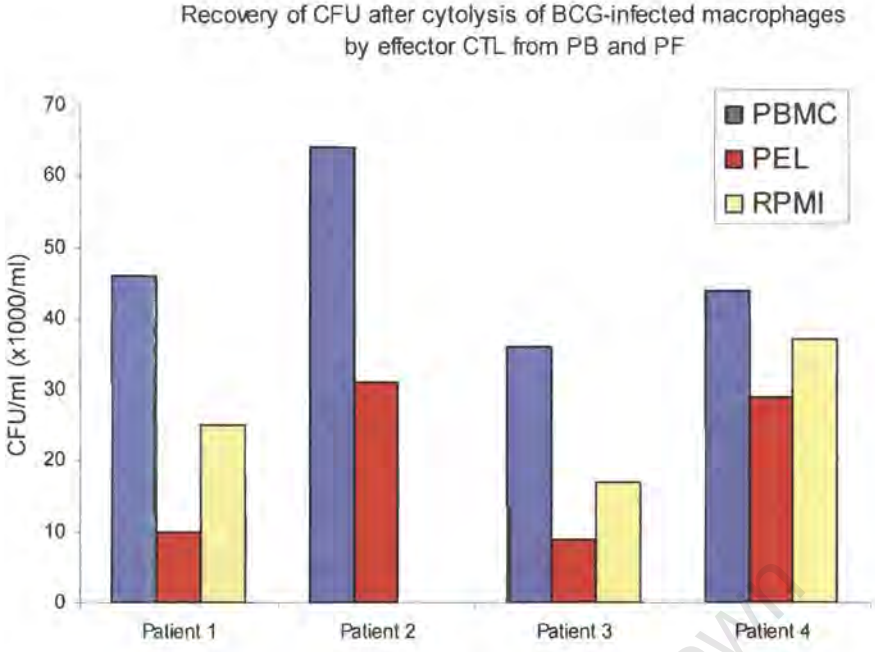

Figure 5.2: Recovery of CFU after cytolysis of infected macrophages by effector PBMC or effector PEL. Mycobacterial viability was assessed by CFU counting after a 16 hour exposure to antigen specific PBMC and PEL at an E;T of 10:1. RPMI represents no effector cells. There was a technical problem with the negative control in the experiment with cells from patient 2.

5.3.3 Granulysin expression correlates with cytotoxicity of CTL and with a reduction of mycobacterial survival

Granulysin and perforin was determined in T-cells and NK cells of PBMC and PEL from 6 patients with active TB. The PEL from two of the six patients were not viable after 2 days and had to be discarded. Table 5.1 shows that *ex vivo* granulysin expression in CD4+ T-cells from PB was slightly higher, or the same, as that of PEL in the four patients, but that more CD8+ T-cells constitutively expressed granulysin in PB (mean of 42.5±8.2%) than in PEL (mean of 27±8%). A mean of 64.5±17.6% of NK cells in PB and 74.8±10.8% of NK cells in PEL expressed granulysin.

A very different picture was seen after 7 days of PPD stimulation. The percentage of CD4+ T-cells from PBMC expressing granulysin after PPD stimulation was no different from unstimulated cells for three of the four patients studied. A similar

picture was seen for CD8+ T-cells (table 5.1). In contrast PPD stimulation resulted in a high percentage of granulysin expressing T-cells from PEL compared to unstimulated cells. Thus, there was a high number of PPD stimulated T-cells expressing granulysin from PEL in patients 1, 2 and 3 and a higher number of granulysin expressing T-cells and NK cells from PBMC and PEL for patient 4 (Table 5.1).

A similar result was found in perforin expressing cells from PBMC and PEL. There were fewer T-cells and NK cells expressing perforin in PEL than PB *ex vivo* but after 7 days of PPD stimulation more T-cells from PEL than PBMC expressed perforin in three of the four patients (Table 5.2). In patient 4 however, there were more CD4+ and CD8+ T-cells expressing perforin in PB than PEL (24% of CD4+ and 35% of CD8+ T-cells from PBMC compared to 2% and 17% respectively in PEL).

In patients 3 and 4, mycobacterial survival was determined by CFU enumeration as previously described. Figure 5.3 shows that PEL from patient 3 had a higher percentage of granulysin and perforin expressing CD4+ and CD8+ T-cells after PPD stimulation than PBMC, and that mycobacterial survival was reduced by 46%. The correlation between expression of granulysin and perforin and mycobacterial survival was also seen in patient 4 where the percentage of T-cells and NK cells expressing granulysin and perforin after PPD stimulation was similar in PBMC and PEL, and that the CFU counts were very similar (figure 5.4). This data suggests that in these two patients, there is a correlation between mycobacteria survival and granulysin and perforin expression.

	Patient 1		Patient 2		Patient 3		Patient 4	
CD4 *ex vivo* PB	18%		14%		7%		14%	
CD4 *ex vivo* PEL	3%		14%		7%		9%	
CD8 *ex vivo* PB	34%		54%		35%		67%	
CD8 *ex vivo* PEL	11%		27%		21%		49%	
CD56 *ex vivo* PB	71%		85%		13%		89%	
CD56 *ex vivo* PEL	80%		86%		43%		90%	
	Unstim	PPD	Unstim	PPD	Unstim	PPD	Unstim	PPD
CD4 Day 7 PB	8%	10%	25%	23%	6%	5%	18%	86%
CD4 Day 7 PEL	4%	45%	7%	37%	5%	73%	7%	51%
CD8 Day 7 PB	20%	42%	46%	12%	8%	8%	60%	67%
CD8 Day 7 PEL	12%	68%	21%	40%	11%	67%	27%	68%
CD56 Day 7 PB	66%	83%	78%	32%	11%	10%	79%	78%
CD56 Day 7PEL	70%	54%	80%	71%	37%	57%	63%	91%

Table 5.1: Percentage of T-cells and NK cells expressing granulysin *ex vivo*, or after PPD stimulation of PBMC or PEL from patients with active TB. Unstimulated (unstim) represents background controls.

	Patient 1		Patient 2		Patient 3		Patient 4	
CD4 *ex vivo* PB	11%		3%		6%		3%	
CD4 *ex vivo* PEL	0%		2%		1%		1%	
CD8 *ex vivo* PB	31%		33%		14%		31%	
CD8 *ex vivo* PEL	3%		8%		7%		12%	
CD56 *ex vivo* PB	83%		83%		63%		80%	
CD56 *ex vivo* PEL	19%		36%		53%		55%	
	Unstim	PPD	Unstim	PPD	Unstim	PPD	Unstim	PPD
CD4 Day 7 PB	2%	3%	4%	3%	0%	1%	1%	24%
CD4 Day 7 PEL	1%	11%	2%	36%	1%	11%	0%	2%
CD8 Day 7 PB	9%	21%	11%	4%	3%	3%	23%	35%
CD8 Day 7 PEL	1%	38%	4%	26%	4%	17%	1%	17%
CD56 Day 7 PB	93%	75%	34%	32%	6%	12%	61%	64%
CD56 Day 7PEL	15%	66%	25%	87%	16%	32%	15%	73%

Table 5.2: Percentage of T-cells and NK cells expressing perforin *ex vivo*, or after PPD stimulation of PBMC or PEL from patients with active TB. Unstimulated (unstim) represents background controls.

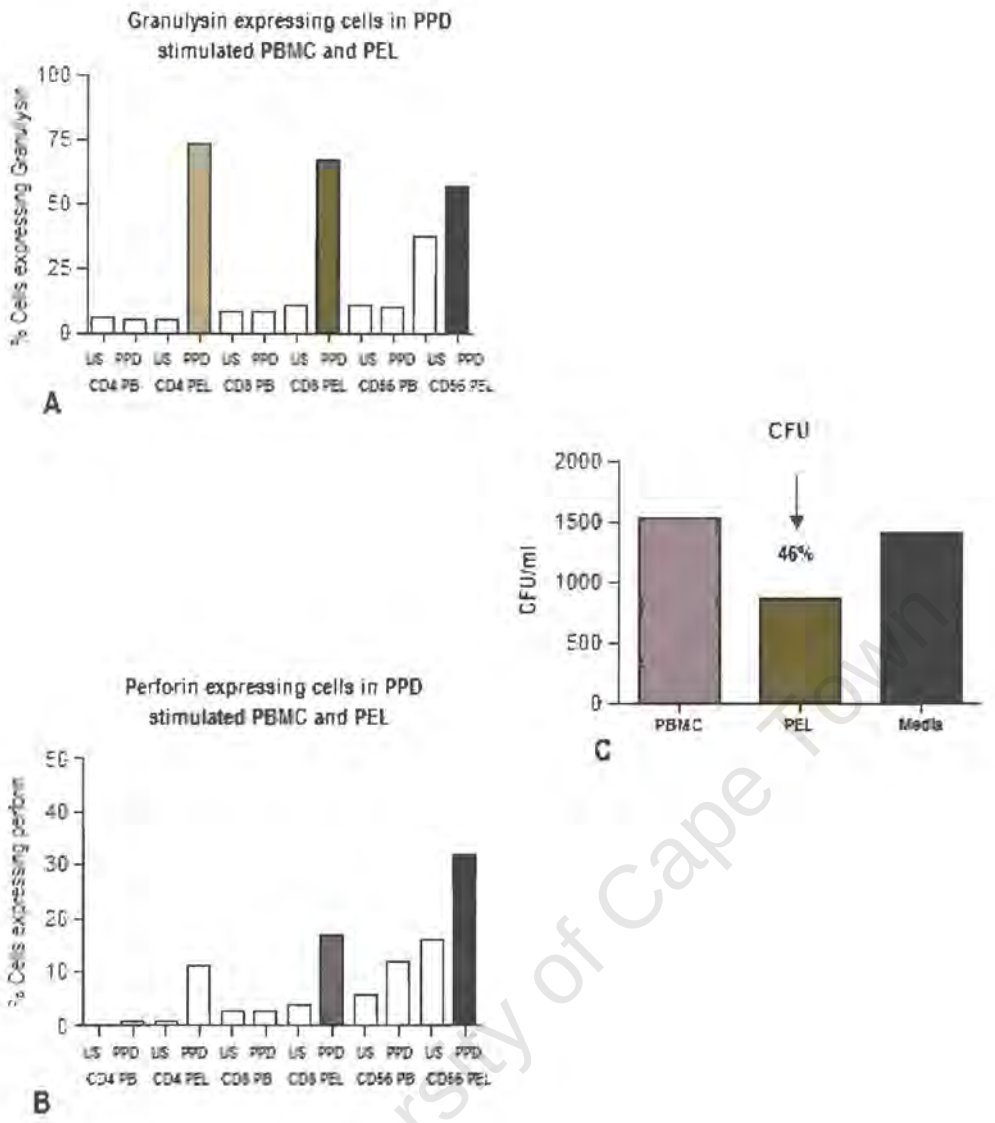

Figure 5.3: Granulysin (A) and perforin (B) expressing cells and mycobacteria survival (C) in patient 3. The percentage of PEL (colour-filled bars) cells expressing granulysin and perforin after 7 days of PPD stimulation was higher than the percentage of PBMC expressing these markers. Infected macrophages cocultured with effector CTL from pleural fluid, resulted in 46% less CFU than infected macrophages cocultured with effector PBMC (C), suggesting that effector PEL are more mycobactericidal to BCG-infected macrophages than effector CTL from PBMC. The media represents CFU obtained when infected macrophages were cocultured with medium only.

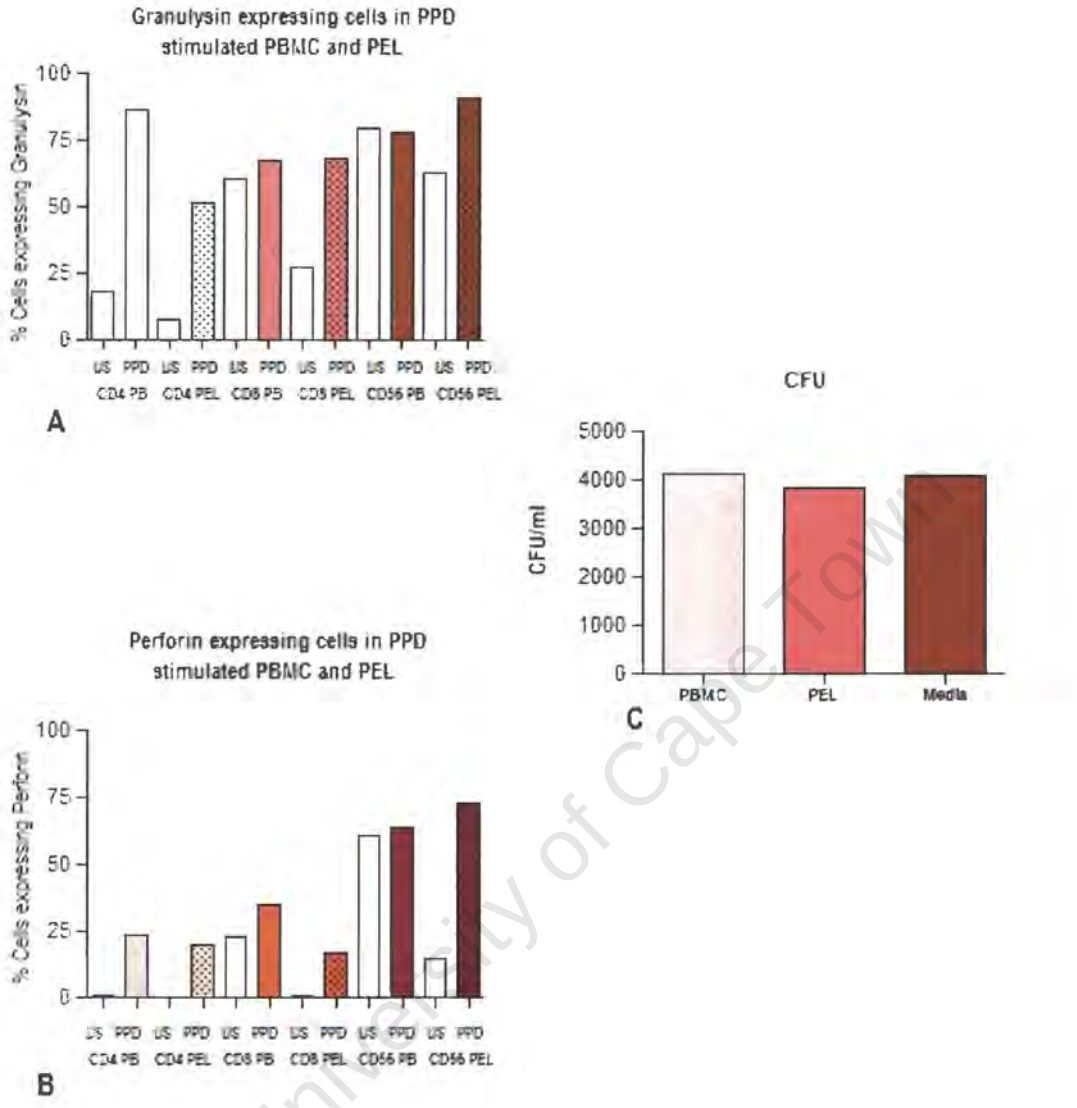

Figure 5.4: Granulysin (A) and perforin (B) expressing cells and mycobacteria survival (C) in patient 4. The percentage of PEL (dotted colour-filled bars) cells expressing granulysin and perforin after 7 days of PPD stimulation was similar to the percentage of PBMC expressing these markers. Infected macrophages cocultured with effector CTL from pleural fluid resulted in almost identical CFU as infected macrophages cocultured with effector PBMC (C), suggesting that the mycobactericidal potential of effector cells from PEL was the same as that for PBMC. The media represents CFU obtained when infected macrophages were cocultured with medium only.

5.3.4 Granulysin is expressed on the surface of PPD stimulated CD3+ and CD3- lymphocytes; perforin is not expressed on the surface of cells

To determine whether the protective effect of granulysin and perforin expression was restricted to the intracellular compartment or, if like TNF-α, a membrane form of either of these markers existed, non-permeabilised and permeabilised PBMC from 6 healthy PPD+ donors were evaluated for granulysin and perforin expression. Surface granulysin was not found on unstimulated cells but a mean of 14.6±5.9% of CD3+ cells expressed surface granulysin after 7 days of PPD stimulation. Intracellular granulysin expression was found in a mean of 5.1 ±0.7% of unstimulated $CD3^+$ cells and in 34.3±8.9% of PPD stimulated cells. There was a statistically significant difference between the percentage of CD3+ cells expressing granulysin inside the cells compared to those expressing surface granulysin ($p=0.04$, figure 5.5). Although there was no difference in the number of CD3- cells expressing granulysin after PPD stimulation in non-permeabilised and permeabilised cells there was a significant difference in the percentage of unstimulated CD3- cells expressing granulysin on the surface of cells compared to intracellular expression (4±1.7 compared to 34.6±8.2 respectively, $p=0.03$, figure 5.5). This is the first report of granulysin being present on the surface of cells.

In contrast, perforin was not expressed on the surface of cells. Table 5.3 shows in three adult volunteers no surface perforin was found on unstimulated or PPD stimulated CD3+ cells while a mean of 4±0.5% of unstimulated and 14±4% of PPD stimulated CD3+ cells had detectable intracellular perforin. Similarly, no perforin was found on the surface of CD3- lymphocytes whilst a mean of 34±7% of CD3- cells had constitutive perforin expression which did not increase significantly after PPD stimulation (39±5%).

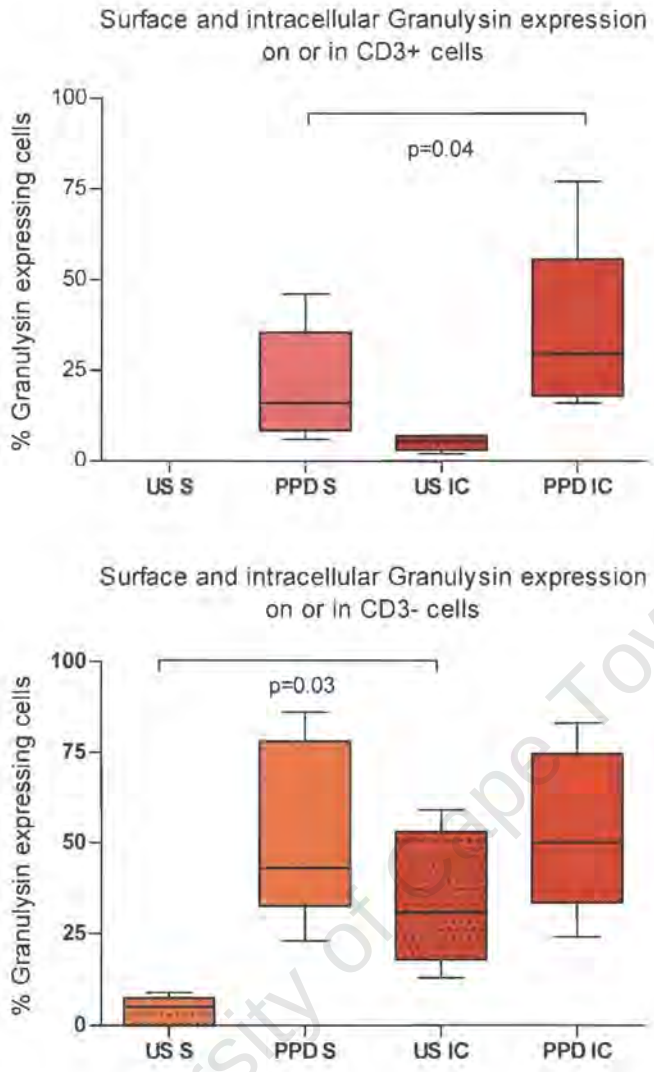

Figure 5.5: Intracellular (IC) and surface (S) granulysin expression in CD3+ and CD3- lymphocytes (n=6). Significantly more T-cells expressed intracellular granulysin compared to surface granulysin (p=0.04, A) whilst constitutive intracellular granulysin was found in significantly more NK cells than surface expression (p=0.03, B). The box extends from the 25th to 75th percentile, the line represents the median and the whiskers represent the maximum and minimum values.

	Surface expression	**Intracellular expression**
CD3+/Unstimulated	0%	4±0.5%
CD3+/PPD stimulated	0%	14±4%
CD3-/Unstimulated	0%	34±7%
CD3-/PPD stimulated	0%	39±5%

Table 5.3 Perforin expression in CD3+ and CD3- lymphocytes of three adult volunteers. No surface expression was found, but intracellular perforin is expressed on PPD stimulated lymphocytes. Constitutive expression is found mainly on CD3- lymphocytes. The mean and SD is shown.

5.3.5 Kinetics of surface and intracellular granulysin expression within T-cells and NK cells

In order to establish at what time-point granulysin could be detected on the surface of cells, non-permeabilised and permeabilised PBMC from one healthy PPD+ donor were evaluated for granulysin expression *ex vivo* and on 4, 5, 6 and 7 days after PPD stimulation. No *ex vivo* surface granulysin was found on T-cells and very little was found on the surface of unstimulated NK cells (3%). In contrast 15% of CD8+ T-cells and 85% of NK cells constitutively expressed intracellular granulysin. After PPD stimulation, the number of T-cells expressing intracellular granulysin increased from day 4 to day 7 (Figure 5.6 A and 5.6 B). Surface granulysin was found on many more CD4+ T-cells than in CD8+ T-cells, whereas intracellular granulysin was expressed in similar numbers of CD4 and CD8 T-cells. Fewer NK cells (33%) expressed surface granulysin compared to intracellular granulysin (85%, Figure 5.6C). Therefore, kinetics of PPD-stimulated expression of intracellular and surface granulysin were similar, peaking on day 6-7. Intracellular granulysin expression is however, expressed on many more cells in all subsets than on the surface of cells.

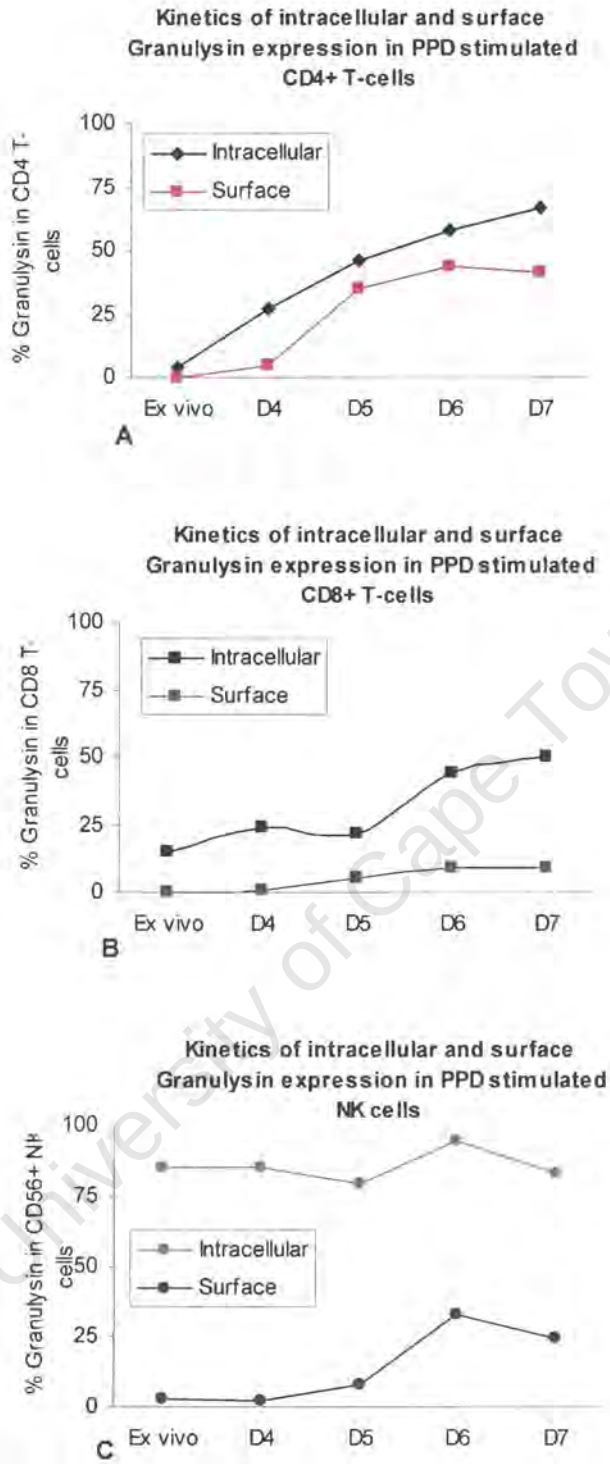

Figure 5.6: Kinetics of surface and intracellular granulysin expression on PPD stimulated CD4 T-cells (A), CD8 T-cells (B) and NK cells (C). Data is from one healthy PPD+ adult volunteer.

5.3.6 The Golgi blocker, Colchicine, inhibits granulysin from being expressed on the surface of cells

Surface and intracellular granulysin expression was determined for unstimulated and PPD-stimulated T-cells and NK cells, with and without the addition of colchicine, to cultures in two healthy PPD+ adult volunteers. The addition of colchicine for 12 hours overnight on day 6 resulted in no significant reduction of intracellular expression of granulysin by PPD stimulated T-cells and NK cells. However, colchicine significantly inhibited surface granulysin expression by 87% and 59% of CD4+ and CD8+T-cells respectively (figure 5.7). Similarly, intracellular granulysin in NK cells was unaffected by colchicine but surface expression was reduced by 69%. 10μM colchicine added daily to the cultures from day 4 to day 7 also resulted in a marked reduction of cells expressing granulysin on the surface.

The inhibition of surface expression could not be attributed to cell death as cell viability was determined by trypan blue staining before granulysin evaluation and there was >90% viability after the addition of colchicine. Lymphocyte proliferation was enumerated in the culture to which colchicine was added from day 4 to day 7 in order to determine whether colchicine was toxic to the cells (figure 5.8) Cell numbers increased from the initial $3x10^6$ to $7.07x10^6$ which supported the viability of the cells.

The cell surface expression of the markers CD107a and CD107b was evaluated within the gated lymphocyte population. A mean of 1.5% of unstimulated, 19.5% of PPD stimulated and 5.5% of PPD stimulated cells to which colchicine had been added, expressed these markers confirming that the presence of colchicine did indeed inhibit degranulation (figure 5.9). Interestingly, 8% of lymphocytes, to which colchicine had been added for three days, expressed these surface markers.

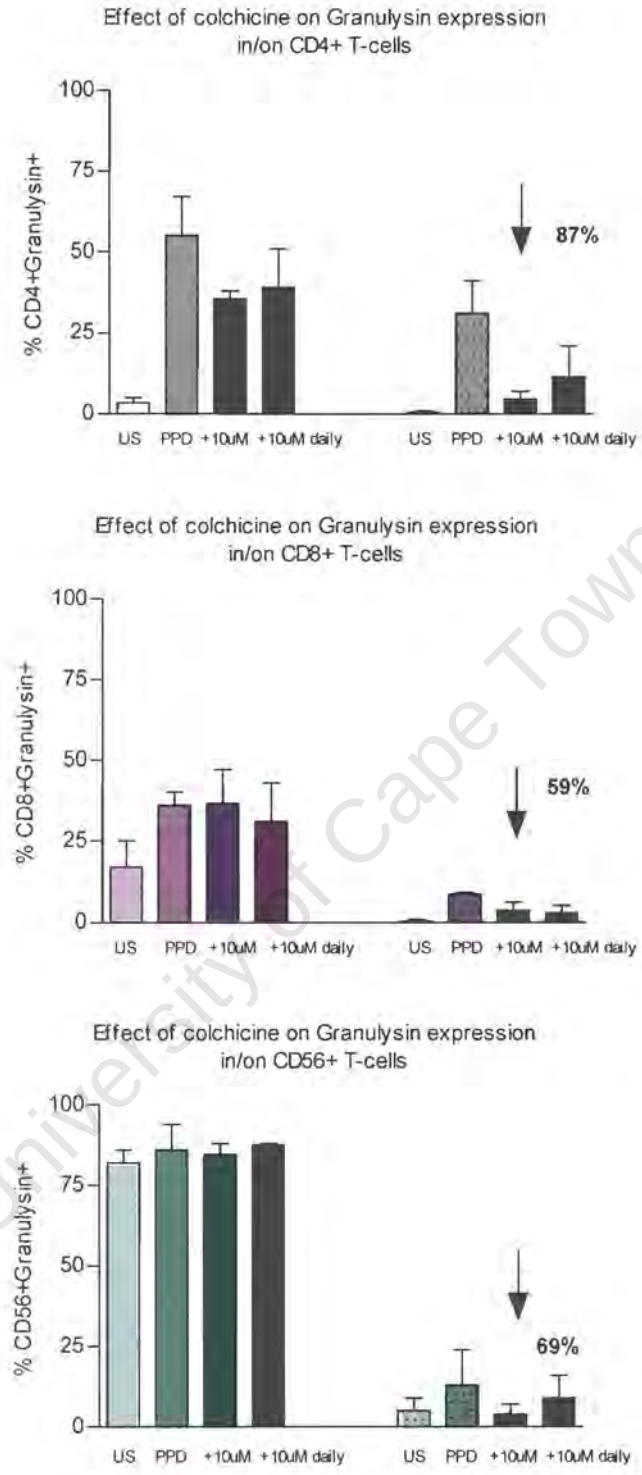

Figure 5.7: The effect of colchicine on surface granulysin expression (dotted colour-filled bars). Intracellular granulysin expression (colour-filled bars) was unaffected by the addition of colchicine but the percentage of CD4+ and CD8+ T-cells that expressed granulysin was reduced by 87% and 59% respectively. The number of NK cells expressing granulysin was reduced by 69%. Data is the mean of two healthy PPD+ volunteers.

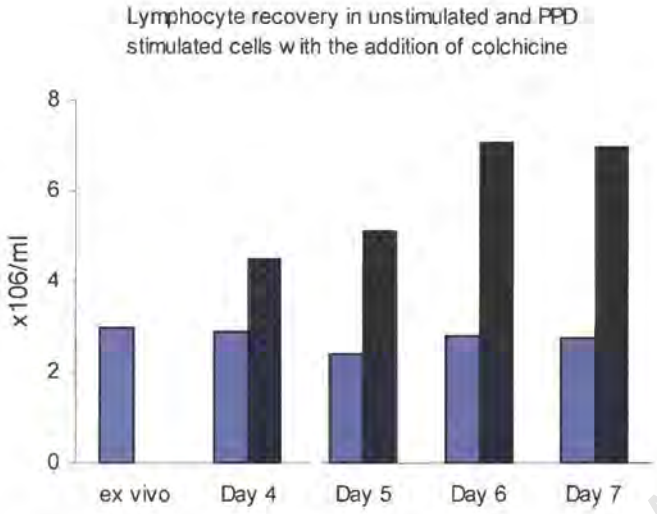

Figure 5.8: Lymphocyte proliferation to PPD in the presence of a Golgi blocker (n=1). 10μM colchicine was added to unstimulated and PPD stimulated (patterned bars) cells from day 4 to day 7. Cell numbers increased from the initial plating of 3 million to a maximum of 7.1 million.

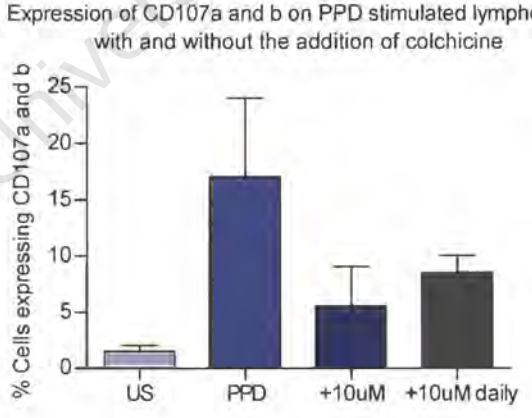

Figure 5.9: Lymphocyte expression of CD107a and b in unstimulated (US) and PPD-stimulated cells with or without the addition of 10μM Colchicine (n=2). There was a marked reduction of cells expressing this surface marker when colchicine was added to the cultures.

5.3.7 Surface granulysin does not result in direct killing of mycobacteria

Granulysin has been shown to directly kill extracellular mycobacteria (Stenger *et al.*, 1998). BCG was added to effector cells with and without colchicine, in order to determine whether surface granulysin was able to directly act on viable mycobacteria. There was no statistical difference to BCG viability, as assessed by colony forming units, if unstimulated or PPD stimulated cells were co-cultured with the mycobacteria (6560cfu/ml compared to 6840cfu/ml respectively). CFU counts of the mycobacteria alone resulted in 7040 cfu/ml whilst PPD stimulated PBMC in the presence of 10μM colchicine had a CFU count of 6710cfu/ml (figure 5.10). As this is preliminary data for one experiment only, it is difficult to interpret these results without optimising the experiment.

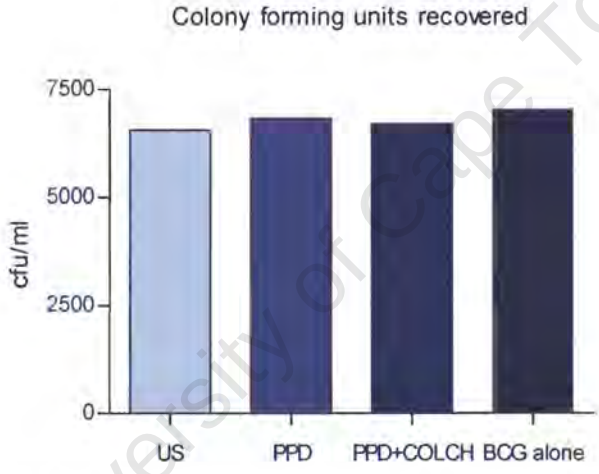

Figure 5.10: The effect of colchicine on BCG survival. There was no significant difference in CFU counts between BCG alone and BCG co-cultured with unstimulated or PPD stimulated cells, with or without the addition of colchicine. Data is in one adult donor.

5.4 DISCUSSION

It is well documented that there is an increase of antigen responsive cells at disease sites, and that CD4+ T-cells are expanded at the sites of infection in TB (Barnes *et al.*, 1990; Pokkali *et al.*, 2008; Prabha *et al.*, 2007). Immunological compartmentalization in pleural fluid in TB was reported as far back as 1990, where it was shown that there was an expansion of PPD-responding T-lymphocytes in PF of tuberculous but not non-tuberculous pleuritis (Lorgat *et al.*, 1992; Ribera *et al.*, 1990). There is a selective concentration of Th 1 cytokines in PF including INF-γ, TNF-α, IL-2 and IL-12, indicating a protective role, as Th1 cytokines induce and maintain high levels of inflammatory cytokines in PF for the continual recruitment of T-cells to the site of infection (Pokkali *et al.*, 2008).

Chemokines mediate their functions by transmigration of various immune cells to the diseased site (Pokkali *et al.*, 2008). Many chemokines associated with M.tb including IP-10, MIG and their receptors (eg CCR1, CCR2, CCR5, CCR7, CXCR2 and CXCR3) are significantly increased in PF compared to PB, allowing for the recruitment of activated T-cells (Pokkali *et al.*, 2008). CCR5 mRNA was higher in PF than PB in patients with pleural TB and four fold higher on CD14+ pleural mononuclear cells than CD4+ lymphocytes from PB (Fraziano *et al.*, 1999; Toossi *et al.*, 2005). In addition, the monocyte chemoattractant protein (MCP)-1 which attracts monocytes and memory cells to tissues, was higher in situ during TB (Toossi *et al.*, 2001).

The data shown in this chapter supports the evidence of Lorgat *et al* (1992), that the CTL from the site of infection have a greater cytolytic potential than cells from the systemic circulation. Due to ethical considerations, low cell numbers, and the exclusion of HIV patients, the numbers were small. In addition, over 50% of the patients recruited for the study had insufficient cells to carry out full experiments, or the PEL died after 6 days in culture. However, there was a dramatic difference in two of the four patients in the ability of CTL from the pleural fluid to lyse BCG-infected macrophages, than CTL from PB. Data from the CFU assay demonstrated that in all four patients, effector PEL were able to reduce bacterial growth as compared to CTL from PB. In three patients, there was a greater than 50% reduction in CFU growth by

the cytotoxic PEL as compared to PBMC. With a reduction in viable bacilli, and the subsequent uptake of surviving mycobacteria by neighbouring macrophages, additional cycles of CTL-mediated lysis should be sufficient to clear the infection at the localized site (Molloy *et al.*, 1994).

M.tb is one of the most resistant pathogens to microbiocidal mechanisms of mononuclear phagocytes (Stenger *et al.*, 1998). Dissemination of the tubercle bacilli from the site of primary infection is a major step in TB pathogenesis. Inhaled bacilli can form primary lesions in the lungs that might not be contained by innate, or emerging acquired immune responses. Multiplying mycobacteria escape these lesions leading to secondary lesions that are more favourable for lesion reactivation (Kraft *et al.*, 2004). The cytolytic mediators, granulysin and perforin, may be imperative for the containment of M.tb in primary lesions. CTL use either the exocytosis or Fas/FasL pathway but killing of M.tb by the Fas/FasL pathway will release the bacteria into the site of infection, and extracellular M.tb could continue to grow and hence propagate the infection (Stenger *et al.*, 1998). Thus, the exocytotic pathway, especially granulysin, is imperative for the host's defense against mycobacteria.

The direct role of granulysin was first shown when purified recombinant granulysin cultured with M.tb, killed the bacilli in a dose dependent manner (Stenger *et al.*, 1998). The extracellular demise of mycobacteria at the local site would prevent the spread of TB and suggests a possible protective role of granulysin. Purified granulysin failed to lyse M.tb-infected macrophages (Stenger *et al.*, 1998) but this was easily explained, as granulysin is unable to access intracellular pathogens without the presence of the pore-forming mediator perforin. Ma *et al.* (2002) reported that CD8+ T-cell mediated killing of *crytococcus neoformans* (CN) required granulysin but interestingly, NK cells used perforin and not granulysin for anticryptococcal activity (Ma *et al.*, 2004). Gamma delta cells used granulysin and perforin to kill M.tb infected macrophages and this resulted in intracellular and extracellular killing of bacilli (Dieli *et al.*, 2001).

The data presented in this chapter demonstrates that, significantly more T-cells expressed granulysin and perforin after PPD stimulation in three of the four patients in the PF, as compared to the PB. Bacterial survival, as demonstrated by CFU, from two

of these patients indicated that there was a correlation between the percentage of cells expressing these markers and the extent of mycobacterial survival. CTL derived from PB have previously been shown to kill infected macrophages without reducing viability (Fazal *et al.*, 1995) which could be due to a low number of granulysin expressing cells in the PB. Granulysin and perforin were expressed in markedly more T-cells in the PEL than in PB in the one patient and there was a 46% reduction of CFU by the effector pleural cells as compared to those in the PB. There was no difference in the cytolytic potential of CTL from PB and PEL in another patient where the percentage of CD4+ and CD8+ T-cells that expressed granulysin was comparable, and the number of CD8+ T-cells expressing perforin was similar. Although these results differ from data of Andersson *et al* (2007) who reported low granulysin and perforin in CD8+ T-cells at the site of infection, there are major differences in the clinical profiles. Andersson's study included chronic TB cases only which were unresponsive to treatment, whereas current data involved active untreated TB. In addition, they used lung tissue whereas the current study involved pleural fluid, and there may be immunological differences between these compartments.

Cell surface expression of granulysin by *in-vitro* activated T-cells was an exciting novel discovery. This finding was specific in that, under identical culture conditions, perforin was not detected on the cell surface. Further evidence for the bona fide nature of this finding was provided from blocking experiments with the microtubule inhibitor, colchicine. The addition of colchicine inhibited surface granulysin expression by 87% and 59% in CD4+ and CD8+ T-cell respectively, and by 69% of NK cells, as compared to surface expression in PPD-stimulated cells without colchicine. As expected, there was no significant difference to intracellular expression of granulysin in PPD- stimulated lymphocytes with the addition of colchicine.

Granulysin was first described to be expressed "late" (3-5 days) after T-cell activation (Pena *et al.*, 1997), and the kinetics of surface granulysin would be in keeping with this in that, after PPD stimulation, surface granulysin was expressed between day 4 and day 5 in all three lymphocyte subsets studied. Although NK cells and, to a lesser degree, CD8 T-cells constitutively express granulysin, surface granulysin was not constitutively expressed. The presence of granulysin on the surface could be due to binding of granulysin to the cell surface following degranulation, or that there is a

novel pathway for cell membrane granulysin expression analogous to membrane TNF-α and IL-1. Further studies, not within the scope of this thesis, are required to define exact mechanisms involved.

The lytic granules of CTL are membrane-bound secretory lysosmes surrounded by a lipid bilayer containing lysosomal associated membrane glycoproteins (LAMPs) which include CD107a (LAMP-1) and CD107b (LAMP-2) (Peters *et al.*, 1991). Degranulation occurs after T-cell receptor (TCR) stimulation as a result of the polarized mobilization of microtubules, that transport the granules to the immunological synapse between the CTL and target cell (Barry and Bleackley, 2002). Betts *et al* (2003) described a novel flow cytometric assay which measured the exposure of CD107a and b on the surface of CD8+ cells as a result of degranulation, which provided an assessment of the capacity, frequency and phenotype of effector CD8+ T-cells. More recently, CD4+ memory cells were shown to degranulate in response to stimulation by cognate antigen, by the presence of CD107a on the cell surface (Casazza *et al.*, 2006). Polyfunctional CD8+ cells co-expressing cytokines and CD107 have recently been implicated in immunity against intracellular infections (Betts *et al.*, 2006). In this chapter, further evidence of the effect of colchicine on surface expression of granulysin was demonstrated when surface expression of CD107a and b, by PPD stimulated lymphocytes, was reduced from 19.5% to 5.5% after the addition of colchicine.

Recombinant granulysin lyses a broad number of microbes including bacteria, fungi and parasites (Stenger *et al.*, 1998). It was capable of killing *Crytococcus neoformans* by acting directly on extracellular *Crytococcus neoformans* at the conjugation synapse of effector and target cells (Sun *et al.*, 2002). CD4+ T-cells were shown to be the major cells for killing *Crytococcus neoformans* in PB, and they use granulysin as the effector molecule (Zheng *et al.*, 2007). Previously, CD8+ T-cells were implicated for the killing of M.tb by the granule exocytosis pathway. Furthermore, sera containing granulysin cocultured with 10^4 M.tb bacilli for 72 hours showed a marked reduction in M.tb CFU counts, and there was a good correlation between the granulysin concentration in sera and the ability to inhibit the growth of M.tb (Liberto *et al.*, 2007).

The possible biological function of surface granulysin was explored by the addition of live BCG to effector cells (PPD stimulated) but no effect on BCG survival was detected. Furthermore, there was no difference in CFU counts if colchicine was added to the effector cells. These results of course need to be treated with caution as only one experiment was performed. No experiments were carried out to determine the optimal length of incubation of mycobacteria, or the optimal number of effector cells needed, or the required MOI. If substantiated, the result would mean that surface granulysin does not have the ability to lyse mycobacteria in the same way that soluble recombinant granulysin does. A possible explanation could be that, anchoring of the molecule to the cell surface may interfere with this activity. Whether this provides evidence that surface granulysin is non-functional or not, needs to be explored.

CHAPTER 6

SUMMARY OF FINDINGS AND FUTURE PROSPECTS

In 1993 WHO declared Tuberculosis (TB) a global health emergency. The disease is responsible for approximately 2 million deaths annually and it is estimated that about a third of the world's population is infected with M.tb. The only vaccination available to combat the disease is the *Bacillus Calmette-Guérin* (BCG) which is effective against the more severe complications of childhood tuberculosis, but which offers limited protection against pulmonary TB. In South Africa approximately 95% of newborn babies are vaccinated with BCG yet TB remains epidemic in the country, and is particularly prevalent in the Western Cape, where the disease incidence in certain areas is above 1500/100,000 per year (Wood *et al.*, 2007).

The development of a more effective vaccine has been hindered by the incomplete understanding of the host immune response to the organism, especially with regard to protective responses. NK cells and CTL are key players in the immune response against bacteria, viruses and tumours, and are also essential components of protective immunity against TB and HIV (Stenger, 2001). In order to develop a more effective vaccine, it is imperative to identify the immune correlates of protection and to evaluate their involvement in clinical TB, and upregulation in response to BCG vaccination. Granulysin, an antimicrobial protein, and perforin, a pore-forming molecule, have been found to co-localise in the granules of CTL and NK cells and could offer protection against TB. These molecules act synergistically and have been implicated in killing of intracellular microbial pathogens, including M.tb, and granulysin has been shown to directly kill extracellular M.tb (Stenger *et al.*, 1998). In addition, expression of both cytolytic mediators has been reported to be reduced and/or defective in HIV-infected individuals (Haridas *et al.*, 2003; Zheng *et al.*, 2008a).

In this thesis, it was shown that granulysin and perforin were constitutively expressed in NK cells and in a low percentage of CD8+ T-cells in healthy PPD+ adult volunteers. The percentage of T-cells and NK cells in the lymphoid population was similar whether PPD or BCG was used as a stimulant. In contrast, although granulysin and perforin were constitutively expressed in NK cells from cord blood, they were not present in unstimulated or PPD stimulated T lymphocytes from cord blood. However, BCG stimulation induced both of these cytolytic markers in CBMC albeit, the percentage of T-cells and NK cells expressing granulysin and perforin was significantly lower, than in healthy PPD+ normal donors. There was a statistically significant increase in cells expressing both granulysin and perforin in PBMC from 10 week-old infants vaccinated at birth with BCG. This upregulation was consistent in most of the infants and was not influenced by the route or strain of the vaccination.

Patients with active PTB had a similar percentage of granulysin and perforin expressing T-cells, following stimulation with PPD, as did healthy PPD+ normal donors. After successful anti-TB therapy, there were significantly more granulysin expressing T-cells compared to healthy controls after PPD stimulation. This is in agreement with two studies involving adults and children in a high TB burden region, where treatment induced an upregulation of granulysin and perforin expression (Sahiratmadja *et al.*, 2007, Caccamo *et al.*, 2006). In contrast, data presented in this thesis showed that active disease resulted in a lower percentage of perforin expressing T-cells following PPD stimulation than healthy controls, which normalized after treatment. Thus, successful anti-TB therapy results in an increase in the percentage of granulysin and perforin expressing T-cells after *in vitro* PPD stimulation. NK cells expressing either granulysin or perforin were reduced in active TB compared to healthy PPD+ individuals, and treatment resulted in an increase in granulysin expressing NK cells, but did not affect the low number of perforin expressing NK cells. The biological significance of the sustained decrease in perforin expressing NK cells after successful chemotherapy is unclear, as this was not the case for T cells which are involved in the adaptive immune response to TB.

CD4+ T-cells in active and treated TB patients were activated *ex vivo* as shown by the expression of the surface activation marker CD25, a phenomenon not seen on CD8+ T-cells and NK cells from those patients. However those CD4+ T-cells did not

express granulysin or perforin *ex vivo*, but CD8+ and NK granulysin and perforin expressing cells were detectable *ex vivo*. This confirmed the finding that granulysin and perforin are constitutively expressed in CD8+ T-cells and NK cells irrespective of whether the subject had active TB or had been successfully treated for TB. In a randomized clinical trial (RCT), there was no significant difference in paired analysis between granulysin and perforin expressing cells in the three different treatment arms before and after treatment. However, in a larger cross-sectional study, patients who had received standard anti-TB drugs and recombinant IFN-γ administered via subcutaneous injection, had a significantly higher percentage of CD8+ T-cells expressing granulysin after they had received treatment, than at baseline. A similar trend was shown with CD4+ granulysin expressing T-cells in the same treatment group, although this did not reach statistical significance. In addition, these patients had significantly more granulysin expressing T-cells after PPD stimulation compared to those of healthy PPD+ adult volunteers. These results suggest that rIFN-γ increases the number of granulysin expressing cells which could be beneficial in abrogation of disease. Condos *et al* (1997) reported that aerosolised rIFN-γ in conjunction with standard anti-TB drugs was effective in MDR-TB, in contrast to Parks *et al* (2007) who found no benefit in any way with the addition of rIFN-γ to the treatment regimen for MDR-TB. My data in drug sensitive TB, favoured subcutaneous administration rather than aerosolised IFN-γ as an adjuvant for TB therapy in a cross-sectional analysis. A further study would be required to confirm this finding by enrolling a greater number of patients in a longitudinal, paired analysis. However, that would be a very expensive and labour intensive undertaking and it is doubtful that another RCT of IFN-γ of that magnitude could be undertaken.

IFN-γ levels in supernatants from PPD stimulated PBMC were significantly lower in active PTB compared to after treatment in the same patients. This was not dependent on the temporal affect of the treatment, as there was no statistical difference in cytokine levels in patients who converted to culture negative early in treatment compared to those who responded later. An interesting observation was a significantly reduced level of TNF-α in the supernatants of PPD stimulated PBMC from patients with a low number of granulysin expressing cells compared to those with a high

percentage of granulysin-expressing cells after treatment. TNF-α has been implicated in a protective role in TB. The correlation between low levels of this cytokine and a low number of granulysin and perforin expressing cells may also suggest its involvement in the resolution of TB infection, but only a small number of patients were evaluated and a larger study is needed to confirm this finding.

Granulysin and perforin have been reported to be reduced and/or defective in patients with HIV (Zheng *et al.*, 2007, Haridas *et al.*, 2003) but the HIV positive patients in this study, who had successfully been treated for TB, had comparable percentages of cells expressing both markers as did healthy PPD+ adult volunteers. Patients who were co-infected with TB and HIV, had significantly less granulysin and perforin expressing CD4+ T-cells than patients with HIV only, but this was not seen in CD8+ T-cells and NK cells. This was not dependent on the CD4 counts, and was in agreement with another South African study which reported that M.tb-specific CD4+ and CD8+ T-cell responses are maintained in the peripheral blood of HIV positive individuals in the absence of active disease and their function was not affected by the disease (Day *et al.*, 2008). In the study included in this thesis, the number of patients that were co-infected with TB and HIV was low, therefore, these findings need to be confirmed and extended in future studies.

Granulysin and perforin were present in many more effectors from PEL than blood, and it was shown that the cytolytic cells from the site of infection had a greater cytolytic potential than cells from the systemic circulation. This correlated with the extent of mycobacterial survival, as effector PEL were able to reduce the viability of mycobacteria more than effector cells from peripheral blood in the same patients. A very exciting novel finding was that granulysin was detected on the surface of T-cells and NK cells and this was supported by the addition of a Golgi blocker which inhibited cell surface expression. This finding was specific in that, under identical culture conditions, perforin was not detected on the cell surface. Granulysin has been shown to kill both intracellular and extracellular M.tb (Stenger *et* al., 1998) so it would be necessary to establish the exact role of surface bound granulysin. More extensive studies would be required to address this, including co-incubation of higher numbers of activated T-cells that express surface granulysin to mycobacteria, an

optimal time and dose response, and use of electron microscopy to detect structural changes in the bacilli (Stenger *et al.*, 1998).

Ag85 stimulation resulted in an increase in granulysin and perforin expressing cells although there was a reduced effect compared to PPD stimulation in the same patients. As Ag85 has been used as a vaccine candidate, this data is encouraging. In conclusion, granulysin and perforin appear to be candidate surrogate markers for studies on correlates of protection in TB, which was demonstrated by the increase in the percentage of cells expressing these cytolytic mediators after successful anti-TB treatment, and the induction of these markers in infants after vaccination with BCG. In addition, these markers are expressed in many more cytolytic cells at the site of infection than further away from the infected area. Future research could include the addition of granulysin and perforin as endpoint assays in studies of novel vaccine candidates. It would be instructive to include measurement of their expression in endpoint immune assays which are pending in the large randomized BCG vaccination study recently completed (presented at the 37th Union World Conference on Lung Health in Paris, 2006). It is imperative that a new vaccine against TB not only enhances but also sustains the protective host response, as it has been postulated that protection due to BCG vaccination wanes with time (Sterne *et al.*, 1998).

The experiments performed in this thesis were limited by the amount of cells obtained from subjects. The patients enrolled in the study were often leucopenic and the cells isolated were insufficient to include all experiments. Pleural effusions often did not contain sufficient cells and pleural lymphocytes were less robust than PBMC, possibly due to hyperactivation. In addition, the vaccinated babies were 10 weeks-old, so sufficient blood could not always be obtained. Nevertheless, the thesis has produced novel findings, including the upregulation of granulysin by BCG vaccination, documentation of cytolytic mediator expression in response to IFN-γ in RCT, cell surface expression of granulysin, relationship between granulysin expression and lytic activity in TB pleuritis, and impact of TB and HIV co-infection on cytolytic mediator expression.

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