THE INVESTIGATION OF PERIPHERAL BLOOD CELLULAR IMMUNE RESPONSES DURING INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS*

HANNELORE F.U. VEENSTRA

Dissertation presented for the degree of Doctor of Philosophy in Medical Sciences at the University of Stellenbosch, South Africa

Proefskrif ingelewer vir die graad Doktor in Filosofie in Mediese Wetenskappe aan die Universiteit Stellenbosch, Suid Afrika

Promoter: Prof. Gerhard Walzl

Co-promoter: Prof. Patrick J.D. Bouic

March 2007

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ..... Date: .....

VERKLARING

Ek, die ondergetekende, verklaar hiermee dat die werk in hierdie proefskrif vervat, my eie oorspronklike werk is en dat ek dit nie vantevore in die geheel of gedeeltelik by enige universiteit ter verkryging van ‘n graad voorgelê het nie.

Handtekening:…… Datum:……

ABSTRACT

Introduction and aims

Despite the ongoing global tuberculosis (TB) problem and extensive research into protective immunity against this intracellular pathogen, mechanisms of protective immunity against *Mycobacterium tuberculosis* (Mtb) in humans have not been fully clarified. Numerous reports have addressed the potential immunological defect(s) in infected individuals that have developed active TB in comparison to those who have remained healthy in spite of infection. Markers of treatment response phenotypes are still elusive. The aims of this study were to define lymphocyte subsets in the peripheral blood of TB patients and controls, to determine intracellular interferon-γ (IFN-γ) and interleukin-4 (IL-4) production and to find correlations of these data with microbiologically-defined treatment response.

Methods

Whole blood tests were done on 30 HIV-negative, smear-positive pulmonary TB patients and 18 healthy skin test positive volunteers resident in the same community. Immunophenotyping was performed by flow cytometry, combined with routine haematology, for the enumeration of peripheral blood immune cell subtypes. Whole blood was also stimulated *in vitro* with anti-CD3 monoclonal antibody and intracellular IFN-γ and IL-4 determined by flow cytometry. Lymphocyte proliferation in response to heat-killed Mtb was determined by tritiated thymidine incorporation. Routine microbiological monitoring by sputum smears and culture was done throughout the patients' 26 weeks of treatment.

Results

Compared to healthy controls, absolute numbers of peripheral blood lymphocytes and lymphocyte subsets were significantly depressed in patients at diagnosis but normalized during treatment with the exception of natural killer (NK) cells and natural killer T (NKT) cells. A novel subset of the latter was found to correlate significantly with treatment response. IFN-γ-producing T cells after a 4-hour T cell receptor stimulation were significantly higher in patients at diagnosis and normalized during treatment. Supplementary kinetic experiments showed that IFN-γ production in patients at diagnosis seemed to be accelerated. Lymphocyte proliferation was lower in patients at diagnosis and normalized during treatment. Neither IFN-γ production nor lymphocyte proliferation correlated with treatment response. Low intracellular IL-4 production was constitutive in patients and controls, was insignificantly lower in patients at diagnosis than in controls and, in the slow responder patient group, it was significantly lower than in the fast responder group. High IL-4 expression was found in low numbers of T cells in patients and controls and supplementary experiments showed co-expression of active caspase-3 in these cells, which signified apoptosis.

Conclusions

Lymphocyte subset phenotypes associated with TB are largely abnormal only during active infection and only a novel subset of NKT cells showed correlation with treatment response. Intracellular IFN-γ production and lymphocyte proliferation is increased and decreased, respectively, only during active infection and does not correlate with treatment response. The T helper 1/T helper 2 (Th1/Th2) hypothesis could not be confirmed in the context of tuberculosis but instead constitutive IL-4 production may play a role as a growth factor.

ABSTRAK

Inleiding en Doelwit

Tenspyte van die wêreldwye tuberkulose (TB) probleem en ekstensiewe navorsing in die veld van beskermende immuniteit teen *Mycobacterium tuberculosis* (Mtb), is die menslike meganisme(s) van beskermende immuniteit teen hierdie intrasellulêre patogeen nog nie duidelik nie. Verskeie verslae adresseer die potensïele immunologiese defek(te) in geïnfekteerde individue met aktiewe TB, in teenstelling met individue wat gesond bly tenspyte van infeksie. Geen definitiewe fenotipiese merkers wat uitkoms van behandeling kan voorspel is nog bekend nie. Die doelwitte van hierdie studie was om limfosiet subgroepe te defineer in TB pasiënte en in kontroles, om intrasellulêre interferon-γ (IFN-γ) en interleukien-4 (IL-4) produksie te bepaal en om korrelasies van hierdie data met mikrobiologies defineerde behandelingsresponse te vind.

Metodes

Heel-bloed toetse is gedoen op 30 MIV-negatief, smeer-positiewe pulmonêre TB pasïente en 18 gesonde veltoets positiewe vrywilliges van dieselfde woongebied. Immunofenotipering is gedoen deur middel van vloeisitometrie en is gekombineer met roetine hematologie vir die identifisering van periferie bloed immuunseltipes. Heel- bloed is ook *in vitro* gestimuleer met ‘n monoklonale anti-liggaam teen CD3 en intrasellulêre IFN-γ en IL-4 bepaal deur vloeisitometrie. Limfosiet proliferasie is bepaal deur middel van getritïeerde timidien inkorporasie na blootstelling aan hitte geïnaktiveerde Mtb. Roetine mikrobiologiese toetse (speeksel smere en kulture), is vir die verloop van die 6 maande behandeling op elke pasïent gedoen.

Resultate

In vergelyking met die van gesonde kontroles, was absolute perifêre bloed limfosiete en limfosietsubtipes beduidend onderdruk in pasïente by diagnose. Hierdie tellings het tydens behandeling genormaliseer, met die uitsondering van natuurlike doderselle en natuurlike doder T selle. ‘n Voorheen onbeskryfde subtipe van laasgenoemde selle het beduidend gekorrelleer met die uitkoms van behandeling. Na ‘n 4 uur stimulasie van die T-selreseptor, was IFN-γ produserende T-selle beduidend meer in pasïente by diagnose. Hierdie verskynsel het genormaliseer tydens behandeling. Bykomende kinetiese eksperimente het gewys dat IFN-γ produksie versnel is in pasïente by diagnose. Limfosiet proliferasie was laer in pasïente by diagnose en het genormaliseer tydens behandeling. Nie IFN-γ produksie of limfosiet proliferasie het gekorrelleer met die uitkoms van behandeling nie. Daar was ‘n konstante laë intrasellulêre IL-4 produksie in pasïente en kontroles, maar dit was nie beduidend laer in pasïente by diagnose in vergelyking met kontroles nie. In die pasïente wat stadiger gereageer het op behandeling, was intrasellulêre IL-4 produksie beduidend laer as in die pasïente wat vinniger gereageer het. Hoë IL-4 uitdrukking is gevind in ‘n klein hoeveelheid T selle in pasïente en kontroles. Bykomende eksperimente het getoon dat uitdrukking van aktiewe kaspase-3 in hierdie selle apoptose voorstel.

Gevolgtrekking

Limfosietsubtipe fenotipes, wat geassosieer word met tuberkulose, is meestal abnormaal slegs tydens aktiewe infeksie en slegs ‘n nuwe subtipe natuurlike doder T sel het ‘n korrellasie getoon met uitkoms van behandeling. Verhoogde intrasellulêre IFN-γ produksie en verlaagde limfosiet proliferasie is slegs waargeneem gedurende aktiewe infeksie en is nie geassosieer met uitkoms van behandeling nie. Die T helper

1/T helper 2 (Th1/Th2) hipotese kon nie bewys word in die konteks van tuberkulose nie, maar konstante IL-4 produksie mag ‘n moontlike rol as groeifaktor speel.

TABLE OF CONTENTS

Abstract iii

Abstrak v

List of figures and tables xi

Acknowledgements xii

Abbreviations xiii

Hypothesis 1

1 General introduction 3

1.1 Tuberculosis and the immune response 3

1.2 The cells of the immune system 3

1.3 The cell-mediated immune response 4

1.4 Treatment of tuberculosis 7

1.5 Flow cytometry as an investigative tool in the evaluation of immune responses 8

2 Study setting, design and subjects 9

2.1 Setting 9

2.2 Patients and controls 9

2.3 Processing of sputum samples for Ziehl-Neelsen smear and culture 10

2.4 Demographic data of study population 11

3 Changes in leukocyte and lymphocyte subsets during tuberculosis treatment; prominence of $CD3^{dim}CD56^{+}$ NKT cells in fast treatment responders 14

3.1 Introduction 14

3.2 Materials and methods 15

3.2.1 Reagents 15

3.2.2 Immunophenotyping by flow cytometry 15

3.2.3 Intracellular cytokine determination 16

3.2.4 Classification of patients into treatment response groups 17

3.2.5 Statistical analysis 17

3.3 Results 18

3.3.1 Longitudinal changes in total and differential white cell count 18

3.3.2 Lymphocyte subsets 21

3.3.3 T lymphocyte subsets 24

3.3.4 A $CD3^{dim}/CD56^{+}$ NKT cell subset was more prominent in patients .. 27

3.3.5 Differences between treatment response groups 32

3.3.6 $CD3^{dim}/CD56^{+}$ NKT cells produce IFN-γ and IL-4 35

3.4 Discussion 38

4 Changes in the kinetics of intracellular IFN-γ production in TB patients during treatment 42

4.1 Introduction 42

4.2 Materials and methods 45

4.2.1 Patients and controls 45

4.2.2 Reagents 45

4.2.3 Intracellular cytokine determination 46

4.2.4 Lymphocyte proliferation 48

4.2.5 Statistical analysis 48

4.3 Results 49

4.3.1 Intracellular IFN-γ 49

4.3.2 Kinetics of IFN-γ production 55

4.3.3 Lymphocyte proliferation 59

4.3.4 Correlation with treatment response 63
4.4 Discussion 63
5 Intracellular interleukin-4 in lymphocytes from patients with tuberculosis – evidence necessitating a review of its role in the immune response 66
5.1 Introduction 66
5.2 Materials and methods 68
5.2.1 Patients and controls 68
5.2.2 Reagents 68
5.2.3 Intracellular cytokine determination: 68
5.2.4 Culture of Jurkat cells and preparation of cell lysate 69
5.2.5 Statistical analysis 70
5.3 Results 70
5.3.1 Intracellular IL-4 expression in T lymphocytes of TB patients. 70
5.3.2 Co-expression of intracellular IL-4 and caspase-3 in Jurkat cells 89
5.3.3 Expression of intracellular IL-4 by apoptotic neutrophils 92
5.3.4 Expression of intracellular IL-4 by apoptotic monocytes 96
5.3.5 Stimulation of neutrophils and monocytes decreases IL-4^{high} expression 99
5.4 Discussion 102
6 General discussion 105
7 References 107
8 Appendix 118
8.1 Suppliers of reagents 118
8.2 Solutions 118
PDF file of publication 120

LIST OF FIGURES AND TABLES

Figure 2.1……..13

Figure 3.1……..19

Figure 3.2……..22

Figure 3.3……..25

Figure 3.4……..28

Figure 3.5……..30

Figure 3.6……..33

Figure 3.7……..36

Figure 4.1……..50

Figure 4.2……..53

Figure 4.3……..57

Figure 4.4……..61

Figure 5.1……..72

Figure 5.2……..75

Figure 5.3……..78

Figure 5.4……..81

Figure 5.5……..84

Figure 5.6……..87

Figure 5.7……..90

Figure 5.8……..94

Figure 5.9……..97

Figure 5.10……..100

Table 2.1……...12

Table 4.1……...43

Table 4.2……...44

Table 4.3……...44

Table 5.1……...92

ACKNOWLEDGEMENTS

I extend my thanks to my supervisor Prof. Gerhard Walzl for his support, knowledge input and time he sacrificed for the completion of this work and to my co-supervisor, Prof. Patrick Bouic, who has advised me for many years in flow cytometry. Dr. Pauline Lukey of GlaxoSmithKline R&D was very involved in the planning and start-up of this study and her input was much appreciated. I also gratefully acknowledge the following for their contributions to the work described in this thesis: Dr. Nora Carroll, Erica Engelke and Marianna de Kock for the routine microbiological data, Ilse Crous, Dr. Shweta Brahmbhatt and Siobhán Harnett for the lymphocyte proliferation data, the clinical and administrative staff of the Department of Paediatrics and Child Health of the University of Stellenbosch under Prof. Nulda Beyers for the blood and sputum collection and patient data management, the patients and the community of Ravensmead and Uitsig for participating in the study, Dr. Martin Kidd of the Centre for Statistical Consultation at the University of Stellenbosch for the Support Vector Machines analysis and general statistical advice, Prof. Robert Gie for CXR analysis and the Department of Haematology at Tygerberg Hospital for performing full blood counts, Dr. Ralf Baumann for help with the

writing up of Chapter 3 and 5 for publication, Liesel Muller for translating the abstract and Hanno Nel for his computer skills, all my colleagues for their support and the laboratory staff for their reliable housekeeping. Finally I would like to thank Prof. Paul van Helden, Head of the DST/NRF Centre of Excellence for Biomedical and TB Research at the University of Stellenbosch Faculty of Health Sciences, where all this work was done.

Funding for this study was provided by GlaxoSmithKline, Stevenage, U.K. and the DST/NRF Centre of Excellence for Biomedical TB Research.

ABBREVIATIONS

Ag	antigen
APC	allophycocyanin
BCG	Bacille Calmette-Guérin
BFA	brefeldin A
BSA	bovine serum albumin
CXR	chest X-ray
DMSO	dimethyl sulphoxide
DOTS	directly observed treatment short course
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FADD	Fas-associated death domain
FC	flow cytometry
cfu	colony-forming units

cpm	counts per minute
ESAT-6	early secretory antigenic target
FCS	foetal calf serum
FSC	forward scatter
FITC	fluorescein isothiocyanate
HIV	human immunodeficiency virus
IFN-γ	interferon-gamma
IL-4	interleukin-4
mAb	monoclonal antibody
Mtb	*Mycobacterium tuberculosis*
NK cells	natural killer cells
NKT cells	natural killer T cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	phycoerythrin
PEG	polyethylene glycol
PerCP	peridinin chlorophyll
PHA	phytohaemagglutinin
PPD	purified protein derivative
RBC	red blood cells
RPMI+	RPMI 1640 medium with antibiotics (see Appendix)
SSC	side scatter
TCR	T cell receptor
Th cells	T helper cells
Tc cells	T cytotoxic cells

TTP	time to positivity
ZN	Ziehl-Nielsen
WCC	white cell count

THE INVESTIGATION OF PERIPHERAL BLOOD CELLULAR IMMUNE RESPONSES DURING INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS*

Null hypothesis

1. Patients with tuberculosis have normal peripheral blood immunophenotypes and cellular immune responses.
2. Treatment response is not influenced by patients' immune status at diagnosis.

Alternative hypothesis

1. Patients' peripheral blood immunophenotypes and cell-mediated immune responses in peripheral blood of patients are altered during active tuberculosis.
2. Treatment response is influenced by patients' immune status at diagnosis.

Aims

1. To define lymphocyte subsets in patients' blood by immune phenotyping at various time points during treatment and compare them with those of healthy control subjects.
2. To define the cytokine production by patients' and control subjects' lymphocytes in response to stimulation in comparison with those of healthy control subjects.

3. To find correlations of immune parameters with routine microbiological data that define fast and slow responders to treatment.

1. GENERAL INTRODUCTION

1.1 TUBERCULOSIS AND THE IMMUNE RESPONSE

TB has been a global health problem for millennia and it is estimated that a third of the world's population is presently infected with *Mycobacterium tuberculosis* (Mtb), its causative organism, which was discovered by Robert Koch in 1882. Evidence for TB has been found in a 2400 year old mummy and was referred to in ancient Greek literature (http://www.state.nj.us/health/cd/tbhistry.htm). Drugs to treat TB were only discovered in 1944. The bacterium has defied enormous efforts to control it and still thrives, particularly in poorer communities, and the appearance of HIV and drug resistance is aggravating the problem. Deaths from TB are estimated to be 2-3 million annually.

For immunologists it has been of great interest that only an estimated 5-10% of Mtb-infected, HIV-uninfected individuals develop active disease, the remainder being protected from illness by their immune system. The Bacille Calmette-Guérin (BCG) vaccine primarily protects young children from disseminated forms of the disease but is not effective in protecting adults from pulmonary TB. The discovery of the mechanisms of the natural protective resistance would be of great benefit in the efforts to stop the spread of the disease and save the lives of those who become ill.

1.2 THE CELLS OF THE IMMUNE SYSTEM

The immune system has two arms, the innate and the adaptive immune response [1]. The innate immune system responds rapidly to foreign invaders, has no memory and

the responding cells are the natural killer (NK) cells, neutrophils and monocyte/macrophages in the blood and the infected tissues. Although the orchestrated response of the innate immune cells plays an important part by engulfing and destroying micro-organisms, the main protective immune response is that of the adaptive immune system which responds more slowly and has a memory that enables it to respond faster after a re-exposure to the same foreign invader of the body. The adaptive immune system, in turn, has two arms, the humoral and cell-mediated immune response. The B lymphocytes mediate the humoral response by the production of antibodies that bind specifically to the invader, whereas in the cell-mediated response the players are the T lymphocytes comprising subpopulations T helper (Th) cells, that express the specific cluster determinant (CD) CD4, T cytotoxic (Tc) cells that express CD8, γδ T cells that express a T cell receptor (TCR) consisting of γ and δ chains instead of the usual α and β chains and NK T cells that express NK cell markers such as CD56 as well as the T cell marker CD3. Dendritic cells and monocyte/macrophages of the innate immune system act as antigen-presenting cells in the cell-mediated adaptive immune response.

1.3 THE CELL-MEDIATED IMMUNE RESPONSE

The adaptive immune response to Mtb infection has been the subject of extensive research because of its memory response which could be harnessed for the development of more effective vaccines. Although TB patients have antibodies to mycobacterial antigens in their circulation, these do not play an important role in the elimination of bacilli but the cell-mediated response and interferon-γ (IFN-γ) production by activated T lymphocytes is crucial [2]. This cytokine also activates

resident macrophages that phagocytose the bacilli in the lungs of an infected subject. During disease progression the mycobacteria replicate in the macrophages, resulting in the formation of granulomas and the attraction of lymphocytes to the perimeter of the granulomas in a chronic cell-mediated response.

In a normal immune response invading micro-organisms are taken up by dendritic cells or macrophages, which are antigen-presenting cells that break down the mycobacterial antigens into peptides in the endosomes. The peptides then associate with major histocompatibility complex (MHC) Class II molecules and are transported to the cell surface where they are presented to the CD4 T helper cells [1]. CD4 T cells bearing the appropriate TCR recognize the presented peptides and enter the effector phase characterized by clonal expansion and production of cytokines which potentiate effector cells to eliminate the bacilli. This cytokine production defines the type 1 (Th1) and type 2 (Th2) helper cell responses, where IFN-γ and interleukin-2 (IL-2) characterize the cell-mediated Th1 response and IL-4 and IL-5 the humoral Th2 response [3]. Many studies with mice have shown that the CD4 T cell subset is essential for the control of the infection (reviewed in [2]) and a cruel experiment of nature with humans has shown the same by the rising incidence of TB in patients infected with the HIV virus that kills its cellular host, the CD4 T cell subset (http://www.who.int/hiv/topics/tb/en/).

The main role of IFN-γ is believed to be macrophage activation [2]. It is produced by CD4 and CD8 T cells and NK cells and, although insufficient alone, is an essential cytokine in the control of Mtb infection as shown by studies with gene knock-out mice (reviewed in [2]) and human subjects with defects in the IFN-γ or IFN-γ receptor genes are susceptible to serious infections with normally non-pathogenic mycobacteria [4].

CD8 T cells classically recognize peptide antigens derived from endogenous cytosolic antigens that are degraded by the proteasome and are transported to the cell surface in association with MHC Class I molecules. Although Mtb antigens located in the phagosome do not appear to have access to the MHC class I processing pathway, there is recent evidence that this is possible and that there is a role for CD8 T cells in the defense against Mtb (reviewed in [2]). Activated CD8 cells also produce cytokines and have been subdivided into Tc1 and Tc2 cells according to their cytokine profiles of IL-2/IFN-γ and IL-4/IL-5 respectively, and they are also capable of cytolysis of the infected cells mediated by the cytotoxic granule proteins perforin and granulysin [2].

A third antigen-presenting pathway involving the CD1 molecules has more recently been identified (reviewed in [5]), in which lipid antigens are presented. Both intracellular and exogenous lipid antigens, which include mycobacterial antigens, can be accessed by CD1 molecules and presented to CD1-restricted CD8 T cells and NKT cells and the effector functions are IFN-γ production and cytotoxic activity.

Following the effector phase of expansion of the antigen-specific T cells and clearance of the infection is the deletion phase during which the majority of the expanded effector cells are removed by programmed cell death or apoptosis while a small number survive and persist as memory cells, capable of rapidly re-starting the response in the event of a repeated exposure to the antigen [1]. Apoptosis is accompanied by distinct morphological changes, decrease in cell volume and fragmentation into apoptotic bodies which are rapidly phagocytosed by macrophages, preventing the release of mediators of a localized inflammatory response [6].

Two types of apoptosis occur in activated T cells that are triggered by different signals [7]: (1) The engagement of death receptors such as Fas which, after their engagement of ligands, transmit intracellular signals by the recruitment of adaptor molecules such as Fas-associated death domain (FADD) which in turn recruit cysteine proteases, first pro-caspases which become activated and in turn activate effector caspases that destroy cell structure and integrity. This type of apoptosis is also termed activation-induced T cell death. (2) Cytokine withdrawal which results in poorly characterized intracellular signals and their transduction via a mitochondrial pathway that utilizes proteins of the Bcl-2 family and cytochrome c to activate the pro-caspases and effector caspases. This latter process is also termed activated T cell autonomous death and is the major mechanism of deletion of T cells responding to foreign antigen [7].

1.4 TREATMENT OF TUBERCULOSIS

The standard therapy for TB is in accordance with the South African National Tuberculosis Program and is based on World Health Organization guidelines [8]. It is designated directly observed treatment short course (DOTS) and is described in detail in Chapter 2. For drug-susceptible TB cure a combination of 3 drugs has to be taken for 6 months and for drug-resistant TB the treatment is even more complex. There is therefore an ongoing search for better drugs to improve and shorten the treatment to prevent the spread of the disease during the early stages of treatment and recurrence in treated patients. To conduct drug trials it is necessary to monitor the infection and the only internationally accepted method of doing this is by staining the acid-fast bacteria in the sputum by means of the Ziehl-Nielsen (ZN) stain and

culturing the bacteria, for instance with the Bactec system. Additional surrogate markers for the infection are still needed and the measurement of immune system parameters of the patients in this study was planned with this in mind.

1.5 FLOW CYTOMETRY AS AN INVESTIGATIVE TOOL IN THE EVALUATION OF THE IMMUNE RESPONSE

Flow cytometry is the detection of cells in suspension that have bound specific antibodies tagged with fluorochromes. The flow cytometer passes the labelled cell suspension through the beam of a laser that excites the relevant fluorescent dyes and the emitted light is measured by photomultipliers. The dedicated software calculates accurate and objective statistics. Two types of cell labelling are used: (1) Extracellular in which fluorochrome-labelled antibodies, usually monoclonal antibodies (mAb), are simply allowed to react with molecules expressed by the cells on the cell membrane. This method is used to identify and quantify lymphocyte subsets in immunophenotyping and is quick and very reproducible. (2) Intracellular labelling in which molecules inside the cells, such as cytokines, are detected with the specific antibodies. As living cells do not allow macromolecules like immunoglobulins to pass through the intact cell membrane, the cells have to be effectively killed to make the plasma membrane permeable to the antibodies. There are many different ways of achieving this permeabilization which is also dependent on the intracellular localization of the molecule of interest. This method is therefore subject to much greater variation in the results obtained [9]. When investigating molecules that are not constitutively expressed, such as cytokines, and have to be induced by a stimulus, additional variables come into play, namely the *in vitro*

culture conditions and the time after the stimulus at which the measurements are made which are like a “snapshot” taken of a short time span in the cells’ response. These variables have to be taken into consideration when interpreting the results.

2. STUDY SETTING, DESIGN AND SUBJECTS

2.1 SETTING

This study was done in the Ravensmead/Uitsig epidemiological field site in metropolitan Cape Town, where the incidence of new smear and/or culture-positive TB was on average 313/100 000 population/year from 1993-1998 [10]. More recently, the number of cases of TB reported in Cape Town in 2005 was 874/100 000 population/year (http://www.capegateway.gov.za/Text/2006/5/tb_stats_2006.pdf).

2.2 PATIENTS AND CONTROLS

The study was approved by the Ethics Committee of the Faculty of Health Sciences at Stellenbosch University (reference number 99/039) and written, informed consent was obtained from all participants. Inclusion criteria included: age 18-65, sputum culture-positive for Mtb, HIV-negative, not pregnant; and for follow-up: no multi-drug resistance, and taking at least 80% of prescribed dosages during the intensive phase of treatment. The presence of helminth infection or atopy in the patient group was not known. Twenty-one patients with first-time TB were enrolled and studied throughout treatment whereas 9 were only studied at diagnosis. Blood samples were taken at diagnosis prior to initiation of treatment and for follow-up at weeks 1, 5, 13,

and 26 after start of treatment (the last blood sample being taken on the last day of chemotherapy). Sputum smears and Bactec cultures were done on day 1 and 3, and week 1, 2, 4, 8, 13 and 26 after start of treatment. A total white cell count (WCC) and differential blood count was done on all blood samples using a Bayer Advia 120.

The patients received standard therapy in accordance with the South African National Tuberculosis Program (based on WHO guidelines). Therapy consisted of a fixed drug combination (depending on body weight) containing isoniazid (320-400mg/day), rifampicin (480-600mg/day), ethambutol (800-1200mg/day) and pyrazinamide (1000-1250mg/day) during the intensive phase (8 weeks) followed by rifampicin and isoniazid during the continuation phase (the remaining 18 weeks) under direct observation. Postero-anterior and lateral chest X-rays (CXR) were taken at commencement of treatment allowing a four week time window on either side of diagnosis. The chest radiographs were evaluated using a standardized method [11] by a physician who had no prior knowledge of the patient's condition. The extent of disease was estimated using a one-dimensional view of the upright posterior-anterior radiograph and by using the right upper lobe as reference area.

One blood sample was taken from each of 18 healthy HIV-negative, PPD skin test-positive (>15mm) volunteers resident in the same community to serve as controls. These participants had no clinical or radiological signs of active TB.

2.3 PROCESSING OF SPUTUM SAMPLES FOR ZIEHL-NEELSEN SMEAR AND CULTURE

Sputum samples were processed for culture using standard methods [12], which included decontamination according to the Bactec 460TB System Procedure Manual

(Becton Dickinson, Maryland, USA) before inoculation into a Bactec 12B vial. The vials were incubated at 37 °C and the growth index (GI) was read daily. Sputum smears, direct and concentrated, were examined for acid-fast bacilli using the Ziehl-Neelsen (ZN) stain and evaluated using the scoring system of the International Union against Tuberculosis and Lung Disease [13]. If multiple smears were done the smear with the highest grade was recorded for that time point.

2.4 DEMOGRAPHIC DATA OF STUDY POPULATION

The 21 patients that were followed up were all cured after 26 weeks of standard DOTS therapy. Three patients were infected with an Isoniazid-monoresistant strain of mycobacteria. After 8 weeks of treatment 15 patients were smear-negative and 6 were smear-positive while only 8 were culture-negative and 13 culture-positive (two of these were Isoniazid-monoresistant). The week 8 Bactec culture was therefore used as the more sensitive indicator of early treatment response. No significant differences between fast and slow responders in CXR findings at diagnosis were found (including extent of disease and presence, number or size of cavities). The age and sex distribution of patients and their responder status is given in Table 2.1.

Table 2.1: Age and sex data of patients and controls

	Patients		**Controls**
	Fast responders[a]	**Slow responders**	
Total (no.)	8	13	14
Male (no.)	3	9	3
Female (no.)	5	4	11
Age (years)	18-51	19-50	20-56

[a]as defined by negative sputum culture at week 8

The time to positivity (TTP) is the number of days that the sputum cultures were incubated until they became positive for Mtb growth and is an indication of the bacterial load of the patient at time of diagnosis. The change in TTP's after initiation of treatment is shown in Fig. 2.1. At week 13 only one patient was still culture-positive.

Figure 2.1

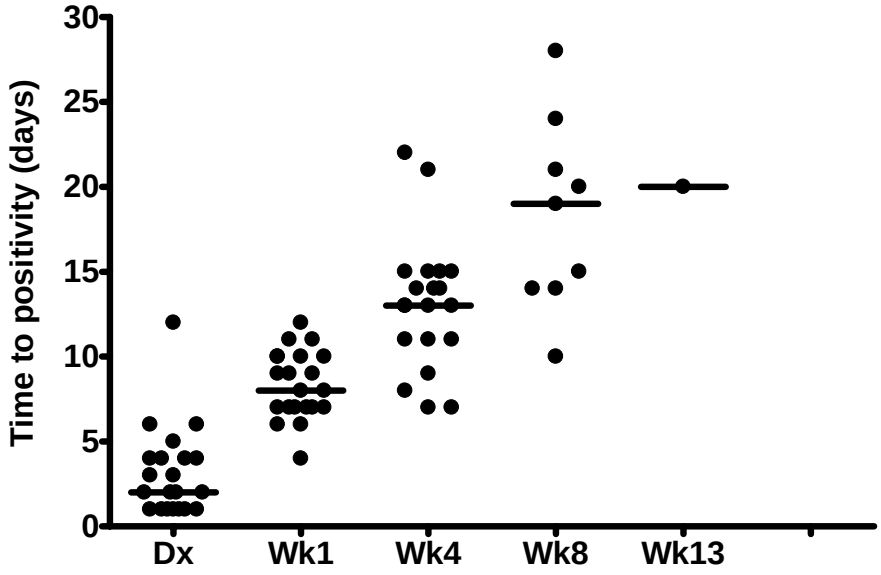

Figure 2.1: Time to positivity in days of sputum cultures of from diagnosis (Dx) to week (Wk) 13.

Each dot represents data from one patient. The lines are at the median values for each time point.

3. CHANGES IN LEUKOCYTE AND LYMPHOCYTE SUBSETS DURING TUBERCULOSIS TREATMENT; PROMINENCE OF $CD3^{dim}CD56^{+}$ NKT CELLS IN FAST TREATMENT RESPONDERS

3.1 INTRODUCTION

To clarify the mechanisms of protective immunity against *Mycobacterium tuberculosis* (Mtb) infection and disease in humans many reports have addressed the potential immunological defect(s) by comparing immune phenotypes in actively diseased patients to those with latent infection. Most of these investigations have focused on T lymphocyte subsets, particularly $CD4^{+}$ and γδ T cells, generally reporting depressed $CD4^{+}$ T cells in peripheral blood of TB patients [14-16] but results are discrepant for γδ T cells, where both elevated [17,18] and normal [19,20] numbers have been found. Only a few but inconclusive reports of B-lymphocyte and NK cell numbers in TB patients exist [14,16,21,22] and NKT cells have, to my knowledge, not been investigated in TB patients. Generally, contributors to TB susceptibility remain unclear and follow-up data during therapy are scanty.

The aim of this study was to investigate immune parameters during therapy and this chapter describes a systematic follow-up of leukocyte counts and lymphocyte subsets in TB patients for the entire 26 week treatment period. Furthermore, due to the fact that the identification of high risk patients for slow response to chemotherapy would have important clinical implications, peripheral blood immunophenotypes

were analyzed as potential surrogate markers of early TB treatment response and a multivariate classification technique applied to identify fast and slow responders to treatment by immunophenotype at diagnosis.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Fluorochrome-labelled mAbs anti-CD45-peridinin chlorophyll (PerCP), CD3-phycoerythrin (PE), CD3-PerCP, CD4-fluorescein isothiocyanate (FITC), CD8-FITC, CD19-FITC, CD56-FITC, γδTCR-FITC, IFN-γ-PE, IL-4-PE and rabbit anti-active caspase-3-FITC were from BD-Bioscience. A rabbit FITC control antibody was not available from the manufacturer. OKT3 anti-CD3 antibody was spent hybridoma medium. The hybridomas were from ATCC. Vα24-PE was purchased from Beckman Coulter, saponin from Sigma and polyethylene glycol 4000 (PEG) from Merck. For the locations of the suppliers see Appendix (p.118).

3.2.2 Immunophenotyping by flow cytometry

Whole blood (50µl per test), anti-coagulated with sodium heparin, was washed once with phosphate-buffered saline (PBS). The cells were suspended in 100µl of 0.1% bovine serum albumin (BSA) in PBS and added to the required antibody mixtures. After 20 minutes at 4°C, cells were washed and red blood cells (RBCs) lysed at the same time by diluting with 3-4ml cold PBS containing 0.05% saponin and 3% PEG (lyse/wash buffer; saponin was chosen as lysing agent because it was noticed by a

colleague previously (J. Adams, personal communication) that RBCs in whole blood from TB patients frequently failed to lyse when treated with commercial lysing solution. The addition of 3% w/v PEG to the saponin buffer prevented damage and clumping of cells in blood obtained at diagnosis and also enhances the formation of antigen/antibody complexes [23]). After centrifugation at 700g the cell pellets were fixed in 4% formaldehyde in PBS and stored at 4°C in the dark until flow cytometric analysis in a Becton-Dickinson FACS Calibur® using CellQuest software®. Lymphocytes were gated in a CD45-PerCP (FL3) versus Side Scatter plot (10 000 events in this gate were acquired) and these were further analyzed for expression of CD3 and CD4 (or CD8, CD19, CD56, γδTCR) in the FL1 and FL2 channels respectively. The lymphocyte sums calculated were all between 95 and 100%. Isotype control antibodies were not routinely used as the background cell surface staining of *ex vivo* blood lymphocytes is very low (not shown).

3.2.3 Intracellular cytokine labelling

This method is described in more detail in Chapter 4. Briefly, whole heparinized blood was mixed 1:1 with RPMI 1640 medium in polypropylene tubes and incubated at 37°C with or without 0.1µg/ml OKT3 antibody for 4 hours, with 10µg/ml Brefeldin A (BFA) present during the last 3 hours. After incubation the blood was diluted with cold lyse/wash buffer, centrifuged in the cold at 700g, and the cells in the pellet were labelled with mAbs in the above buffer containing 0.1% BSA for 20 minutes in the cold. After one wash with cold lyse/wash buffer, the cell pellets were fixed in 4% formaldehyde in PBS and analyzed in the flow cytometer.

3.2.4 Classification of patients into treatment response groups

In order to find possible differences between fast and slow responders to treatment, patients were divided into two responder groups according to Bactec culture status at week 8 after start of treatment. Of the 21 enrolled patients 8 were culture-negative (fast responders) and 13 culture-positive (slow responders) (Table 2.1).

3.2.5 Statistical analysis

Data for patients at diagnosis and at the end of treatment were analyzed for significant differences from those for healthy subjects by means of the Mann-Whitney test. The Friedman test with Dunn's post test was used to analyze longitudinal changes in parameters with respect to the diagnosis time point values. (* or #: p=0.01-0.05, ** or ##: p=0.001-0.01, *** or ###: p<0.001; asterisks refer to the Mann-Whitney test and hashes to the Friedman test). The Pearson Chi-square test and Fisher's exact test were used to analyze categorical CXR data.

To find the best combination of variables at diagnosis that may have potential for the prediction of early treatment response, as defined by the week 8 Bactec sputum culture, a Support Vector Machines analysis was performed, a multivariate discriminant classification technique that has received much attention in the statistical literature in the past few years [24]. Combinations of up to a maximum of 5 variables were analyzed and, using the variables included in the optimal classification model, a leave-one-out cross validation table was constructed.

3.3 RESULTS

3.3.1 Longitudinal changes in total and differential white cell count

The total white cell count (WCC) and absolute neutrophil counts were significantly elevated in patients at diagnosis relative to controls (Fig. 3.1A, B) but returned to normal levels by the end of treatment. The absolute monocyte counts were also significantly elevated at diagnosis but then dropped dramatically to significantly depressed levels at week 26 (Fig. 3.1C). The absolute lymphocyte count of patients at diagnosis was significantly depressed at diagnosis but counts were no longer significantly different from controls at the end of treatment (Fig. 3.1D).

Figure 3.1

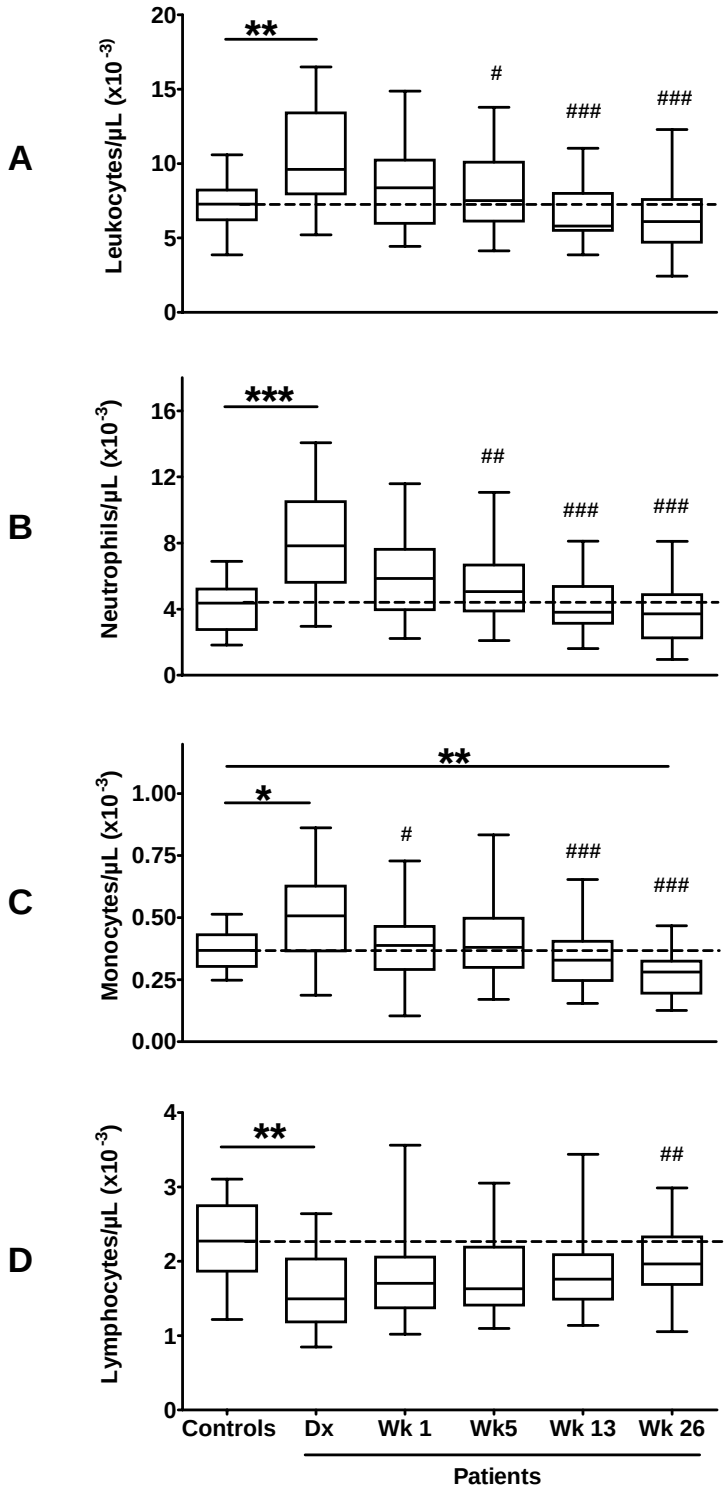
A
Leukocytes/µL (x10-3)
20
15
10
5
0
**
#
###
###
B
Neutrophils/µL (x10-3)
16
12
8
4
0
***
##
###
###
C
Monocytes/µL (x10-3)
1.00
0.75
0.50
0.25
0.00
**
*
#
###
###
D
Lymphocytes/µL (x10-3)
4
3
2
1
0
**
##
Controls
Dx
Wk 1
Wk5
Wk 13
Wk 26
Patients

Figure 3.1: Absolute leukocyte counts of healthy controls and TB patients.

Counts were calculated from the total white cell count and differential blood count. A: Total WCC, B: neutrophils, C: monocytes, D: lymphocytes. The boxes extend from the 25th to the 75th percentile with a line at the median and the whiskers show the highest and lowest values. Data for patients at diagnosis (Dx) and at the end of treatment at week (Wk) 26 were analysed for significant differences from those for healthy subjects by means of the Mann-Whitney test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). The Friedman test with Dunn's post test was used to analyze changes in parameters during the patients' follow-up with respect to values at diagnosis (# $p<0.05$, ## $p<0.01$, ### $p<0.001$).

3.3.2 Lymphocyte subsets

Percentages of T lymphocytes and NK cells were not significantly different from those of controls at diagnosis or at week 26 while percentages of B lymphocytes were depressed in patients at diagnosis ($p<0.05$) and recovered during treatment (not shown). The absolute lymphocyte subset counts were calculated from the subset percentages and absolute lymphocyte counts (Fig. 3.2). The absolute $CD3^+$ T cell and absolute $CD19^+$ B cell counts were significantly depressed in patients at diagnosis but at week 26 these were not significantly different from those of control subjects (Fig. 3.2A, B). Absolute $CD56^+/CD3^-$ NK cell counts at diagnosis showed a trend towards lower numbers ($p=0.06$) and remained depressed until week 26 ($p<0.05$, Fig. 3.2C).

Figure 3.2

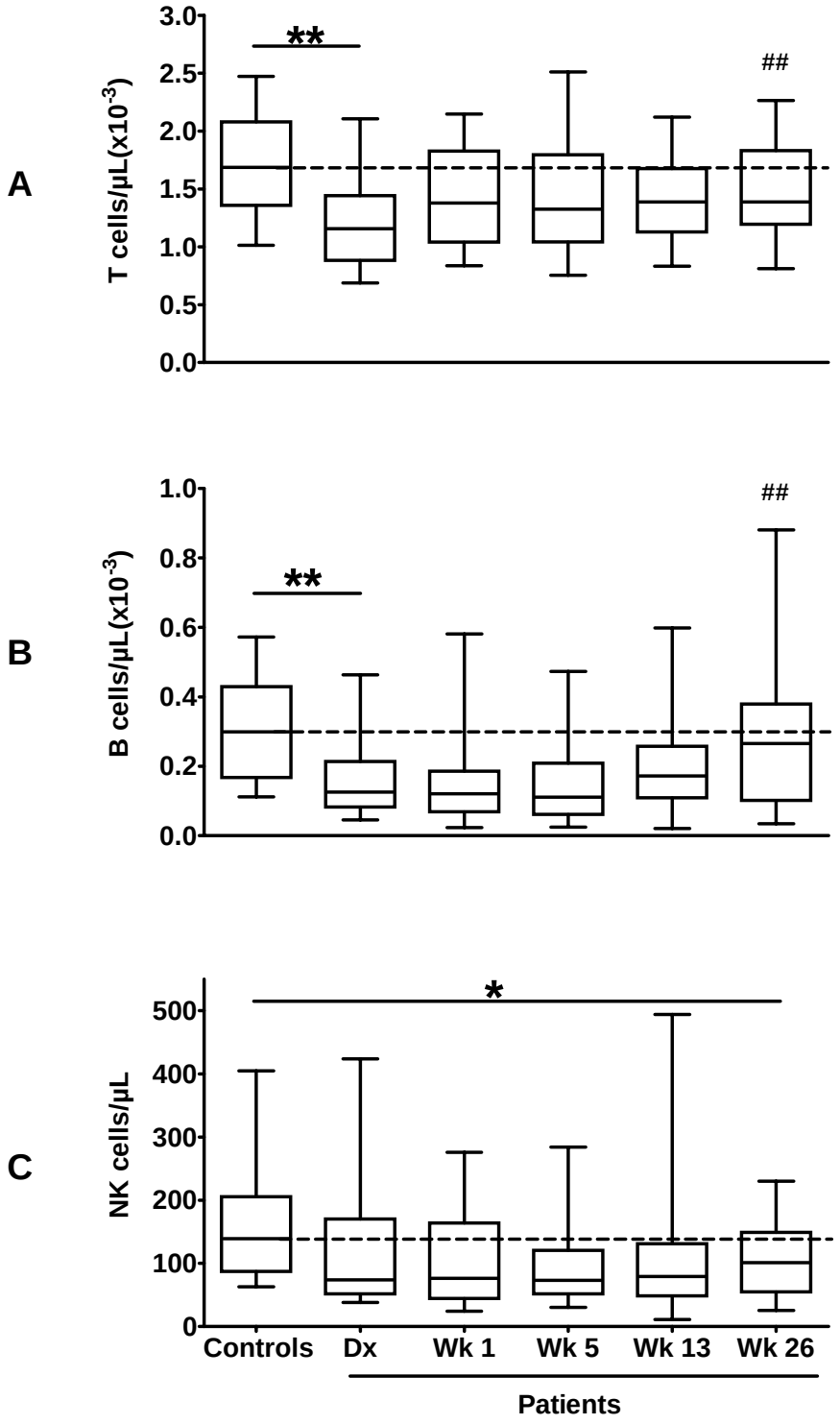
A
B
C
T cells/µL(x10-3)
B cells/µL(x10-3)
NK cells/µL
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.8
0.6
0.4
0.2
500
400
300
200
100
0
**
*
##
Controls
Dx
Wk 1
Wk 5
Wk 13
Wk 26
Patients

Figure 3.2: Absolute lymphocyte subset counts of healthy controls and TB patients.

Counts were calculated from the absolute lymphocyte counts and the percentages of subsets determined by flow cytometric immunophenotyping. A: T lymphocytes ($CD3^+$), B: B lymphocytes ($CD19^+$), C: NK cells ($CD3^-CD56^+$). Box and Whisker plots and statistical analyses as for Fig. 3.1 (Mann-Whitney test * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Dunn's post-test # $p<0.05$, ## $p<0.01$, ### $p<0.001$).

3.3.3 T lymphocyte subsets

The percentages of $CD4^+$, $CD8^+$ and γδ T cells and the CD4:CD8 ratio at diagnosis and at week 26 were not significantly different from those of control individuals and only small fluctuations were detected during follow-up. Two populations of NKT cells were detected that differed in their levels of expression of CD3: a $CD56^+$ cell population which expressed CD3 levels comparable to conventional T cells ($CD3^{bright}/CD56^+$ NKT cells) and one that expressed reduced levels ($CD3^{dim}/CD56^+$ NKT cells). The percentages of $CD3^{bright}/CD56^+$ NKT cells in patients at diagnosis and at week 26 were not significantly different from those of controls (not shown) and $CD3^{dim}/CD56^+$ NKT cells are described in detail below. Absolute numbers of T cell subsets, calculated from the absolute lymphocyte count and the percentages determined by immunophenotyping are illustrated in Fig. 3.3. $CD4^+$ T cell numbers (Fig. 3.3A) were significantly depressed at diagnosis relative to control subjects ($p<0.01$) and, while numbers increased significantly during treatment, they were still lower at week 26 than in controls ($p=0.06$). $CD8^+$ T cell counts (Fig. 3.3B) showed no significant differences or variation and γδ T cell counts were significantly depressed (Fig. 3.3C, $p<0.05$) at diagnosis but recovered during treatment to normal levels at week 26. Absolute numbers of $CD3^{bright}/CD56^+$ NKT cells were lower at diagnosis ($p=0.06$) and at the end of treatment ($p<0.05$, Fig. 3.3D).

Figure 3.3

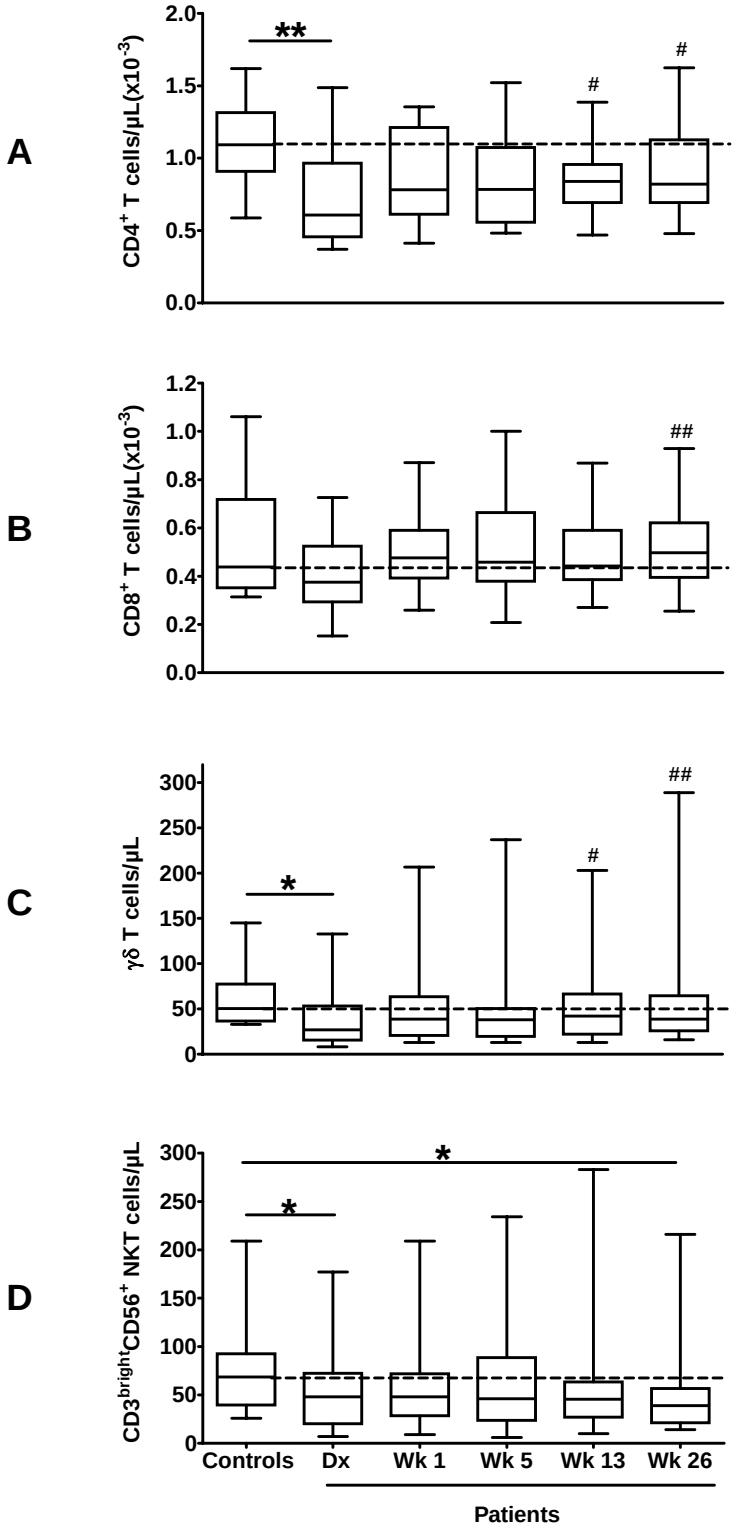
A
B
C
D
CD4+ T cells/µL(x10-3)
CD8+ T cells/µL(x10-3)
γδ T cells/µL
CD3brightCD56+ NKT cells/µL
**
*
#
##
Controls
Dx
Wk 1
Wk 5
Wk 13
Wk 26
Patients

Figure 3.3: Absolute T cell subset counts of healthy controls and TB patients. The subset counts were calculated from the absolute T cell counts and the percentages of the subsets determined by flow cytometric immunophenotyping. A: $CD4^+$ T cells, B: $CD8^+$ T cells, C: $\gamma\delta TCR^+$ T cells, D: $CD3^{bright}/CD56^+$ NKT cells. Box and Whisker plots and statistical analyses as for Fig. 3.1

3.3.4 A $CD3^{dim}/CD56^{+}$ NKT cell subset was more prominent in patients

An unusual subset of lymphocytes was detected more frequently in patients (9 of the 21 patients had ≥2% at diagnosis) than in controls (2 of 14 had ≥2%). In the flow cytometric analyses, of which Fig. 3.4 is an example, these cells were weakly $CD3^{+}$ ($CD3^{dim}$), $CD4^{-}$, weakly $CD8^{+}$ or $CD8^{-}$, and $CD56^{+}$, shown in region R2 in Fig. 3.4D, and also $\gamma\delta TCR^{-}$ (not shown). The number of cells in region R2, as illustrated in Fig. 3.4, expressed as a percentage of the cells in the CD45 gate, was determined for all blood samples. Fig. 3.5A shows increased percentages of $CD3^{dim}/CD56^{+}$ NKT cells in patients at diagnosis relative to control subjects although this was not statistically significant (p=0.23). Very low or undetectable numbers remained so during follow-up while higher numbers persisted and sometimes increased after start of treatment (shown for fast and slow responders in Fig. 3.5C); the highest recorded was 20.3% at week 1.

Figure 3.4

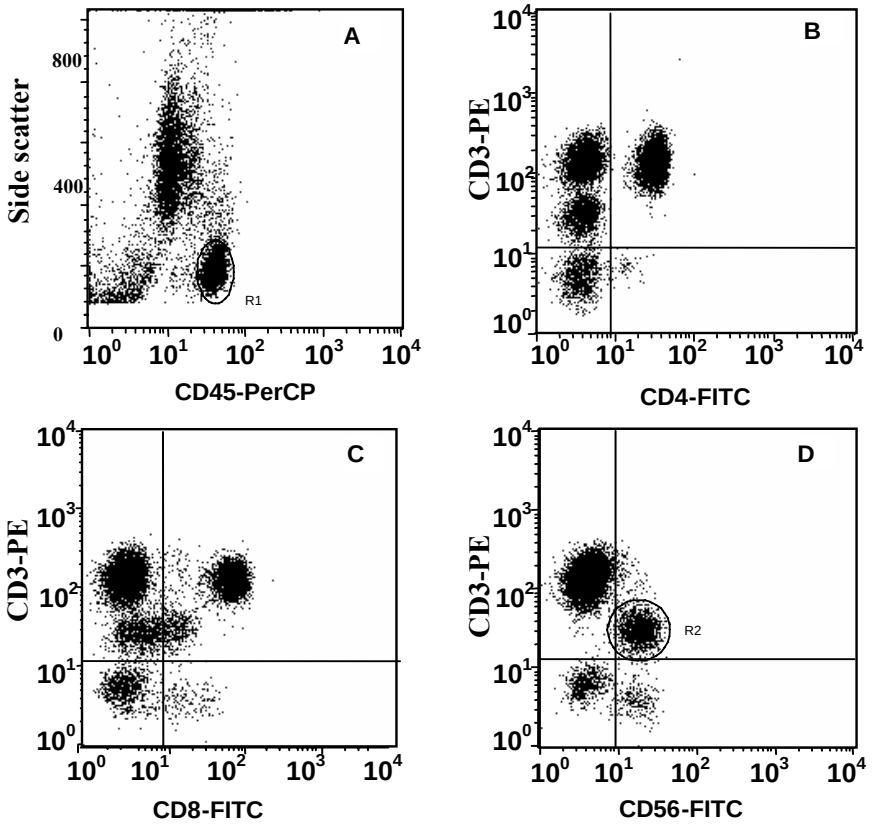
A
Side scatter
800
400
0
R1
CD45-PerCP
B
CD3-PE
CD4-FITC
C
CD3-PE
CD8-FITC
D
CD3-PE
R2
CD56-FITC

Figure 3.4: A representative lymphocyte subset analysis of flow cytometric data from a patient with a prominent $CD3^{dim}/CD56^{+}$ NKT cell population.

A: Gating of the $CD45^{bright}$ low side scatter total lymphocyte population.

B, C, D: The gated lymphocytes analyzed for CD3 and CD4, CD8 and CD56 expression, respectively. Region R2 in Fig. 4D contains the $CD3^{dim}/CD56^{+}$ NKT cells.

Figure 3.5

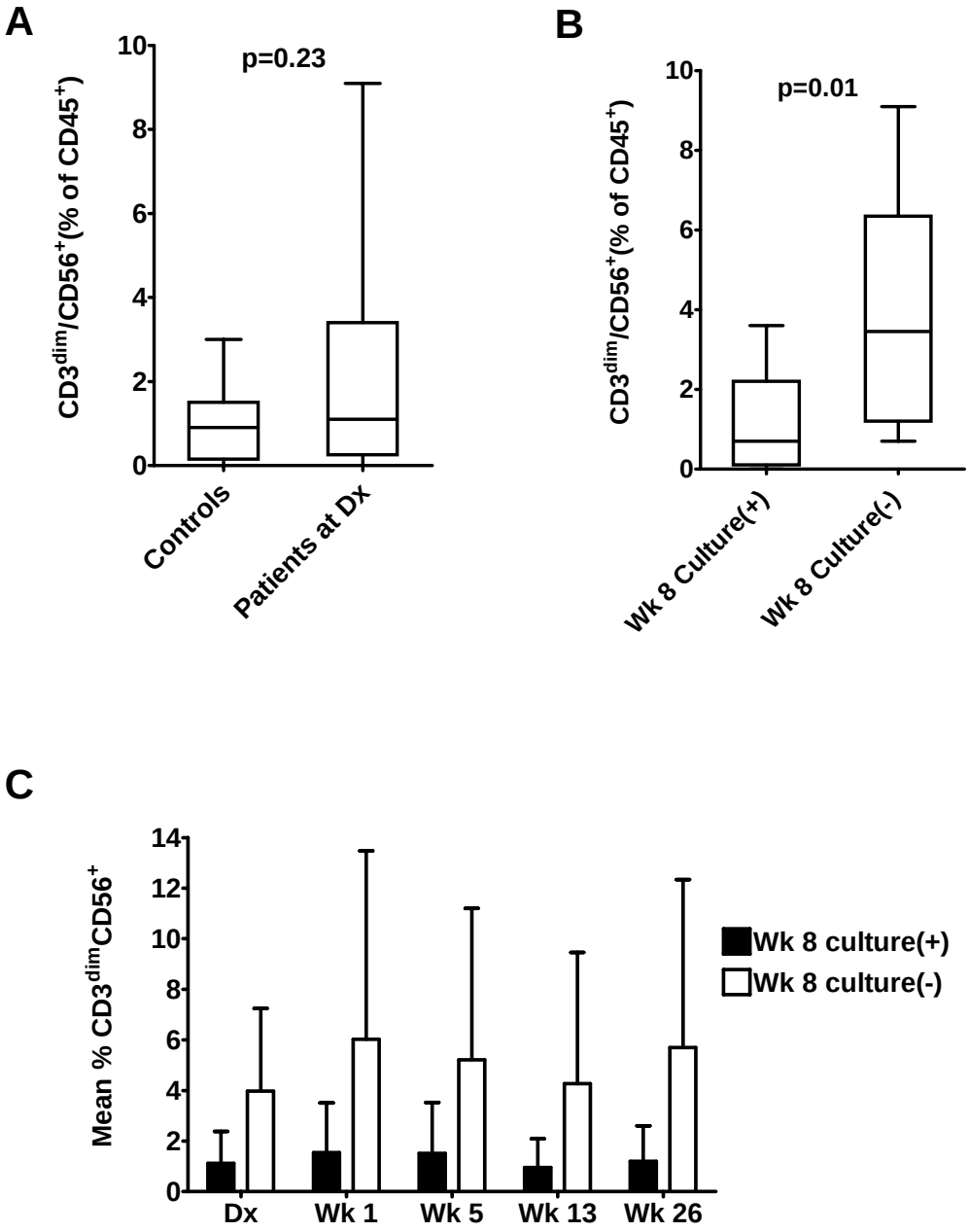
A
p=0.23
CD3dim/CD56+(% of CD45+)
10
8
6
4
2
0
Controls
Patients at Dx
B
p=0.01
CD3dim/CD56+(% of CD45+)
10
8
6
4
2
0
Wk 8 Culture(+)
Wk 8 Culture(-)
C
Mean % CD3dimCD56+
14
12
10
8
6
4
2
0
Dx
Wk 1
Wk 5
Wk 13
Wk 26
Wk 8 culture(+)
Wk 8 culture(-)

Figure 3.5: **$CD3^{dim}/CD56^{+}$ NKT cell percentages in the lymphocyte gate.**

A: Controls and patients at diagnosis compared with the Mann-Whitney test. B: Patients at diagnosis grouped into slow responders to treatment (culture(+) at week 8) and fast responders (culture(-) at week 8), compared with the Mann-Whitney test. C: Mean percentages of $CD3^{dim}/CD56^{+}$ NKT cell counts with standard deviation error bars in the slow and fast responder patient groups from diagnosis to end of treatment.

3.3.5 Differences between treatment response groups

When percentages and absolute numbers of each cell type at diagnosis in fast responders were compared to those at diagnosis of slow responders with a Mann-Whitney test, the percentage and absolute count of $CD3^{dim}/CD56^{+}$ NKT cells at diagnosis were the only single variables that correlated significantly with treatment response – they were significantly higher at diagnosis in fast responders ($p=0.01$, Fig. 3.5B). The percentages of $CD3^{dim}/CD56^{+}$ NKT cells did not change significantly during follow-up and are shown for the fast and slow responding patients in Fig. 3.5C.

As the $CD3^{dim}/CD56^{+}$ NKT cell numbers at diagnosis did not correlate with treatment response in all patients, a multivariate classification technique was used to find combinations of variables that may more accurately classify patients into fast and slow responders. Differences between early response phenotypes were most prominent at diagnosis and the variables at diagnosis that were used for the analysis were the absolute numbers of leukocyte, lymphocyte and T cell subsets. The Support Vector Machines discriminant analysis showed that the best classification of patients into the two treatment response groups could be obtained with just two variables (Fig. 3.6): absolute $CD3^{dim}/CD56^{+}$ NKT cells and absolute NK cells which correctly classified all 13 slow responders and 5 of 8 fast responders in a leave-one-out cross validation. Absolute NK cell counts at diagnosis alone did not correlate with treatment response (not shown).

Figure 3.6

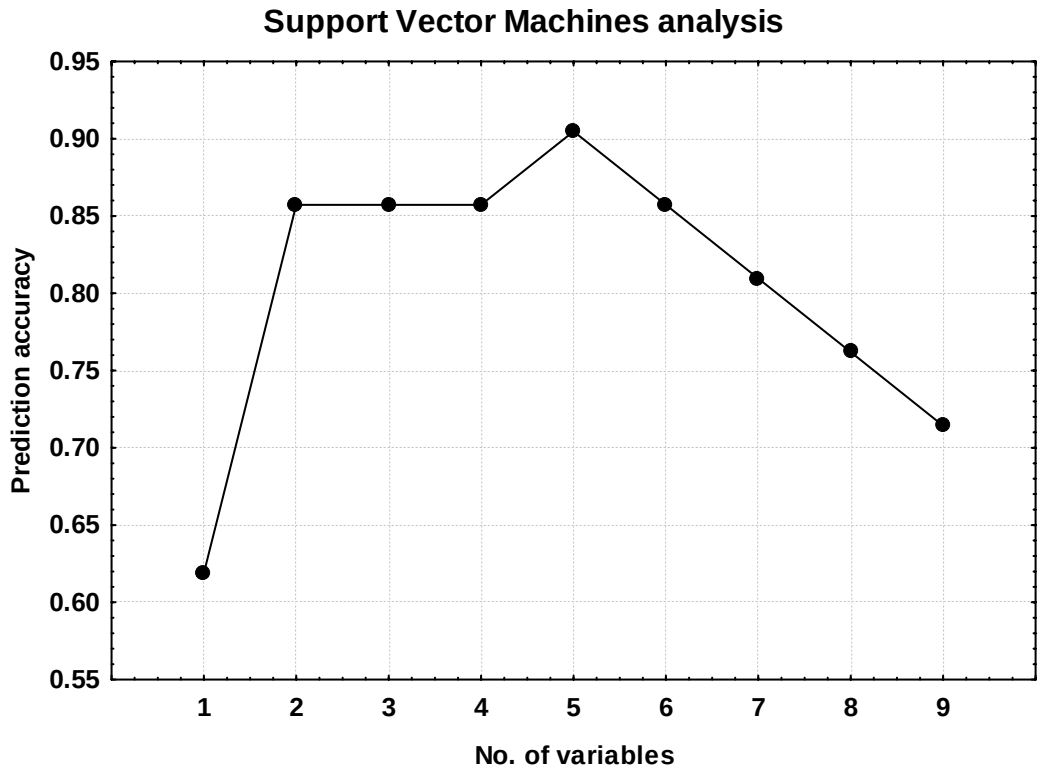
Support Vector Machines analysis
Prediction accuracy
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
1
2
3
4
5
6
7
8
9
No. of variables

Figure 3.6: Support Vector Machines analysis of phenotyping variables for the prediction of treatment response as defined by week 8 sputum culture.

The graph shows the overall prediction accuracy versus the number of variables included in the analysis. The two variables that gave a prediction accuracy of 0.85 were absolute $CD3^{dim}$ NKT cells and absolute NK cells. The analysis was performed by Dr Martin Kidd.

3.3.6 $CD3^{dim}/CD56^{+}$ NKT cells produce IFN-γ and IL-4

To assess functional aspects of $CD3^{dim}/CD56^{+}$ NKT cells an analysis was done of flow cytometric data of intracellular IFN-γ and IL-4 measurements in saponin-permeabilized T cells after a 4-hour stimulation of whole blood with anti-CD3 mAb, described in detail in Chapter 4. In samples from patients with a prominent $CD3^{dim}/CD56^{+}$ NKT cell population these cells were discernible in the CD3-PerCP versus side scatter plots used for gating the T lymphocytes. The $CD3^{dim}$ and $CD3^{bright}$ cells were analyzed separately in all diagnosis blood samples that had high numbers of $CD3^{dim}$ T cells. A CD56 mAb was not routinely used in the intracellular cytokine determinations but was included on two occasions and these analyses showed that approximately 90% of the $CD3^{dim}$ T cells were $CD56^{+}$. IFN-γ was only produced by some of the patients and the $CD3^{dim}$ and $CD3^{bright}$ cells produced comparable low levels of this cytokine (Fig. 3.7B,C). All patients showed IL-4 production by both stimulated and unstimulated T cells and this tended to be higher in $CD3^{dim}$ T cells (Fig. 3.7D,E). The $CD3^{dim}$ population contains more cells that express active caspase-3, an indicator of apoptosis, and this expression correlates with higher levels of intracellular IL-4 (Fig. 3.7F,G). It is however not known if these cells are also $CD56^{+}$.

Figure 3.7

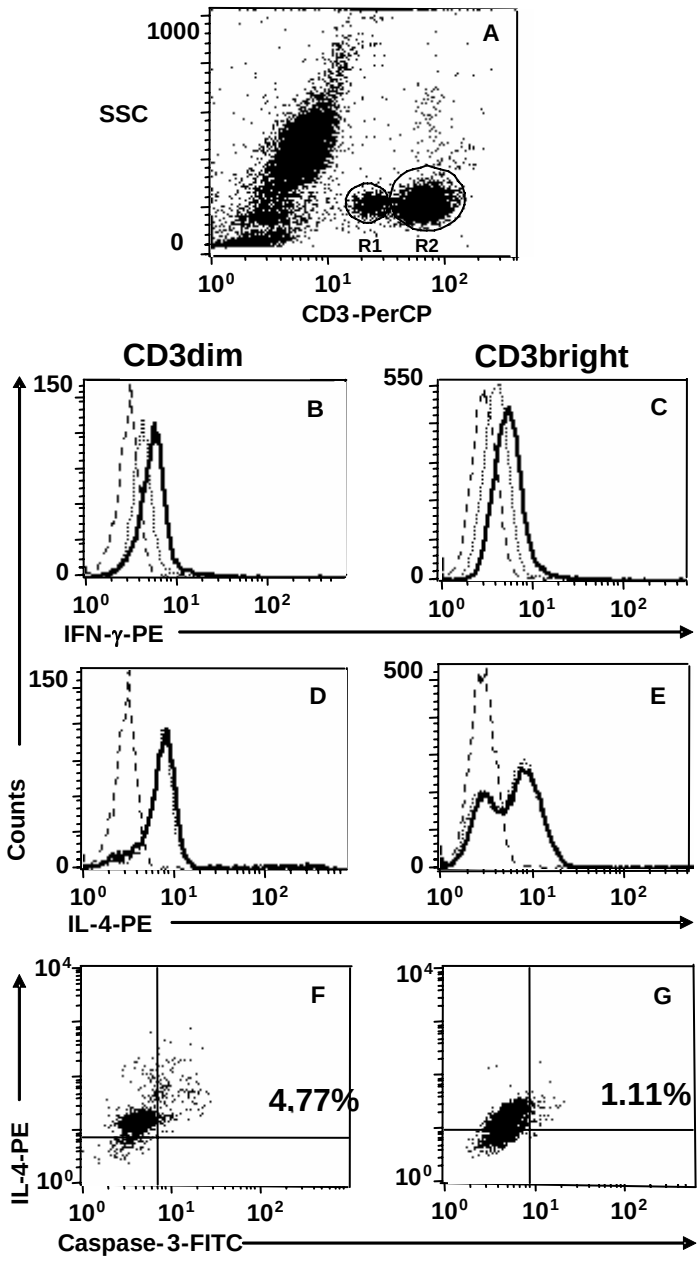
A
1000
SSC
0
R1
R2
10^0
10^1
10^2
CD3-PerCP
CD3dim
CD3bright
B
C
150
550
0
IFN-γ-PE
D
E
500
Counts
IL-4-PE
F
G
10^4
4.77%
1.11%
Caspase-3-FITC

Figure 3.7: Intracellular cytokine analysis of permeabilized lymphocytes from whole blood.

Blood from two patients at diagnosis was incubated for 4 hours with or without stimulation with 0.1μg/ml anti-CD3 and the lymphocytes were permeabilized with saponin and labelled with mAbs as described in Materials and Methods of Chapter 4. A: Gating of $CD3^{dim}$ (R1) and $CD3^{bright}$ (R2) T cells in a CD3-PerCP versus SSC plot of leukocytes from the first patient. B-E: Histograms of the gated cells showing IFN-γ (B, C) and IL-4 (D, E) expression. Overlaid histograms are: (▬) stimulated, specific antibody, (·····) unstimulated, specific antibody, (----) stimulated, control antibody. F-G: Dot plots of similarly gated unstimulated T cells from the second patient showing co-expression of caspase-3 and IL-4. The position of the quadrant markers was determined by a PE-labelled control antibody (not shown).

3.4 DISCUSSION

The data in this chapter show significant changes in absolute numbers of neutrophils, monocytes and lymphocyte subsets during active TB. That these changes occur already during the first weeks of treatment is important as it strongly suggests that TB patients tested at different time points during their treatment should not be grouped together in the analysis of results. A $CD3^{dim}/CD56^{+}$ subset of NKT cells was found to be more prominent in TB patients and correlates with a faster treatment response. A multivariate classification technique identified $CD3^{dim}/CD56^{+}$ NKT cells, in combination with NK cells, at diagnosis as variables indicating the likelihood of culture conversion early during TB treatment. NKT cells have, to my knowledge, not been reported in the context of TB disease and the reported findings support the future inclusion of these cells in the search for surrogate markers for treatment response.

The interesting subset of NKT cells that was detected expressed CD56 and reduced levels of CD3 and was either double negative (DN) or weakly $CD8^{+}$. NKT cells, which express CD3 and to a variable degree the NK cell markers CD56, CD57 and CD161 [25-27], are a heterogeneous population in mice and humans with several subsets that differ in phenotype, TCR repertoire, MHC restriction and cytokine profile, as reviewed in [27]. “Classical” NKT cells express an invariant TCR with Vα24 (Vα14Jα281 in the mouse, now Vα14-Jα18), are CD1d restricted and express the NK cell marker CD161 or NKR-P1A. Two subsets of non-classical NKT cells do not express this invariant TCR. Human $CD56^{+}$ NKT cells are abundant in the liver, are predominantly $CD8^{+}$ or DN and Vα24 TCR-negative, have cytotoxic capacity and produce Th1 and Th2 cytokines when stimulated *in vitro* [28].

As the detection of the $CD3^{dim}/CD56^{+}$ NKT cells was unexpected, a Vα24 antibody was not routinely included in the panel but some additional phenotyping with this antibody indicated that these cells did not express the invariant TCR (not shown). The possibility of artefactual $CD3^{dim}$ staining of NK cells due to non-specific binding to Fc receptors must be considered but this is unlikely as the antibodies to CD4, CD19 and γδTCR were of the same (IgG1) isotype and did not stain the cells. Furthermore, NK cells do not express the high affinity Fcγ receptors CD32 and CD64 and can be seen as a clearly CD3-negative population in Fig. 4D.

The reduced expression of CD3 could be the result of TCR downregulation [29] and the $CD3^{dim}/CD56^{+}$ NKT cells could be an activated subset of $CD3^{bright}/CD56^{+}$ NKT cells, but only a weak inverse correlation between the percentages of these NKT cell subsets (Spearman correlation coefficient -0.34, not shown) was found. Takayama et al [30] demonstrated that a $CD122^{+}$ subset of human $CD8^{+}$ T cells with intermediate TCR expression in the peripheral blood that produce high levels of IFN-γ and are also potently cytotoxic.

Peripheral blood $CD56^{+}$ T cells are increased during the early phase of *Plasmodium falciparum* or *Plasmodium vivax* infections in humans [31], suggesting an important role in the immune response to intracellular pathogens. Slifka et al. [32] found that 90% of virus-specific $CD8^{+}$ and $CD4^{+}$ T cells from choriomeningitis virus-infected mice co-express one or more NK cells markers for more than 500 days post-infection. In the patients of our study not much variation was found in the percentages of $CD3^{dim}/CD56^{+}$ NKT cells over time and they could represent a similar persistent population specific for mycobacterial antigens.

The observation of the often higher numbers and percentages of $CD3^{dim}/CD56^{+}$ NKT cells in patients indicates that this cell population is expanded in

the blood of some TB patients, and that these patients are able to clear the infection more efficiently after the initiation of chemotherapy. As $CD3^{dim}/CD56^{+}$ NKT cells appear to produce variable IFN-γ and IL-4, it can be postulated that they are cells that have been activated, as could be indicated by their reduced CD3 expression, and are at variable stages between activation and apoptosis. This is supported by the finding that they contain a higher percentage of cells expressing active caspase-3 and that they produce more intracellular IL-4. Previous findings have associated intracellular IL-4 expression in lymphocytes with mitochondrial apoptosis markers [33]. Therefore $CD3^{dim}/CD56^{+}$ NKT cells could be indicators of an active immune system in TB patients and would accelerate clearance of the infection by antibiotics.

The other variable that, together with $CD3^{dim}/CD56^{+}$ NKT cells, had predictive value according to the multivariant discriminative analysis, was the absolute NK cell count. Interestingly, a higher NK cell count is partially indicative of a slow response to treatment. A higher NK cell count in the peripheral blood may be the result of an inability of these cells to migrate into infected tissues. In humans NK cells are present in tuberculous pleural effusions [34] and in mice infected with Mtb NK cell numbers in the lung increase over the first 21 days of infection although their removal does not affect host resistance. A role of NK cells in the control of TB has been suggested by the results of *in vitro* studies with human NK cells and Mtb-infected monocytes [35-37].

Monocytes/macrophages are important components of the innate immune response to mycobacterial infections and the dramatic change in the absolute monocyte counts in the patients between diagnosis and week 26 should be noted. The surprising finding here is that their numbers are significantly depressed in fully

treated patients and it is unknown what causes this depressed absolute monocyte count.

To determine whether the depressed absolute monocyte, NK cell and $CD3^{bright}/CD56^{+}$ NKT cell counts at the end of treatment could contribute to increased susceptibility to TB relapse [10], phenotyping needs to be performed on larger numbers of blood samples taken after cessation of antibiotic treatment with subsequent long-term clinical follow up.

A drawback of this study is that the patient numbers in the two treatment response groups are small and therefore the accuracy of the statistical classifications is limited. It is also not optimal that, for logistical reasons, the week 26 blood samples were taken on the day of the last dose of antibiotics and not after cessation of drug therapy. It is unknown whether drug treatment directly affects cell counts.

In summary, peripheral blood white cell counts change rapidly during treatment and some counts at diagnosis hold promise as surrogate markers of treatment response. Further prospective studies with larger numbers of patients are now needed to evaluate the role of immunophenotyping in general and of $CD3^{dim}/CD56^{+}$ NKT cells specifically, including their functional characterization. The role of these cells in predicting differential outcomes at month six and the development of recurrence after cure needs to be assessed.

4. CHANGES IN THE KINETICS OF INTRACELLULAR IFN-γ PRODUCTION IN TB PATIENTS DURING TREATMENT

4.1 Introduction

Despite the ongoing global TB problem and extensive research into protective immunity against this intracellular pathogen, mechanisms of protective immunity against *Mycobacterium tuberculosis* (Mtb) in humans have yet to be fully clarified. Immunological parameters that contribute to TB susceptibility remain unclear and markers of treatment response phenotypes are still elusive.

Numerous reports have addressed the potential immunological defect(s) in infected individuals that have developed active TB in comparison to those who have remained healthy in spite of infection. As IFN-γ is required for a Th1 immune response to Mtb infection, this cytokine has been measured *ex vivo* in serum [38], bronchoalveolar lavage fluids [39] and pleural effusions of TB patients [40] or in culture supernatants of lymphocytes isolated from these body fluids and stimulated *in vitro* with mycobacterial antigens. In the majority of the latter studies secreted IFN-γ was measured by ELISA, less frequently by ELISPOT, and in some cases intracellular IFN-γ was measured by flow cytometry (FC). The results of these studies have varied considerably. Of 33 such studies using mycobacterial antigen stimulation of isolated lymphocytes or whole blood, 13 found that patients produced less IFN-γ than controls, 13 found that they produced more, and 7 found no difference (summarized with assay variables in Tables 4.1-3). Another paradox is added by findings of high IFN-γ levels in serum [38], pleural fluids [40,41] and lungs

[39] of TB patients. There is therefore still a need for more work to clarify these discrepant results.

Table 4.1: Reports that found IFN-γ production lower in patients compared to controls

Reference	Assay	Stimulant	Incubation	Serum	Endotoxin
[42]	ELISA	Mtb	4 days	human	?
[43]	ELISA	Mtb 10,30,38, 65kDa Ag	4 days	human	50pg/ml
[44]	ELISA	30kDa Mtb Ag	48 hrs	FCS	?
[45]	FC	Mtb	48 hrs	human	?
[46]	ELISA	H37Ra Mtb	4 days	FCS	<10pg/ml
[47]	FC ELISA	PPD,Mtb Ag85	4 days	FCS	?
[48]	ELISA	Mtb 30,32kDa Ag	4 days	FCS	<1.5pg/ml
[49]	ELISA	Mtb	4 days	human	?
[50]	ELISPOT	Mtb	4 days	human	?
[51]	ELISA	PPD	4 days	FCS	<0.1pg/ml
[52]	FC	BCG	6 days	autologous plasma	?
[38]	ELISA	Mtb	5 days	human	?
[53]	ELISA	CFP10	5 days	whole blood	?

Table 4.2: Reports that found IFN-γ production higher in patients compared to controls

Reference	Assay	Stimulant	Incubation	Serum	Endotoxin
[54]	ELISA	ESAT-6 PPD	5 days	human	?
[55]	ELISPOT	ESAT-6 H37Ra lysate	48 hrs	?	?
[56]	ELISA	Mtb sonicate	4 days	human	?
[57]	ELISPOT	ESAT-6	12 hrs	FCS	?
[58]	ELISA	ESAT-6, PPD	24 hrs	whole blood	?
[59]	ELISA	ESAT-6	5 days	autologous plasma	?
[60]	FC	30kDa Mtb Ag	6 days	FCS	?
[61]	ELISA	TB27.4 Mtb Ag	4 days	human	<1pg/ml
[62]	ELISPOT	ESAT-6	1 and 6 days	autologous plasma	?
[63]	ELISPOT	PPD	24 hrs	calf serum	?
[64]	FC	ESAT-6	6 hrs	whole blood	?
[65]	ELISPOT	ESAT-6 peptides	40 hrs	FCS	?
[66]	ELISA	Mtb 9.8,39A,40 Ag85B	6 days	human	<5pg/ml

Table 4.3: Reports that found no difference in IFN-γ production in patients and controls

Reference	Assay	Stimulant	Incubation	Serum	Endotoxin
[67]	ELISPOT	Mtb extract	4 days	FCS	?
[59]	ELISA	PPD, Mtb Ag85	5 days	autologous plasma	?
[60]	ELISA	30kDa Mtb Ag, Mtb extract	6 days	FCS	?
[68]	ELISA	ESAT-6 and peptides	3 days	human	?
[69]	ELISA	10, 30, 85A, 85B Mtb Ag	5 days	autologous plasma	negative
[65]	ELISPOT	ESAT-6	40 hrs	FCS	?
[66]	ELISA	Mtb culture filtrate, cell wall	6 days	human	?

4.2 MATERIALS AND METHODS

4.2.1 Patients and controls

The same 21 patients described in Chapter 3 were followed up at the same time points and compared to the same controls. Bloods from an additional 6 HIV-negative patients at diagnosis and 4 HIV-negative, skin test-positive additional controls were used for the IFN-γ kinetics experiments

4.2.2 Reagents

Fluorochrome-labelled mAbs CD3-PerCP, CD8-FITC, and IFNγ-PE (clone 4S.B3) were from BD-Bioscience. Anti-CD3 mAb for stimulation was OKT3 in the form of hybridoma medium (hybridomas obtained from ATCC), diluted in tissue culture medium; the concentration of mouse immunoglobulin determined by ELISA. RPMI 1640 medium was from Gibco-BRL, saponin and Brefeldin A from Sigma, polyethylene glycol 4000 from Merck and purified protein derivative (PPD) was from Weybridge Veterinary Institute (UK). A stock solution of 2mg/ml in PBS (endotoxin <0.125EU/ml) was diluted in RPMI 1640 for adding to cultures to a final concentration of 3μg/ml. Heat-killed Mtb for stimulation was prepared from H37Rv strain of Mtb cultured in 7H9 medium. The bacteria were washed twice in 0.01% Tween 80 in saline, resuspended in Tween/saline, heated in a heating block preheated to 101°C for 20 minutes and frozen at -80°C in aliquots. After thawing the stock was diluted in RPMI 1640 medium and added to cultures to give a final concentration of $5x10^5$ cfu/ml.

4.2.3 Intracellular cytokine determination

In a pilot study and subsequent optimization experiments for this study clumping of cells was consistently observed in whole blood from patients at diagnosis after stimulation with live Mtb or PPD in overnight culture. The light scatter properties of the leukocytes in these cultures were abnormal, the cell number being reduced and cell debris increased compared to those of control individuals and patients that had received treatment (not shown). These observations suggested that there may be rapid cell death in blood cultures from patients at diagnosis when stimulated with mycobacterial antigen and possible loss of the very cells that were to be analyzed by flow cytometry. Soluble anti-CD3 mAb was therefore used as stimulus and intracellular IFN-γ production could be detected in T cells after a four-hour stimulation with no change in light scatter or clumping of cells obtained from patients at diagnosis.

Blood was collected in sodium heparin tubes and processed within 3 hours of venesection. For the stimulation, 750µl of whole blood was mixed with 750µl RPMI 1640 with bicarbonate, 25mM HEPES, penicillin/streptomycin and 50µM 2-mercaptoethanol (RPMI+) in 12ml round-bottom polypropylene tubes. After 30mins in a 37°C water bath, OKT3 mAb was added to a final concentration of 0.1µg/ml. Incubation was continued for another 4 hours, with 10µg/mL Brefeldin A (BFA) present during the last 3 hours to stop secretion of cytokines.

As commercial RBC lysing solution was previously found to be ineffective with blood from TB patients at diagnosis and the commercial intracellular cytokine labelling kit protocol was too long for the restricted working conditions in the category 3 containment TB laboratory, a rapid method without fixation of cells was

used, based on a method described by Holmes et al [70] for the labelling of intracellular antigens. It was modified for use with whole blood and simultaneous cell surface staining by reducing the saponin concentration from 0.3% to 0.05% and by the addition of 3% w/v PEG 4000 to the saponin buffer to prevent clumping and loss of leukocytes in TB patients' diagnosis blood samples. As this concentration of PEG has also been shown to enhance the formation of antigen/antibody complexes [23], it did not interfere with the labelling of cellular antigens with monoclonal antibodies.

After incubation/stimulation the blood was diluted to 20ml with cold PBS containing 0.05% saponin, and 3% PEG (lyse/wash buffer) and centrifuged in the cold at 700g after RBC lysis was complete. The cell pellet was resuspended in 500µl of 0.1% bovine serum albumin (BSA) in lyse/wash buffer, 100µl aliquots of the suspension were added to premixed antibodies (CD3-PerCP, CD8-FITC and IFNγ-PE (or control mAb)) and left for 20 minutes in ice water. After one wash with lyse/wash buffer at 4^{o}C, the cell pellets were suspended in cold, freshly diluted 4% formaldehyde in PBS and stored at 4°C in the dark until flow cytometric analysis the following day on a Becton-Dickinson (BD) FACS Calibur® with CellQuest software®.

T lymphocytes were gated in a CD3-PerCP versus SSC plot (50 000 events in this gate were acquired) and CD8-positive and –negative cells were then gated in a CD3-PerCP versus CD8-FITC plot of the T cells. In separate plots of IFNγ-PE versus CD8-FITC the percentage of cytokine-positive cells in the CD8-positive and CD8-negative population (assumed to be $CD4^{+}$ and henceforth referred to as CD4 T cells) were obtained from the quadrant statistics. Because the staining of the negative cells with the isotype control antibody was slightly different to that of the specific

antibody (see Fig. 4.1), the quadrant marker was first placed in the plot for the unstimulated cells and the same position was used for the plot of the stimulated cells.

4.2.4 Lymphocyte proliferation

A whole blood lymphocyte proliferation test with mycobacterial antigens as stimulus was performed in parallel with intracellular IFN-γ. These assays were done as part of the same study by Ilse Crous and Shweta Brahmbhatt, two colleagues in the Department of Biomedical Sciences at the University of Stellenbosch Faculty of Health Sciences, and the results are included here for the sake of completeness. Whole blood was diluted 1 in 10 with RPMI+. Aliquots of 180µl were dispensed into wells of 96-well round-bottom tissue culture plates with 20µl of the stimulants PPD (3µg/ml final concentration) or heat-killed Mtb ($5x10^5$cfu/ml final concentration) and incubated for 6 days at 37°C in 5% carbon dioxide. Tritiated (^3H) thymidine was then added at 0.5µCi/well and the plates incubated for another 6 hours before harvesting with a custom-built harvester onto glass fibre discs which were counted in a scintillation counter. The medians of quadruplicate counts per minute (cpm) of unstimulated cultures were subtracted from the medians of stimulated cultures.

4.2.5 Statistical analysis

Data for patients at diagnosis and at the end of treatment were analyzed for significant differences from those for healthy subjects by means of the Mann-Whitney test. The patients were divided into two groups according to whether they were sputum culture-positive (slow responders) or -negative (fast responders) after 8

weeks of treatment and data for patients in these two groups were also compared by means of the Mann-Whitney test. The Spearman correlation coefficient was determined to test for correlation between two data sets (*: p=0.01-0.05, **: p=0.001-0.01, ***: p<0.001).

4.3 RESULTS

4.3.1 Intracellular IFN-γ

The unstimulated blood samples mostly contained insignificant numbers of IFNγ-positive T cells at or near background staining which varied around approximately 50 events or 0.2%. These insignificant percentages were not subtracted from those in the stimulated cultures. Irrelevant mouse monoclonal antibody (hybridoma supernatant) did not stimulate IFN-γ production and was not routinely added to unstimulated control cultures. Fig. 4.1 is a representative flow cytometric analysis plot of the lymphocytes in the blood from a patient at diagnosis, labelled with mAbs to IFN-γ, CD3 and CD8.

Figure 4.1

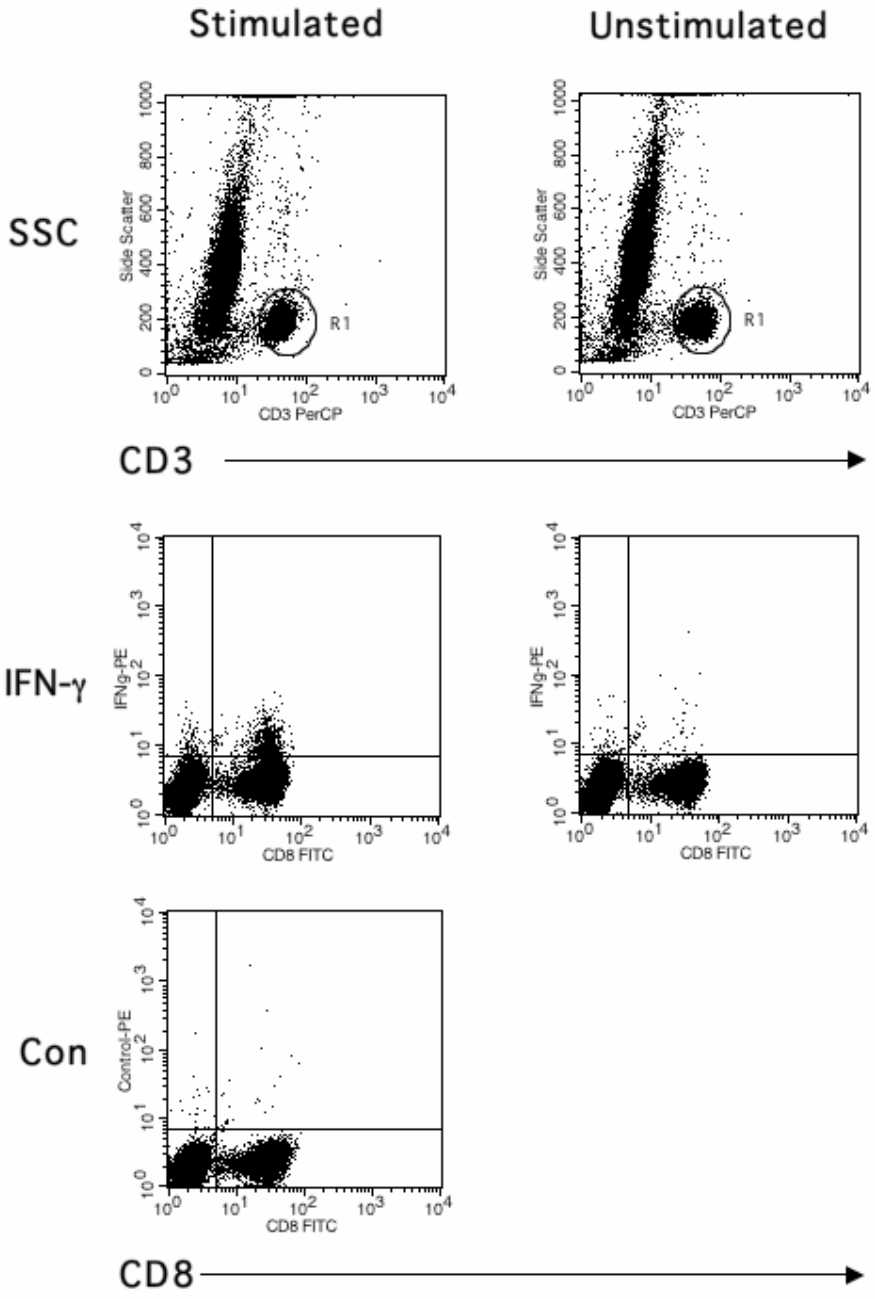
Stimulated
Unstimulated
SSC
Side Scatter
R1
CD3 PerCP
CD3
IFN-γ
IFNg-PE
CD8 FITC
Con
Control-PE
CD8

Figure 4.1: Representative flow cytometric data of intracellular IFN-γ staining of leukocytes from whole blood.

T lymphocytes gated in a CD3-PerCP versus SSC plots (region R1) and the CD8-FITC and IFN-γ-PE staining of the gated cells is shown. To obtain the percentages of IFN-γ^+ cells in the CD8 and CD4 subpopulations, the cells were gated further as described in Materials and Methods (not shown). The quadrant markers of the unstimulated cells were used as a reference for demarcating IFN-γ-negative and -positive cells in the stimulated samples.

Patients' blood samples at diagnosis contained very variable percentages (up to 13%) of CD8 IFN-γ-producing T cells (Fig. 4.2A), which were significantly higher than in control subjects ($p<0.001$). In the week 1 blood samples the percentages of IFN-γ-producing cells were already reduced and decreased to normal levels at week 26 when treatment was completed.

Numbers of IFN-γ^+ CD4 T cells were lower (Fig. 4.2B) but there was still a significant difference ($p<0.01$) between the percentages in patients' blood at diagnosis and control subjects and the numbers in patients decreased from week 1 onwards similar to those in the CD8 population and at week 26 there was no significant difference between the percentages in patients and controls. There was a significant correlation between the percentages of IFN-γ^+ cells in the CD4 and CD8 T cell populations at diagnosis (Spearman correlation coefficient 0.6149, $p<0.01$, not shown). The decrease in the percentage of IFN-γ^+ CD4 and CD8 T cells over time mirrored that of the decrease in bacterial load in the patients as indicated by the increase in time to positivity (TTP) of their sputum cultures (Fig. 2.1).

Figure 4.2

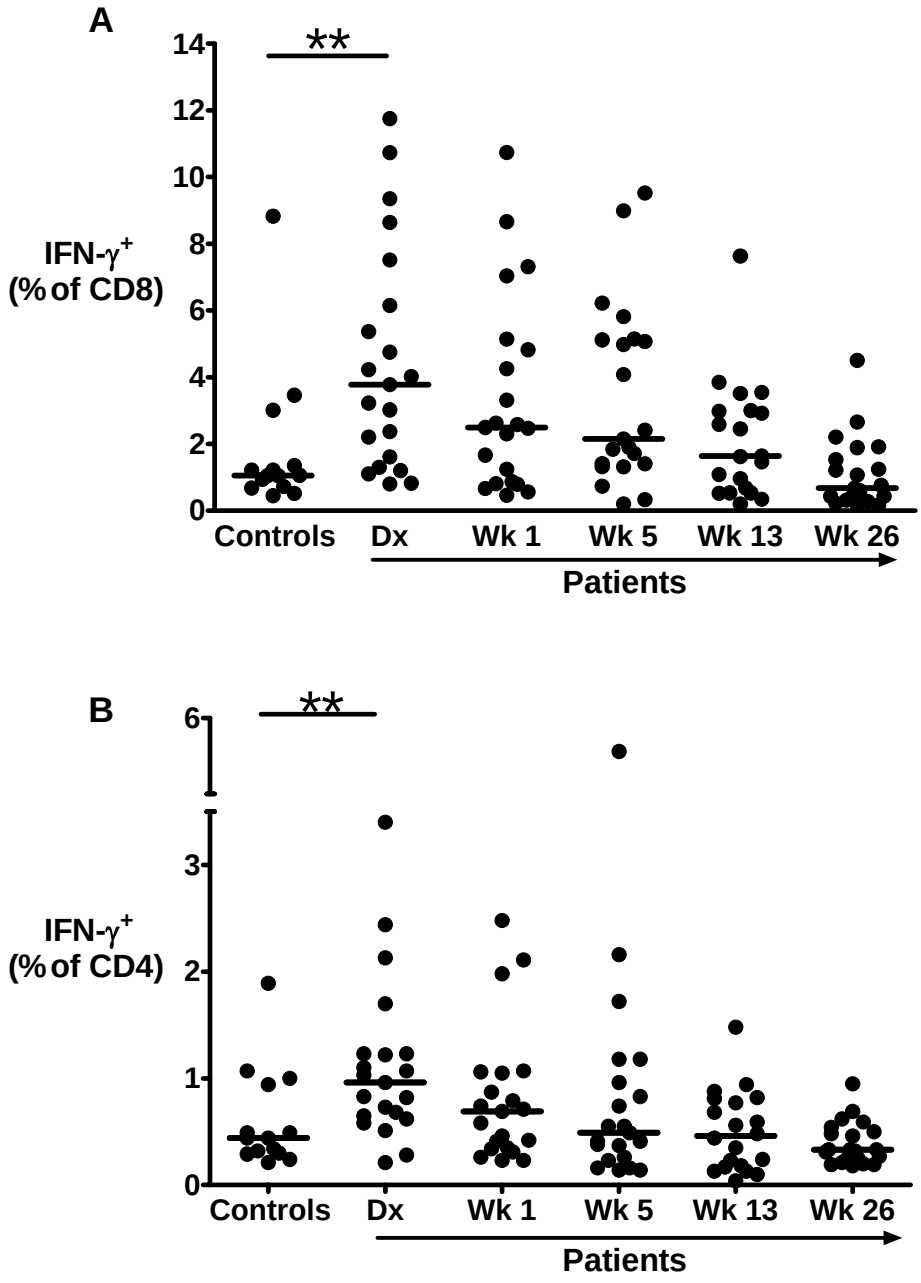
A
**
14
12
10
8
6
4
2
0
IFN-γ+
(% of CD8)
Controls
Dx
Wk 1
Wk 5
Wk 13
Wk 26
Patients
B
**
6
3
2
1
0
IFN-γ+
(% of CD4)
Controls
Dx
Wk 1
Wk 5
Wk 13
Wk 26
Patients

Figure 4.2: Intracellular IFN-γ expression in T cells of TB patients and healthy skin test-positive control subjects after 4 hours stimulation of whole blood with anti-CD3 mAb.

IFN-γ^+ CD8 T cells (A) and IFN-γ^+ CD4 T cells (B), expressed as % of gated CD8 and CD4 cells respectively. The gating is described in Materials and Methods. Time points for patients are in weeks from diagnosis until treatment end at week 26. Horizontal lines represent median values for each set of data. Percentages in control subjects and patients at diagnosis were compared by means of a Mann-Whitney test (*: p=0.01-0.05, **: p=0.001-0.01, ***: p<0.001)

4.3.2 Kinetics of IFN-γ production

The above findings of non-antigen-specific increased IFN-γ production during active TB are in agreement with those of others who have reported increased IFN-γ production in response to stimulation with various mycobacterial antigens by means of ELISA, ELISPOT or flow cytometry (FC), as shown in Table 4.2. However, a large number of studies have found the opposite or no difference (Table 4.1 and 4.3 respectively).

One of the reasons for this discrepancy could be the kinetics of the IFN-γ production. For logistical reasons, and to avoid *in vitro* artefacts, a short incubation of 4 hours with stimulant was chosen, whereas most other protocols used overnight or longer incubations. If the kinetics of IFN-γ production in patients at diagnosis were faster than in control subjects, measurement and comparison of IFN-γ production at different time points would lead to markedly different results. Increasing production in one group may coincide with declining levels in another group. To substantiate this hypothesis, blood of patients and controls was stimulated for 4 hours and 8 hours with anti-CD3 antibody and intracellular IFN-γ production was determined at both time points. BFA was added to both cultures for the last 3 hours of incubation. Patients' bloods taken at week 26 after completion of treatment were tested as well, although the patients tested at diagnosis and at week 26 were not the same individuals.

The results (Fig. 4.3A) show a substantial drop in IFN-γ^+ CD8 T cells from 4 hours to 8 hours in 5 of the 7 patients tested at diagnosis. In the blood samples taken at week 26 and in healthy controls no clear kinetic pattern was observed. IFN-γ production in healthy controls could not be determined after overnight or longer

stimulation due to severe down regulation of CD3, which would prevent gating of T cells. The kinetics of IFN-γ production by CD4 T cells (Fig. 4.3B) was variable, with percentages of IFN-γ^+ cells higher after 8 hours than after 4 hours in half the patients tested at diagnosis. Fig. 4.3 shows that there is variation in the kinetics of IFN-γ production not only between groups of patients and controls but also between individuals in each group and the results obtained would therefore depend on the chosen stimulation time.

Figure 4.3

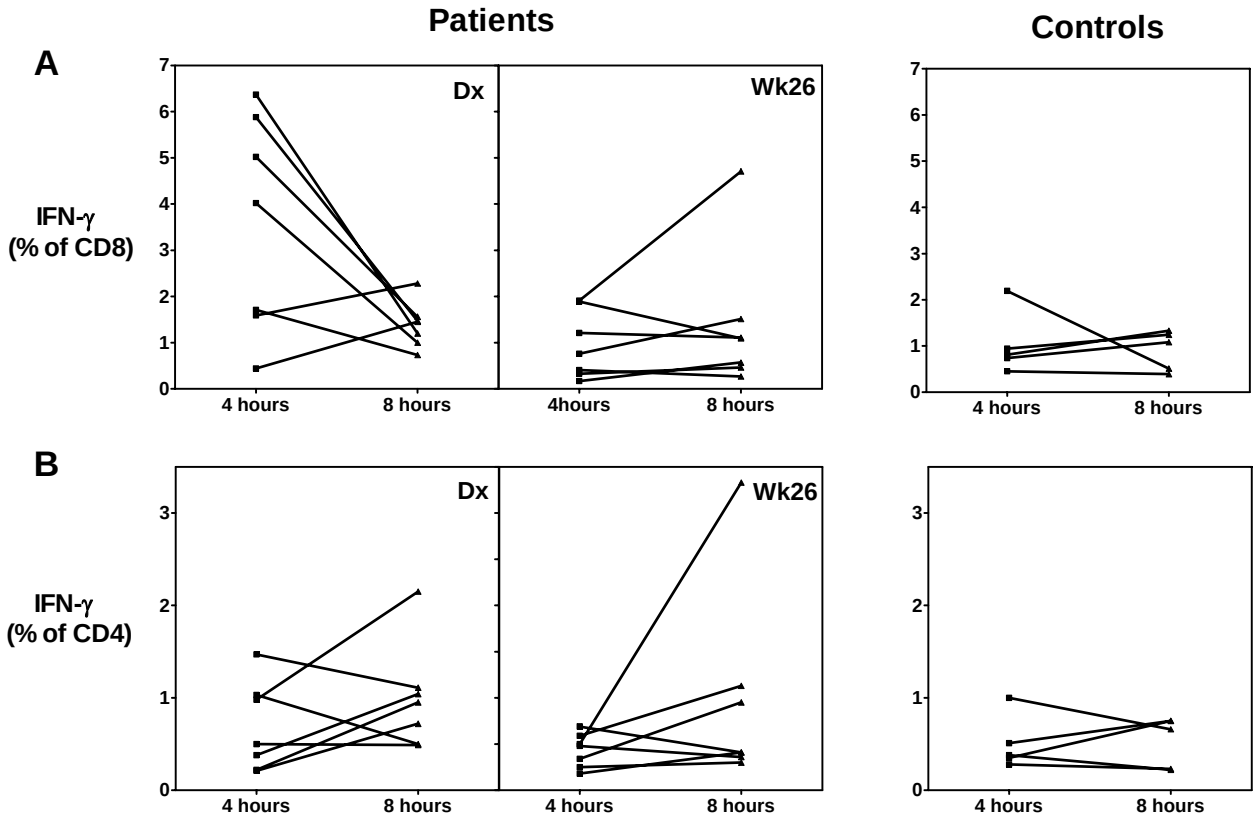
Patients
Controls
A
IFN-γ
(% of CD8)
Dx
Wk26
4 hours
8 hours
4hours
8 hours
B
IFN-γ
(% of CD4)
Dx
Wk26
4 hours
8 hours
4 hours
8 hours
4 hours
8 hours

Figure 4.3: Kinetics of IFN-γ production by CD8 and CD4 T cells in TB patients and controls during the first 8 hours of stimulation.

Whole blood was stimulated for 4 hours or 8 hours with anti-CD3 mAb, of which the last 3 hours was in the presence of BFA, and intracellular IFN-γ was labelled as in Materials and Methods. Kinetics in patients at diagnosis are compared with those in patients at the end of treatment (not the same individuals) and with healthy skin test-positive controls in CD8 T cells (A) and CD4 T cells (B).

4.3.3 Lymphocyte proliferation

These tests were performed by Ilse Crous and Shewta Brahmbhatt as part of this study and I gratefully include them here as the results help to explain the other findings.

^{3}H-thymidine uptake of lymphocytes stimulated in whole blood with heat-killed Mtb was significantly depressed in patients at diagnosis relative to controls (Fig. 4.4A, $p<0.05$). Patients' counts then increased rapidly to above normal during weeks 5-13 of treatment, when they generally became sputum culture-negative, and again fell to normal levels at the end of treatment. As this assay was a whole blood assay and it was also found that patients' lymphocyte counts were depressed at diagnosis relative to control subjects (Chapter 3) the ^{3}H-thymidine uptake of 20µl blood was normalized to $2x10^{4}$ T cells, calculated from the total white cell count, differential count and CD3 immunophenotyping. The normalized counts followed a similar pattern during the follow-up period (not shown) but the difference in counts between control subjects and patients at diagnosis was no longer significant ($p=0.083$). Overall, while lymphocyte proliferation increased during treatment the percentages of IFN-γ-producing T cells decreased (as shown in Fig. 3.2), together with the reduction of bacterial load as indicated by a longer TTP (Fig. 2.1). In individual patients there was no significant inverse correlation between lymphocyte proliferation and intracellular IFN-γ but the TTP at week 4 correlated significantly with lymphocyte proliferation at the week 5 time point (Fig. 4.4B). This correlation was significant when ^{3}H-thymidine uptake was expressed as median cpm per 20µl of blood (Spearman $r=0.4455$, $p< 0.05$) or as cpm normalized to $2x10^{4}$ T cells ($r=0.4576$, $p< 0.05$). When PPD was used as stimulant in the cultures, there was no

difference in ^{3}H-thymidine uptake between patients at diagnosis and control subjects and very little change in the counts during the follow-up period (not shown).

Figure 4.4

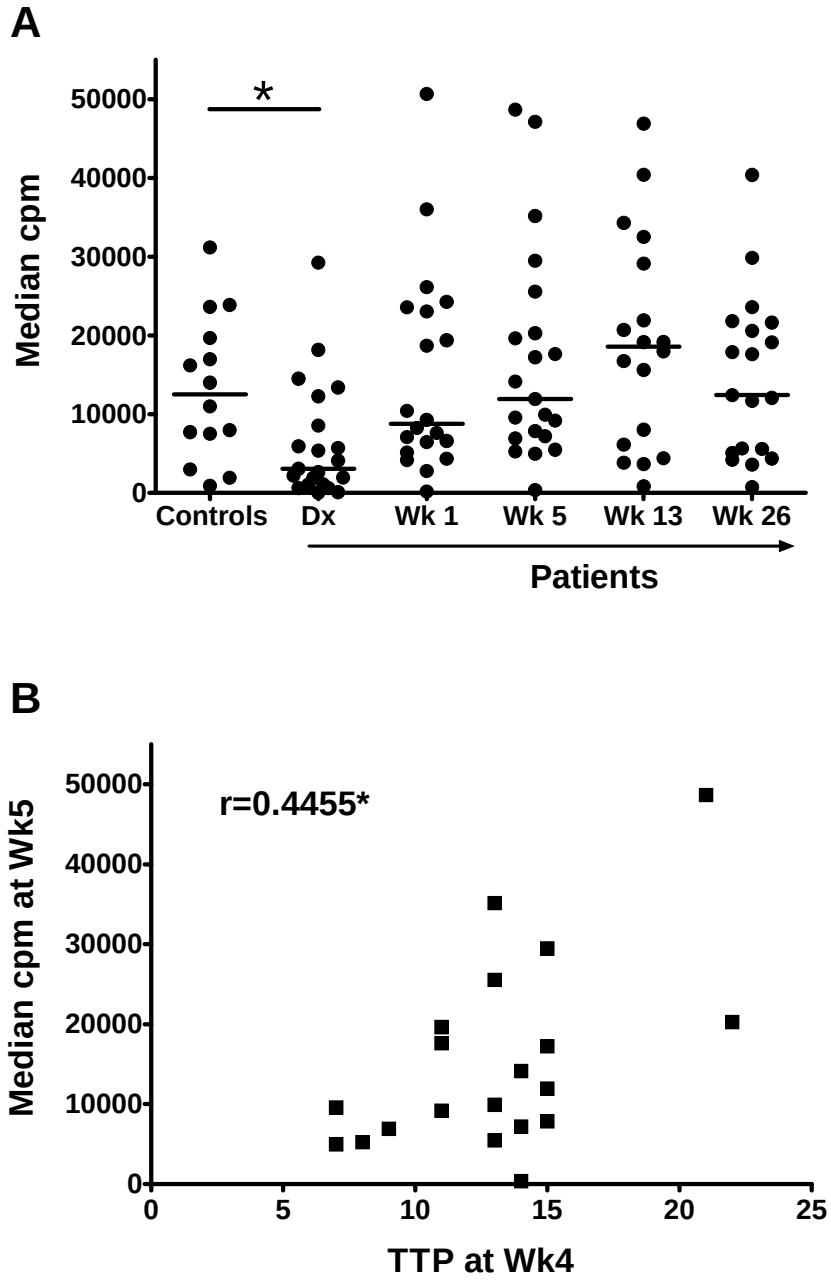
A
Median cpm
50000
40000
30000
20000
10000
0
*
Controls
Dx
Wk 1
Wk 5
Wk 13
Wk 26
Patients
B
Median cpm at Wk5
50000
40000
30000
20000
10000
0
r=0.4455*
0
5
10
15
20
25
TTP at Wk4

Figure 4.4: ^{3}H-thymidine uptake after 7 days incubation of whole blood with heat-killed Mtb in TB patients and healthy skin test-positive controls.

A: Median cpm for controls and patients at diagnosis and time points (weeks from diagnosis) during treatment. Horizontal lines represent median values for each set of data. Counts in control subjects and patients at diagnosis were compared by means of a Mann-Whitney test (*: p=0.01-0.05, **: p=0.001-0.01, ***: p<0.001).

B: Correlation of time to positivity (TTP) of patients' sputum cultures at week 4 with lymphocyte proliferation at week 5. TTP in days is plotted against median cpm and the Spearman correlation coefficient (r) is calculated.

4.3.4 Correlation with treatment response

In order to find possible correlations of intracellular IFN-γ production or lymphocyte proliferation with treatment response, the patients were divided into two groups according to whether they were sputum culture-positive (slow responders) or -negative (fast responders) after 8 weeks of treatment, as in Chapter 3. There was no difference between CD8 or CD4 IFN-γ production or ^{3}H-thymidine uptake at diagnosis in the two groups of patients.

4.4 DISCUSSION

Previously reported findings of IFN-γ production *in vitro* by stimulated lymphocytes of patients with TB have been contradictory and are summarized in Tables 4.1-3. Stimulation of lymphocytes in culture is subject to a large number of variables in the methodology. For example, the use of foetal calf serum (FCS), human AB serum or autologous plasma could have an effect, or the type of culture plates. Only 7 of the 33 cited studies specify the endotoxin levels in the stimulants used. Endotoxin contamination in pg/ml concentrations could stimulate the monocytes in whole blood and in isolated PBMC's and this could have secondary effects on the lymphocytes. The time in culture is another variable, which is examined in the present study.

The data reported here, which show that stimulated lymphocytes from TB patients at diagnosis make significantly more intracellular IFN-γ than those from healthy controls after 4 hours of stimulation, agree with the cited studies in Table 4.2 which used mycobacterial antigens as stimulus. Similar to our work, another study reported the use of soluble anti-CD3 mAb [71] and found similar results. These

authors assayed IFN-γ in the medium of 4-day lymphocyte cultures and showed increased levels in cultures from TB patients relative to healthy controls although they did not give comparative statistics. The focus of their study was the comparison of TB with pulmonary disease caused by non-tuberculous mycobacteria.

Some explanation of the discrepant results of previous studies may be provided by the kinetic studies included in this thesis which have shown that patients' CD8 lymphocytes at diagnosis are able to produce IFN-γ rapidly but that this production is short-lived and wanes after 8 hours. One could therefore postulate that, if IFN-γ levels were measured in culture supernatants after several days, the overall production would be low whereas in cultures from healthy subjects with a later onset of production, sustained for longer, it would be higher. It was shown here that IFN-γ kinetics show large inter-individual variation within each subject group and that the kinetics and the measured IFN-γ production change during treatment.

The lack of correlation between lymphocyte proliferation and intracellular IFN-γ that was found here supports previous findings [67]. The overall depressed lymphocyte proliferation of patients' lymphocytes at diagnosis in response to Mtb stimulation that was found by my colleagues in this study is associated with increased IFN-γ production measured after 4 hours of stimulation. These results can be explained if one postulates that the kinetics of the response to mycobacterial antigens is also accelerated, like that to non-specific TCR stimulation with anti-CD3 mAb. In that case some stimulated lymphocytes would already have gone into apoptosis by day 7, when the cell cultures are pulsed with ^{3}H-thymidine, and incorporation of the label would be reduced. This view is supported by my observations during optimization experiments of cell clumping in patients' blood at diagnosis in the presence of mycobacterial antigens in overnight culture. The finding

of increased ^{3}H-thymidine uptake with increased TTP, i.e. lower bacterial load, at week 4 could also indicate that there is reduced cell death *in vitro* with the clearance of the infection.

Increased apoptosis of T cells in TB patients has been reported previously [45,46]. Hirsch et al [46] found an inverse correlation between increased apoptosis of $CD4^{+}$ and $CD4^{-}$ T cells induced by Mtb in patients at diagnosis and IFN-γ in culture supernatants after 96 hours of culture, but there was no significant difference between treated patients and controls. Another study found a loss of IFN-γ positive $CD4^{+}$ and $CD4^{-}$ T cells in 40 hour Mtb-stimulated PBMC cultures from of TB patients relative to controls and a concomitant decrease of IFN-γ in culture supernatant as well as increased apoptosis [45]. These studies and ours suggests that pre-activated T cells are present in the peripheral blood of patients with active TB which is also supported by the finding of increased numbers of CD4 T cells expressing the activation marker CD38 in the peripheral blood of patients with active TB relative to cured patients and healthy, skin test-positive controls [15]. The presence of significant numbers of IFN-γ^{+} T cells in some of the skin test-positive control subjects in this study may be an indication that they have been recently infected with Mtb but have remained asymptomatic. However, because anti-CD3 is a non-specific stimulus, it could also mean that the T cells have been activated by another infectious agent.

In summary, the data in this chapter have shown that T lymphocytes of patients with active TB proliferate below normal levels in response to stimulation with mycobacterial antigens and have the capacity for rapid production of IFN-γ after TCR stimulation. However, the kinetics of the response tends to be accelerated relative to controls and cured patients, but not sustained. As there was wide inter-

individual variation in the kinetics of cytokine production in each group, it is difficult to recommend a single optimal time point for analysis of IFN-γ production. The detailed follow-up results also show that the measured parameters are affected by the bacterial load of the patients and change rapidly within the first weeks of treatment. The implications of the increased rapid intracellular IFN-γ responses during active mycobacterial infection need to be investigated as it is presently unclear whether this is a beneficial reaction or whether it indicates a brief and non-sustained and therefore inadequate effector mechanism.

5. INTRACELLULAR INTERLEUKIN-4 IN LYMPHOCYTES FROM PATIENTS WITH TUBERCULOSIS – EVIDENCE NECESSITATING A REVIEW OF ITS ROLE IN THE IMMUNE RESPONSE

5.1 INTRODUCTION

The Th1/Th2 Hypothesis postulates two different types of lymphocyte responses to stimulation with environmental or bacterial antigens that are defined by the cytokine production of the stimulated cells [3]. The Th1 response, characterized by, amongst other cytokines, IFN-γ production, is required in microbial infections while the Th2 response, characterized by IL-4, IL-5, IL-10, IL-13 and others, is associated with humoral responses to helminth infections and allergens. The two responses are thought to have a reciprocally inhibitory effect on each other and an imbalance could cause inappropriate immune responses. In the context of TB in humans it has been

postulated that excessive IL-4 production may inhibit the required Th1 response to the microbial infection, which has been shown experimentally in mice infected with Leishmania parasites [72].

IL-4 is a pleiotropic growth factor produced by T cells, mast cells and activated basophils but protein and/or mRNA has also been reported to be produced by neutrophils [73], alveolar macrophages [74], dendritic cells [75], and human lymphoid and myeloid cell lines [76]. IL-4 receptors have been detected on many cell types, including T and B lymphocytes, monocytes, granulocytes, fibroblasts, epithelial and endothelial cells [77] and IL-4 could be an autocrine/paracrine growth factor for these cells. Raised levels of IL-4 have been detected in cultures of T and B cells from patients with chronic lymphocytic leukaemia (CLL) thus maintaining the leukaemic cells' extended viability in culture and *in vivo* (reviewed by Kay [78]).

Levels of secreted IL-4 in cultures of stimulated T cells are low and notoriously difficult to measure in supernatants of stimulated PBMC's. Some studies have attempted to do so in the context of TB but have not been able to detect it in significant concentrations [42-44,47], whereas others have found no difference in patients and controls [68], or higher levels in patients [56,67]. Intracellular IL-4 measured by flow cytometry was found to be no different in patients and controls [64], lower in patients [47], higher in patients' CD8 T cells [52] or CD4 T cells [45].

The Th1/Th2 hypothesis has thus not been confirmed in the context of human pulmonary TB and this study attempted to find additional evidence for its validity. As the findings could not be explained with the Th1/Th2 hypothesis, some supplementary *in vitro* experimental work was conducted to find an alternative explanation for the results.

5.2 MATERIALS AND METHODS

5.2.1 Patients and controls

The 21 patients for the follow-up study and 14 healthy controls for comparison were described in Chapter 3. Blood from three additional patients at diagnosis and two additional healthy control subjects was used for the supplementary experiments.

5.2.2 Reagents

Fluorochrome-labelled mAbs anti-CD3-PerCP, CD8-FITC, IL-4-PE (clone 8D4-8), HLA-DR-FITC, HLA-DR-allophycocyanin (APC) and rabbit anti-active caspase-3-FITC were from BD-Biosciences. A rabbit control-FITC Ab is not supplied by the manufacturer. Anti-CD3 mAb for stimulation was OKT3 as in Chapter 4. RPMI 1640 medium was from Gibco-BRL, saponin, BFA, lipopolysaccharide (LPS) and protease inhibitor cocktail from Sigma. The high sensitivity IL-4 ELISA kit was from R&D Systems and the Jurkat E6.1 T cell line was from ATCC. Recombinant IL-4 was a gift from Prof Bernard Ryffel of the University of Cape Town.

5.2.3 Intracellular cytokine determination:

The stimulation of whole blood with anti-CD3 antibody and the intracellular cytokine labelling has been described in Chapter 4.

For the supplementary experiments with Jurkat cells and T cells, neutrophils and monocytes in whole blood incubated overnight with BFA, the labelling method

was modified to include a fixation step before the saponin treatment for the preservation of the light scatter of the cells: Jurkat cells or cultured whole blood was washed once with PBS and suspended in 0.1% BSA in PBS. An equal volume of 1% formaldehyde in PBS was added and after 15 minutes at room temperature the cell suspension was diluted with cold 0.1% BSA in lyse/wash buffer (see Appendix). When RBC lysis was complete, the samples were centrifuged, the supernatant aspirated and the pellet resuspended in the residual buffer. The cells were then labelled with mAbs as in Chapter 4. In the analyses of the flow cytometric data T lymphocytes were gated in a CD3-PerCP versus SSC plot (50 000 events in this gate were acquired). The IL-4 expression was bimodal and strongly IL-4-positive cells ($IL\text{-}4^{high}$) were determined in the conventional way, using quadrant markers as for the IFN-γ expression (Chapter 4). The weakly IL-4-positive cells ($IL\text{-}4^{low}$) were analysed by gating T cells as above and then CD8-positive and –negative cells in a CD8-FITC versus IL-4-PE plot. The $IL\text{-}4^{+}$ cells in these gates were then plotted as histograms. The histogram of the cells labelled with control-PE antibody was overlaid on that of the IL-4 labelled cells and scaled to the same number of gated cells. Using the dedicated software, the control histogram was subtracted from the IL-4 histogram and the number of cells in the subtracted histogram was obtained from the histogram statistics after placement of an appropriate marker.

5.2.4 Culture of Jurkat cells and preparation of cell lysate.

Jurkat cells were cultured in RPMI 1640 medium with 5% FCS and penicillin and streptomycin at 10^6/ml with 1μg/ml BFA or the equivalent concentration of the solvent dimethyl sulphoxide (DMSO) overnight. After a sample was removed for

flow cytometry they were centrifuged and the cell pellets resuspended at $30x10^6$/ml in cold lysis buffer consisting of 1.75% v/v Triton-X100 in 0.15M NaCl, 20mM sodium phosphate buffer pH 7.4 and 1% v/v protease inhibitor cocktail. After 30mins on ice the lysates were microfuged in the cold and the supernatants frozen at -80°C. Before testing in the IL-4 ELISA the lysates were diluted with an equal volume of lysis buffer without Triton-X.

5.2.5 Statistical analysis

Data for patients at diagnosis and control subjects were analyzed for significant differences from those for healthy subjects by means of the Mann-Whitney test whereas the Wilcoxon matched pairs test was used for the analysis of data for patients at diagnosis and at the end of treatment as well as data for stimulated and unstimulated cells (*: p=0.01-0.05, **: p=0.001-0.01, ***: p<0.001).

5.3 RESULTS

5.3.1 Intracellular IL-4 expression in T lymphocytes of TB patients.

T cells and their CD8-positive and negative subsets were gated as in Fig. 5.1A. A small but a distinct strongly IL-4 positive T cell population was detected in blood from patients and healthy controls which could be accurately separated from the negative population ($IL\text{-}4^{high}$, shown in Fig. 5.1B). The labelling was specific as it could be inhibited by pre-incubation of the mAb with recombinant IL-4 (comparable to that with isotype control-PE mAb, not shown). Background labelling with control

antibody was low, usually less than 50 events or 0.2% (Fig. 5.1B). The numbers of IL-4^{high} cells in the CD8 population were mostly not significant, i.e. less that 100 events, and were not analyzed. Significant numbers were however found in the CD8-negative population, referred to as CD4 T cells, as they would comprise mainly CD4^{+} T cells but could also include some double-negative and NKT cells. When comparing the IL-4 antibody-labelled cells with the control antibody-labelled cells, it was apparent that all cell samples contained a large population that expressed low levels of IL-4 (IL-4^{low}, shown in Fig. 5.1B) and overlapped with the negative population. Accurate percentages of these cells could not be obtained by the conventional method of analysis i.e. copying the quadrant marker from the control sample into the test sample, as the placement of the marker is subjective and small variations lead to large differences in the percentages of cells obtained. Therefore histogram subtraction according to the BD flow cytometry user manual was used to analyze these cells as described in Materials and Methods and shown in Fig. 5.1C. This mathematical method is absolutely objective and the results are only subject to some inaccuracy due to biological variation relating to the variable binding of the control antibody to different cell samples, which was greater in patients than in controls.

Figure 5.1

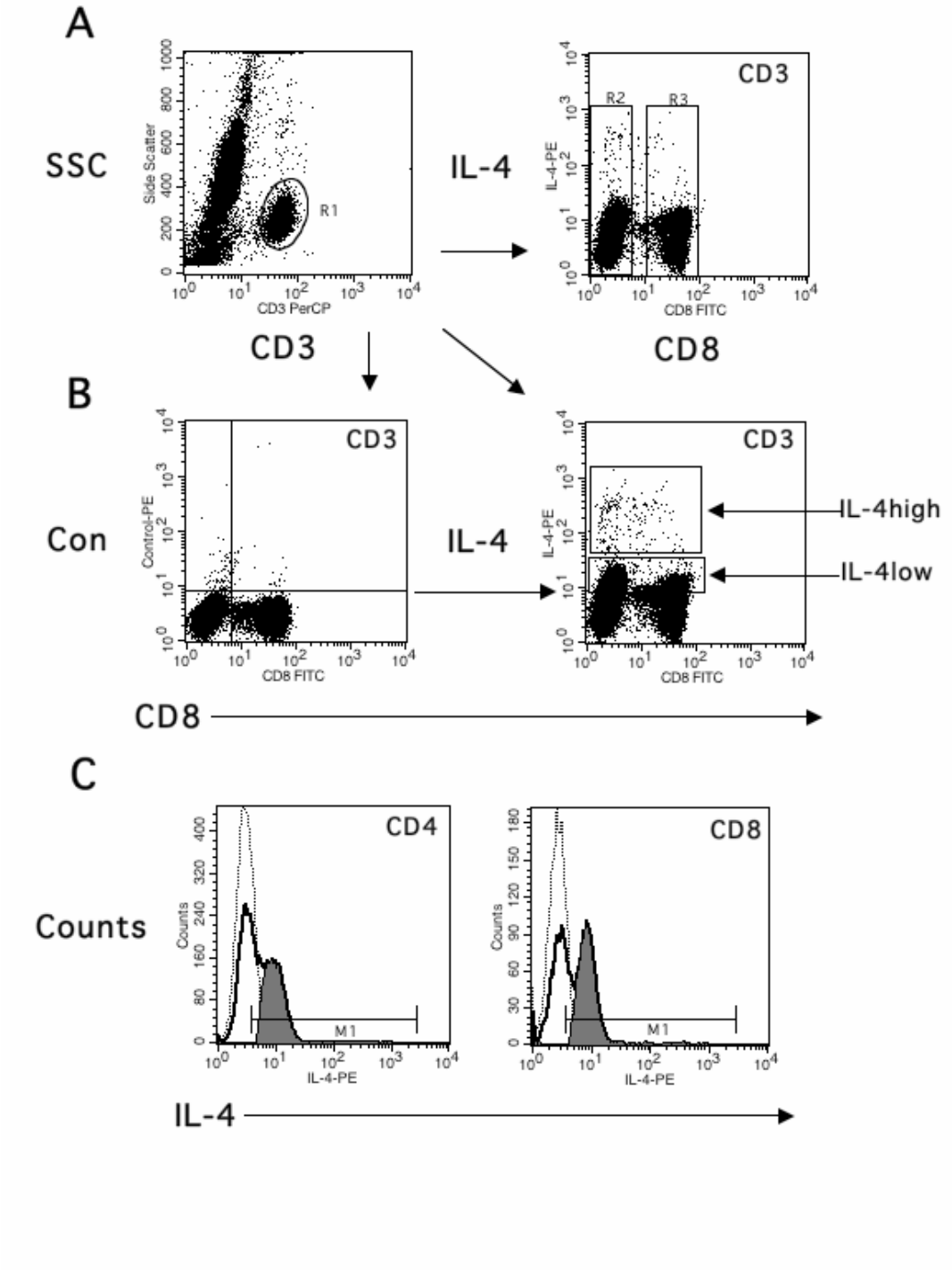
A
SSC
Side Scatter
CD3 PerCP
R1
IL-4
IL-4-PE
CD8 FITC
CD3
R2
R3
CD3
CD8
B
Con
Control-PE
CD8 FITC
CD3
IL-4
IL-4-PE
CD8 FITC
CD3
IL-4high
IL-4low
CD8
C
Counts
Counts
IL-4-PE
CD4
M1
CD8
M1
IL-4

Figure 5.1: A representative flow cytometric analysis showing the bimodal expression of intracellular IL-4 by T cells from a TB patient at diagnosis.

Whole blood was diluted 1:1 with RPMI+ medium prior to stimulation for 4 hours with anti-CD3 mAb at 0.1μg/ml, with BFA present for the last 3 hours. Labelling of intracelluar IL-4 and cell surface CD3 and CD8 was performed as described in Materials and Methods. The gating of CD3 T cells and CD8-positive and –negative subsets is shown in A and the IL-4^{high} and IL-4^{low} T cells in B. The histogram subtraction described in Materials and Methods, which was used to determine percentages of IL-4^{low} CD4 and CD8 T cells, is illustrated in C where the shaded histogram represents the result of subtracting the control histogram (dotted line) from the IL-4 histogram (solid line). As the IL-4 histogram is bimodal and the control histogram unimodal, the subtracted histogram coincides almost exactly with the second peak of the IL-4 histogram.

Significant numbers of IL-4^{high} CD4 T cells were not only found in the stimulated blood cultures but also in the unstimulated cultures of both patients and controls (shown for patients in Fig. 5.2A) and the percentages in the stimulated blood were significantly lower than in the unstimulated samples ($p<0.05$, Wilcoxon matched pairs test). In TB patients at diagnosis the percentages in unstimulated blood were higher (not significantly) than those in unstimulated blood of controls (Fig. 5.2B) and there was a significant decrease in IL-4^{high} CD4 T cells in patients between diagnosis and end of treatment at week 26 ($p<0.01$, Wilcoxon matched pairs test). Numbers of IL-4^{high} cells in the CD8 subset were mostly below the detection limit of the assay.

Figure 5.2

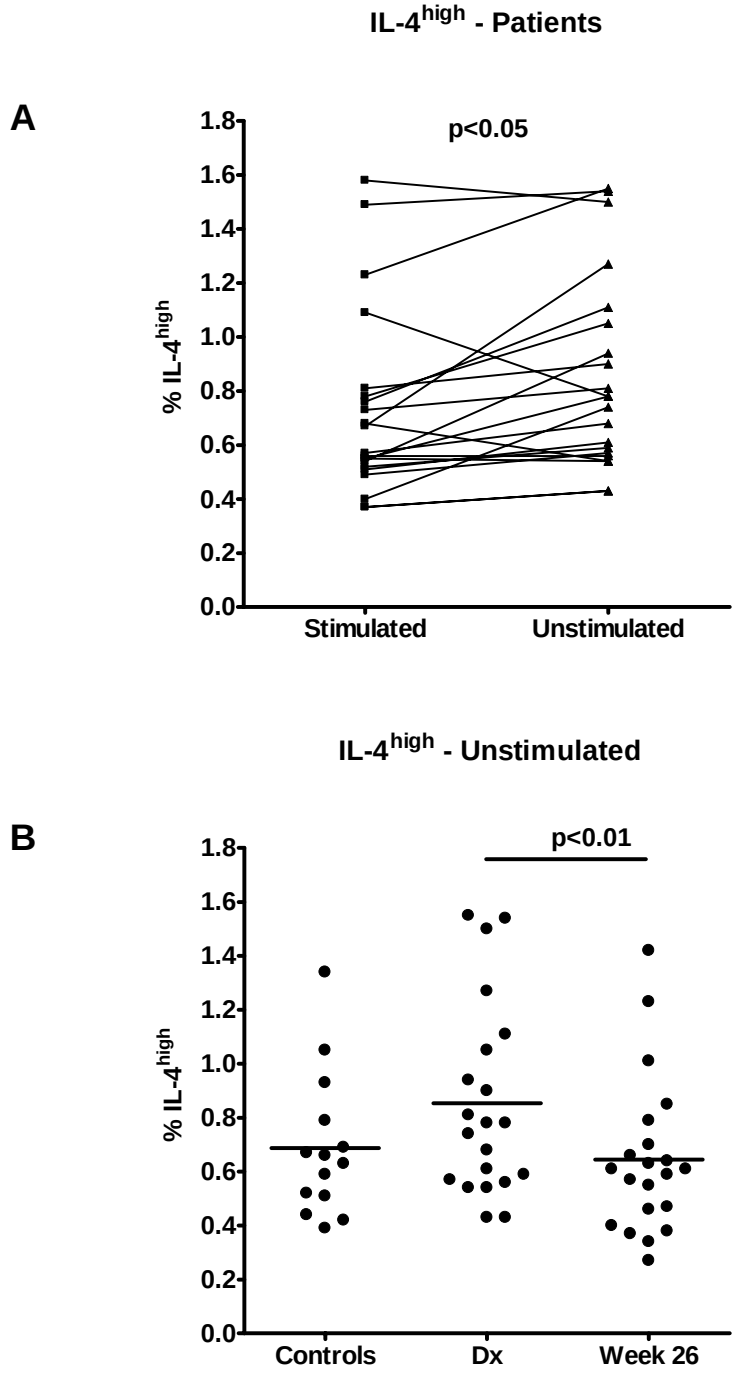
IL-4high - Patients
A
p<0.05
% IL-4high
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Stimulated
Unstimulated
IL-4high - Unstimulated
B
p<0.01
% IL-4high
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Controls
Dx
Week 26

Figure 5.2: High level IL-4 expression in the CD4 T cell subset.

Expression in stimulated blood of patients at diagnosis is compared with that of unstimulated blood in Fig. 5.2A. The higher percentages in unstimulated blood are compared in controls, patients at diagnosis and at treatment end in Fig. 5.2B.

The percentages of IL-4^{low} T cells in the CD4 T cell subset (Fig. 5.3A and B) and CD8 subset (C and D) were slightly higher or no different in stimulated compared to unstimulated blood from patients and controls (shown for patients in A and C). Percentages of IL-4^{low} CD4 T cells were lower than normal in stimulated blood from patients at diagnosis but at week 26 they were comparable to normal (B) whereas in CD8 T cells they were lower than normal in patients at diagnosis and at week 26 (D). Percentages in unstimulated blood were similar and are not shown. The differences were not statistically significant but p values close to 0.05 are given.

Figure 5.3

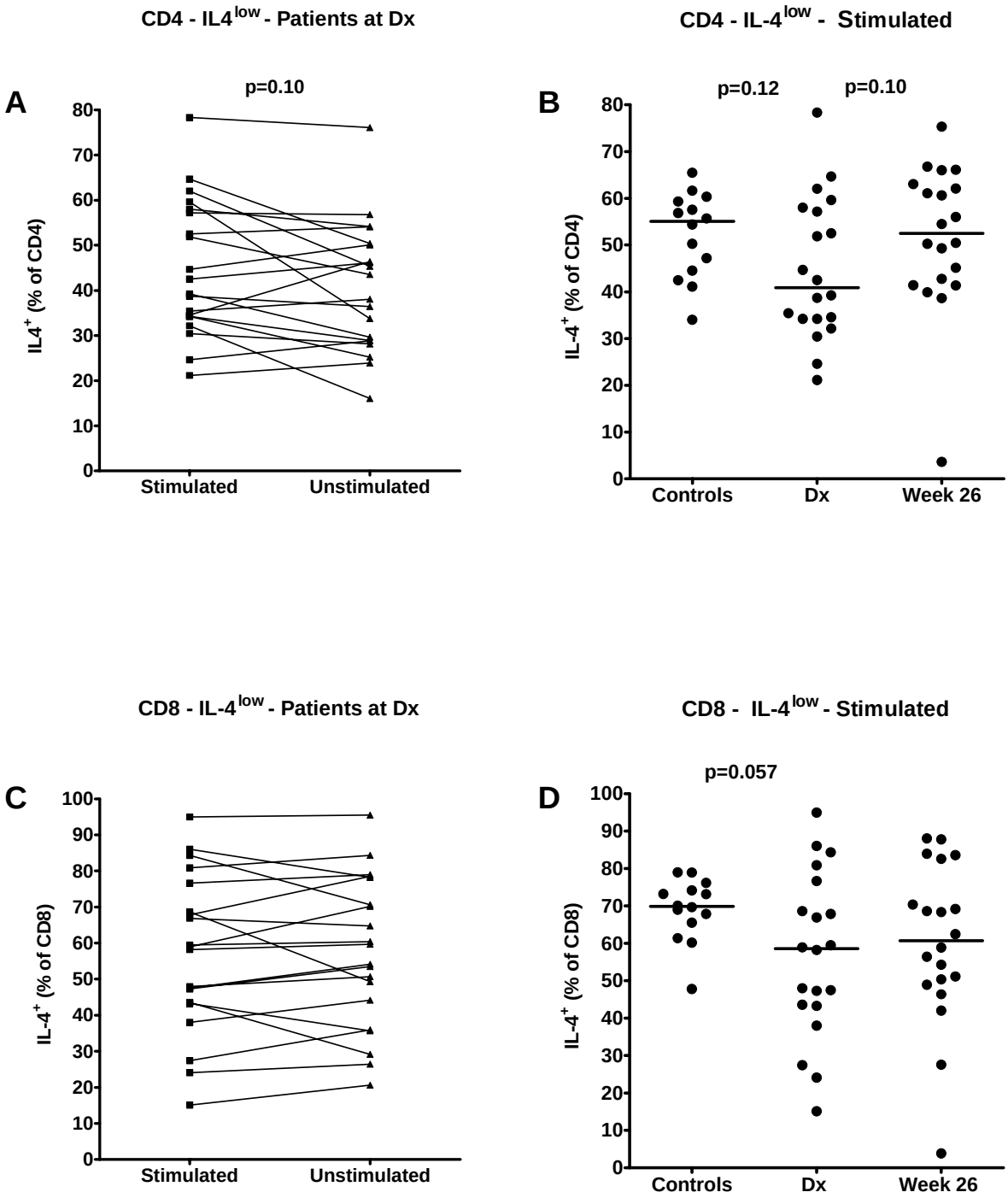
CD4 - IL4low - Patients at Dx
A
p=0.10
IL4+ (% of CD4)
Stimulated
Unstimulated
CD4 - IL-4low - Stimulated
B
p=0.12
p=0.10
IL-4+ (% of CD4)
Controls
Dx
Week 26
CD8 - IL-4low - Patients at Dx
C
IL-4+ (% of CD8)
Stimulated
Unstimulated
CD8 - IL-4low - Stimulated
D
p=0.057
IL-4+ (% of CD8)
Controls
Dx
Week 26

Figure 5.3: Low level IL-4 expression determined by histogram subtraction.

Percentages of $IL4^{low}$ T cells is shown for the CD4 subsets (A and B) and CD8 subset (B and C). The differences between stimulated and unstimulated cells (shown for patients at diagnosis in A and C) are not significant (Wilcoxon matched pairs test). The percentages in stimulated blood of controls, patients at diagnosis and at week 26 are compared in B and D and are not significant (Mann-Whitney test). Near-significant p values close to 0.05 are shown.

When the patients were divided into fast and slow responder groups according to sputum culture positivity at week 8 (as in the previous chapters), and their IL-4^{low} percentages compared, the fast responders had more IL-4^{low} cells that the slow responders, both in the CD4 and CD8 subset (Fig. 5.4 A and B respectively) and the difference in the CD4 subset was significant ($p<0.05$, Mann-Whitney test).

Figure 5.4

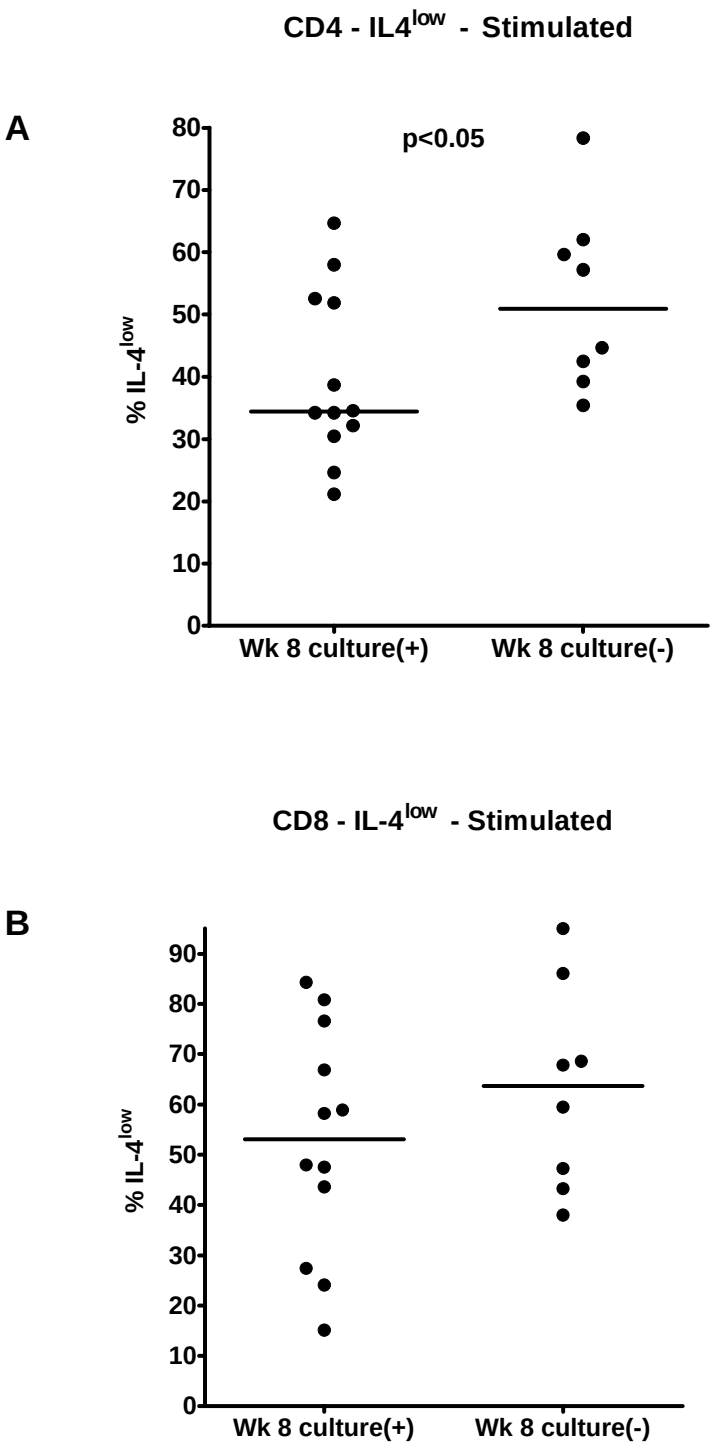
A
CD4 - IL4low - Stimulated
p<0.05
% IL-4low
80
70
60
50
40
30
20
10
0
Wk 8 culture(+)
Wk 8 culture(-)
B
CD8 - IL-4low - Stimulated
% IL-4low
90
80
70
60
50
40
30
20
10
0
Wk 8 culture(+)
Wk 8 culture(-)

Figure 5.4: Low level IL-4 expression in fast and slow responder patients.

$IL\text{-}4^{low}$ expression in stimulated T cells is compared by means of the Mann-Whitney test in fast responder and slow responder patients, defined by negative or positive sputum culture at week 8 respectively, in the CD4 T cell subset (A) and CD8 subset (B).

If the Th1/Th2 hypothesis were true for the cellular immune response to TB, patients' T cells should produce more IL-4 in response to TCR stimulation than those of controls. Instead, constitutive bimodal intracellular IL-4 expression was found in both patients and controls in this study and the results were not explicable in this context. An alternative explanation had to be considered and is possibly offered by a previous report of an association of intracellular IL-4 expression with apoptosis of human lymphocytes [33]. This report showed co-labelling of cells with antibody to IL-4 and the apoptosis-related molecules Apo2.7 and Bcl-2. The authors did not investigate or postulate a mechanism of this IL-4 co-expression. If intracellular IL-4^{high} expression was an indicator of apoptosis this could explain the higher percentages of IL-4^{high} cells in patients compared to controls as an increase in apoptotic cells has been found in the circulation of TB patients [45,46]. The present study has found, by kinetic studies and intracellular IFN-γ labelling after anti-CD3 stimulation (Chapter 4) that TB patients at diagnosis have more pre-activated T cells in the circulation than controls. These cells, when cultured *in vitro*, would have a shorter lifespan and would go into apoptosis sooner than T cells from uninfected donors. Re-stimulation of these pre-activated cells could prolong their lifespan and this could explain why lower numbers of IL-4^{high} T cells were found in the stimulated cultures. To substantiate this thinking, unstimulated blood from 3 new TB patients at diagnosis was labelled for IL-4 and active caspase-3, the main effector caspase in apoptosis, using the same 4-hour culture conditions and labelling method as for the main study. This showed that most of IL-4^{high} T cells co-expressed active caspase-3 (shown for one patient in Fig. 5.5).

Figure 5.5

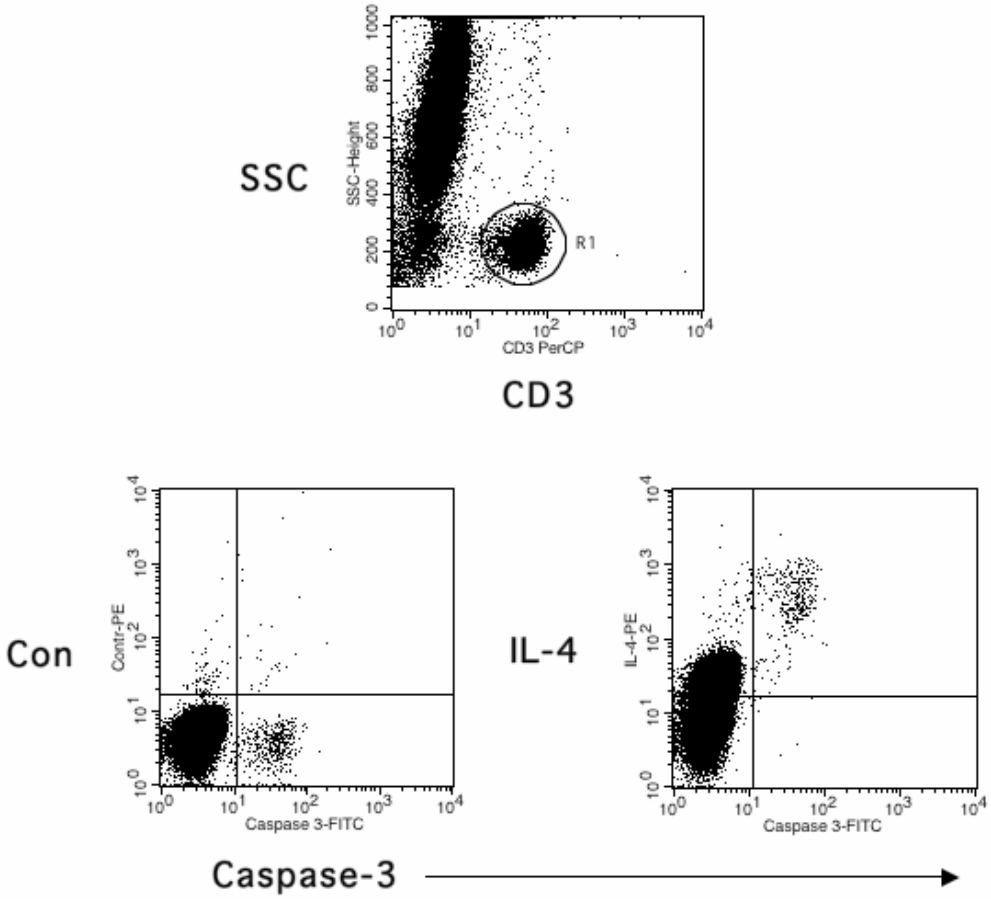
SSC
SSC-Height
CD3 PerCP
R1
CD3
Con
Contr-PE
Caspase 3-FITC
IL-4
IL-4-PE
Caspase 3-FITC
Caspase-3

Figure 5.5: Co-expression of IL-4^{high} and active caspase-3 in T cells from a TB patient at diagnosis.

Whole blood was incubated for 4 hours without stimulation as in Materials and Methods. CD3-PerCP expression was used to gate the T cells of which the IL-4-PE (or control-PE) co-labelling with caspase-3-FITC is shown. The quadrant marker from the control-PE plot was copied into the caspase-3-FITC versus IL-4-PE plot.

To obtain additional evidence for the association of IL-4 expression with apoptosis induced by *in vitro* culture, whole blood from a healthy laboratory staff volunteer was diluted 1:1 with RPMI+ medium as in the tests of patients, but was incubated at 37°C in 5% carbon dioxide overnight in the presence of 5μg/ml BFA, which has been shown to induce apoptosis in the Jurkat T cell line [79], or the solvent dimethyl sulphoxide (DMSO). The leukocytes were then labelled for intracellular IL-4 and active caspase-3, using the method that includes a fixation step to obtain better flow cytometric separation of lymphocytes from neutrophils and monocytes by light scatter characteristics. As shown in Fig. 5.6, the T cells, gated by their low side scatter and CD3 expression, showed a clear population that co-expressed IL-4 and caspase-3.

Figure 5.6

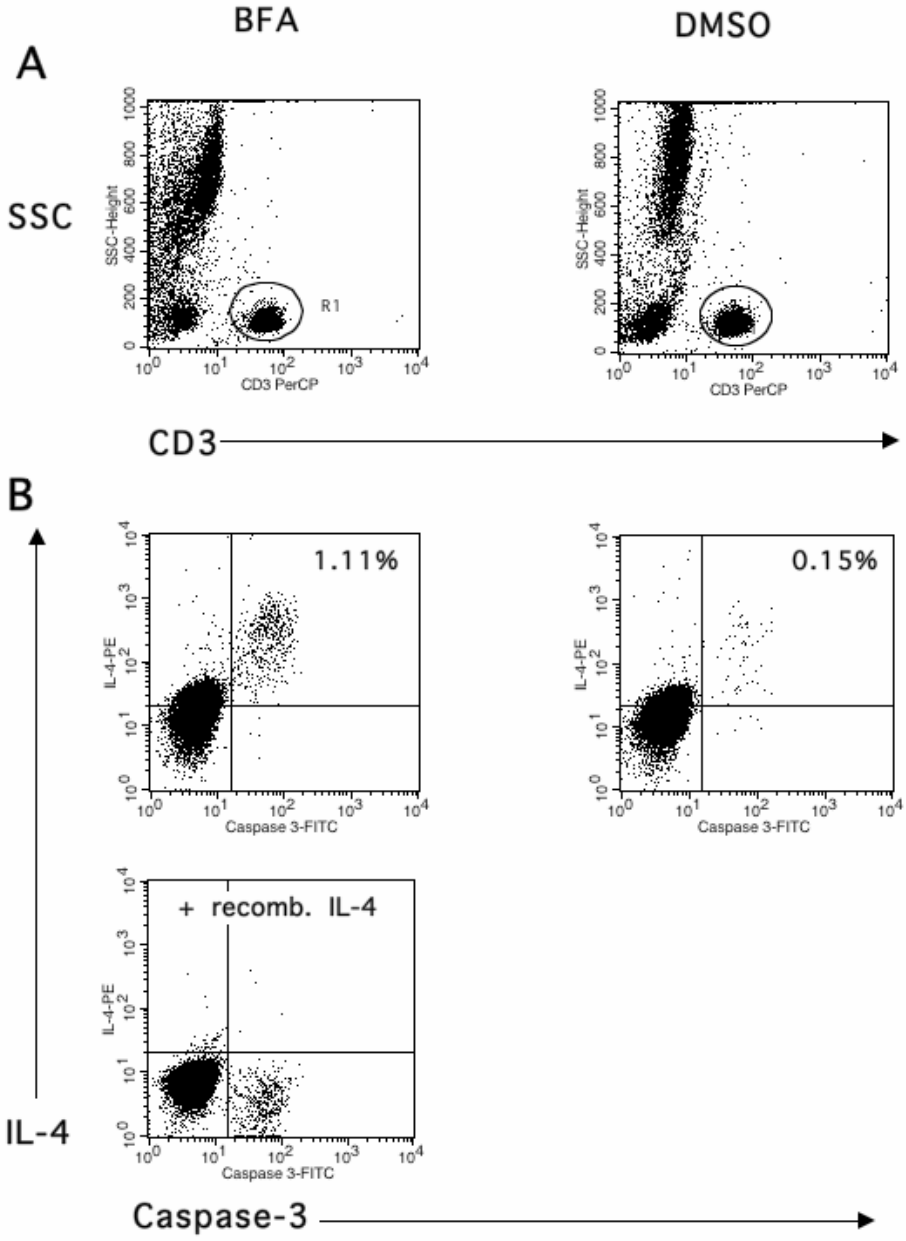
BFA
DMSO
A
SSC
SSC-Height
CD3 PerCP
R1
CD3
B
1.11%
0.15%
IL-4-PE
Caspase 3-FITC
+ recomb. IL-4
IL-4
Caspase-3

Figure 5.6: Induction of apoptotic T cells by prolonged culture with BFA.

Whole blood from a healthy volunteer was diluted 1:1 with RPMI+ medium and was incubated in polypropylene tubes for 24 hours at 37°C and 5% CO_2 in the presence of 5µg/ml BFA (DMSO for the control). Leukocytes were then labelled for IL-4 and active caspase-3 as described in Materials and Methods, using the method that includes a fixation step. The gating according to low side scatter and CD3 expression is shown in A and IL-4 and caspase-3 co-expression in B. As a negative control for the specificity of the IL-4 labelling, 5µg recombinant IL-4 was added to the IL-4 antibody 15 minutes before the addition to the cells. The quadrant marker from this plot was copied into the other two plots in B.

5.3.2 Co-expression of intracellular IL-4 and active caspase-3 in Jurkat cells

To find further evidence of the association of IL-4 with apoptosis, the Jurkat T cell line was chosen for supplementary experiments. It has been shown that apoptosis is readily induced in these cells by low concentrations of BFA [79]. After incubating the cells with BFA at 1μg/ml for 24 hours the cells were labelled for IL-4 and active caspase-3. Live and apoptotic cells were gated in light scatter dot plots as shown in Fig. 5.7A. Co-expression of high levels of IL-4 and active caspase-3 is shown in Fig. 5.7B where 40.9% of BFA-treated cells and only 1.5% of the control cells, treated with only the solvent DMSO, co-expressed these molecules. The quadrant marker was copied from the plot showing the IL-4 labelling inhibited by the addition of recombinant IL-4. The BFA-treated cells that expressed only caspase-3 could be in a more advanced stage of cell death.

An alternative method of inducing apoptosis in cells is culture in medium containing low concentrations of serum. Jurkat cells were cultured overnight in medium containing 0.5% FCS instead of the normal 5% and labelled for IL-4 and caspase-3 (Fig. 5.7C). The number of IL-4^+ cells was 3% in 0.5% serum and 0.9% in 5% serum and the majority of the IL-4^+ cells co-expressed active caspase-3.

Figure 5.7

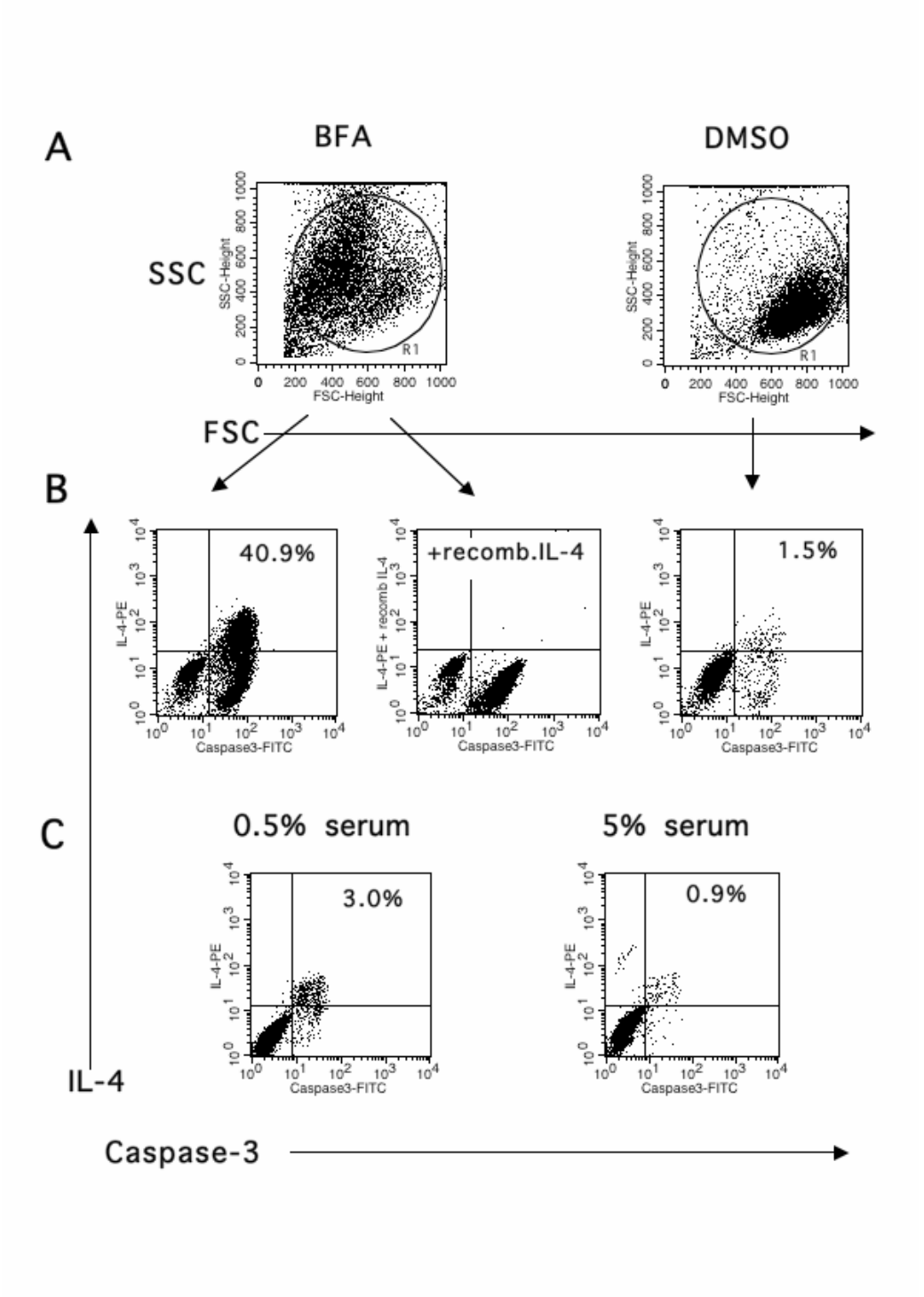
A
BFA
DMSO
SSC
SSC-Height
FSC-Height
R1
FSC
B
40.9%
+recomb.IL-4
1.5%
IL-4-PE
IL-4-PE + recomb IL-4
Caspase3-FITC
C
0.5% serum
5% serum
3.0%
0.9%
IL-4
Caspase-3

Figure 5.7: Expression of intracellular IL-4 and active caspase-3 in Jurkat cells. The cells were gated in a light scatter plot shown in (A) to include both the high FSC viable cells and the low FSC apoptotic cells. The IL-4/caspase-3 co-expression is shown in (B) for cells treated for 24 hours with BFA at 1μg/ml and control cells treated with DMSO. The addition of recombinant IL-4 to the IL-4 antibody before addition to the cells abolished the labelling, confirming the specificity of antibody binding. In (C) the co-expression of IL-4 and caspase-3 is shown in Jurkat cells incubated for 24 hours in 0.5% FCS and control cells grown in 5% FCS.

To confirm the findings in Jurkat cells an alternative method, i.e. ELISA, was chosen to show the IL-4 production by these cells. Jurkat cells were incubated for 24 hours in the presence of 1µg/ml BFA or an equivalent concentration of DMSO and approximately 1ml of the spent culture supernatants saved. Detergent lysates of the cells were prepared which were subsequently tested by means of a high sensitivity IL-4 ELISA kit. A sample of the cells was also labelled for flow cytometry and the results were comparable to those shown in Fig. 5.7. The ELISA showed just detectable levels of secreted IL-4 of 0.30pg/ml in the control supernatant but not in the cell lysate. In the BFA-treated culture secreted IL-4 was undetectable (<0.25pg/ml) but in the cell lysate 1.55pg/ml IL-4 was detected, thus confirming the intracellular staining results (summarized in Table 5.1). A repeat experiment gave similar results.

Table 5.1: Parallel tests for IL-4 in Jurkat cells by flow cytometry and ELISA

	Flow cytometry (% IL-4^{high} cells)	**ELISA (pg/ml)**	
		Supernatant	Lysate
Control	1.8	0.30	<0.25
BFA	50.6	<0.25	1.55

5.3.3 Expression of intracellular IL-4 by apoptotic neutrophils

During the flow cytometric data acquisition of the whole blood samples it was apparent that a subset of neutrophils, which die rapidly when cultured *in vitro*, were

labelled by the IL-4 antibody. The expression of IL-4 in neutrophils has been reported before [73] and it has also been shown that BFA increases the constitutive apoptosis of human neutrophils [80]. To investigate if the co-expression of IL-4 and caspase-3 holds true for neutrophils as well, whole blood was diluted with an equal volume of RPMI+ medium in polypropylene tubes as for the main study but was incubated in a 37° incubator with 5% carbon dioxide overnight in the presence of 5μg/ml BFA (as for Fig. 5.6). The blood cells were labelled for flow cytometry using the method with a fixation step to preserve the light scatter characteristics of the neutrophils and monocytes for gating as shown in Fig. 5.8A. Monocytes in cultured blood have similar light scatter to neutrophils which were separated from monocytes by their lack of DR expression. The number of IL-4^{high} cells, which co-expressed active caspase-3, was increased in the BFA-treated cells and a smaller population expressed only caspase-3 (Fig. 5.8B). The addition of recombinant IL-4 to the anti-IL-4 antibody before reaction with the cells inhibited the antibody binding, thus confirming the specificity of the labelling. The quadrant marker in this plot was copied into the other plots of Fig. 5.8B.

Figure 5.8

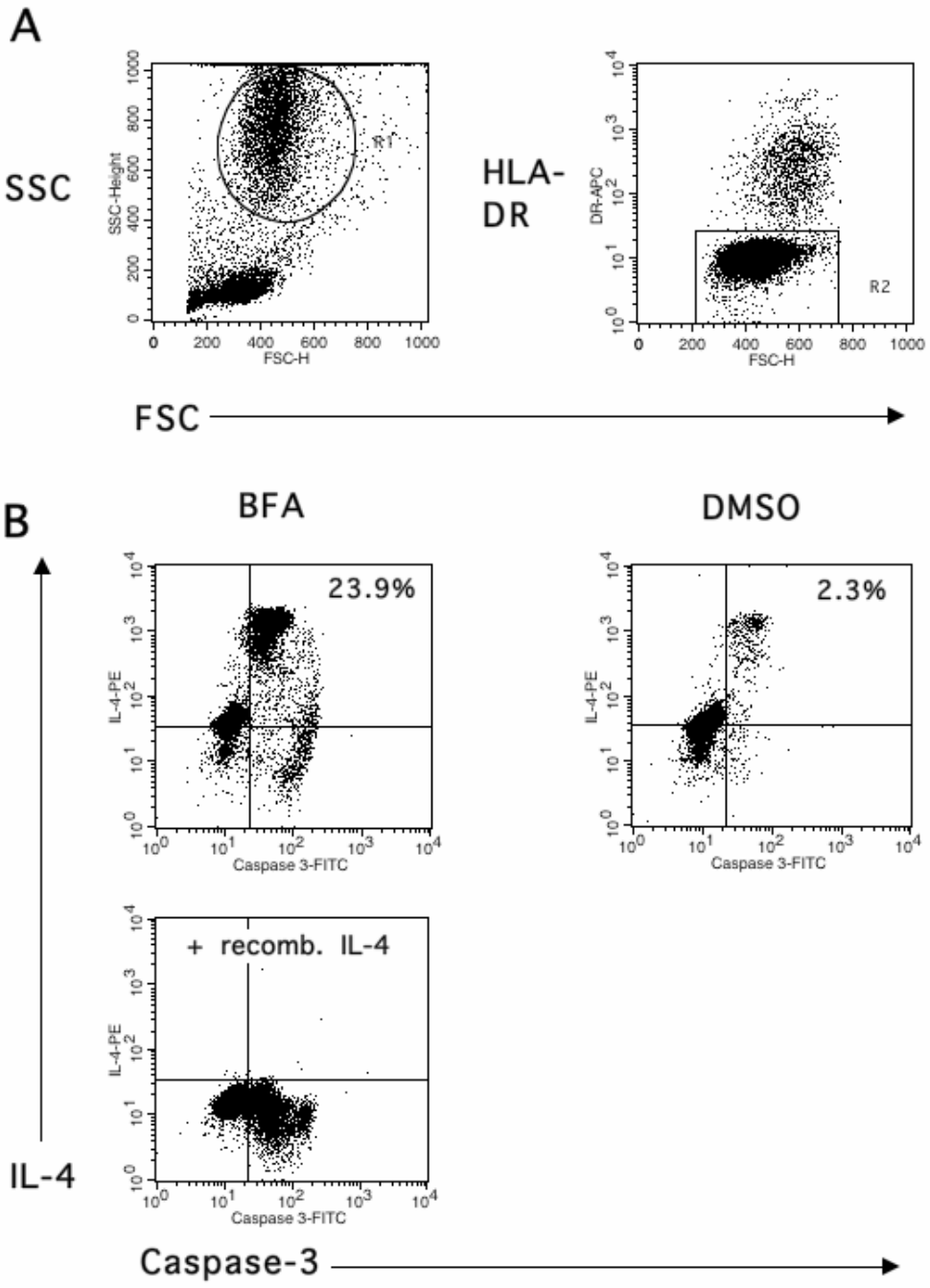
A
SSC
SSC-Height
FSC-H
R1
HLA-DR
DR-APC
R2
FSC
B
BFA
DMSO
23.9%
2.3%
IL-4-PE
Caspase 3-FITC
+ recomb. IL-4
IL-4
Caspase-3

Figure 5.8: Expression of intracellular IL-4 and active caspase-3 in peripheral blood neutrophils.

Whole heparinized blood from a healthy control was diluted 1:1 with RPMI+ medium and incubated 24 hours with 5µg/ml BFA in polypropylene tubes. Intracellular IL-4, active caspase-3 and cell surface HLA-DR were labelled as described in Materials and Methods with the inclusion of a fixation step to preserve the light scatter characteristics of neutrophils and monocytes. Neutrophils were gated as shown in A by their high SSC and lack of HLA-DR-APC binding and then analyzed for IL-4 and caspase-3 co-expression (B). For the control IL-4 labelling was inhibited by the addition of recombinant IL-4. The quadrant marker from the control plot was copied into the other plots.

5.3.4 Expression of intracellular IL-4 by apoptotic monocytes

As Pouliot et al [74] demonstrated IL-4 protein and mRNA expression in alveolar macrophages of all their normal test subjects and asthma patients, peripheral blood monocytes were included in the analysis of whole blood incubated in the presence of 5μg/ml BFA for 24 hours. Monocytes in the high side scatter population were separated from neutrophils by their DR expression (Fig. 5.9A) and labelled for IL-4 and caspase-3 (Fig. 5.9B). In the DMSO control most monocytes produced low levels of IL-4 and a low percentage produced high levels and co-expressed caspase-3. In the BFA-treated blood the IL-4^{high} cells co-expressed caspase-3 and there was also a significant population that expressed caspase-3 only. The quadrant marker in the control plot, where IL-4 labelling was inhibited by recombinant IL-4, was copied into the other plots in Fig. 5.9B.

Figure 5.9

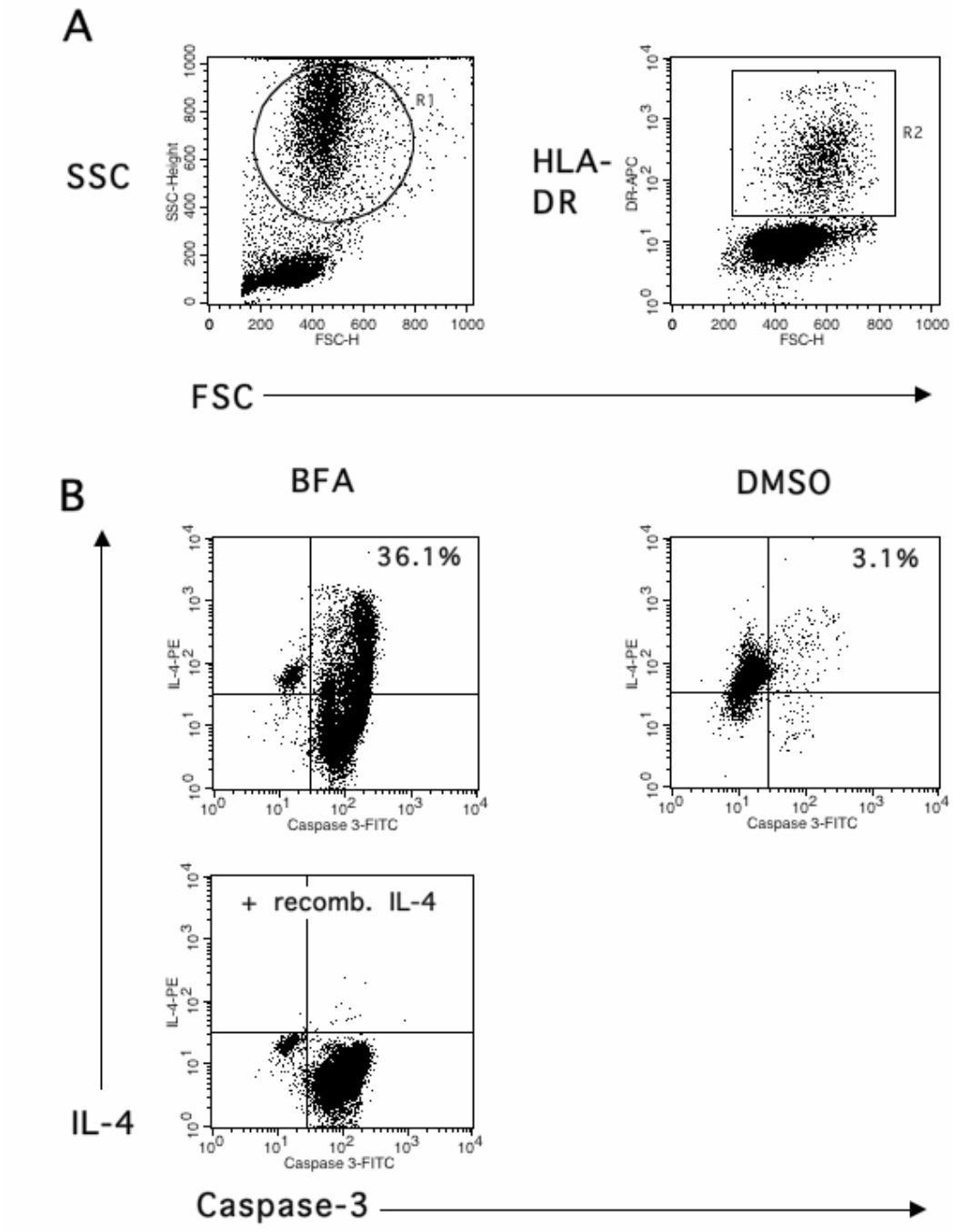
A
SSC
SSC-Height
FSC-H
R1
HLA-DR
DR-APC
FSC-H
R2
FSC
B
BFA
DMSO
36.1%
3.1%
IL-4-PE
Caspase 3-FITC
+ recomb. IL-4
IL-4
Caspase-3

Figure 5.9: Expression of IL-4 and active caspase-3 by peripheral blood monocytes.

Heparinized whole blood from a healthy control was incubated with BFA and labelled with mAbs as for Fig. 5.8. Monocytes were gated by their high SSC and HLA-DR expression as shown in A and then analyzed for IL-4 and caspase-3 co-expression with the control as in Fig. 5.8. DR expression is down regulated in apoptotic monocytes but still sufficient for gating.

5.3.5 Stimulation of neutrophils and monocytes decreases IL-4^{high} expression

As lipopolysaccharide (LPS) is known to save neutrophils from apoptosis [81], IL-4 expression in neutrophils and monocytes in whole blood treated with 50pg/ml LPS overnight was compared with that in untreated cells, shown in Fig. 5.10. As for Figs 5.8 and 5.9, the high SSC cells were separated into neutrophils and monocytes by their DR expression. The percentage of IL-4^{high} neutrophils and monocytes, shown in the relevant quadrants, was markedly lower in the LPS-treated blood than in the control suggesting that stimulation of these cells delays their apoptosis *in vitro*.

Figure 5.10

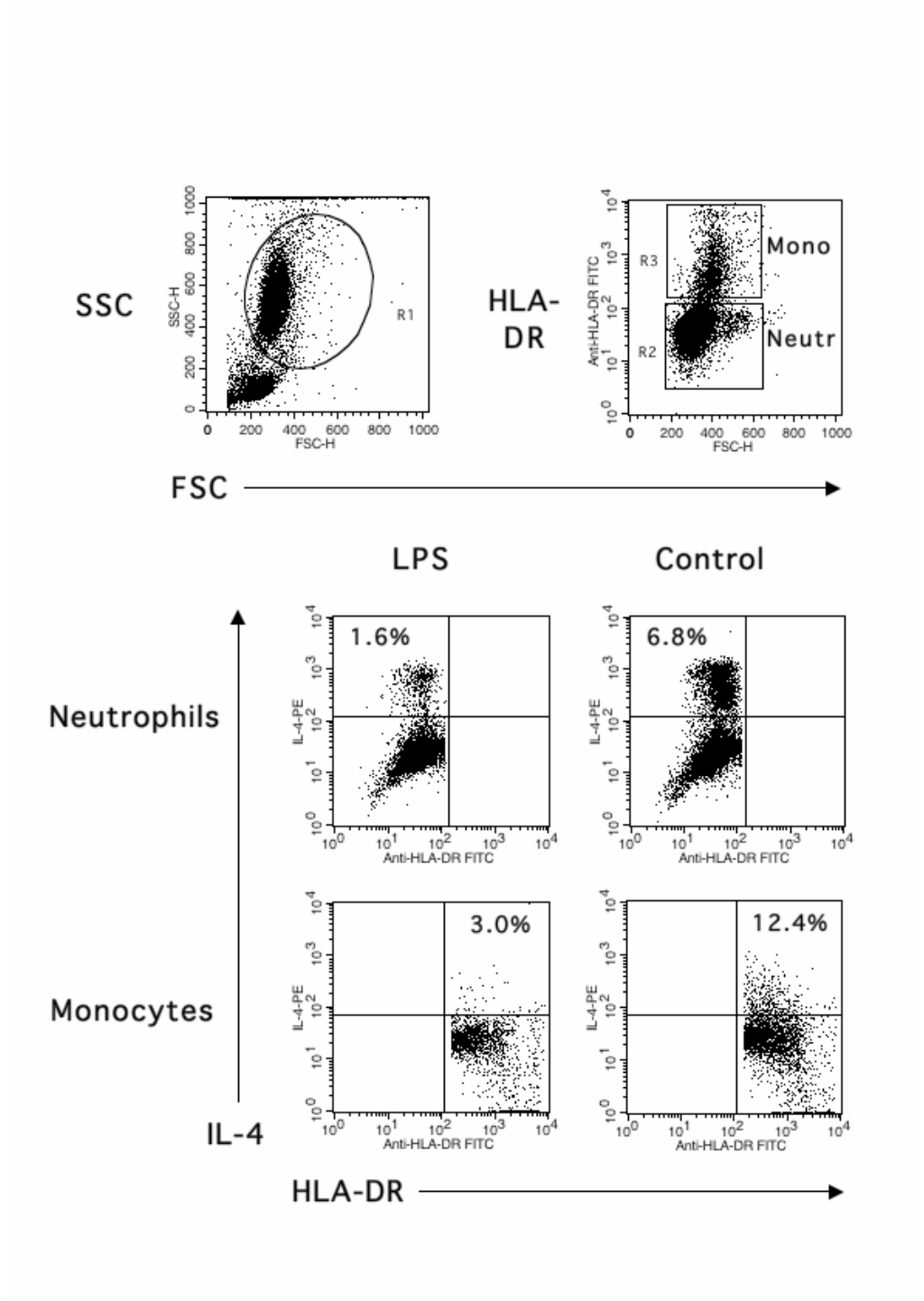
SSC
SSC-H
FSC-H
R1
HLA-DR
Anti-HLA-DR FITC
R3
R2
Mono
Neutr
FSC
LPS
Control
Neutrophils
Monocytes
IL-4
IL-4-PE
Anti-HLA-DR FITC
1.6%
6.8%
3.0%
12.4%
HLA-DR

Figure 5.10: Inhibition of intracellular IL-4 expression in neutrophils and monocytes by stimulation with LPS.

Heparinized whole blood from a healthy control was diluted 1:1 with RPMI+ medium in polypropylene tubes and stimulated overnight with 50pg/ml LPS. Leukocytes were labelled with IL-4-PE and HLA-DR-FITC using the method with a fixation step. Monocytes and neutrophils in the high SSC population were gated according to whether they expressed HLA-DR or not, respectively, and the percentages of IL-4^+ cells compared. Active caspase-3 was not labelled in these cells.

5.4 DISCUSSION

As outlined in the introduction, attempts to prove the Th1/Th2 hypothesis in the clinical setting of tuberculosis have not been very conclusive which may, in part, be due to the fact that many cells express IL-4 receptors and IL-4 secreted by cells into the medium during *in vitro* culture may be consumed by the same or other cells in an autocrine or paracrine manner. The detection of mRNA for IL-4 does not necessarily indicate that the cytokine is expressed and secreted, as was shown in human B lymphocytes and lymphoid cell lines [76]. An additional confounding factor is the existence of an alternatively spliced form of IL-4, IL-4δ2 [82], that acts as a competitive antagonist binding to the α chain of the IL-4 receptor and cannot be distinguished from IL-4 by currently available antibodies.

Secreted IL-4 has been detected by ELISA in the medium of PHA- or PMA+ionophore-stimulated PBMCs [76], Mtb sonicate-stimulated and unstimulated PBMCs [56], and PBMCs stimulated with Mtb recombinant antigens and peptides [68], as well as by ELISPOT in Mtb antigen-stimulated PBMCs [67]; IL-4 mRNA could also be detected in *ex vivo* unstimulated PBMCs [45,46,83]. In a recent report it was shown that IL-4 acts as an autocrine growth factor in dendritic cells [75]. The authors detected very low levels in the cells by means of an amplification step in their labelling protocol and rarely detected it in the medium as a result of its binding to cell surface receptors.

With the methods used here such low level secretion could just be detected in spent medium of Jurkat cells with a very sensitive ELISA assay, and for the detection of intracellular IL-4 by flow cytometry a specially optimized labelling protocol was

used in which the addition of 3% PEG to the buffers achieved additional sensitivity when compared to buffers without PEG (not shown). Intracellular IL-4 was also detected in unstimulated T cells as did Dlugovitzky et al [56] in unstimulated supernatants. A comparison of labelled peripheral blood lymphocytes from a patient in *ex vivo* blood with those in 4-hour stimulated blood showed the presence of IL-4^{low} but not IL-4^{high} T cells in the *ex vivo* blood (not shown), suggesting that the low intracellular IL-4 levels are steady-state levels of IL-4 whereas IL-4^{high} T cells are cells that are induced by *in vitro* culture. The percentages of IL-4^{low} T cells in patients tested at diagnosis in this study were lower than in control subjects and lower than in the patients at the end of treatment. They were also lower in the patients that responded more slowly to treatment. This suggests that this IL-4 production is an advantage and would confirm a growth factor function for IL-4 in the response to Mtb infection.

The bimodal expression of intracellular IL-4 has, to my knowledge, not been reported before but IL-4 has been associated with apoptosis [33]. Apoptosis is cell death that occurs naturally during tissue turnover and after activation in the terminal phase of the cell-mediated immune response. In lymphocytes it is triggered by either the engagement of death receptors, such as Fas, or by cytokine withdrawal (reviewed in [7]). Signals from death receptors are transmitted via an intracellular signalling complex involving adaptor molecules while cytokine withdrawal initiates stress-induced signals within the cell that are transmitted via the mitochondria and the Bcl-2 family proteins. Both these pathways lead to the activation of caspases, with caspase-3 being a key player in the effector phase and the loss of cell integrity.

The demonstrated co-expression of high levels of IL-4 with active caspase-3 in a subset of peripheral blood T lymphocytes, neutrophils and monocytes and in

Jurkat cells indicates that these cells are in the process of apoptosis. This is in agreement with the findings of Stein et al [33] who demonstrated the co-expression of IL-4 and the apoptosis-associated molecule Apo2.7 in PBMC, the T cell line MOLT-4 and the monocytic line THP-1 after inducing apoptosis with a variety of drugs. Like these authors, it was found here that the kinetics of the co-expression of IL-4 and caspase-3 vary in the different cell types, the co-expression in lymphocytes being longer than in neutrophils or monocytes, with a greater likelihood of detecting it at the end of the culture period.

The findings of this study have led me to postulate that IL-4 is produced as an autocrine/paracrine growth factor in various cell types and that the secretion ceases at the onset of apoptosis as a result of a stimulus or lack of a stimulus which could be either a soluble factor or a ligand(s) on the surface of other cells. Intracellular accumulation of IL-4 then takes place during the apoptotic process until cell death. The neutrophils labelled for IL-4 shown in Fig. 5.10 were not treated with BFA to stop secretion of the cytokine which would support this view. Whether the accumulated intracellular IL-4 is released into the medium by dead cells before their engulfment by phagocytic cells is unknown. If IL-4 is an essential growth factor, my finding that T cells with constitutive low level production of IL-4 were reduced in TB patients at diagnosis when compared to those from healthy controls, may indicate impaired function. The higher numbers of apoptotic cells that express higher levels of IL-4 in patients is in line with previous findings of increased numbers of apoptotic T cells in the blood TB patients [45,46].

In summary, the presented data relating to intracellular IL-4 expression and the conflicting large volume of published data of IFN-γ production by T cells of TB patients (reviewed in Chapter 4) suggests that a reconsideration of the role of IL-4 is

warranted and that a continued search for other defects of cell-mediated immunity in these patients is needed.

6. GENERAL DISCUSSION

The findings of this thesis have pointed out several important aspects in the study of immune parameters during active TB disease which have to be taken into consideration when performing similar investigations.

The immune cells of the TB patients are in an abnormal state and accurate comparisons of test results for patients at or soon after diagnosis, when the bacterial load is high, cannot be made with those from healthy subjects. The results of tests performed in this study changed rapidly within the first few weeks of treatment and if groups of patients are tested, this has to be done at accurately matched time points after start of treatment. In this study, with the exception of the absolute monocyte, NK and NKT cells counts, the parameters in patients at treatment end were comparable to normal subjects.

The immunophenotyping was found to be an accurate and reproducible test and the detection of a novel NKT cell subset should be confirmed and their significance investigated in larger number of patients. Phenotyping is also a relatively low cost and quick assay and may therefore be more useful in large scale applications.

The stimulation of patients' blood in *in vitro* culture and the measurement of cytokine production and proliferation is complicated by the abnormal immune status of the patients in that they seem to have relatively large numbers of cells previously activated by mycobacterial antigens which, when re-activated, rapidly progress

towards apoptosis, after which no more functional assays can be done on them. The obvious cell clumps in blood of TB patients at diagnosis stimulated with live Mtb or PPD that I observed in optimization experiments for this study pointed out this problem. This was also shown experimentally *in vitro* by Soruri et al [84] who produced PPD-activated T cells in culture and then re-activated them. They found that re-activation with PPD caused apoptosis in a concentration-dependent manner and the authors postulate that this happens *in vivo* in caseous tuberculous granulomas in the lungs. To my knowledge, the variation in the kinetics of IFN-γ production has not been shown before, and could partially explain the extremely variable results and paradoxes that characterize the investigations of this essential cytokine, illustrated in Tables 4.1, 4.2 and 4.3 (pages 43-44) compiled from my literature search. The results of this study indicate that the intracellular IFN-γ assay after a short stimulation of approximately 6 hours may be helpful in the identification of Mtb-infected individuals.

The role of IL-4 in the context of the Th1/Th2 hypothesis, analogous to its role in the mouse, but not proved in human TB, could not be confirmed in this study. On the contrary, the results point to a beneficial and normal function of IL-4 as a growth factor and an interesting mechanism of secretion or retention of this cytokine in various cell types in response to apoptosis-inducing conditions. The demonstration of high levels of intracellular IL-4 expression after a relatively short exposure to BFA leads one to cautious interpretation of results of assays that measure the intracellular expression of this cytokine in which BFA is usually added to cultures to prevent the secretion of the cytokine.

In summary, regarding the hypothesis formulated for this study, point (1) of the alternative hypothesis can be said to be true for patients at diagnosis when their

immune responses were found to be abnormal. At the end of treatment, when there was no evidence of infection, they were essentially normal. The exceptions of the low absolute monocyte, NK and $CD3^{bright}$ NKT cell counts at the end of treatment could be an effect of the treatment itself. Point (2) of the alternative hypothesis was found not to be true for the conventional immune parameters but was true for a newly described NKT cell subset and possibly constitutive production of IL-4 by T cells of patients at diagnosis. Both these are, to my knowledge, new findings and would have to be confirmed by more investigations on larger numbers of patients.

7. REFERENCES

1. Goldsby RA, Kindt TJ, Osborne BA, and Kuby J. Immunology. New York: W.H. Freeman and Company, 2003.
2. Flynn JL and Chan J. Immunology of tuberculosis. Annu Rev Immunol 2001; 19:93-129.
3. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, and Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; 136:2348-57.
4. Ottenhoff THM, Kumararatne D, and Casanova JL. Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to intracellular bacteria. Immunol Today 1998; 19:491-4.
5. Vincent MS, Gumperz JE, and Brenner MB. Understanding the function of CD1-restricted T cells. Nat Immunol 2003; 4:517-23.
6. Maderna P and Godson C. Phagocytosis of apoptotic cells and the resolution of inflammation. Biochim Biophys Acta 2003; 1639:141-51.

7. Hildeman DA, Zhu Y, Mitchell TC, Kappler J, and Marrack P. Molecular mechanisms of activated T cell death in vivo. Curr Opin Immunol 2002; 14:354-9.
8. World Health Organization. Treatment of Tuberculosis:guidelines for national programmes. WHO/CDS/TB/2003.313, 2004.
9. Millard I, Degrave E, Phillipe M, and Gala J. Detection of intracellular antigens by flow cytometry: comparison of two chemical methods and microwave heating. Clin Chem 1998; 44:2320-30.
10. Verver S, Warren RM, Beyers N et al. Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. Am J Respir Crit Care Med 2005; 171:1430-5.
11. Simon G. Radiology in epidemiological studies and some therapeutic trials. Br Med J 1966; 5512:491-4.
12. Carroll NM, Richardson M, Engelke E, de Kock M, Lombard C, and van Helden PD. Reduction of the rate of false-positive cultures of Mycobacterium tuberculosis in a laboratory with a high culture positivity rate. Clin Chem Lab Med 2002; 40:888-92.
13. Enarson DA, Rieder HL, Arnadottir T, and Trébucq A. Management of Tuberculosis. A Guide for Low Income Countries. International Union Against Tuberculosis and Lung Disease (IUATLD), 2000.
14. Onwubalili JK, Edwards AJ, and Palmer L. T4 lymphopenia in human tuberculosis. Tubercle 1987; 68:195-200.
15. Rodrigues DSS, Medeiros EAS, Weckx LY, Bonnez W, Salomao R, and Kallas EG. Immunophenotypic characterization of peripheral T lymphocytes in

Mycobacterium tuberculosis infection and disease. Clin Exp Immunol 2002; 128:149-54.

16. Swanson Beck J, Potts RC, Kardjito T, and Grange JM. T4 lymphopenia in patients with active pulmonary tuberculosis. Clin Exp Immunol 1985; 60:49-54.
17. Balbi B, Valle MT, Oddera S et al. T-lymphocytes with γδ+ Vδ2+ antigen receptors are present in increased proportions in a fraction of patients with tuberculosis or with sarcoidosis. Am Rev Respir Dis 1993; 148:1685-90.
18. Ito M, Kojiro N, Ikeda T, Ito T, Funada J, and Kokubu T. Increased proportions of peripheral blood γδ T cells in patients with pulmonary tuberculosis. Chest 1992; 102:195-7.
19. Barnes PF, Grisso CL, Abrams JS, Band H, Rea TH, and Modlin RL. γδ T lymphocytes in human tuberculosis. J Infect Dis 1992; 165:506-12.
20. Li B, Rossman MD, Imir T et al. Disease-specific changes in γδ T cell repertoire and function in patients with pulmonary tuberculosis. J Immunol 1996; 157:4222-9.
21. Corominas M, Cardona V, Gonzalez L et al. B-lymphocytes and co-stimulatory molecules in *Mycobacterium tuberculosis* infection. Int J Tuberc Lung Dis 2004; 8:98-105.
22. Nirmala R, Narayanan PR, Mathew R, Maran M, and Deivanayagam CN. Reduced NK activity in pulmonary tuberculosis patients with/without HIV infection: identifying the defective stage and studying the effect of interleukins on NK activity. Tuberculosis (Edinb) 2001; 81:343-52.
23. Harrington JC, Fenton II JW, and Pert JH. Polymer-induced precipitation of antigen-antibody complexes: 'precipiplex' reactions. Immunochemistry 1971; 8:413-21.

24. Hastie T, Tibshirani R, and Friedman J. Support vector machines and flexible discriminants. In: The Elements of Statistical Learning. Data Mining, Inference and Prediction. New York: Springer, 2001:371-391.

25. Loza MJ, Metalitsa LS, and Perussia B. NKT and T cells: coordinate regulation of NK-like phenotype and cytokine production. Eur J Immunol 2002; 32:3453-62.

26. Ohkawa T, Seki S, Dobashi H et al. Systematic characterization of human $CD8^+$ T cells with natural killler cell markers in comparison with natural killer cells and normal $CD8^+$ T cells. Immunology 2004; 103:281-90.

27. Godfrey DI, McDonald HR, Kronenberg M, Smyth MJ, and Van Kaer L. NKT cells: what's in a name? Nat Rev Immunol 2004; 4:231-7.

28. Doherty DG, Norris S, Madrigal-Estebas L et al. The human liver contains multiple populations of NK cells, T cells, and $CD3^+CD56^+$ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. J Immunol 1999; 163:2314-21.

29. Müller S, Demotz S, Bulliard C, and Valitutti S. Kinetics and extent of protein tyrosine kinase activation in individual T cells upon antigenic stimulation. Immunology 1999; 97:287-93.

30. Takayama E, Koike Y, Ohkawa T et al. Functional and Vβ repertoire characterization of human $CD8^+$ T-cell subsets with natural killer cell markers, $CD56^+$ $CD57^-$ T cells, $CD56^+$ $CD57^+$ T cells and $CD56^-$ $CD57^+$ T cells. Immunology 2003; 108:211-9.

31. Watanabe H, Weerasinghe A, Miyaji C et al. Expansion of unconventional T cells with natural killer markers in malaria patients. Parasitol Int 2003; 52:61-70.

32. Slifka MK, Pagarigan RR, and Whitton JL. NK markers are expressed on a high percentage of virus-specific $CD8^+$ and $CD4^+$ T cells. J Immunol 2000; 164:2009-15.

33. Stein GM, Pfüller U, Schietzel M, and Büssing A. Expression of interleukin-4 in apoptotic cells: stimulation of the type-2 cytokine by different toxins in human peripheral blood mononuclear and tumour cells. Cytometry 2000; 41:261-70.

34. Arruda S, Chalhoub M, Cardoso S, and Barrol-Netto M. Cell-mediated immune responses and cytotoxicity to mycobacterial antigens in patients with tuberculous pleurisy in Brazil. Acta Tropica 1998; 71:1-15.

35. Brill KJ, Li Q, Larkin R et al. Human natural killer cells mediate killing of intracellular *Mycobacterium tuberculosis* H37Rv via granule-independent mechanisms. Infect Immun 2001; 69:1755-65.

36. Yoneda T and Ellner JJ. $CD4^+$ T cell and natural killer cell-dependent killing of *Mycobacterium tuberculosis* by human monocytes. Am J Respir Crit Care Med 1998; 158:395-403.

37. Vankayalapati R, Klucar P, Wizel B et al. NK cells regulate $CD8^+$ T cell effector function in response to an intracellular pathogen. J Immunol 2004; 172:130-7.

38. Vankayalapati R, Wizel B, Weis SE et al. Serum cytokine concentrations do not parallel *Mycobacterium tuberculosis*-induced cytokine production in patients with tuberculosis. Clin Infect Dis 2003; 36:24-8.

39. Barry SM, Lipman MC, Bannister B, Johnson MA, and Janossy G. Purified protein derivative-activated Type 1 cytokine-producing $CD4^+$ T lymphocytes in the lung: a characteristic feature of active pulmonary and nonpulmonary tuberculosis. J Infect Dis 2003; 187:243-50.

40. Hirsch CS, Toossi Z, Johnson JL et al. Augmentation of apoptosis and interferon-γ production at sites of active *Mycobacterium tuberculosis* infection in human tuberculosis. J Infect Dis 2001; 183:779-88.

41. Jalapathy KV, Prabha C, and Das SD. Correlates of protective immune response in tuberculous pleuritis. Fems Immunol Med Mic 2004; 40:139-45.

42. Zhang M, Lin Y, Iyer DV, Gong J, Abrams JS, and Barnes PF. T-cell cytokine responses in human infection with *Mycobacterium tuberculosis*. Infect Immun 1995; 63:3231-4.

43. Mehra V, Gong J, Iyer D et al. Immune response to recombinant mycobacterial proteins in patients with tuberculosis infection and disease. J Infect Dis 1996; 174:431-4.

44. Torres M, Herrera T, Villareal H, Rich EA, and Sada E. Cytokine profiles for peripheral blood lymphocytes from patients with active pulmonary tuberculosis and healthy household contacts in response to the 30-kilodalton antigen of *Mycobacterium tuberculosis*. Infect Immun 1998; 66:176-80.

45. Bhattacharyya S, Singla R, Dey AB, and Prasad HK. Dichotomy of cytokine profiles in patients and high-risk healthy subjects exposed to tuberculosis. Infect Immun 1999; 67:5597-603.

46. Hirsch CS, Toossi Z, Vanham G et al. Apoptosis and T cell hyporesponsiveness in pulmonary tuberculosis. J Infect Dis 1999; 179:945-53.

47. Jo E-K, Kim H-J, Lim J-H et al. Dysregulated production of interferon-γ, interleukin-4 and interleukin-6 in early tuberculosis patients in response to antigen 85B of *Mycobacterium tuberculosis*. Scand J Immunol 2000; 51:209-17.

48. Song C-H, Kim H-J, Park J-K et al. Depressed interleukin-12 (IL-12), but not IL-18, production in response to a 30- or 32-kilodalton mycobacterial antigen in patients with active pulmonary tuberculosis. Infect Immun 2000; 68:4477-84.

49. Samten B, Thomas EK, Gong J, and Barnes PF. Depressed CD40 ligand expression contributes to reduced gamma interferon production in human tuberculosis. Infect Immun 2000; 68:3002-6.

50. Shams H, Wizel B, Weis SE, Samten B, and Barnes PF. Contribution of $CD8^+$ T cells to gamma interferon production in human tuberculosis. Infect Immun 2001; 69:3497-501.

51. Lee J-S, Song C-H, Kim C-H et al. Profiles of IFN-γ and its regulatory cytokines (IL-12, IL-18 and IL-10) in peripheral blood mononuclear cells from patients with multidrug-resistant tuberculosis. Clin Exp Immunol 2002; 128:516-24.

52. Smith SM, Klein MR, Malin AS, Sillah J, McAdam KPWJ, and Dockrell HM. Decreased IFN-γ and increased IL-4 production by human $CD8^+$ T cells in response to *Mycobacterium tuberculosis* in tuberculosis patients. Tuberculosis 2002; 82:7-13.

53. Hussain R, Kaleem A, Shahid F et al. Cytokine profiles using whole-blood assays can discriminate between tuberculosis patients and healthy endemic controls in a BCG-vaccinated population. J Immunol Methods 2002; 264:95-108.

54. Ravn P, Demissie A, Eguale T et al. Human T cell responses to the ESAT-6 antigen from *Mycobacterium tuberculosis*. J Infect Dis 1999; 179:637-45.

55. Ulrichs T, Anding P, Porcelli S, Kaufmann SHE, and Munk ME. Increased numbers of ESAT-6- and purified protein derivative-specific gamma interferon-

producing cells in subclinical and active tuberculosis infection. Infect Immun 2000; 68:6073-6.

56. Dlugovitzky D, Bay ML, Rateni L et al. Influence of disease severity on nitrite and cytokine production by peripheral blood mononuclear cells (PBMC) from patients with pulmonary tuberculosis (TB). Clin Exp Immunol 2000; 122:343-9.
57. Lalvani A, Pathan AA, McShane H et al. Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. Am J Respir Crit Care Med 2001; 163:824-8.
58. Brock I, Munk ME, Kok-Jensen A, and Andersen.P. Performance of whole blood IFN-γ test for tuberculosis diagnosis based on PPD or the specific antigens ESAT-6 and CFP-10. Int J Tuberc Lung Dis 2001; 5:462-7.
59. Cardoso FLL, Antas PRZ, Milagres AS et al. T-Cell responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 in Brazilian tuberculosis patients. Infect Immun 2002; 70:6707-14.
60. Portales-Pérez DP, Baranda L, Layseca E et al. Comparative and prospective study of different immune parameters in healthy subjects at risk for tuberculosis and in tuberculosis patients. Clin Diag Lab immunol 2002; 9:299-307.
61. Agger EM, Brock I, Okkels LM et al. Human T-cell responses to the RD1-encoded protein TB27.4 (Rv3878) from *Mycobacterium tuberculosis*. Immunology 2003; 110:507-12.
62. Ferrand.R.A., Bothamley GH, Whelan A, and Dockrell HM. Interferon-gamma responses to ESAT-6 in tuberculosis patients early into and after anti-tuberculosis treatment. Int J Tuberc Lung Dis 2005; 9:1034-9.
63. Morosini M, Meloni F, Uccelli M, Marone Bianco A, Solari N, and Fietta AM. *Ex vivo* evaluation of PPD-specific IFN-γ or IL-5 secreting cells in the

peripheral blood and lungs of patients with tuberculosis. Int J Tuberc Lung Dis 2005; 9:753-9.

64. Hughes AJ, Hutchinson P, Gooding T, Freezer NJ, Holdsworth SR, and Johnson PDR. Diagnosis of *Mycobacterium tuberculosis* infection using ESAT-6 and intracellular cytokine cytometry. Clin Exp Immunol 2005; 142:132-9.
65. Vincenti D, Carrara S, De Mori P et al. Identification of Early Secretory Antigen Target-6 epitopes for the immunodiagnosis of active tuberculosis. Mol Med 2003; 9:105-11.
66. Al-Attiyah R, Mustafa AS, Abal AT, El-Shamy ASM, Dalemans W, and Sheiky YAW. *In vitro* cellular immune responses to complex and newly defined recombinant antigens of *Mycobacterium tuberculosis*. Clin Exp Immunol 2004; 138:139-44.
67. Surcel HM, Troye-Blomberg M, Paulie S et al. Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. Immunology 1994; 81:171-6.
68. Lopez-Vidal Y, de Leon-Rosales SP, Castanon-Arreola M, Rangel-Frausto MS, Melendez-Herrada E, and Sada-Diaz E. Response of IFN-γ and IgG to ESAT-6 and 38 kDa recombinant proteins and their peptides from *Mycobacterium tuberculosis* in tuberculosis patients and asymptomatic household contacts may indicate possible early-stage infection in the latter. Arch Med Res 2004; 35:308-17.
69. Antas PRZ, Cardoso FLL, Pereira KC et al. T cell immune responses to mycobacterial antigens in Brazilian tuberculosis patients and controls. Trans R Soc Trop Med Hyg 2005; 99:699-707.

70. Holmes K, Fowlkes BJ, Schmid I, and Giorgi JV. Preparation of cells and reagents for flow cytometry. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, and Strober W eds. Current Protocols in Immunology, Vol 1. New York: John Wiley & Sons, Inc., 1991:5.3.9-5.3.10.

71. Greinert U, Schlaak M, Rüsch-Gerdes S, Flad H-D, and Ernst M. Low *in vitro* production of interferon-γ and tumour necrosis factor-α in HIV-seronegative patients with pulmonary disease caused by nontuberculous mycobacteria. J Clin Immunol 2000; 20:445-52.

72. Scott P, Natovitz P, Coffman RL, Pearce E, and Sher A. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. J Exp Med 1988; 168:1675-84.

73. Brandt E, Woerly G, Younes AB, Loiseau S, and Capron M. IL-4 production by human polymorphonuclear neutrophils. J Leukoc Biol 2000; 68:125-30.

74. Pouliot P, Turmel V, Gelinas E, Laviolette M, and Bissonnette EY. Interleukin-4 production by human alveolar macrophages. Clin Exp Allergy 2005; 35:804-10.

75. Maroof A, Penny M, Kingston R et al. Interleukin-4 can induce interleukin-4 production in dendritic cells. Immunology 2006; 117:271-9.

76. Klein SC, Golverdingen JG, van Wichen DF et al. Expression of two interleukin-4 mRNA isoforms in B lymphoid cells. Cell Immunol 1996; 167:259-68.

77. Park LS, Friend D, Sassenfeld HM, and Urdal DL. Characterization of the human B cell stimulatory factor 1 receptor. J Exp Med 1987; 166:476-88.

78. Kay NE and Pittner BT. IL-4 biology: impact on normal and leukemic CLL B cells. Leuk Lymphoma 2003; 44:897-903.

79. Guo H, Tittle TV, Allen H, and Maziarz RT. Brefeldin A-mediated apoptosis requires the activation of caspases and is inhibited by Bcl-2. Exp Cell Res 1998; 245:57-68.

80. Lee MJ, Park JY, Lee SY et al. Modulation of constitutive and delayed apoptosis by brefeldin A in human neutrophils. Int Immunopharmacol 2003; 3:835-43.

81. Lee A, Whyte MK, and Haslett C. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J Leukoc Biol 1993; 54:283-8.

82. Alms WJ, Atamas SP, Yurovsky VV, and White B. Generation of a variant of human interleukin-4 by alternative splicing. Mol Immunol 1996; 33:361-70.

83. Seah GT, Scott GM, and Rook GAW. Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis. J Infect Dis 2000; 181:385-9.

84. Soruri A, Schweyer S, Radzun HJ, and Fayyazi A. Mycobacterial antigens induce apoptosis in human purified protein derivative-specific αβ T lymphocytes in a concentration-dependent manner. Immunology 2002; 105:222-30.

8. APPENDIX

8.1 SUPPLIERS OF REAGENTS

BD-Biosciences (Erembodegem, Belgium).

ATCC (Rockville, MD, USA)

Gibco-BRL (Paisley, Scotland),

Sigma (Kempton Park, South Africa).

R&D Systems (Minneapolis, US)

Beckman Coulter (Johannesburg, South Africa),

Merck (Cape Town, South Africa).

8.2 SOLUTIONS

Phosphate-buffered saline (PBS):

NaCl	8.0g	per litre
KCl	0.2g	
$Na_2HPO_4.2H_2O$	1.44g	
KH_2PO_4	0.24g	
NaN_3	0.5g	

Lyse/wash buffer:

PBS containing 0.05% saponin and 3% PEG 4000

RPMI+

RPMI 1640 medium (Gibco) with GlutaMAX, bicarbonate and 25mM HEPES, to which 100µg/ml Streptomycin, 100U/ml Penicillin and 50µM 2-Mercaptoethanol is added just before use. The same medium, but without HEPES, was used for the overnight incubations, the culture of Jurkat cells and the lymphocyte proliferation.

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03144.x

Changes in leucocyte and lymphocyte subsets during tuberculosis treatment; prominence of $CD3^{dim}CD56^{+}$ natural killer T cells in fast treatment responders

H. Veenstra,* R. Baumann,* N. M. Carroll,* P. T. Lukey,¶ M. Kidd,‡ N. Beyers,† C. T. Bolliger,§ P. D. van Helden* and G. Walzl*

*Division of Molecular Biology and Genetics and MRC Centre for Molecular and Cellular Biology, University of Stellenbosch, †Desmond Tutu TB Centre, Department of Paediatrics and Child Health, University of Stellenbosch, ‡Centre for Statistical Consultation, University of Stellenbosch, §Lung Unit, Internal Medicine, University of Stellenbosch, South Africa, and ¶GlaxoSmithKline R&D, Stevenage, UK

Accepted for publication 18 May 2006
Correspondence: Mrs H. Veenstra, Division of Molecular Biology and Genetics and MRC Centre for Molecular and Cellular Biology, University of Stellenbosch, PO Box 19063, Tygerberg, 7505, South Africa.
E-mail: hveen@sun.ac.za

Summary

The immune responses against pulmonary tuberculosis are still poorly defined. This study describes changes in leucocyte and lymphocyte subsets during treatment to find reliable immunological markers for the disease and treatment response. Flow cytometric peripheral blood immune phenotyping, routine haematology and sputum microbiology were performed on 21 HIV-negative adult tuberculosis (TB) patients with positive sputum cultures during therapy in comparison with 14 healthy purified protein derivative (PPD)-positive volunteers. Patients at diagnosis showed high absolute neutrophil and monocyte counts which fell during treatment but low lymphocyte subset counts which increased [except natural killer (NK) and NK T cells]. High counts of a population of $CD3^{dim}/CD56^{+}$ NK T cells at diagnosis correlated significantly with negative sputum culture after 8 weeks of treatment. A multivariate classification technique showed improved correlation when NK cells were taken into account. In conclusion, peripheral blood white cell counts change significantly during treatment and counts at diagnosis, especially $CD3^{dim}/CD56^{+}$ NK T cells, hold promise in predictive models of TB treatment response.

Keywords: immunophenotyping, NK T cells, treatment response, tuberculosis

Introduction

The mechanisms of protective immunity against *Mycobacterium tuberculosis* (Mtb) infection and disease in humans have not been fully clarified. Many reports have addressed the potential immunological defect(s) by comparing immune phenotypes in actively diseased patients to those with latent infection. Most of these investigations have focused on T lymphocyte subsets, particularly $CD4^{+}$ and γδ T cells, generally reporting depressed $CD4^{+}$ T cells in peripheral blood of tuberculosis (TB) patients [1–3], but results are discrepant for γδ T cells, where both elevated [4,5] and normal [6,7] numbers have been found. Only a few but inconclusive reports of B lymphocyte and natural killer (NK) cell numbers in TB patients exist [1,3,8,9] and NK T cells have, to our knowledge, not been investigated in TB patients. Generally, contributors to TB susceptibility remain unclear and follow-up data during therapy are scanty.

The aim of our study was to investigate immune parameters during therapy and this report describes a systematic follow-up of leucocyte counts and lymphocyte subsets in TB patients for the entire 26-week treatment period. Furthermore, due to the fact that the identification of high-risk patients for slow response to chemotherapy would have important clinical implications, we analysed peripheral blood immunophenotypes as potential surrogate markers of early TB treatment response and applied a multivariate classification technique to identify fast and slow responders to treatment by immunophenotype at diagnosis.

Materials and methods

Setting

This study was conducted in an epidemiological field site in metropolitan Cape Town, where the incidence of new smear and/or culture-positive TB was on average 313/100 000 population/year (1993–98) [10].

Patients and controls

The study was approved by the Ethics Committee of the Faculty of Health Sciences at Stellenbosch University and written, informed consent was obtained from all participants.

Twenty-nine new smear-positive pulmonary TB patients were screened for this study. Inclusion criteria included: sputum culture-positive for Mtb, no multi-drug resistance, HIV-negative, taking at least 80% of prescribed doses during the intensive phase of treatment. Eight patients were excluded for the following reasons: non-compliance, multi-drug-resistant TB, negative sputum culture, refusal of HIV testing or incomplete follow-up visits. Twenty-one patients with first-time TB were enrolled and studied throughout treatment. Blood samples were taken at diagnosis prior to initiation of treatment and at weeks 1, 5, 13, and 26 after start of treatment (the last blood sample being taken on the last day of chemotherapy). Sputum smears and Bactec cultures were performed on days 1 and 3, and weeks 1, 2, 4, 8, 13 and 26 after start of treatment. A total white cell count (WCC) and differential blood count was performed on all blood samples using a Bayer Advia 120. The patients received standard therapy in accordance with the South African National Tuberculosis Program [based on World Health Organization (WHO) guidelines]. Therapy consisted of a fixed drug combination (depending on body weight) containing isoniazid (320–400 mg/day), rifampicin (480–600 mg/day), ethambutol (800–1200 mg/day) and pyrazinamide (1000–1250 mg/day) during the intensive phase (8 weeks) followed by rifampicin and isoniazid during the continuation phase (the remaining 18 weeks) under direct observation. Posterior–anterior and lateral chest X-rays (CXR) were taken at commencement of treatment allowing a 4-week time window on either side of diagnosis. The chest radiographs were evaluated using a standardized method [11] by a physician who had no prior knowledge of the patient's condition. The extent of disease was estimated using a one-dimensional view of the upright posterior–anterior radiograph and by using the right upper lobe as reference area.

One blood sample was taken from each of 14 healthy HIV-negative, purified protein derivative (PPD) skin test-positive (> 15 mm) volunteers resident in the same community to serve as controls. These participants had no clinical or radiological signs of active TB.

Processing of sputum samples for Ziehl–Nielsen smear and culture

Sputum samples were processed for culture using standard methods [12], which included decontamination according to the Bactec 460TB System Procedure Manual (Becton Dickinson, Sparks, MD, USA) before inoculation into a Bactec 12B vial. The vials were incubated at 37°C and the growth index (GI) was read daily. Sputum smears, direct and concentrated, were examined for acid-fast bacilli using the Ziehl–Nielsen (ZN) stain and evaluated using the scoring system of the International Union against Tuberculosis and Lung Disease [13]. If multiple smears were performed the smear with the highest grade was recorded for that time-point.

Reagents

Fluorochrome-labelled monoclonal antibodies (mAb) anti-CD45-peridinin chlorophyll (PerCP), CD3-phycoerythrin (PE), CD3-PerCP, CD4-fluorescein isothiocyanate (FITC), CD8-FITC, CD19-FITC, CD56-FITC, γδ T cell receptor (TCR)-FITC, interferon (IFN)-γ-PE, interleukin (IL)-4-PE and rabbit anti-active caspase 3-FITC were from BD-Bioscience (Erembodegem, Belgium). A rabbit FITC control antibody was not available from the manufacturer. OKT3 anti-CD3 antibody was spent hybridoma medium. The hybridomas were from the American Type Culture Collection (ATCC, Rockville, MD, USA). Vα24-PE was purchased from Beckman Coulter (Johannesburg, South Africa), saponin from Sigma (Kempton Park, South Africa) and polyethylene glycol 4000 (PEG) from Merck (Cape Town, South Africa).

Immunophenotyping by flow cytometry

Whole blood (50 μl per test), anti-coagulated with sodium heparin, was washed once with phosphate-buffered saline (PBS), suspended in 100 μl of 0·1% bovine serum albumin (BSA), 0·05% sodium azide in PBS and added to the required antibody mixtures. After 20 min at 4°C, cells were washed and red blood cells (RBCs) lysed at the same time by diluting with 3–4 ml cold PBS containing 0·05% saponin, 0·05% sodium azide and 3% PEG. (We have noted previously that RBCs in whole blood from TB patients frequently failed to lyse when treated with commercial lysing solution and therefore used saponin as alternative lysis solution. The addition of 3% w/v PEG to the saponin buffer prevents damage and clumping of cells in blood obtained at diagnosis and also enhances the formation of antigen/antibody complexes [14]). After centrifugation at 700 ***g*** the cell pellets were fixed in 4% formaldehyde in PBS and stored at 4°C in the dark until flow cytometric analysis in a Becton-Dickinson fluorescence activated cell sorter (FACS)Calibur using CellQuest software. Lymphocytes were gated in a CD45-PerCP *versus* side scatter plot (10 000 events in this gate were acquired) and these were analysed further for expression of CD3 and CD4 (or CD8, CD19, CD56, γδTCR) in the FL1 and FL2 channels, respectively. The lymphocyte sums calculated were all between 95 and 100%. Isotype control antibodies were not used routinely as the background cell surface staining of *ex vivo* blood lymphocytes is very low (not shown).

Intracellular cytokine labelling

Briefly, whole heparinized blood was mixed 1 : 1 with RPMI-1640 medium with antibiotics in polypropylene tubes and incubated at 37°C with or without 0·1 μg/ml OKT3 antibody for 4 h, with 10 μg/ml Brefeldin A present during the last 3 h. After incubation the blood was diluted with cold PBS containing 0·05% saponin, 0·05% sodium azide and 3% PEG

(lyse/wash buffer), centrifuged in the cold at 700 ***g***, and the cells in the pellet were labelled with mAbs in the above buffer containing 0·1% BSA for 20 min in the cold. After one wash with cold lyse/wash buffer, the cell pellets were fixed in 4% formaldehyde in PBS and analysed in the flow cytometer.

Classification of patients into treatment response groups

In order to find possible differences between fast and slow responders to treatment, patients were divided into two responder groups according to Bactec culture status at week 8 after start of treatment. Of the 21 enrolled patients eight were culture-negative (fast responders) and 13 culture-positive (slow responders).

Statistical analysis

Data for patients at diagnosis and at the end of treatment were analysed for significant differences from those for healthy subjects by means of the Mann–Whitney test. The Friedman test with Dunn's post-test was used to analyse longitudinal changes in parameters with respect to the diagnosis time-point values (* or #: $P = 0{\cdot}01$–$0{\cdot}05$, ** or ##: $P = 0{\cdot}001$–$0{\cdot}01$, *** or ###: $P < 0{\cdot}001$; asterisks refer to the Mann–Whitney test and hashes to the Friedman test). The Pearson χ^2 test and Fisher's exact test were used to analyse categorical CXR data.

To find the best combination of variables at diagnosis that may have potential for the prediction of early treatment response, as defined by the week 8 Bactec sputum culture, a support vector machines analysis was performed, a multivariate discriminant classification technique that has received much attention in the statistical literature in the past few years [15]. Combinations of up to a maximum of five variables were analysed and, using the variables included in the optimal classification model, a leave-one-out cross-validation table was constructed.

Results

Demographic data of study population

The 21 patients were all cured after 26 weeks of standard directly observed treatment short course (DOTS) therapy. Three patients were infected with an Isoniazid-monoresistant strain of mycobacteria. After 8 weeks of treatment 15 patients were smear-negative and six were smear-positive, while only eight were culture-negative and 13 culture-positive (two of these were Isoniazid-monoresistant). The week 8 Bactec culture was therefore used as the more sensitive indicator of early treatment response. No significant differences between fast and slow responders in CXR findings at diagnosis were found (including extent of disease and presence, number or size of cavities). The age and sex distribution of patients is given in Table 1.

Table 1. Age and sex data of patients and controls.

	Patients		
	Fast responders[a]	Slow responders	Controls
Total (no.)	8	13	14
Male (no.)	3	9	3
Female (no.)	5	4	11
Age (years)	18–51	19–50	20–56

[a]As defined by negative sputum culture at week 8.

Longitudinal changes in total and differential WCC

The total WCC and absolute neutrophil counts were significantly elevated in patients at diagnosis relative to controls (Fig. 1a,b) but returned to normal levels by the end of treatment. The absolute monocyte counts were also significantly elevated at diagnosis but then dropped dramatically to significantly depressed levels at week 26 (Fig. 1c). The absolute lymphocyte count of patients at diagnosis was significantly depressed at diagnosis, but counts were no longer significantly different from controls at the end of treatment (Fig. 1d).

Lymphocyte subsets

Percentages of T lymphocytes and NK cells were not significantly different from those of controls at diagnosis or at week 26, while percentages of B lymphocytes were depressed in patients at diagnosis ($P < 0{\cdot}05$) and recovered during treatment (not shown). The absolute lymphocyte subset counts were calculated from the subset percentages and absolute lymphocyte counts (Fig. 2). The absolute $CD3^+$ T cell and absolute $CD19^+$ B cell counts were significantly depressed in patients at diagnosis, but at week 26 these were not significantly different from those of control subjects (Fig. 2a,b). Absolute $CD56^+/CD3^-$ NK cell counts at diagnosis showed a trend towards lower numbers ($P = 0{\cdot}06$) and remained depressed until week 26 ($P < 0{\cdot}05$, Fig. 2c).

T lymphocyte subsets

The percentages of $CD4^+$, $CD8^+$ and γδ T cells and the CD4 : CD8 ratio at diagnosis and at week 26 were not significantly different from those of control individuals and only small fluctuations were detected during follow-up. We detected two populations of NK T cells that differed in their levels of expression of CD3: a $CD56^+$ cell population which expressed CD3 levels comparable to conventional T cells ($CD3^{bright}/CD56^+$ NK T cells) and one that expressed reduced levels ($CD3^{dim}/CD56^+$ NK T cells). The percentages of $CD3^{bright}/CD56^+$ NK T cells in patients at diagnosis and at week 26 were not significantly different from those of controls (not shown) and $CD3^{dim}/CD56^+$ NK T cells are described in detail below. Absolute numbers of T cell subsets, calculated from the absolute lymphocyte count and the

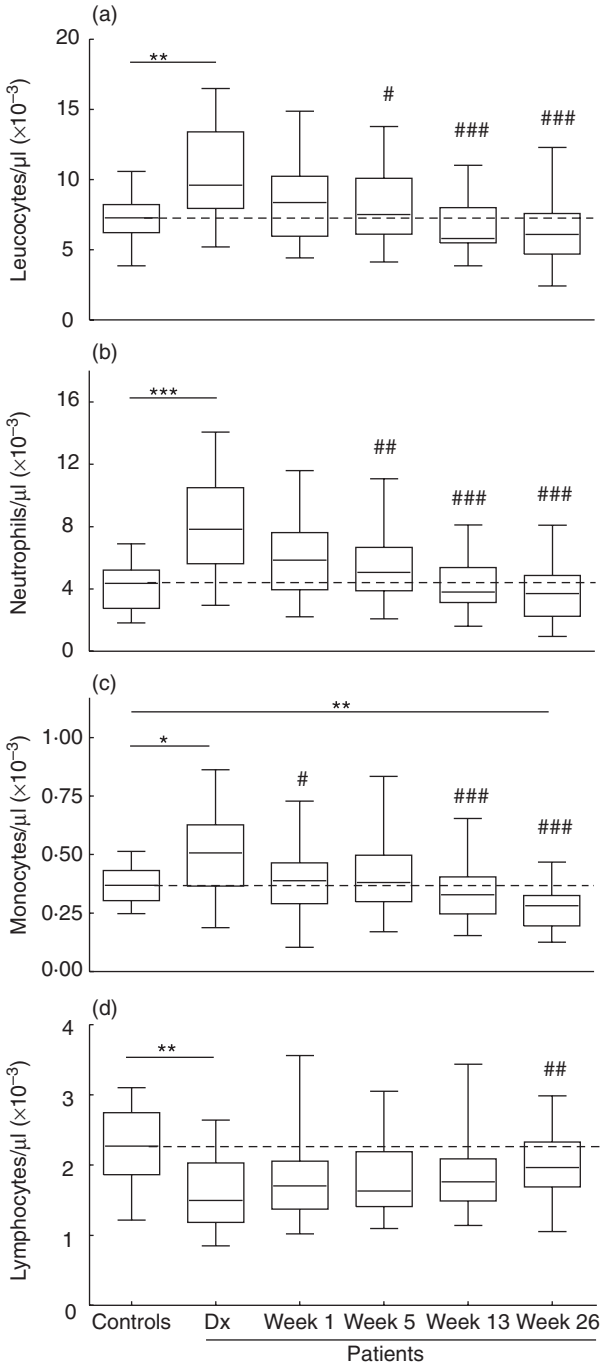

Fig. 1. Absolute leucocyte counts of healthy control subjects and tuberculosis (TB) patients, calculated from the total white cell count and differential blood count. (a) Total white cell count (WCC), (b) neutrophils, (c) monocytes, (d) lymphocytes. The boxes extend from the 25th to the 75th percentile with a line at the median and the whiskers show the highest and lowest values. Data for patients at diagnosis (Dx) and at the end of treatment at week 26 were analysed for significant differences from those for healthy subjects by means of the Mann–Whitney test (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$). The Friedman test with Dunn's post-test was used to analyse changes in parameters during the patients' follow-up with respect to values at diagnosis (#$P < 0{\cdot}05$, ##$P < 0{\cdot}01$, ###$P < 0{\cdot}001$).

percentages determined by immunophenotyping are illustrated in Fig. 3. $CD4^+$ T cell numbers (Fig. 3a) were significantly depressed at diagnosis relative to control subjects ($P < 0{\cdot}01$) and, while numbers increased significantly during treatment, they were still lower at week 26 than in controls ($P = 0{\cdot}06$). $CD8^+$ T cell counts were lower at diagnosis, although not significantly so (Fig. 3b, $P = 0{\cdot}13$) and γδ T cell counts were significantly depressed (Fig. 3c, $P < 0{\cdot}05$) but both subsets recovered during treatment to normal levels at week 26. Absolute numbers of $CD3^{bright}/CD56^+$ NK T cells were lower at diagnosis ($P = 0{\cdot}06$) and were significantly low at the end of treatment ($P < 0{\cdot}05$, Fig. 3d).

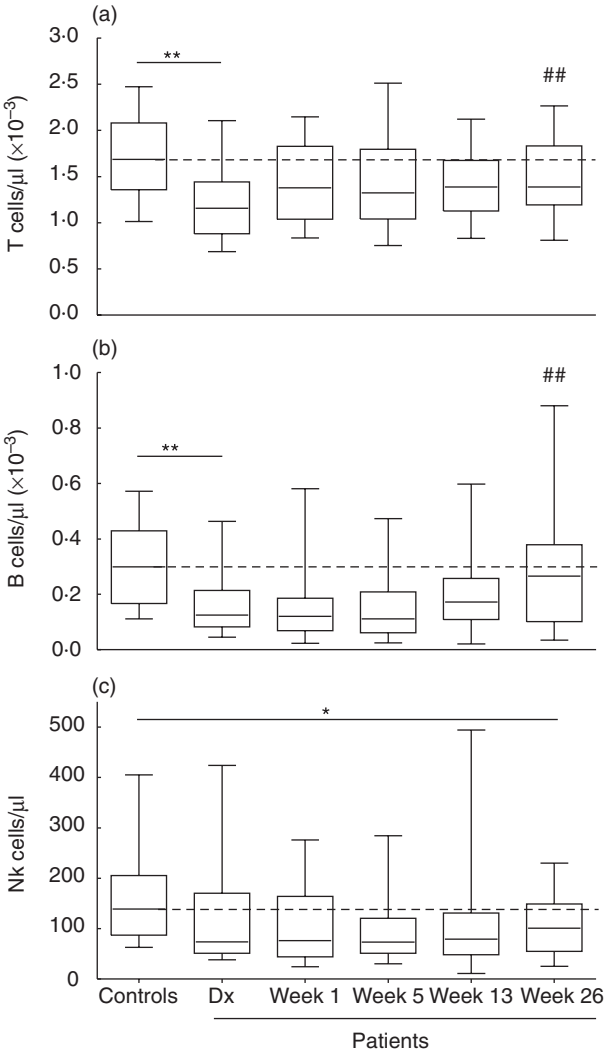

Fig. 2. Absolute lymphocyte subset counts of healthy control subjects and tuberculosis (TB) patients, calculated from the absolute lymphocyte counts and the percentages of subsets determined by flow cytometric immunophenotyping. (a) T lymphocytes ($CD3^+$), (b) B lymphocytes ($CD19^+$), (c) natural killer (NK) cells ($CD3^-CD56^+$). Box and whisker plots and statistical analyses as for Fig. 1 (Mann–Whitney test *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$, Dunn's post-test #$P < 0{\cdot}05$, ##$P < 0{\cdot}01$, ###$P < 0{\cdot}001$).

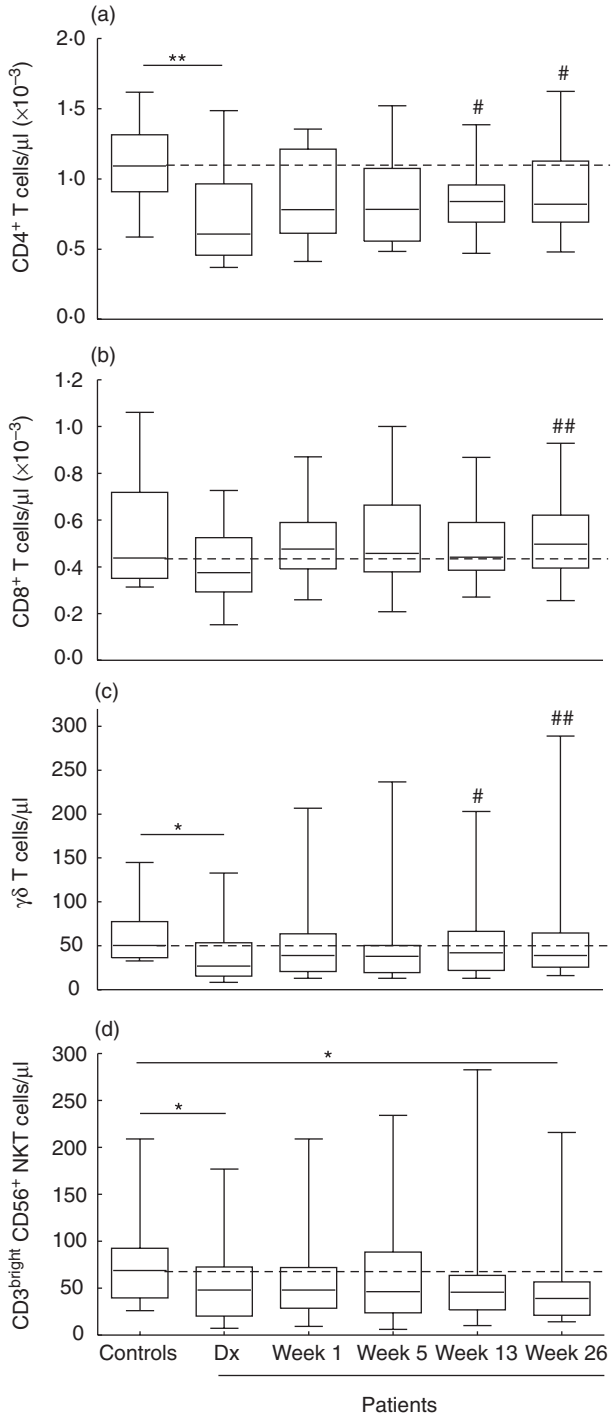

Fig. 3. Absolute T cell subset counts of healthy control subjects and tuberculosis (TB) patients, calculated from the absolute T cell counts and the percentages of the subsets determined by flow cytometric immunophenotyping. (a) $CD4^+$ T cells, (b) $CD8^+$ T cells, (c) γδ T cell receptor (TCR^+) T cells, (d) $CD3^{bright}/CD56^+$ natural killer (NK) T cells. Box and whisker plots and statistical analyses as for Fig. 1 (Mann–Whitney test $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, Dunn's post-test $\#P < 0{\cdot}05$, $\#\#P < 0{\cdot}01$, $\#\#\#P < 0{\cdot}001$).

A $CD3^{dim}/CD56^+$ NK T cell subset was more prominent in patients

We detected an unusual subset of lymphocytes more frequently in patients (nine of the 21 patients had ≥ 2% at diagnosis) than in controls (two of 14 had ≥ 2%). In the flow cytometric analyses, of which Fig. 4 is an example, these cells were weakly $CD3^+$ ($CD3^{dim}$), $CD4^-$, weakly $CD8^+$ or $CD8^-$ and $CD56^+$, shown in region R2 in Fig. 4d, and also $\gamma\delta TCR^-$ (not shown). The number of cells in region R2, as illustrated in Fig. 4, expressed as a percentage of the cells in the CD45 gate, was determined for all blood samples. Figure 5a shows increased percentages of $CD3^{dim}/CD56^+$ NK T cells in patients at diagnosis relative to control subjects, although this was not statistically significant ($P = 0{\cdot}23$). Very low or undetectable numbers remained so during follow-up, while higher numbers persisted and sometimes increased after start of treatment (shown for fast and slow responders in Fig. 5c); the highest recorded was 20·3% at week 1.

Differences between treatment response groups

When percentages and absolute numbers of each cell type at diagnosis in fast responders were compared to those at diagnosis of slow responders with a Mann–Whitney test, the percentages and absolute counts of $CD3^{dim}/CD56^+$ NK T cells at diagnosis were the only parameters that correlated significantly with treatment response – they were significantly higher at diagnosis in fast responders ($P = 0{\cdot}01$, Fig. 5b). The percentages of $CD3^{dim}/CD56^+$ NK T cells did not change significantly during follow-up and are shown for the fast and slow responding patients in Fig. 5c.

As the $CD3^{dim}/CD56^+$ NK T cell numbers at diagnosis did not correlate with treatment response in all patients, we used a multivariate classification technique to find combinations of variables that may classify patients more accurately into fast and slow responders. Differences between early response phenotypes were most prominent at diagnosis and the variables at diagnosis that were used for the analysis were the absolute numbers of leucocyte, lymphocyte and T cell subsets. In the support vector machines discriminant analysis the best classification of patients into the two treatment response groups could be obtained with just two variables: absolute $CD3^{dim}/CD56^+$ NK T cells and absolute NK cells which correctly classified all 13 slow responders and five of eight fast responders in a leave-one-out cross-validation.

$CD3^{dim}/CD56^+$ NK T cells produce IFN-γ and IL-4

To assess functional aspects of $CD3^{dim}/CD56^+$ NK T cells we analysed flow cytometric data of intracellular IFN-γ and IL-4 measurements in saponin-permeabilized T cells after a 4-h stimulation of whole blood with anti-CD3 antibody. In samples from patients with a prominent $CD3^{dim}/CD56^+$ NK T cell population, these cells are found in the CD3-PerCP

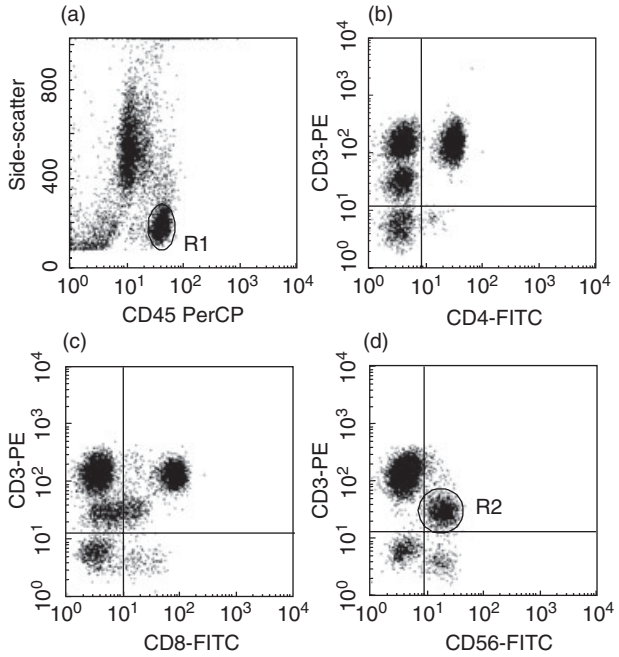

Fig. 4. A representative lymphocyte subset analysis of flow cytometric data from a patient with a prominent $CD3^{dim}/CD56^{+}$ natural killer (NK) T cell population. (a) Gating of the $CD45^{bright}$ low side scatter total lymphocyte population; (b,c,d) the gated lymphocytes analysed for CD3 and CD4, CD8 and CD56 expression, respectively. Region R2 in (d) contains the $CD3^{dim}/CD56^{+}$ NK T cells.

versus side scatter plots used for gating the T lymphocytes. The $CD3^{dim}$ and $CD3^{bright}$ cells were analysed separately. IFN-γ was only produced by some patients and the $CD3^{dim}$ and $CD3^{bright}$ cells produced comparable low levels of this cytokine. All patients showed IL-4 production by both stimulated and unstimulated T cells and this tended to be higher in $CD3^{dim}$ T cells (Fig. 6). The $CD3^{dim}$ population contains more cells that express active caspase 3, an indicator of apoptosis, and this expression correlates with higher levels of intracellular IL-4.

Discussion

In this study we have shown significant changes in absolute numbers of neutrophils, monocytes and lymphocyte subsets during active TB. Our finding that these changes occur already during the first weeks of treatment is important, as it suggests strongly that TB patients tested at different timepoints during their treatment should not be grouped together in the analysis of results. We detected a $CD3^{dim}/CD56^{+}$ subset of NK T cells that is more prominent in TB patients and correlates with a faster treatment response. A multivariate classification technique identified $CD3^{dim}/CD56^{+}$ NK T cells, in combination with NK cells, at diagnosis as variables indicating the likelihood of culture conversion early during TB treatment. NK T cells have, to our knowledge, not been reported in the context of TB disease and we believe that our findings support the future inclusion of these cells in the search for surrogate markers for treatment response.

The interesting subset of NK T cells found in this study expressed CD56 and reduced levels of CD3 and was either double-negative (DN) or weakly $CD8^{+}$. NK T cells, which express CD3 and to a variable degree the NK cell markers CD56, CD57 and CD161 [16–18], are a heterogeneous population in mice and humans with several subsets that differ in phenotype, TCR repertoire, MHC restriction and cytokine profile, as reviewed in [18]. 'Classical' NK T cells express an invariant T cell receptor (TCR) with Vα24 (Vα14–Jα281 in the mouse, now Vα14–Jα18), are CD1d restricted and express the NK cell marker CD161 or NKR-P1A. Two subsets of non-classical NK T cells do not express this invariant TCR. Human $CD56^{+}$ NK T cells are abundant in the liver, are pre-

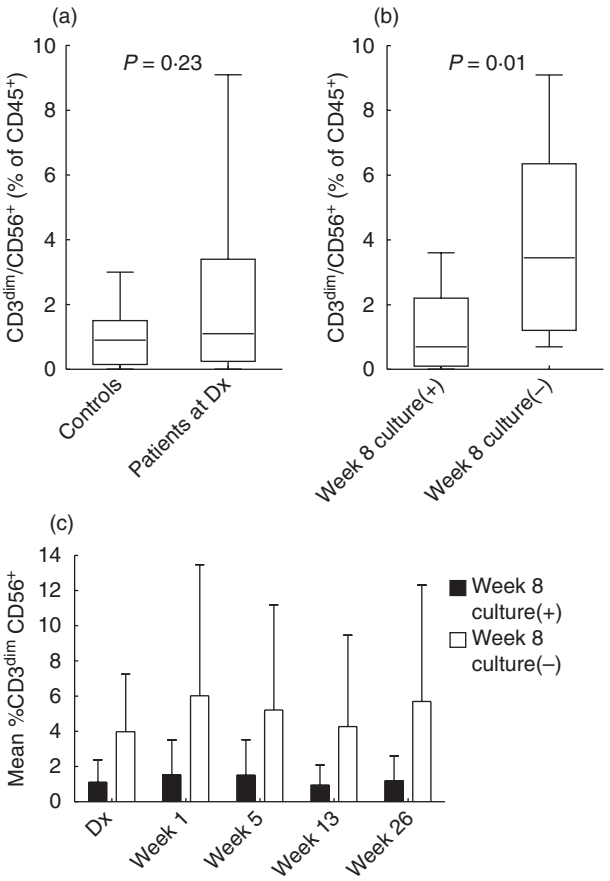

Fig. 5. $CD3^{dim}/CD56^{+}$ natural killer (NK) T cells. (a) Percentages in the lymphocyte gate in controls and patients at diagnosis compared with the Mann–Whitney test. (b) Percentages in the lymphocyte gate in patients at diagnosis grouped into slow responders to treatment [culture(+) at week 8] and fast responders [culture(–) at week 8], compared with the Mann–Whitney test. (c) Mean percentages of $CD3^{dim}/CD56^{+}$ NK T cell counts with s.d. error bars in the slow and fast responder patient groups from diagnosis to end of treatment.

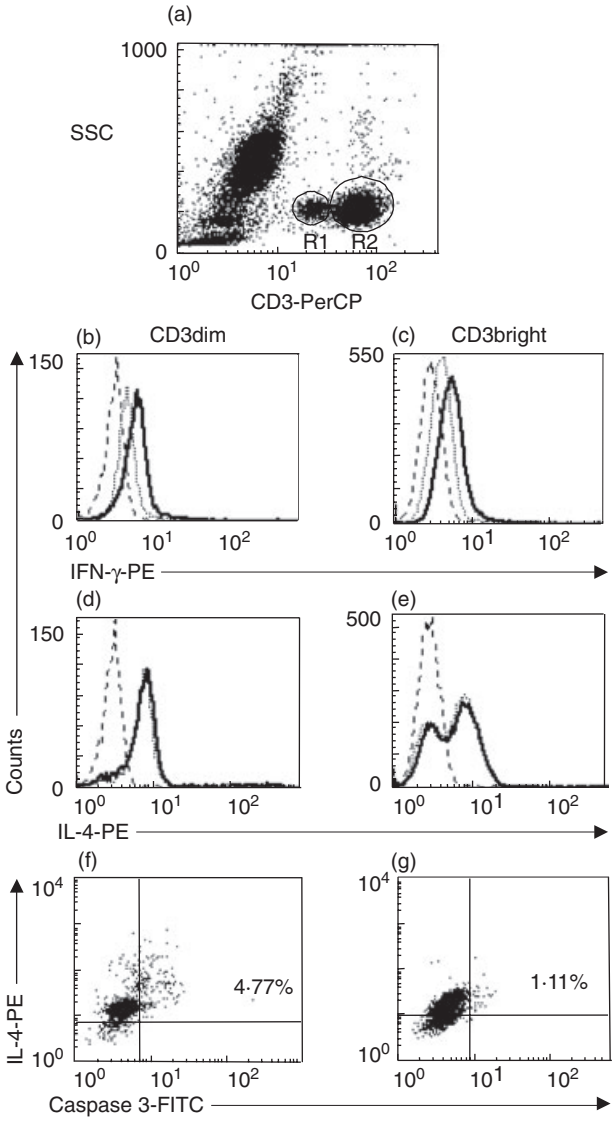

Fig. 6. Intracellular cytokine analysis of saponin-permeabilized lymphocytes from whole blood of two patients at diagnosis incubated for 4 h with or without stimulation with 0·1 μg/ml anti-CD3. (a) Gating of $CD3^{dim}$ (R1) and $CD3^{bright}$ (R2) T cells in a CD3-PerCP *versus* SSC plot. (b–e) Histograms of the gated cells of one patient showing IFN-γ (b,c) and interleukin (IL)-4 (d,e) expression. Overlaid histograms are: (–) stimulated, specific antibody, (···) unstimulated, specific antibody, (---) stimulated, control antibody. (f–g) Dot plots of similarly gated unstimulated T cells from another patient showing co-expression of caspase 3 and IL-4. The position of the quadrant markers was determined by a phycoerythrin-labelled control antibody (not shown).

dominantly $CD8^+$ or DN and Vα24 TCR-negative, have cytotoxic capacity and produce Th1 and Th2 cytokines when stimulated *in vitro* [19].

As our detection of the $CD3^{dim}/CD56^+$ NK T cells was unexpected, a Vα24 antibody was not included routinely in our panel, but some additional phenotypings with this antibody indicated that these cells did not express the invariant TCR (not shown). The possibility of artefactual $CD3^{dim}$ staining of NK cells due to non-specific binding to Fc receptors must be considered but this is unlikely, as all the antibodies used were of the IgG1 isotype and the $CD3^{dim}$ cells would be double-labelled with the CD4, CD19 and γδTCR antibody as well, which was not the case and, furthermore, NK cells do not express the high affinity Fcγ receptors CD32 and CD64 and can be seen as a clearly CD3-negative population in Fig. 4d.

The reduced expression of CD3 could be the result of TCR down-regulation [20] and the $CD3^{dim}/CD56^+$ NK T cells could be an activated subset of $CD3^{bright}/CD56^+$ NK T cells, but we found only a weak inverse correlation between the percentages of these NK T cell subsets (Spearman's correlation coefficient −0·34, not shown). Takayama *et al.* [21] demonstrated that a $CD122^+$ subset of human CD8 T cells with intermediate TCR expression in the peripheral blood produce high levels of IFN-γ and are also potently cytotoxic.

Peripheral blood $CD56^+$ T cells are increased during the early phase of *Plasmodium falciparum* or *P. vivax* infections in humans [22], suggesting an important role in the immune response to intracellular pathogens. Slifka *et al.* [23] found that 90% of virus-specific $CD8^+$ and $CD4^+$ T cells from choriomeningitis virus-infected mice co-express one or more NK cells markers for more than 500 days post-infection. In our patients we did not detect much variation in the percentages of $CD3^{dim}/CD56^+$ NK T cells over time and they could represent a similar persistent population specific for mycobacterial antigens.

Our observation of the often higher numbers and percentages of $CD3^{dim}/CD56^+$ NK T cells in patients indicates that this cell population is expanded in the blood of some TB patients, and that these patients are able to clear the infection more efficiently after the initiation of chemotherapy. As $CD3^{dim}/CD56^+$ NK T cells appear to produce variable IFN-γ and IL-4, we postulate that they are cells that have been activated, as could be indicated by their reduced CD3 expression, and are at variable stages between activation and apoptosis. This is supported by our finding that they contain a higher percentage of cells expressing active caspase 3 and that they produce more intracellular IL-4. Previous findings have associated intracellular IL-4 expression in lymphocytes with mitochondrial apoptosis markers [24]. Therefore $CD3^{dim}/CD56^+$ NK T cells could be indicators of an active immune system in TB patients and would accelerate clearance of the infection by antibiotics.

The other variable that, together with $CD3^{dim}/CD56^+$ NK T cells, had predictive value according to our multivariant discriminative analysis, was the absolute NK cell count. Interestingly, a higher NK cell count is partially indicative of a slow response to treatment. A higher NK cell count in the peripheral blood may be the result of an inability of these cells to migrate into infected tissues. In humans NK cells are present in tuberculous pleural effusions [25], and in mice infected with Mtb NK cell numbers in the lung increase over the first 21 days of infection, although their removal does not

affect host resistance. A role of NK cells in the control of TB has been suggested by the results of *in vitro* studies with human NK cells and Mtb-infected monocytes [26–28].

Monocytes/macrophages are important components of the innate immune response to mycobacterial infections and the dramatic change in the absolute monocyte counts in our patients between diagnosis and week 26 should be noted. The surprising finding here is that their numbers are significantly depressed in fully treated patients and it is unknown what causes this depressed absolute monocyte count.

To determine whether the depressed absolute monocyte, NK cell and $CD3^{bright}/CD56^{+}$ NK T cell counts at the end of treatment could contribute to increased susceptibility to TB relapse [10], phenotyping needs to be performed on larger numbers of blood samples taken after cessation of antibiotic treatment with subsequent long-term clinical follow-up.

A drawback of our study is that the patient numbers in the two treatment response groups are small and therefore the accuracy of the statistical classifications is limited. It is also not optimal that in our study, for logistical reasons, the week 26 blood samples were taken on the day of the last dose of antibiotics and not after cessation of drug therapy. It is unknown whether drug treatment directly affects cell counts.

In summary, peripheral blood white cell counts change rapidly during treatment and some counts at diagnosis hold promise as surrogate markers of treatment response. Further prospective studies with larger numbers of patients are now needed to evaluate the role of immunophenotyping in general and of $CD3^{dim}/CD56^{+}$ NK T cells specifically, including their functional characterization. The role of these cells in predicting differential outcomes at month 6 and the development of recurrence after cure needs to be assessed.

Acknowledgements

We gratefully acknowledge the contributions by Erica Engelke, Marianna de Kock, Ilse Steyn and the clinical and administrative staff of the Department of Paediatrics and Child Health of the University of Stellenbosch, Professor Robert Gie for CXR analysis and the Department of Hematology at Tygerberg Hospital for performing full blood counts. Funding assistance for this study were provided by GlaxoSmithKline, Stevenage, UK and the DST/NRF Centre of Excellence for Biomedical and TB Research.

References

1 Onwubalili JK, Edwards AJ, Palmer. L. T4 lymphopenia in human tuberculosis. Tubercle 1987; **68**:195–200.
2 Rodrigues DSS, Medeiros EAS, Weckx LY, Bonnez W, Salomao R, Kallas EG. Immunophenotypic characterization of peripheral T lymphocytes in *Mycobacterium tuberculosis* infection and disease. Clin Exp Immunol 2002; **128**:149–54.
3 Swanson Beck J, Potts RC, Kardjito T, Grange JM. T4 lymphopenia in patients with active pulmonary tuberculosis. Clin Exp Immunol 1985; **60**:49–54.
4 Balbi B, Valle MT, Oddera S *et al.* T-lymphocytes with γδ+ Vδ2+ antigen receptors are present in increased proportions in a fraction of patients with tuberculosis or with sarcoidosis. Am Rev Respir Dis 1993; **148**:1685–90.
5 Ito M, Kojiro N, Ikeda T, Ito T, Funada J, Kokubu T. Increased proportions of peripheral blood γδ T cells in patients with pulmonary tuberculosis. Chest 1992; **102**:195–7.
6 Barnes PF, Grisso CL, Abrams JS, Band H, Rea TH, Modlin RL. γδ T lymphocytes in human tuberculosis. J Infect Dis 1992; **165**:506–12.
7 Li B, Rossman MD, Imir T *et al.* Disease-specific changes in γδ T cell repertoire and function in patients with pulmonary tuberculosis. J Immunol 1996; **157**:4222–9.
8 Corominas M, Cardona V, Gonzalez L *et al.* B-lymphocytes and co-stimulatory molecules in *Mycobacterium tuberculosis* infection. Int J Tuberc Lung Dis 2004; **8**:98–105.
9 Nirmala R, Narayanan PR, Mathew R, Maran M, Deivanayagam CN. Reduced NK activity in pulmonary tuberculosis patients with/without HIV infection: identifying the defective stage and studying the effect of interleukins on NK activity. Tuberculosis (Edinb) 2001; **81**:343–52.
10 Verver S, Warren RM, Beyers N *et al.* Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. Am J Respir Crit Care Med 2005; **171**:1430–5.
11 Simon G. Radiology in epidemiological studies and some therapeutic trials. Br Med J 1966; **5512**:491–4.
12 Carroll NM, Richardson M, Engelke E, de Kock M, Lombard C, van Helden PD. Reduction of the rate of false-positive cultures of *Mycobacterium tuberculosis* in a laboratory with a high culture positivity rate. Clin Chem Lab Med 2002; **40**:888–92.
13 Enarson DA, Rieder HL, Arnadottir T, Trébucq A. Management of tuberculosis. A guide for low income countries. Paris: International Union Against Tuberculosis and Lung Disease (IUATLD), 2000.
14 Harrington JC, Fenton IIJW, Pert JH. Polymer-induced precipitation of antigen-antibody complexes: 'precipiplex' reactions. Immunochemistry 1971; **8**:413–21.
15 Hastie T, Tibshirani R, Friedman J. Support vector machines and flexible discriminants. In: The elements of statistical learning. Data mining, inference and prediction. New York: Springer, 2001:371–91.
16 Loza MJ, Metalitsa LS, Perussia B. NK T and T cells: coordinate regulation of NK-like phenotype and cytokine production. Eur J Immunol 2002; **32**:3453–62.
17 Ohkawa T, Seki S, Dobashi H *et al.* Systematic characterization of human $CD8^{+}$ T cells with natural killler cell markers in comparison with natural killer cells and normal $CD8^{+}$ T cells. Immunology 2004; **103**:281–90.
18 Godfrey DI, McDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NK T cells: what's in a name? Nat Rev Immunol 2004; **4**:231–7.
19 Doherty DG, Norris S, Madrigal-Estebas L *et al.* The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. J Immunol 1999; **163**:2314–21.
20 Müller S, Demotz S, Bulliard C, Valitutti S. Kinetics and extent of protein tyrosine kinase activation in individual T cells upon antigenic stimulation. Immunology 1999; **97**:287–93.
21 Takayama E, Koike Y, Ohkawa T *et al.* Functional and Vbeta repertoire characterization of human CD8+ T-cell subsets with

natural killer cell markers, CD56+ CD57- T cells, CD56+ CD57+ T cells and CD56- CD57+ T cells. Immunology 2003; **108**:211–19.

22 Watanabe H, Weerasinghe A, Miyaji C *et al.* Expansion of unconventional T cells with natural killer markers in malaria patients. Parasitol Int 2003; **52**:61–70.

23 Slifka MK, Pagarigan RR, Whitton JL. NK markers are expressed on a high percentage of virus-specific $CD8^+$ and $CD4^+$ T cells. J Immunol 2000; **164**:2009–15.

24 Stein GM, Pfüller U, Schietzel M, Büssing A. Expression of interleukin-4 in apoptotic cells: stimulation of the type-2 cytokine by different toxins in human peripheral blood mononuclear and tumour cells. Cytometry 2000; **41**:261–70.

25 Arruda S, Chalhoub M, Cardoso S, Barrol-Netto M. Cell-mediated immune responses and cytotoxicity to mycobacterial antigens in patients with tuberculous pleurisy in Brazil. Acta Trop 1998; **71**:1–15.

26 Brill KJ, Li Q, Larkin R *et al.* Human natural killer cells mediate killing of intracellular *Mycobacterium tuberculosis* H37Rv via granule-independent mechanisms. Infect Immun 2001; **69**:1755–65.

27 Yoneda T, Ellner JJ. $CD4^+$ T cell and natural killer cell-dependent killing of *Mycobacterium tuberculosis* by human monocytes. Am J Respir Crit Care Med 1998; **158**:395–403.

28 Vankayalapati R, Klucar P, Wizel B *et al.* NK cells regulate $CD8^+$ T cell effector function in response to an intracellular pathogen. J Immunol 2004; **172**:130–7.