

**IMMUNE RESPONSES OF PATIENTS WITH
TUBERCULOSIS AND HEALTHY CONTROLS
OF DIFFERENT AGES**

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DECLARATION

I, the undersigned, hereby declare the work contained in this thesis is my own original work and has not, to my knowledge, previously in its entirety or in part been submitted at any university for a degree.

Signature:

Date:

SUMMARY

The immune system matures progressively from infancy to adulthood, thus children may differ from adults in their immune function. The immature immune system demonstrates a higher naïve to memory T cell ratio, defective macrophage function and antigen presentation which, cumulatively, results in diminished production of cytokines such as IFN- γ . This cytokine has been shown to play a pivotal role in protection against *Mycobacterium tuberculosis* (*M.tuberculosis*) disease. Other cytokines, such as IL-12 and TNF- α , are also involved in the defence against *M.tuberculosis*. Epidemiological evidence suggests an age-related incidence of tuberculosis (TB) irrespective of prevalence in a given region. Reports in the literature also demonstrate depressed immune responses in TB patients, at diagnosis, (before TB therapy) with subsequent improvement after TB therapy.

The aims of this study were to optimise a whole blood assay in order to characterise immune responses, as measured by proliferation and cytokine production, in TB patients (after TB therapy) and healthy controls of different ages. Immune responses of TB patients would also be compared, before, and after TB therapy.

A total of 68 subjects were included in this study. These comprised 27 TB patients and 41 healthy Mantoux positive controls. All subjects were stratified into two age groups: <12 years and >12 years.

Diluted whole blood was cultured and stimulated with the mitogen, phytohaemagglutinin (PHA) and the specific mycobacterial antigen, purified protein derivative (PPD) to measure proliferation and IFN- γ , IL-2, TNF- α and IL-10 production in the supernatant of cultures.

Age was a significant variable for the following PHA-stimulated cytokines: IFN- γ , TNF- α and IL-10. Proliferation and IL-2 production after PHA stimulation did not demonstrate any relationship with age. None of the PPD-stimulated proliferative or cytokine responses demonstrated any correlation with age.

Concentrations of PHA- and PPD-induced IFN- γ for all subjects (patients and controls) were increased “after therapy”, compared to “before therapy”. This phenomenon could possibly be due to maturation in the capacity of the immune system to produce this cytokine.

Patients >12yrs demonstrated improvement in all proliferative and cytokine responses (except for PPD-induced IL-2 and TNF- α) “after therapy”, compared to “before therapy”. This is probably a valid finding and is thus in accordance with the literature.

The whole blood assay is a simple, non-laborious assay that, according to the literature, produces results that seem to correlate well with that of conventionally used PBMCs.

Age appears to be an important variable in the quantitative assessment of cellular immune responses (when the mitogen, PHA is used as a stimulant) and immune responses of older TB patients appear to improve after TB therapy, compared to before TB therapy.

OPSOMMING

Die immuunsisteem matureer stelselmatig van kind na volwassene. Dus sal kinders se immunititeit verskil van volwassenes s'n. Die immature immuunsisteem het 'n hoër naïwiteit vir geheue T-sel verhouding, defektiewe makrofaag funksie en antigeen presentering wat gesamentlik lei tot verminderde produksie van sitokiene soos byvoorbeeld IFN- γ . Daar is bewys dat hierdie sitokien 'n deurslaggewende rol speel in die beskerming teen *Mycobacterium tuberculosis* (*M. tuberculosis*). Ander sitokiene, soos IL-12 en TNF- α speel ook 'n rol in die beskerming teen *M. tuberculosis*. Epidemiologiese data dui aan dat daar 'n ouderdomverwante insidensie van tuberkulose (TB) is sonder dat dit beïnvloed word deur die voorkoms van TB in 'n sekere area. Verslae in die literatuur wys ook op onderdrukte immunitetrespons in TB-pasiënte by diagnose (vóór TB-behandeling) met uiteindelijke verbetering ná TB-behandeling.

Die doel van hierdie studie was om 'n volbloed metode te optimaliseer in 'n poging om die immuunrespons te karakteriseer soos gemeet met behulp van proliferasie en sitokien produksie by TB-pasiënte (ná TB-behandeling) en gesonde kontrole persone van verskillende ouderdomme. Die immuunrespons van TB-pasiënte word ook vergelyk vóór en ná TB-behandeling.

'n Totaal van 68 gevalle is vir die studie gebruik. Dit sluit in 27 TB-pasiënte en 41 gesonde Mantoux positiewe kontroles. Al die gevalle is in twee ouderdomsgroepe verdeel: <12 jaar en >12 jaar.

Kulture is gemaak van verdunde volbloed en gestimuleer met phytohaemagglutinin (PHA) en gesuiwerde proteien derivaat (purified protein derivative-PPD) om proliferasie en IFN- γ , IL-2, TNF- α en IL-10- produksie in die supernatant van die kulture te meet.

Ouderdom was 'n beduidende veranderlike vir die volgende PHA-gestimuleerde sitokiene: IFN- γ , TNF- α en IL-10. Daar was geen korrelasie tussen proliferasie en IL-2-produksie ná PHA-stimulasie aan die een kant en ouderdom aan die ander kant nie. Geen van die PPD-gestimuleerde proliferasie response of sitokien response het enige korrelasie met ouderdom getoon nie.

Konsentrasies van PHA- en PPD-geïnduseerde IFN- γ vir alle gevalle (pasiënte en kontrole) was verhoog “ná behandeling”, vergeleke met “vóór behandeling”. Hierdie fenomeen kan moontlik toegeskryf word aan maturasie in die vermoë van die immuunsisteem om sitokiene te vervaardig.

Pasiënte >12 jaar het bewyse getoon van verbetering in alle proliferasie en sitokien response (behalwe vir PPD-geïnduseerde IL-2 en TNF- α) “ná behandeling”, vergeleke met “vóór behandeling”. Dit is waarskynlik ‘n geldige bevinding en is dus in ooreenstemming met verslae in die literatuur.

Die volbloed metode is ‘n eenvoudige metode wat nie baie arbeidsintensief is nie, wat volgens die literatuur, resultate lewer wat goed korreleer met die konvensionele gebruik van perifere bloed mononukliêre selle (PBMC’s).

Dit wil voorkom asof ouderdom ‘n belangrike veranderlike is in die kwantitatiewe beoordeling van sellulêre immuunrespons (wanneer PHA gebruik word as ‘n stimulant), en of die immuunrespons van ouer TB-pasiënte verbeter ná TB-behandeling in vergeleke met die respons vóór TB-behandeling.

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LIST OF ABBREVIATIONS

Ag	antigen
AIDS	acquired immunodeficiency syndrome
AM	alveolar macrophage
APC	antigen presenting cell
BCG	bacille Calmette-Guérin
BMC	bone marrow cell
BSA	bovine serum albumin
cpm	counts per minute
CR	chest radiograph
CR3	complement receptor 3
DOTS	direct observation therapy short course strategy
ELISA	enzyme-linked immunosorbant assay
Fc	Fragment crystallizable
FCS	fetal calf serum
HIV	human immunodeficiency virus
HCL	hydrochloric acid
Ig	immunoglobulin
INOS	inducible nitric oxide synthase
IFN- γ	interferon-gamma
IL-2	interleuken-2
IL-4	interleuken-4
IL-5	interleuken-5
IL-10	interleuken-10
IL-12	interleuken-12
IL-18	interleuken-18
KCL	potassium chloride
KH ₂ PO ₄	potassium di-hydrogen phosphate
LAM	lipoarabinomannan
LPS	lipopolysaccharide
Man pos	mantoux positive

MHC	major histocompatibility complex
MØ	macrophage
MDR	multi-drug resistant
<i>M.tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MW	molecular weight
NaCl	sodium chloride
Na ₂ CO ₃	di-sodium carbonate
NaHCO ₃	sodium hydrogen carbonate
Na ₂ HPO ₄	di-sodium hydrogen phosphate
NK	natural killer cell
NO	nitric oxide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PPD	purified protein derivative
Pt	patient
RNO	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RPMI	Rosewall Park Memorial Institute
SD	standard deviation
SNF	supernatant fluid
TAP	transporter associated with antigen processing
TB	tuberculosis
TCR	T cell receptor
TGF-β	transforming growth factor-beta
Th1	type 1 helper cell
Th2	type 2 helper cell
TNF-α	tumour necrosis factor-alpha
TST	tuberculin skin test
WBA	whole blood assay
yrs	years
ZN	Ziehl Neelsen

CHAPTER 1

INTRODUCTION

1.1 IMMUNOBIOLOGY OF THE IMMUNE SYSTEM

The human body is constantly besieged by a multitude of microbes, which include bacteria, viruses, fungi and parasites. Healthy individuals protect themselves against the replication and spread of these microbes by means of their immune system. Immunity to microbes is the result of the interaction between the two components of the immune system: innate (non-specific) immunity and adaptive (specific) immunity. In order for the immune system to destroy invading pathogens, it must be able to distinguish “self” from “non-self”, thereby destroying the pathogen without damaging the host itself. This concept is fundamental to the normal functioning of the immune system.

1.1.1 Innate Immunity

The innate immune system consists of defences which are already in place and do not require memory in order to prevent spread and replication of many common infectious agents. The first line of defense is the tough barrier of the skin, which, if intact, is impenetrable to most infectious microbes. Lysozyme (present in most secretions), stomach acids or entrapment in the sticky mucous of the nose or throat, with subsequent expulsion by sneezing or coughing, are additional non-specific defence mechanisms responsible for repelling most infectious agents. Spermine and zinc in semen, lactoperoxidase in milk and commensal organisms in the gut and vagina also form part of innate immune defences. If infectious organisms manage to penetrate the outer defences of the body, the cells of the innate immune system are the next line of defence. These include phagocytes (neutrophils, monocytes and macrophages) and natural killer (NK) cells. Macrophages and neutrophils have surface receptors (pattern recognition receptors) that have evolved to recognize and bind common components (elements) of many bacterial surfaces. Binding of bacterial molecules to these receptors triggers these cells to engulf (phagocytose) the bacterium with subsequent killing by oxygen-dependent and oxygen-independent microbicidal mechanisms. Phagocytosis induces the secretion of chemical mediators called cytokines, which produce a range of effects known collectively as the inflammatory response. Important components of the innate immune

system include cytokines, collectins (bind to bacterial cells via carbohydrates), NK cells (cytotoxic lymphocytes), acute phase proteins (bind and neutralize toxins and limit tissue damage) and complement (a multi-component enzyme cascade that enhances phagocytosis by coating microorganisms).

Persistent microorganisms, not destroyed by components of the innate immune system, activate the adaptive or specific immune system. Innate immunity mechanisms are vitally important, not only to hold the infectious agent in check while the adaptive immune response is being activated, but also to direct the immune response that subsequently develops.

1.1.2 Adaptive Immunity

The cells that play a major role in the adaptive immune response are T lymphocytes (responsible for cell mediated immunity and also important in humoral immunity) and B lymphocytes (responsible for humoral immunity). Each of these lymphocytes carries cell surface receptors of a single specificity. When the lymphocyte encounters and binds to a specific antigen (in conjunction with a co-stimulatory signal), it proliferates and gives rise to many identical progeny, called clones. This process, known as clonal selection, is probably the single most important principle of adaptive immunity (Janeway, 1992). Another very important property of adaptive immunity is immunological memory: the ability of the immune system to respond faster and more specifically when encountering a pathogen on a repeat occasion.

1.1.2.1 B Lymphocytes

B lymphocytes, like T lymphocytes, arise from a common progenitor stem cell in the bone marrow. The B cells remain in the bone marrow until they are mature and subsequently migrate through the blood stream into peripheral lymphoid organs. Each mature B cell has specific cell-surface immunoglobulin (Ig) molecules that are specific for a specific antigen. When these antigens come into contact with their specific immunoglobulin receptors (in conjunction with a signal provided by T-cells), they are activated and secrete immunoglobulins as soluble antibodies. Various isotypes of immunoglobulin can be secreted (IgM, IgD, IgG, IgE, IgA), each with different functions, resulting in a variety of responses to the same antigen under different conditions. These antibodies subsequently bind to the pathogen, resulting in engulfment and destruction by phagocytes. B-cells are thus extremely important in providing defences against extracellular pathogens.

1.1.2.2 T Lymphocytes

After production in the bone marrow, T lymphocytes migrate to the thymus where they mature. These antigen naïve T cells subsequently circulate through the blood stream and lymphoid organs. Most T cells in peripheral blood express a T cell receptor (TCR) that consists of a heterodimeric $\alpha\beta$ chain. Similar to the B cell receptor, each TCR is also a specific receptor for a specific antigen. A complex of three cell-surface proteins, collectively called CD 3 is associated with the TCR receptor. In addition, T-cells co-express the cell-surface proteins CD 4 or CD8, which together with CD 3, are very important in signal transduction.

Cells that are infected with pathogens process foreign proteins (from these pathogens) into peptide fragments that are transported to the cell surface, stably bound to specialized glycoproteins called major histocompatibility complex (MHC) proteins. These MHC proteins present antigens to T cells. There are two classes of MHC proteins: MHC class I (present on all nucleated cells and recognized by CD 8 positive T cells), and MHC class II (present on B cells, macrophages and dendritic cells and recognized by CD 4 positive T cells). When the specific TCR of antigen-naïve cells recognizes specific MHC-bound antigen (in conjunction with a co-stimulatory signal), the cells are activated through the TCR with subsequent proliferation and differentiation into armed effector cells. On subsequent encounters with their specific antigen, these armed effector cells perform a number of functions, the most important being killing of infected cells (by CD8 cytotoxic T cells) and activation of microbicidal properties of macrophages (by a subset of CD4 positive T cells). Together, these make up the most important components of cell mediated immunity.

A small number of T cells express a different T cell receptor. This receptor is called a $\gamma\delta$ receptor and is expressed on 1-5% of T cells present in peripheral blood and lymphoid organs. Generally, $\gamma\delta$ T cells are thought to be non-MHC-restricted and function largely as cytotoxic T cells.

1.1.2.3 Cytokines

Cytokines are low molecular weight soluble proteins involved in cell growth and differentiation, inflammation, immunity and repair. Cytokines play a pivotal role in facilitating communication between immune cells and coordinating both the activation and

dampening of the immune response to infection or injury. Cytokines are secreted upon stimulation (for example, in infection), and, once released, their half-life is short. Cytokines normally act in the local milieu in a paracrine or autocrine manner, unlike hormones that are transported through the blood stream to their target organs (Hamblin, 1993). Following stimulation, many cells (for example: T cells, B cells, macrophages and NK cells), can secrete a variety of cytokines, which exert an array of functions. Table 1.1 shows a few well-defined cytokines with some known functions.

Table 1.1 Cytokines: sources and functions

Cytokine Name	MW (kDa)	Cellular Source	Major Functions
IL-2	15-20	T cells	↑ growth and differentiation of T cells, B cells and NK cells
IL-4	15-19	T cells, mast cells and basophils	↑ differentiation of B cells and Th2 cells; ↑ IgG ₄ and IgE synthesis; ↓ proinflammatory Th1 cell and MØ function
IL-5	45; homo-dimer	T cells, mast cells and eosinophils	↑ growth and differentiation of eosinophils and B cells (IgA synthesis)
IL-10	35-40	T cells, B cells and MØ	↓ Th1, NK cells and MØ function including cytokine synthesis; ↑ B cell and mast cell proliferation
IL-12	35,40 subunits; heterodimer	B cells and MØ	↑ NK cells, CTL and Th1 generation, ↑ IFN-γ production by NK cells and T cells; ↑ NK and ADCC activity; costimulates T cell proliferation
TGF-β	25; homo-dimer	Many cell types including T cells and monocytes/MØ	↑ IgA production and activation of naïve T cells; ↓ activation of monocytes and memory T cells; active in fibroblast growth and wound healing
IFN-γ	40-70	T cells and NK cells	Antiviral; ↑ MØ and NK cell function; ↑ MHC class I and II surface Ag expression
TNFα	17; homo-dimer	Many cell types including monocytes/MØ, B cells, T cells	Expressed as cell surface homotrimer, also shed in soluble form; ↑ fever and septic shock; cytotoxic for many tumour cell types

(From: Cytokine/Chemokine Manual. Application Manual. 2nd Ed. April 1998. Pharmingen Bectin/Dickinson Company)

Abbreviations:

↑	- increases	Th1	- type 1 helper cell
↓	- decreases	Th2	- type 2 helper cell
MØ	- macrophage	MHC	- major histocompatibility complex
NK	- natural killer	BMC	- bone marrow cell

Cytokines are usually grouped into 2 classes, depending on their function. T helper cell type 1 (Th1) cytokines activate macrophages, cytotoxic T cells and NK cells and play a vital role in the generation of cell mediated responses to intracellular organisms such as mycobacteria, viruses and protozoa. T helper cell type 2 (Th2) cytokines mediate the stimulation and differentiation of B cells, eosinophils and mast cells, all of which are involved in the generation of the humoral response, which is important for defense against large extracellular parasites such as helminthes.

1.1.2.4 Th1 and Th2 Cytokines

Th Phenotype	Cytokines Produced
Th1	IFN- γ , TNF- β , IL-2
Th2*	IL-4, IL-5, IL-6, IL-13

* IL-10 is usually classed as a Th2 cytokine in the mouse, but both Th1 and Th2 phenotypes can secrete IL-10 in humans.

The Th phenotypes, which were first described in the mouse model, are characterized by the cytokines they produce. Mouse Th1 cells were reported to secrete IFN- γ , while Th2 cells secreted IL-4 (Mosmann et al., 1986). Subsequent studies have also identified Th1 and Th2 cells in humans. These studies have established that Th1 cells produce IFN- γ , TNF- β , IL-2, while Th2 cells secrete IL-4, IL-5, IL-6, IL-13 (Romagnani et al., 1997; Delespesse et al., 1997). IL-10 is usually classed as a Th2 cytokine in the mouse, but both Th1 and Th2 phenotypes can secrete IL-10 in humans (Abbas et al., 1996; Zhai et al., 1999). Th1 and Th2 cells negatively cross-regulate each other. IFN- γ inhibits the development and response of Th2 cells, while IL-4 and IL-10 have strong anti-inflammatory effects and inhibit the development and response of Th1 cells (Abbas et al., 1996). Other Th cell types include Th0 cells, (precursors of Th1 and Th2), producing a mixed array of cytokines (Romagnani et al.,

1997), and Th3 cells, (CD 4 positive immune regulatory T cells) that secrete TGF- β (Weiner et al., 1997).

1.2.2.5 Measurement of cellular immune responses

Assessment of cells involved in immune responses can be of a quantitative as well as a qualitative nature. The quantitation of the various cells and subpopulations of cells, for example, T cells and B cells, is usually done by automated cell counting procedures and flow cytometrical analysis respectively.

Assessment of the qualitative function of cellular immune responses, specifically in-vitro T cell responses, can be done by measuring the ability of these cells to proliferate and produce cytokines in response to a variety of stimulants. For in-vitro analysis of proliferative and cytokine responses by human cells, most investigators have traditionally used isolated peripheral blood mononuclear cells (PBMC) suspended in RPMI supplemented with fetal calf serum (FCS) (Hirsch et al., 1999a) or human AB serum (Battacharryya et al., 1999).

More recently, the use of diluted whole blood has been implemented to analyse these responses, (Weir et al., 1994; Elliot et al., 1999; van Crevel et al., 1999). Results appear to parallel those obtained using PBMC, provided cell counts are within the normal ranges (Weir et al., 1994; De Groote et al., 1992). Whole blood assays have a number of advantages over PBMC cultures, these include: 1) a small volume of blood is required, 2) the whole blood assay is rapid and simple, 3) no unphysiological cell separation is involved and 4) there is no requirement for FCS which is known to contain TGF- β and growth factors produced by platelets that could alter T cell cytokine responses in vitro (Daynes et al., 1992). Furthermore, investigators have demonstrated that erythrocytes and neutrophils, which are not present in PBMC cultures, can provide supplementary cytokines and factors that contribute to lymphocyte and monocyte responses (Kalechman et al., 1993; Van der Pouw Kraan et al., 1995).

The whole blood assay thus appears to be a useful model to quantify cellular immune responses and since it was not established in our laboratories, one of the aims of this project was to set up the assay and establish optimal conditions.

1.2 IMMUNOBIOLOGY OF TUBERCULOSIS (TB)

1.2.1 Introduction

Tuberculosis (TB) infection in humans and mammals continues to be a major cause of morbidity and mortality throughout the world. The disease in man is usually caused by infection by the obligate human pathogen, *Mycobacterium tuberculosis* (*M.tuberculosis*). TB is one of the most important infections in man and is responsible for causing approximately 2 million deaths and 8 million new cases each year (World Health Organization, 1999).

In recent years, the global TB epidemic has worsened. At least four factors have contributed to the re-emergence of TB in industrialized nations such as the United States. These include: (1) increased homelessness and substance abuse in inner cities; (2) the growing epidemic of the acquired immunodeficiency syndrome (AIDS); (3) the migration of infected individuals from high-prevalence countries and (4) the decline of tuberculosis control programs. In addition, the situation is worsened by the increasing incidence of multi-drug resistant (MDR) TB.

The largest burden of TB is found in Southeast Asia (3 million cases per year) (World Health Organization, 2000). Sub-Saharan Africa has 1.6 million cases per year and the overall notification rate for South Africa specifically was reported to be 366 new cases/100 000 population (Beyers et al., 1996). The highest notification rate within South Africa was reported in the Western Cape (718 new cases/100 000 population) (Beyers et al., 1996).

1.2.2 The bacillus

Robert Koch first identified the tubercle bacillus more than a century ago. It is a small, non-motile rod, measuring between 1 and 4 μm in length and 0.3 to 0.6 μm in diameter. Three aspects of mycobacterial physiology strongly influence the pathogenesis of TB disease. First, the tubercle bacillus is an obligate aerobe, which is probably partly responsible for the tissue tropism, growing well in oxygen-rich tissues such as lungs and more slowly in oxygen-poor tissues, such as the liver (Corper et al., 1927). Second, in optimal conditions, the bacillus grows very slowly, with a doubling time of about 20-24 hours. This contributes to the slowly progressing, chronic nature of the disease. Third, the bacillus has an extremely complex and lipid-rich cell wall. This lipid-rich wall is responsible for many of the unusual characteristics

of the mycobacterium, for example, chemical and stress resistance, ability to resist the killing mechanisms of macrophages and to replicate intracellularly, anti-microbial resistance and ability to stimulate a potent adjuvant response. This last characteristic may contribute to immunopathological manifestations of TB disease.

1.2.3 Susceptibility to TB

The host immune response, as well as the innate virulence of the TB bacillus itself, plays a major role in determining the clinical manifestation and final outcome of the infection. There is marked heterogeneity in the immune response of different individuals to *M.tuberculosis*. Resistance and control of the initial infection appears to occur in about 95% of normal hosts, while the other 5% develop progressive primary TB (Ellner, 1997). These heterogeneous immune responses to *M.tuberculosis* are determined by genetic, socio-economic as well as environmental factors. The innate immune system appears to play a pivotal role in susceptibility versus resistance to infection with *M.tuberculosis*, while the adaptive immune response appears to be involved in the control of the outcome of infection. In mice, IFN- γ is crucial for control of *M.tuberculosis* infection (Cooper et al., 1993; Flynn et al., 1993), as are IL-12 (Cooper et al., 1997), and CD 4 positive cells (Scanga et al., 2000). Polymorphic determinants of susceptibility to mycobacterial infection in mice have been identified as *nramp 1* and the *sst1* locus (Kramnik et al., 2000). Individuals who have been identified with single defects in the IFN- γ receptor and IL-12 receptor genes show an increased susceptibility to mycobacterial infections (Newport et al., 1996). In most individuals, however, multiple genetic factors have subtle effects on the immune response. These susceptibility genes or loci include the vitamin D receptor gene and others located on chromosomes 3, 5, 6, 8, 9, 15 and X (Hill et al., 1998).

Socio-economic factors also have an impact on susceptibility to *M.tuberculosis*. Since the beginning of the 19th century, there has been a sharp decline in the incidence of TB disease in industrialized countries. This steady decline coincided with an increase in the standard of living and has been interrupted by temporary increases during the two world wars.

Age is also an important risk factor for the development of TB disease. Young children who are exposed to *M.tuberculosis* are at increased risk of developing TB disease, especially disseminated and meningeal disease, compared to adults. This is more commonly seen in children younger than 1 year of age (discussed in more detail in section 1.3.3).

1.2.4 The diagnosis of tuberculosis

The diagnosis of TB is a vitally important element in the control of the disease. Not only should it be fast, but it should be accurate as well. The gold standard remains clinical examination, combined with the microscopic examination of sputum and culture of bacteria. *M.tuberculosis* culture can take up to 8 weeks and is not always successful, as the bacillus cannot be cultured in 10-20% of cases (Pottumarthy et al., 1999). Patients with subclinical infection present a further problem as current diagnostic strategy depends on patients presenting with clinical symptoms. Therefore the rapid and accurate diagnosis of *M.tuberculosis* infection can be problematical in many cases. There is thus an urgent need for better and faster diagnostic tests in TB. Assessment of human in-vitro cellular immune responses to TB infection could help in the development of such tests, as well as identify possible correlates of protection.

Various types of blood tests have been suggested as an alternative method of diagnosing TB. Antibody responses have not been helpful in this respect as they are directed at a broad set of antigens. In addition, responses vary among patients and the serological assays are not sensitive enough. As *M.tuberculosis* is an intracellular pathogen, an alternative approach is the assessment of whether a patient's T cells have been sensitised to *M.tuberculosis* antigens. This is the principal on which the tuberculin skin test (TST) or Mantoux skin test is based. Tuberculin or purified protein derivative (PPD) has generally been used as the *M.tuberculosis* antigen in this test, but the greatest disadvantage of PPD is that most of the protein components are shared between mycobacterial species or with unrelated species of bacteria (Anderson et al., 1994). This results in a decreased specificity of the TST. Many researchers have attempted to identify and isolate specific antigens from *M.tuberculosis* for use as diagnostic reagents (Nagai et al., 1991). This will be hopefully be achieved by the identification of regions in the *M.tuberculosis* genome that are absent in BCG and most non-tuberculous mycobacteria (Behr et al., 1999).

PPD has also been used for in-vitro detection of specific IFN- γ -producing lymphocytes that have been sensitised to mycobacterial antigens.

1.2.5 Initial TB infection

The route of entry of the mycobacterium into the host is usually via inhalation of aerosolised bacilli into the respiratory tract. Only particles smaller than 5-10 μ m in diameter, containing 1-10 bacilli, are small enough to reach the alveoli (Sonkin, 1951). Less than 10% of aerosolised bacilli will reach the alveoli, while the majority (droplets of a larger size) are expelled from the lower respiratory tract by the physical barriers of the nasopharynx and upper respiratory tract (reviewed by Fenton and Vermeulen, 1996).

After inhalation into the alveoli, the *M.tuberculosis* organism may either be killed, multiply (causing primary TB) or become dormant (causing latent infection). These so-called dormant bacteria may reactivate later, causing postprimary TB.

1.2.6 Interaction of *M.tuberculosis* with monocytes and macrophages

The *M.tuberculosis* bacterium binds to alveolar macrophages via many receptors. These include Fc receptors, complement receptors, mannose receptors, CD 14 and scavenger receptors (reviewed by Schluger and Rom, 1998). The choice of receptor used can also influence the cellular response. For example, entry of IgG-opsonised mycobacteria via Fc receptors promotes production of reactive oxygen intermediates and allows phagosome-lysosome fusion (Armstrong et al., 1975) while internalisation via CR3 inhibits the activation of the respiratory burst (Le Cabec et al., 2000).

Once the organism gains entry into the cell, it is internalized into a phagosome where it can potentially be killed by a number of mechanisms, many of which are poorly understood. In mice, reactive nitrogen intermediates (RNI), particularly nitric oxide (NO), are a prerequisite for the destruction of the organism. It has been reported that inducible nitric oxide synthase-(iNOS)-knock out mice demonstrate more rapid *M.tuberculosis* replication than wild type mice (MacMicking, 1997). In humans, macrophages can also produce NO, although at lower levels than that of mice and RNI probably play a role in the killing of the organism in human macrophages (reviewed by Schluger and Rom, 1998 and Fenton and Vermeulen, 1996). Reactive oxygen intermediates (ROI) alone have been shown to be insufficient to kill the *M.tuberculosis* organism, but they enhance killing by RNI (reviewed by Fenton and Vermeulen, 1996). Apoptosis of infected macrophages, induced by hydrogen peroxide, purinergic receptors (Lammas et al., 1997) or cytotoxic T cells, is another mechanism that the

host can potentially use to kill the organism. Apoptosis (by CD 8 T cells), mediated by the secretion of granules, but not via the fas pathway, contributes directly to mycobacterial killing (Stenger et al., 1997). Finally, cytotoxic T cells can also secrete small proteins called TIA-1 that have been shown to be involved in apoptosis (Law et al., 1996).

The *M.tuberculosis* bacillus utilizes a number of strategies to avoid being killed within the macrophage. It appears to modify the maturation of the phagosomal compartment via alterations in the protein content, including altered Rab GTPase composition (Clemens et al., 2000) and exclusion of the vacuolar ATPase with consequent lack of acidification (Sturgill-Koszycki et al., 1994). In addition, mycobacterial products such as sulphatides and lipoarabinomannan (LAM) have the ability to scavenge free radicals or inhibit the respiratory burst (reviewed by Fenton and Vermeulen, 1996). Finally, *M.tuberculosis* induces the secretion of immunosuppressive cytokines such as TGF- β and IL-10 which results in the down regulation of many responses that are necessary for the elimination of the *M.tuberculosis* organism (discussed in more detail in 1.2.8).

Mycobacteria also deploy a number of strategies to acquire essential nutrients. One of these nutrients is iron, which is required for intracellular survival. Macrophages also require iron as a co-factor in the induction of microbicidal effector mechanisms and this iron is acquired from the transferrin receptor, which internalizes extracellular iron bound to transferrin and lactoferrin. This complex is transported to an early endosomal recycling compartment where the mildly acidic conditions assist in the release of iron from the receptor. *M.tuberculosis* competes for this iron in a number of ways. For example, the mycobacterial phagosome is restricted in its maturation state to that of an early endosome. This endosome resides in the recycling endosomal pathway, which results in free access to the transferrin receptor with its iron bound to transferrin (Russel et al., 1996). In addition, mycobacteria have also developed specialized iron-binding molecules called siderophores. These molecules have a high affinity for intracellular iron and transfer iron from host proteins to specific mycobactin molecules in the mycobacterial cell wall (Gobin et al., 1996).

1.2.7 Regulation of immune responses by T cells

Before the development of a specific immune response, macrophages infected with *M.tuberculosis* are stimulated to produce IL-12, which subsequently induces NK cells to

secrete IFN- γ . This cytokine, together with TNF- α (also produced by infected macrophages), stimulates the production of more IL-12 from the infected macrophages, creating a positive feedback loop.

Macrophages infected with *M.tuberculosis*, also present processed antigens via MHC class II to CD 4 positive T cells, while some antigens, which escape to the cytosol of the macrophage, can be presented via MHC class I to CD 8 positive cells. Lipid antigens from mycobacterial cell walls (mycolic acid and LAM) are also recognized by T cells when associated with the non-polymorphic CD 1b molecules found on APCs. On recognition of the antigen, naïve T cells are stimulated to produce a mixture of cytokines (Th 0 response). After prolonged stimulation, CD 4 cells tend to polarize to either type 1 cells secreting mainly IFN- γ and IL-2, or type 2 cells secreting mainly IL-4, IL-5, IL-6 IL-10 and IL-13. A number of factors can influence the polarization of type 1 and type 2 cells. Infected macrophages also produce the immunosuppressive cytokines TGF- β and IL-10, which are capable of down-regulating type 1 responses.

CD 4 positive T cells play a crucial role in amplification of the host immune response by the activation of effector cells and recruitment of additional cells to the site of disease. The importance of this subset of T cells was confirmed in a number of experimental models using antibody depletion or knock-out mouse strains deficient in CD 4 positive T cells (Scanga et al., 2000). Also, the increased susceptibility of HIV-infected patients to TB, further demonstrates the critical role of CD 4 positive T cells. The main function of these cells is proposed to be the production of cytokines, specifically IFN- γ , which plays a crucial role in macrophage activation with subsequent induction of microbicidal effector mechanisms. In order to be activated, CD 4 cells require the help of certain co-stimulatory molecules that are present on APCs. Although little is known about the status of these co-stimulatory molecules in TB, one such molecule, CTLA-4, has been reported to be down-regulated in TB patients (Gong et al., 1996).

CD 8 positive T cells appear to be involved in the direct lysis and induction of apoptosis of infected cells. The importance of their role in cellular immunity was demonstrated in β 2-microglobulin-knockout mice that were unable to express MHC class I molecules and were highly susceptible to mycobacterial infection (Flynn et al., 1993). In addition, mice deficient

in the transporter associated with antigen processing (TAP) (Behar et al., 1999), CD 8 α and perforin (Cooper et al., 1997) were all more susceptible to *M.tuberculosis* infection than wild-type mice. Finally, CD 8 positive cells are capable of secreting cytokines such as IFN- γ and IL-4 and thus may play a role in regulating the balance of type 1 and type 2 cells.

The role of $\gamma\delta$ T cells in host immune response has not been well clarified, but there has been some evidence that these cells do play a role. They appear to accumulate early, both in the lesions of leprosy patients (Modlin et al., 1989) and in experimental *M.tuberculosis* infection of mice (Griffen et al., 1991). In addition, mycobacterial phospholipids stimulate all V γ 2 δ 2-bearing cells. The manner in which this activation occurs is not fully understood, but it results in a high frequency of mycobacteria-specific $\gamma\delta$ cells, which appears to be of the same magnitude as the clonal expansion of $\alpha\beta$ T cells (Kaufmann, 1996).

1.2.8 Regulation of immune responses by cytokines

The presence of cytokines, secreted by T cells, macrophages and NK cells, ultimately leads to the recruitment and activation of monocytes and macrophages that demonstrate enhanced microbicidal activity. The profile produced (type 1 versus type 2) could play an important role in the outcome of infection (protection versus disease). This is demonstrated in a related mycobacterial disease, leprosy, in which the T cells of patients with progressive lepromatous disease secrete little IFN- γ and IL-2 (type 1 cytokines) and increased amounts of IL-4 (type 2 cytokines), when compared to patients with tuberculous leprosy, the more benign form of disease. (Bloom et al., 1992). Other diseases with clinical manifestations that may be related to the cytokine profile present at the site of disease are asthma (Kay, 1996), Crohns disease (Parronchi et al., 1997) and organ transplantation (Piccotti et al., 1997).

IFN- γ appears to play a vital role in the control of mycobacterial infection. Lymphocytes present at the sites of mycobacterial infection produce IFN- γ , especially at those sites where effective T cell-mediated immunity is observed (i.e. pleuritis) (Barnes et al., 1993). Mice deficient in IFN- γ itself, or IL-12, a critical IFN- γ -inducing cytokine, were highly susceptible to *M.tuberculosis* challenge (Cooper et al., 1993; Cooper et al., 1997). In addition, patients from families with mutations in the α chain of the IFN- γ receptor are highly susceptible to infections with atypical mycobacteria. (Newport et al., 1996; Jouanguy et al., 1996). Other patients, also with severe atypical mycobacterial infections, appear to have defective IL-12 or

IFN- γ production (Frucht et al., 1996). In mice, IFN- γ may fully activate macrophages to kill *M.tuberculosis* in vitro by NOS induction. In humans, however, IFN- γ alone does not fully activate macrophages to kill or inhibit growth of *M.tuberculosis*. It may act together with TNF- α and 1,25-dihydroxyvitamin D₃ to induce killing or growth inhibition of *M.tuberculosis* by macrophages (Denis et al., 1991).

IL-2 promotes an increase in macrophage-activating factors by inducing proliferation of antigen-reactive T cells. Treatment with recombinant IL-2 in mice infected with *M.bovis* reduces bacillary load and improves clinical status (Jeevan et al., 1988). Similar findings were seen in humans with lepromatous leprosy and MDR TB (Kaplan et al., 1991; Johnson et al., 1995).

Activated macrophages and T cells produce large amounts of TNF- α . In *M.bovis* BCG-infected mice, treatment with anti-TNF antibodies resulted in lack of granuloma formation and failure to contain infection. TNF- α or 55 kd TNF receptor knock-out mice showed similar findings (Kindler et al., 1990). In humans, alveolar macrophage-derived TNF- α appears to inhibit the in-vitro growth of *M.tuberculosis* more than alveolar monocyte-derived TNF- α (Hirsh et al., 1994a). Excessive amounts of TNF- α contribute to immunopathology such as fever, cachexia and tissue damage. The TNF inhibitor, thalidomide, appears to be able to reverse some of the tissue necrosis in mice (Moreira et al., 1997).

IL-12 is an important promoter of type 1 responses and increased levels have been found in pleural fluids and lymph nodes of TB patients (Zhang et al., 1994). Mutations in the IL-12 β 1 receptor gene or IL-12p40 genes, resulting in absence of functional IL-12 receptor complexes, have been shown to result in poor granuloma formation in patients with disseminated atypical mycobacterial disease (Altare et al., 1998; de Jong et al., 1998).

Human and murine macrophages and human T cells secrete IL-10 in-vitro in response to *M.tuberculosis* infection. IL-10 also suppresses T cell function and de-activates macrophages (Othieno et al., 1999). In addition, it down-regulates the effects of inflammatory cytokines such as IFN- γ and TNF- α and thereby limits the extent of tissue damage. PBMCs from TB patients have been shown to produce higher levels of IL-10 than controls (Torres et al., 1998)

and patients with active TB also demonstrate elevated serum levels of this cytokine (Verbon et al., 1999). In a study of tuberculous pleural fluids (Barnes et al., 1993), the presence of IL-10 was shown at the site of pathology. In contrast, no IL-10 was demonstrated in BAL fluids from patients with intrapulmonary TB (Schwander et al., 1998). IL-10 has recently been shown to act together with TGF- β to suppress PPD-induced IFN- γ production in PBMCs (Othieno et al., 1999). In addition, IL-10 production by monocytes was shown to be moderately up-regulated by the presence of TGF- β (Othieno et al., 1999).

TGF- β is secreted by macrophages and monocytes in response to infection by *M.tuberculosis* and is regarded as a macrophage inactivator. Most of its effects are anti-inflammatory and these include: interference with NK and CD 8 T cell function, inhibition of T cell proliferation and interference of macrophage production of reactive oxygen and nitrogen-intermediates. The presence of TGF- β enhances the growth of *M.tuberculosis* in human macrophages and induction of this cytokine may be a strategy by which the bacillus manipulates the host immune response to its own advantage (Hirsh et al., 1994b). However, TGF- β production may benefit the host by limiting the extent of tissue damage as well as containing the infection by inducing fibrosis and angiogenesis. TGF- β , induced by *M.tuberculosis*, both directly and indirectly (through IL-10 induction) may be responsible for the down-regulation of T cell function and macrophage de-activation (Othieno et al 1999).

IL-4 production by lymphocytes from susceptible mice was increased compared to resistant mice and mice with disrupted IL 4 genes showed normal resistance to infection with *M.tuberculosis* (North et al., 1998). In humans, the measurement of IL-4, an important type 2 cytokine, has not been well documented. This is probably due to technical problems (very low levels) involved in the accurate assessment of IL-4 concentration in samples. However, increased levels of IL-5, another type 2 cytokine, have been found in a cohort of individuals in Uganda, a TB endemic area (Elliot et al., 1999). The authors speculate that this cytokine, which may relate to a high prevalence of helminth infestation, may suppress type 1 cytokines and thereby have a possible deleterious effect on protective immunity against mycobacterial pathogens.

Taken together, susceptibility to *M.tuberculosis* may be due to number of defects including abnormal IFN- γ receptors, defects in signal transduction, inefficient killing or antigen

presentation, deficient expression of co-stimulatory molecules or of protective cytokines (such as IL-12), or excessive secretion of suppressive cytokines such as TGF β and IL-10. Reduced killing by cytotoxic T cells, abnormal T cell function (as demonstrated by increased susceptibility to *M.tuberculosis* infection by HIV-infected people) or insufficient or inappropriate cytokine secretion may all play a role in susceptibility to *M.tuberculosis*.

In order to investigate this last possibility, many researchers have compared cytokine production in patients with active pulmonary TB to that of healthy sensitised individuals. A number of studies have documented differences in cytokine production between TB patients and healthy controls. The most well-documented difference is seen in IFN- γ production, which has repeatedly been shown to be decreased in PPD-stimulated PBMC from TB patients as opposed to healthy controls (Turner et al., 2000, Torres et al., 1994, Hirsch et al 1999b). Although the majority of studies confirm the diminished production of this cytokine in the periphery, there have been a few reports where this was not the case (Surcel et al., 1994). When IFN- γ production was measured at the site of disease, it was found to be abundant, suggesting compartmentalization of cytokines (Schwander et al., 1998; Barnes et al., 1993). IL-2 production has also been shown to be depressed in patients with active pulmonary TB (Hirsch et al., 1996; Barnes et al., 1992). Type 1 responses therefore appear to be depressed in TB patients as opposed to healthy controls. A number of reports have demonstrated increased levels of TGF- β and IL-10 from PPD-stimulated PBMC of patients with active disease as compared with healthy sensitised individuals (Hirsh et al., 1999; Toozi et al., 1995). TGF- β and IL-10 are known to down-regulate type 1 responses therefore providing a possible explanation for depressed type 1 responses in TB patients.

Although a variety of cytokines, chemokines and other soluble factors have been investigated in *M.tuberculosis*, information concerning the role that they play is still limited and often controversial. Many conflicting results have been found in different studies. For example, high levels (Hirsch et al., 1999b) and normal levels (Hirsch et al., 1996) of IL-10 were found in *M.tuberculosis* -stimulated PBMC from TB patients. Some studies have also shown a heterogeneous pattern of type 1 and type 2 cytokines when stimulated with *M.tuberculosis* antigens (Barnes et al., 1993; Orme et al 1993). These conflicting results have also been reported in different patient populations (Hirsh et al., 1996). While different culture conditions may be responsible for some of the differences, variations in the intrinsic immune

response of patient populations may also be important. For example, malnutrition (unrelated to TB) can drastically alter the production of cytokines (Dai et al., 1998). Secondly, TB patients, with asymptomatic parasite infection can have up-regulated production of type 2 cytokines which in turn could down-regulate type 1 cytokine responses (Bentwich et al., 1999). Thirdly, the severity of TB disease could affect the cytokine response (Seah et al., 2000) and lastly, cytokine responses are altered during and after TB therapy (Hirsch et al., 1999b).

1.2.9 Immune responses after tuberculosis therapy

Many reports of immune responses of adult TB patients at the time of diagnosis have been published. On the other hand, very few studies have re-examined the immune responses of these patients after TB therapy. Hirsch et al., (1999b) followed patients and healthy sensitised controls eighteen months after TB diagnosis and re-assessed PBMC cytokine production after PPD stimulation. IFN- γ levels were still depressed even though the immunosuppressive cytokines, TGF- β and IL-10, had returned to baseline levels. The authors speculate that this persistent IFN- γ depression may relate to reactivation of latent TB. Verbon et al., (1999) measured serum cytokines in TB and healthy sensitised controls at the time of TB diagnosis and again after TB therapy was completed. He showed that, compared to controls, IFN- γ and IL-10 levels were increased in TB patients, at the time of diagnosis and decreased after TB treatment. The increased IFN- γ level in patients was not related to increased IL-12 levels, as this was similar to those found in controls.

The status of the immune response in TB patients after treatment is largely unknown. Therefore, a further aim of this study was to quantify immune responses of TB patients after therapy, and compare it to data found before TB therapy.

1.3 MATURATION OF THE IMMUNE SYSTEM, AGE AND TB

1.3.1 Introduction

A number of components of the immune system that seem to be important in host defence are different in adults and infants. These differences, both qualitative and quantitative, probably contribute in part to the greater risk of disease in children of particular ages.

The development of the immune system in the human begins during the first month of gestation with haemopoietic stem cells in the yolk sac (Huston et al., 1997). Up to three months, haematopoiesis occurs mainly in the liver until the skeletal components are developed. Thereafter, the bone marrow becomes the major site of haematopoiesis. The immune system develops throughout life and although the basic requirements for the development of immune competence are present at birth, the complete expansion of the immune system requires learning or maturation. This is achieved only by encounter with pathogens, which induce a response as well as memory for a specific encounter.

1.3.2 Maturation of the immune system

1.3.2.1 Enumeration of Immune cells during maturation

The absolute numbers of leukocytes, total lymphocytes, as well NK cells, declines with age (Erkeller-Yuksel et al., 1992; Comans-Bitter et al., 1997), while the percentage of T cells within the total lymphocyte population increases with age (Erkeller-Yuksel et al., 1992). Studies of CD 4/CD 8 ratios, however, revealed controversial results. Wiener et al., (1990) found no significant changes across age groups, while other researchers reported increased CD 4/CD 8 ratios in cord blood (Solinger et al., 1985) or in the first year of life (Comans-Bitter et al., 1997). Peripheral blood monocyte numbers, in neonates, appear to be similar to that of adults (Weinberg et al., 1985).

1.3.2.2 $\alpha\beta$ TCR T cells

a) Immature surface phenotype

Neonatal peripheral blood T cells usually contain a high frequency of T cells which double-stain for CD 4/CD 8, as well CD 1, in contrast to adult peripheral blood T cells, which do not (Griffiths-Chu et al., 1984). In addition, more than 75% of CD 4 + cord blood T cells express CD 38 (common thymocyte) surface antigen, compared to a small number of adult cells

(Clement et al., 1990). CD 40 ligand, a receptor involved in T-cell-mediated macrophage activation, is generally expressed at lower levels on activated neonatal T cells in contrast to adult T cells (Noelle et al., 1996), but levels increase within the first few months of life (Durandy et al., 1995).

b) “Memory versus Naïve” as detected by surface expression of the common leukocyte molecule, CD 45

Differential expression of CD 45 iso-forms is usually used to identify functionally heterogeneous CD 4⁺ T cell subsets. “Naïve” precursor -T cells are CD 45 RA⁺RO⁻, while recall-antigen responsive T cells (memory T cells) are CD 45 RA⁻RO⁺ (Hassan et al., 1993). CD 45 RA expression occurs in up to 90% of cord blood CD 4 positive T cells, compared to approximately 50% in adults (Hannet et al., 1992). CD 45 RA⁻RO⁺ is expressed on <5% of the peripheral blood T cell pool in the cord and steadily increases throughout childhood toward adult levels (30-45%), presumably as a result of repeated antigen exposure (Hannet et al., 1992). Adult levels of CD 45 RA⁻RO⁺ expression are generally reached between 15-20 years of age and are comparable between CD 4 and CD 8 subsets as well as $\alpha\beta$ TCR and $\gamma\delta$ TCR T cell populations (Hayward et al., 1989). Researchers have also identified a unique CD 45 RA⁻RO⁻ subset in cord blood, which may be the immediate precursor of unprimed CD 45 RA⁺RO⁻ T cells (Bofill et al., 1994).

c) Cytotoxic effector functions

In the neonate, anti-viral immunity is diminished compared to adults during primary infection. Neonatal T cell cytotoxic activity was shown to be reduced after stimulation with PHA (reviewed by Holt, 1995).

d) T- cell suppressor activity

Non-specific in-vitro T cell “suppressor” activity in various cell types in experimental animals has been shown to be more active during the immediate post-natal period than in subsequent adulthood (Strober, 1984). This appears to be the case in humans as well. Adult T cell proliferation in-vitro is inhibited by both foetal and neonatal lymphocytes (Olding et al., 1976) and this immunosuppressive activity has been suggested to play an important role in the “maintenance” of normal pregnancy.

e) Cytokines

Differences in cytokine production between neonates and adults have been observed. The underlying causes for these differences are mostly unclear, although a crucial factor is probably a lack of exposure to antigen. This is supported by the finding of a predominantly “naïve “ T cell phenotype in neonatal peripheral blood, (CD 45 RA⁺RO⁻), which decreases with age (Hassen et al., 1996). However, other factors, such as inefficient antigen presentation and/or T cell help may be important in influencing the level of cytokines produced.

It is generally accepted that the capacity to produce IFN- γ is markedly reduced (approximately 10% of adult capacity) in neonatal T cells. (Holt et al., 1995; reviewed by Smith, 1997). One contributing factor to this observation is that naïve T cells, compared to memory T cells, are deficient in the production of this cytokine (Ehlers et al., 1991). Other possible reasons have been suggested: neonatal T cells are more sensitive to prostaglandin E2 (Wakasugi et al., 1985), or inefficient accessory cell activity of neonatal macrophages (Taylor and Brysan et al., 1985). It has also been found that variations in the rate of postnatal maturation of T cell competence, as shown by IFN- γ and IL-4 production pattern, may be associated with the development of atopy (Elsässer-Beile et al., 1991). The human foetus and neonate is unusually susceptible to infection with intracellular pathogens and this susceptibility has been proposed to be due to the inability of neonates to produce sufficient IFN- γ (Wilson et al., 1986). IFN- γ production increases within the first few months of life and the majority of children acquire levels equivalent to adults 2-5 years after birth (Frenkel et al., 1987).

Reports on the production of IL-2 by neonatal T cells are controversial. It has been reported to be similar to that of adults (Miyawaki et al., 1985), higher than in adults (Wilson et al., 1986), or significantly lower (Lilic et al., 1997). These disparities may be due to the use of different stimulating agents (Miyawaki et al., 1985). IL-2 receptor expression in neonates is comparable to that found in adults (Wilson et al., 1986).

Neonates and young children (under the age of 10) produce less TNF- α (with non-specific mitogenic stimulation), than older individuals (Wilson et al., 1986; Elsässer-Beile et al.,

1995). However, during active disease episodes, eg, bacterial meningitis (van Furth et al., 1995), young children are quite capable of producing increased levels of TNF- α .

Studies on IL-10 production also report contradictory results. Chheda et al., (1996) claimed increased production at birth compared to adults, while Seghaye et al., (1998), using a whole blood assay, report similar levels of IL-10 in cord blood compared to adult blood, after LPS stimulation.

Mitogenic stimulation of IL-4 has been shown to be lower in younger children compared to adults (Tang et al., 1995). Possible mechanisms suggested may involve naïve/memory subpopulations as well as other inhibitory cellular or serum factors.

Reports on age-related differences between IL-12 or TGF- β production have been not been documented.

1.3.2.3 $\gamma\delta$ T cells

$\gamma\delta$ T cells account for 1 to 5% of adult human peripheral blood T cells, whereas negligible numbers are found in the cord blood of newborn infants (Smith et al., 1990). These numbers increase progressively with age (Pecchold et al., 1994). $\gamma\delta$ T cells that recognize isoprenyl pyrophosphates of mycobacteria ($v\gamma 2v\delta 2$ T cells) are abundant in adults, but infrequent in neonates (Tsuyuguchi et al., 1991; Smith et al., 1997).

1.3.2.4 B cells

The number of CD 19 positive B lymphocytes has been shown to increase from birth until five months of age. This remains stable until about 5 years, after which there is a gradual decline to adult age (Comans-Bitter et al., 1997).

1.3.2.5 NK cells

The number and percentages of NK cells has been found to be highest in cord blood (Erkeller-Yuksel et al., 1992). Cord blood NK cells are functionally less active than those of adults and have reduced IFN- γ production (Hannet et al., 1992; Abo et al., 1983). This is possibly due to their immature state or due to a delay in the development of other cytokine-generating cells, which is necessary for complete NK activation.

1.3.2.6 Antigen-presenting macrophages and monocytes

Alveolar macrophages (AM) in neonatal animals have been relatively well-studied. They appear to be deficient in number as well as function, compared to their adult counterparts. The numbers increase during the early postnatal period, while the effector functions increase postnatally at varying rates in the different species (Weiss et al., 1986; D'Ambola et al., 1988). Although there is minimal data on humans, it appears that the human AM population also expands during early postnatal life (Alenghat et al., 1984) and demonstrates depressed anti-microbial function compared to adults (D'Ambola et al., 1988).

Neonatal and adult monocytes are similar in terms of absolute numbers and antimicrobial activity, but chemotaxis of neonatal monocytes is reduced in-vitro (Raghunathan et al., 1982). Recruitment of monocytes into tissues is also reduced in neonates (Bullock et al., 1969). Adult levels of monocyte chemotaxis are attained at 6-10 years of age (Raghunathan et al., 1982). These relative monocyte recruitment deficiencies may play a role in the reduced ability of neonates and children < 2 years of age to demonstrate delayed type hypersensitivity (DTH) after microbial infection. IL-6 (Yachie et al., 1992) and TNF- α (Burchett et al., 1988) production by neonatal monocytes is decreased. This may contribute to defective inflammatory activity and may also play a role the decreased febrile response in neonates (Klein et al., 1983).

The capacity of neonatal monocytes and macrophages to mediate IFN- γ production by NK cells or T cells after lipopolysaccharide (LPS) or mitogen stimulation is reportedly only half that of adult cells (Taylor and Bryson, 1985). In general, the capacity of neonatal monocytes and macrophages to secrete, and respond to, a number of cytokines is similar or modestly reduced compared to adults (Rowen et al., 1995).

1.3.2.7 Antigen presentation

MHC class II-restricted presentation of peptide antigens appears to be intact, as monocytes from human neonates can present antigen to maternal T cells as efficiently as maternal monocytes (Lewis and Wilson, 1995). These studies, however, examined antigen presentation to memory maternal T cells and not to neonatal T cells. Human cord blood dendritic cells have been shown to be less efficient APCs and stimulators of allogeneic responses than adult dendritic cells (Hunt et al., 1994). Dendritic cells from infants appear to have decreased

antigen presentation to naïve, neonatal T cells which does not reach adult levels until 1 year of age (Clerici et al., 1993).

The dendritic cell is a particularly important cell in neonates and infants, as the initial response to antigen is most dependent on efficient antigen presentation by these cells. Reduced dendritic cell function may therefore cause a delay in the initiation of an appropriate antigen-specific response, especially in the draining lymph nodes of the infant respiratory tract (Holt, 1995), and may therefore play a role in the development of disease.

1.3.2.8 Age-related risk of infectious diseases

Infancy and early childhood are generally acknowledged to be high-risk periods for infectious diseases, for example: gram negative and group B streptococcus infections are common during the first month of life. Thereafter, between 1 and 6 months of age, viral infections often occur. During the next 12 months, the major causative agents of infections are viruses and bacteria, particularly inflammatory cocci. Progressive development of immunologic memory and function occurs from the age of two years (reviewed by Pabst et al., 1980). The same authors hypothesized that a peak resistance period (to a number of infections), occurs around 10 years of age. They also demonstrated that overall human mortality followed a parabolic curve, with the “nadir” at 12 years of age, the time when total lymphoid tissue mass in the human body peaked. After this, overall mortality (due to infectious diseases), rises again, with a parallel decline in lymphoid tissue mass and immunoglobulin-producing cells (reviewed by Pabst et al., 1990).

It is generally accepted that, under neutral conditions, neonatal T cells are biased towards a type 2 profile. This bias could possibly play a role in the increased susceptibility to certain infections early in life and could be due to the type 2-cytokine milieu of pregnancy and/or altered APC/T cell interactions.

1.3.3 Age and TB

1.3.3.1 Age-related risk of TB

It is well known that there is an age-dependent risk for developing active TB, irrespective of the prevalence of infection in a given region. Children younger than the age of five years are highly susceptible to the development of active TB (especially disseminated and meningeal disease) and it is estimated that 20-40% of the infected children develop signs and/or symptoms of TB. Between the ages of 5 and 12 years the incidence is at a lifetime low. Thereafter, during adolescence, the lifetime risk of developing TB disease is approximately 15% until early adulthood where it remains at about 10% until late in life (Donald and Beyers, 1996).

1.3.3.2 Assessment of immune responses and diagnosis of TB in children

The diagnosis of TB disease in children is usually problematical. It is often difficult to obtain sputum from children with TB and the number of bacteria is generally low as TB is not usually a pulmonary cavitary disease in children. This leads to a low yield of cultures. There is thus an urgent need for the development of improved diagnostic tests for children. Studies comparing cellular immune responses in children with TB to healthy sensitized controls of the same age would provide important information, which could be used for the development of such improved diagnostic tests, as well as identify possible correlates of protection to TB. Unfortunately, very few studies involving children and the cellular immune responses to TB have been published. Diele et al., (2000) assessed lymphocyte proliferation after stimulation of PBMC with PPD and found no difference between patients and healthy sensitized controls. Swamathan et al., (1999) showed that *M.tuberculosis*-stimulated PBMC, from children with TB, produced less IFN- γ compared to controls, whereas IL-12, IL-4 and IL-10 production was similar between the two groups. Studies conducted in young BCG-vaccinated infants in the Gambia have shown that their PBMC are capable of producing IFN- γ after in-vitro stimulation with BCG (Marchant et al., 1999).

As so little is known about cellular immune responses in children with TB, it is vital that more studies be conducted. In addition, no studies on cellular immune responses in children who have completed TB therapy have been published. This information could be important with respect to the reactivation of latent TB.

1.3.3.3 Differences in clinical manifestations of TB disease

In children, TB disease is usually the result of first contact with the *M.tuberculosis* bacillus (primary TB), while in adults, disease (postprimary TB) is thought to be due to reactivation of a latent infection (the Koch phenomenon). Children younger than the age of 12 years usually present with primary TB. Most often the disease is a complication of enlarged hilar and paratracheal lymph nodes which could cause local compression of a lung segment and /or ulcerative erosion of the bronchi. Complications of disseminated disease, such as miliary TB and tuberculous meningitis occur most commonly under the age of five. During adolescence the clinical manifestations of the disease change to adult type chronic pulmonary TB. More pronounced systemic effects such as severe weight loss, high fever, tissue necrosis and the development of cavities characterize this form of the disease. Postprimary TB develops either from reactivation of dormant bacilli or by re-infection from a new contact. The change from primary to post-primary TB in adolescence has been hypothesized to be due to hormones, but this is still under investigation (Donald and Beyers, 1996).

1.3.3.4 Differences in immunological components in TB disease

A number of components of the immune system that may be important in host defense against *M.tuberculosis* differ in adults and infants. As already mentioned, chemotaxis of neonatal monocytes is depressed (Raghunathan et al., 1982) and monocyte recruitment into tissues is also decreased (Bullock et al., 1969). These defects may delay recruitment of activated cells to *M.tuberculosis*-infected sites. This delay would permit the bacillus to grow and thereby overwhelm early local host immune responses.

Less effective antigen presentation by dendritic cells in regional lymph nodes to naïve T cells may also result in a delay of initiation of T cell specific responses. This could result in decreased production of IFN- γ , TNF- α and CD 40L, which in turn could limit macrophage activation, allowing increased replication of the bacillus in these cells. Although many of these differences may be small on their own, the “cumulative” effect would result in an inefficient host response. Although there has been a considerable amount of research in the developing immune response to mycobacterial disease in adults and animals, additional information regarding the host response in children is required.

Therefore, while various components of the immune system are known to be different at different ages, information regarding the in-vitro production of certain cytokines in children, compared to adults, is still either controversial or not documented at all. The final aim of this study was thus to quantify and compare in-vitro cytokine immune responses, as well as proliferative responses, in subjects of different ages.

The previous section has highlighted some of the knowledge and gaps in our understanding of TB. This study aims to address some of the gaps in our knowledge, as outlined below:

1.4 HYPOTHESIS

1. The whole blood assay is a useful model to quantify cellular immune responses.
2. Cellular immune responses to mitogens (PHA) and mycobacterial antigens (PPD) can differ at different ages.
3. The immune responses of patients with acute TB (at diagnosis, before treatment) may be different from patients with a history of TB (after treatment).

1.5 AIMS

1. Establish whole blood assay method in order to quantify cellular immune responses (by the measurement of proliferation as well cytokine production, in response to PHA and PPD).
2. Quantify and compare immune responses of patients with a past history of TB and healthy Mantoux positive subjects of different ages.
3. Compare immune responses of TB patients before treatment (at diagnosis), to immune responses after TB treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1 REAGENTS

2.1.1 Tissue culture reagents

Tissue culture reagents were obtained as follows:

Rosewall Park Memorial Institute (RPMI) 1640 - GIBCO BRL, Grand Island, NY

L-Glutamine - 200mM- Highveld Biologicals, Kelvin, Johannesburg

Penicillin/streptomycin - 10mg/ml - Highveld Biologicals, Kelvin, Johannesburg

KSLMS Serum Free Medium - Highveld Biologicals, Kelvin, Johannesburg

β - Mercaptoethanol - Merck, Darmstadt, Germany

Phytohaemagglutinin (PHA) - Sigma Chemicals, St.Louis, Missouri, USA

Purified protein derivative (PPD) - a gift from Dr. S. Ress, Department of Clinical Immunology, University of Cape Town, South Africa

2.1.2 Radiochemicals

[methyl-³H] Thymidine ([³H] thymidine), specific activity 25Ci/mmol - AEC Amersham, Buckinghamshire, England

2.1.3 Media and buffers

RPMI tissue culture medium

500ml of RPMI was supplemented with L-glutamine (2mM), penicillin/streptomycin (0.1mg/ml) and β -mercaptoethanol (0.02 μ M)

Phosphate buffered saline (PBS) - pH 7.3

136mM NaCl, 2.6mM KCL, 8mM Na₂HPO₄, 1.4mM KH₂PO₄

Carbonate Buffer - pH 9.5

30mM Na₂CO₃, 69mM NAHCO₃

Tris buffered saline (TBS) - pH 7.3

20mM Tris, 150mM NaCl

2.1.4 Enzyme-linked immunosorbant assay (ELISA) DuoSet kits

Each kit contained a capture antibody (mouse anti-human), a mass calibrated standard, a secondary biotinylated antibody (either rabbit, chicken or goat anti-human) and a peroxidase-labelled streptavidin reagent.

ELISA duoSet kits were obtained as follows:

IFN- γ , IL-2, TNF- α , IL-4 - Genzyme Corporation, USA

IL-12p40, IL-10, TGF- β - R&D Systems Europe, Abingdon, UK

2.1.5 Analytical grade reagents

These reagents were obtained as follows:

Hydrochloric acid (HCL), Potassium chloride (KCL), Potassium di-hydrogen phosphate (KH_2PO_4), Sodium hydrogen carbonate (NaHCO_3), Sulphuric acid (H_2SO_4) - BDH Chemicals, Poole, England

Bovine serum albumin (BSA), Tween 20 - Sigma Chemicals, St.Louis, Missouri, USA

Tris(hydroxymethyl)aminomethane (Tris), Sodium chloride (NaCl), di-Sodium hydrogen phosphate (Na_2HPO_4), di-Sodium carbonate (Na_2CO_3) - Merck, Darmstadt, Germany

2.1.6 Other reagents

Insta-Gel Plus scintillation fluid - Packard Bioscience Company, Groningen, The Netherlands

Tetramethylbenzidine/peroxide (TMB) substrate - Kirkegaard and Perry (KPL), Maryland, USA

2.2 STUDY SUBJECTS

2.2.1 Setting

The majority of participating individuals resided in two adjacent Cape Town suburbs, namely, Ravensmead and Uitsig. The remaining individuals resided in areas in close proximity to these suburbs. The TB notification rate in the Ravensmead and Uitsig communities is more than 700/100 000 (Beyers et al., 1996), representing a typical very high incidence community, characteristic of those in the Western Cape.

2.2.2 Recruitment

The subjects of this study were recruited at the time of TB diagnosis in patients, from May 1998 to November 1999 (for the “before therapy” study) and again approximately 1 year later (6 months after patients had completed TB therapy), from May 1999 to November 2000 (for this follow-up “after therapy” study). They were recruited from the local authority health clinics in the City of Tygerberg (with permission from Doctor I. Toms, Head of Health Services, City of Tygerberg) and the paediatric wards of the Tygerberg Hospital (with permission from Professor P.B. Hesseling, Head of the department of Paediatrics and Child Health).

2.2.3 Consent and ethical approval

Informed written consent was obtained from every subject 14 years and older or his/her guardian when the participant was younger than 14 years. All subjects volunteered to participate in this study and did not receive any compensation for participation. HIV-test counselling was given to each subject and his/herguardian.

The Ethics Committee, Faculty of medicine, University of Stellenbosch, has approved this study (registration number: 98/117).

2.2.4 Inclusion criteria

- I. A) Patients with TB (before commencement of TB therapy):
 - TB patients were recruited before initiation of treatment. No patient was known to have any disease other than TB or was on treatment with corticosteroids at the time of recruitment

- After diagnosis of TB, the patients were treated with standard combination therapy under direct supervision (DOTS) in accordance with the South African National TB Control Programme (South African TB Control Programme, February 1996)

- A case of TB fulfilled one of the following criteria:

- A positive sputum-smear for acid-fast bacilli and symptoms of TB
- A positive culture for *M.tuberculosis* in sputum, gastric aspirate cerebrospinal fluid or lymph node in a person with symptoms of TB
- A history of a recent contact with a known and identified TB patient in combination with signs of TB on the chest radiograph and clinical symptoms of TB (cough, weight loss)

B) Patients with TB (after completion of TB therapy):

- Successful completion of a full course of combination TB therapy (with a negative Ziehl Neelsen (ZN) stain for *M.tuberculosis* in adolescent and adult patients)

II. Healthy control subjects who had a positive tuberculin skin test (Mantoux):

- A person with a Mantoux skin test induration $>$ or equal to 15mm and no recent history or clinical symptoms of any disease
- A chest radiograph (CR) was evaluated upon entry to the study to exclude radiological signs of TB disease for all persons under the age of 12 years
- In accordance with the South African National TB Control Programme, all healthy Mantoux positive children under the age of five, who were living in the same house as a patient with TB disease, received supervised prophylactic treatment

2.2.5 Exclusion criteria

The exclusion criteria for subjects to enter the study were:

- Previous TB treatment, including prophylactic chemotherapy
- Numerous periods (>3 /year) of purulent otorrhea
- Skin infection with scabies or eczema at the time of recruitment
- Asthma, if on treatment with oral steroids during the previous 3 months

- Measles during the previous 3 months or a measles contact (2 weeks prior to recruitment)
- Chickenpox or chickenpox contact (3 weeks prior to recruitment)
- Oral or intramuscular hormonal contraception
- Pregnancy and the 3 month period after giving birth, if breastfeeding
- Known immunodeficiency or a positive HIV-1/HIV-2 test
- Signs of any overt infection other than TB
- Fever ($>38^{\circ}\text{C}$) and /or malnutrition

2.3 METHODS

2.3.1 Treatment of patients

Children diagnosed with TB were treated with daily isoniazid, rifampicin and pyrazinamide for 4 months while adult patients received all the afore-mentioned drugs plus ethambutol, administered daily for 2 months, followed by a 4 month continuation phase of isoniazid and rifampicin, administered twice weekly. To confirm cure, sputa from adolescent and adult patients were tested for presence of *M. tuberculosis* by ZN staining.

2.3.2 Tuberculin skin testing (Mantoux)

0.1ml (5TU) tuberculin (Japan BCG Laboratory) was injected intradermally by trained healthcare workers. The size of the transverse induration was read 48-72 hours after administration and an area of $>15\text{mm}$ was regarded as positive.

2.3.3 Whole blood culture

Blood was collected (5ml in a sodium heparin tube) from subjects between 9.00 and 12.00am and transported to the laboratory at room temperature. All blood was processed under laminar flow conditions within 2-3 hours of venous puncture.

Blood was diluted 1:10 in RPMI and $180\mu\text{l}$ /well was pipetted (in triplicate for proliferation studies and quadruplicate for harvesting of supernatant fluid for cytokine analysis) into 96-well, U-bottom plates (Corning Costar, Cambridge, MA). Twenty μl of the polyclonal stimulant, PHA (diluted to a final concentration of $10\mu\text{g}/\text{ml}$ in RPMI), or $20\mu\text{l}$ of the specific mycobacterial antigen, PPD (diluted to a final concentration of $3.3\mu\text{g}/\text{ml}$ in RPMI), was added to each well serving as a stimulated culture, while cultures incubated with RPMI alone

served as negative controls. All cultures were subsequently placed in a humidified 5% CO₂, 37°C incubator.

PHA- and PPD- stimulated cells were cultured for a maximum of 4 and 7 days respectively, depending on the optimum time of proliferation and cytokine production.

2.3.4 Proliferation assay

Cellular proliferation was assessed by [³H] thymidine uptake. This assay measures [³H] thymidine uptake into the DNA of dividing cells and is a commonly used method for assessing cellular proliferation. Diluted blood (section 2.3.3) was cultured in triplicate with RPMI (negative control), PHA (final concentration: 10µg/ml) and PPD (final concentration: 3,3µg/ml) for 3 and 6 days respectively, before pulsing by the addition of [³H] thymidine (0.5µCi/well) for 20 hours. Thereafter, each culture was transferred into a well in a 96-well flat-bottom plate and frozen. When sufficient assays were accumulated, the frozen plates were allowed to thaw at room temperature and transported to the cell harvester which was located in the department of Microbiology at Tygerberg hospital. Thereafter, non-soluble radioactivity was precipitated with a Skatron Cell Harvester (Flow Laboratories, Bonn) onto glass fiber filter mats (Macherey-Nagel, Germany), dried and immersed in scintillation fluid. Radioactivity was subsequently read on a β-scintillation counter (Beckman multi-purpose scintillation counter, Beckman Instruments, Fullerton, CA) and expressed as counts per minute (cpm). Triplicate cpm readings were averaged and background from unstimulated cultures was subtracted from stimulated cultures.

2.3.5 Harvesting of supernatant fluid (SNF) for cytokine analysis

SNF from cultures was carefully removed (harvested) from each well, taking care not to disturb the sedimented red blood cells. The harvested SNF from each identical well was then pooled, mixed, aliquotted and frozen at -20°C for subsequent measurement of IFN-γ, TNF-α, IL-10, IL-12p40, IL-2 and TGF-β levels by ELISA. The harvesting procedure was performed at various time points for the different cytokines (Table 2.1) as was determined in optimization studies (section 3.1.4).

Table 2.1**Harvesting time points of SNF for cytokine assays**

Cytokine	PHA	PPD
IFN- γ	Day 4	Day 7
IL-2	Day 3	Day 3
IL-12	Day 4	Day 7
IL-10	Day 3	Day 7
TNF- α	Day 3	Day 7
TGF- β	Day 4	Day 7

2.3.6 ELISA

A sandwich ELISA was used to quantify levels of cytokines in culture SNF. Briefly, 100 μ l of capture antibody (see table 3.19 for concentration), diluted in carbonate buffer (pH 9.5), was coated onto the wells of a 96-well maxisorb microtiter plate (Nunc) by overnight incubation at 4°C. After washing in PBS containing 0.05% Tween 20 (wash buffer), free antibody binding sites were blocked. This was done by adding 250 μ l of blocking buffer (PBS containing 1% BSA) to each well. Blocking buffer was then discarded and 100 μ l of serially diluted recombinant standard or sample (see Table 2.2 for dilutions) was added in duplicate (samples for TGF- β analysis were first transiently treated with hydrochloric acid in order to release TGF- β from its noncovalent association with latency-associated peptide).

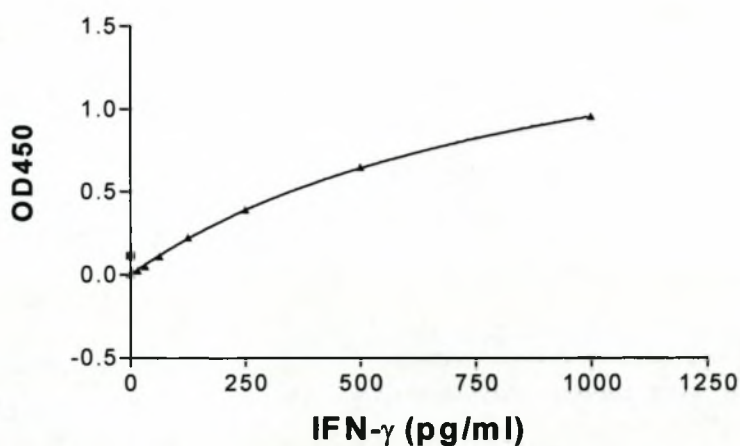
Table 2.2**Dilutions for samples for measurement of cytokines with ELISA**

Cytokine	PHA	PPD
IFN- γ	Subjects < 12 yrs – 1:20 Subjects > 12 yrs – 1:40	Subjects < 12 yrs – 1:20 Subjects > 12 yrs – 1:40
IL-2	1:2	1:2
IL-12	1:2	1:2
IL-10	1:10	1:2
TNF- α	1:10	1:5
TGF- β	1:2.8	1:2.8

The specific cytokine, if present, binds to the capture antibody on the micro-titer plate. Thereafter, plates were washed with wash buffer (see above) and 100µl of secondary biotinylated antibody which was diluted in PBS containing 1% BSA and 0.05% Tween 20 was added (see table 3.20 for concentration). This antibody binds to the captured cytokine. Unbound secondary antibody was washed off with wash buffer (see above) and 100µl of peroxidase-labelled streptavidin reagent was added. This reagent attaches to biotin in the immune complex. Plates were again washed with wash buffer and 100µl of TMB substrate solution was added, which produces a blue color in the presence of peroxidase. The colour reaction was stopped after 10 minutes by the addition of 100µl of 2N H₂SO₄, which changes the blue colour to yellow. The intensity of the yellow colour is proportional to the amount of cytokine present in the standards or samples. The absorbance of each well was read at 450nm (A₄₅₀) on a Titertek spectrophotometer and the background values (obtained from the A₄₅₀ of the zero standard) were subtracted from all the values. A standard curve was constructed using Prism graph pad software, by plotting the mean, background-subtracted absorbance for each standard on the vertical axis (y-axis) versus the corresponding standard concentration on the horizontal axis (x-axis). The software package automatically calculated the concentration of the unknown samples from the standard curve. The lower limits of detection for all the cytokines were 15pg/ml. If the absorbance of the undiluted sample fell below the absorbance of 15pg/ml, the concentration was calculated using the formula: $X=Y.KD/B_{max}-Y$ (Y=absorbance of sample; B_{max} and KD values were automatically calculated by the software package). Figure 2.1 depicts a typical standard curve used for the calculation of the cytokine concentrations in the samples.

Figure 2.1

Example of a typical standard curve used for calculation of unknown cytokine concentrations



2.3.7 Statistical analysis

Statistical analysis was performed using SPSS software. Since the variables tested did not show a normal distribution, they were compared by the non-parametric Mann-Whitney U test for the comparison of 2 independent groups, and the Wilcoxon test, for the comparison of 2 related samples. Correlation analysis was conducted using Spearman rank correlation coefficient test. *P* values of <0.05 were considered significant.

CHAPTER 3

OPTIMIZATION AND ESTABLISHMENT OF WHOLE BLOOD CULTURE AND ELISA CONDITIONS

3.1 Optimization of whole blood culture conditions

3.1.1 Background

Most investigators have traditionally used isolated peripheral blood mononuclear cells (PBMC) for the in-vitro analysis of proliferative and cytokine responses by human cells. More recently, diluted whole blood has been used to analyse these responses, (Weir et al., 1994; Elliot et al., 1999; van Crevel et al., 1999) which have been shown to parallel those obtained using PBMC (Weir et al., 1994; De Groote et al., 1992). Whole blood assays (WBA) have a number of advantages over PBMC cultures. These include: 1) only a small volume of blood is required, 2) the WBA is rapid and simple, 3) no unphysiological cell separation is involved and 4) there is no requirement for FCS which is known to contain TGF- β and growth factors produced by platelets that could alter T cell cytokine responses in vitro (Daynes et al., 1992). Furthermore, investigators have demonstrated that erythrocytes and neutrophils, which are not present in PBMC cultures, can provide supplementary cytokines and factors that contribute to lymphocyte and monocyte responses (Kalechman et al., 1993; Van der Pouw Kraan et al., 1995).

Since the WBA was not established in our laboratories, the work described in this section was done to set up the assay and establish optimal conditions. In order to do this, it was necessary to compare a number of factors, for example, two available cell culture media, namely, RPMI and KSLMS (KSLMS was previously used by another researcher in the laboratory). Whole blood proliferative responses (see section 2.3.4), in the absence and presence of stimulants (PHA: 10 μ g/ml and PPD: 3.3 μ g/ml) were therefore compared over time, using the two different media.

In these experiments, after the cultures were pulsed with ^3H thymidine, they were immediately transported to the only available cell harvester, which was located in the Department of Microbiology at Tygerberg Hospital (approximately 1 km away). Non-soluble

^3H thymidine was precipitated onto glass fibre filters by the cell harvester, dried, placed into scintillation fluid and counted. Because of the distant location of the harvester it was decided to evaluate whether freezing and later thawing of the cultures (approximately 1 week), before harvesting on the harvester, would influence the results. Another proliferation assay, over time, in the absence and presence of PHA (10 $\mu\text{g/ml}$) was therefore performed.

The measurement of cytokine levels following PHA- and PPD-stimulation, was also controversial with respect to time points for supernatant fluid harvesting (Barnes et al., 1993; Zhang et al., 1994). Furthermore, it was not known whether the kinetics of cytokine production in children is different from that of adults. A WBA over time, to determine the optimum harvesting time points of supernatant fluid, was thus done on blood obtained from four individuals. These individuals included two patients diagnosed with TB (1 child, aged 3, and 1 adult) and two Mantoux positive individuals (both adults).

3.1.2 Comparison of proliferative responses using RPMI and KSLMS

Heparinised blood was obtained from a healthy volunteer and diluted 1:10 with either RPMI or KSLMS. Diluted blood was plated, in triplicate, into a 96-well round bottom plate and stimulated with PHA or PPD for 5 and 7 days respectively (section 2.3.3). PHA and PPD were both diluted in RPMI to give final concentrations of 10 $\mu\text{g/ml}$ and 3.3 $\mu\text{g/ml}$ respectively. Blood incubated with medium alone served as a negative control. Proliferative responses were measured daily by ^3H thymidine incorporation (section 2.3.4) and results were expressed as cpm stimulated minus unstimulated cultures (means of triplicate cultures).

Table 3.1 and Figure 3.1 shows the respective results and kinetic curves following PPD stimulation. As seen in Table 3.1 and Figure 3.1 proliferative responses for the two media showed similar results and kinetic curves.

(Note: the experiment was performed twice and results are from one representative experiment).

Table 3.1**PPD-stimulated proliferative response of whole blood diluted in RPMI and KSLMS media, over time**

Results are expressed as mean cpm of stimulated cultures (triplicate measurements) minus mean cpm of unstimulated cultures (triplicate measurements)

Day	RPMI (cpm)	KSLMS (cpm)
1	-140	-79.0
2	1023	1203
3	6373	6171
4	15593	14644
5	38725	39512
6	79131	48013
7	74358	77991

Figure 3.1**PPD-stimulated proliferative response of whole blood diluted in RPMI and KSLMS media, over time**

Results are expressed as mean cpm of stimulated cultures (triplicate measurements) minus mean cpm of unstimulated cultures (triplicate measurements)

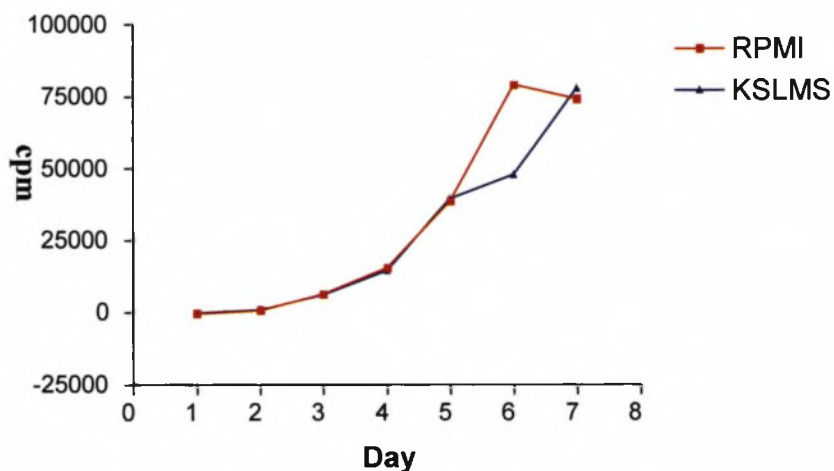


Table 3.2 and Figure 3.2 show the respective results and kinetic curves following PHA stimulation. As seen in Table 3.2 and Figure 3.2, proliferative responses for the two media showed equivalent results and kinetic curves. There was no result available for day 5 for blood diluted in KSLMS due to a technical problem. (Note: the experiment was performed twice and results are from one representative experiment).

Table 3.2

PHA-stimulated proliferative response of whole blood diluted in RPMI and KSLMS media, over time

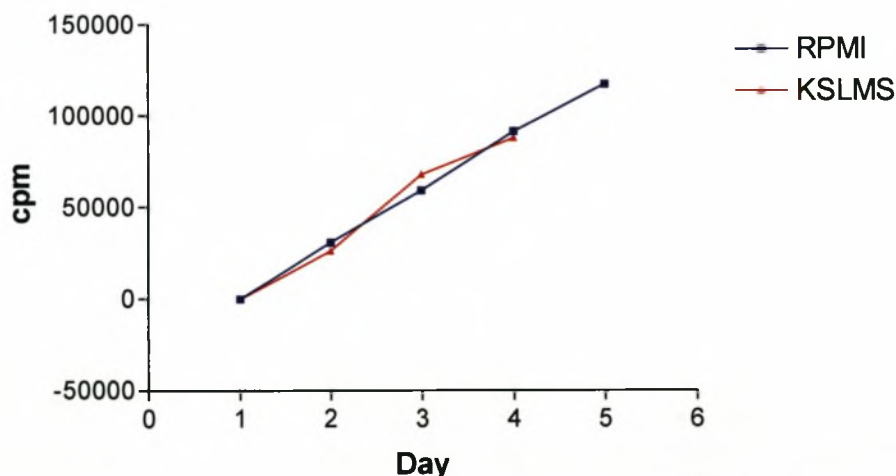
Results are expressed as mean cpm of stimulated cultures (triplicate measurements) minus mean cpm of unstimulated cultures (triplicate measurements)

Day	RPMI (cpm)	KSLMS (cpm)
1	-2	-1
2	30990	26230
3	59429	68145
4	91550	88022
5	117486	Technical error

Figure 3.2

PHA-stimulated proliferative response of whole blood diluted in RPMI and KSLMS media, over time

Results are expressed as mean cpm of stimulated cultures (triplicate measurements) minus mean cpm of unstimulated cultures (triplicate measurements)



3.1.3 Evaluation of the freeze/thaw process on proliferative responses

Heparinised blood was obtained from the same volunteer whose blood was used for the experiment described in section 3.1.2 and diluted 1:10 with RPMI. Diluted blood (180µl/well) was cultured (in triplicate wells) with PHA (10µg/ml). Blood incubated with medium alone served as a negative control. Each day the cultures were pulsed by the addition of [³H] thymidine (0.5µCi/well) for 20 hours. The experiment was performed in duplicate, whereby, each day, after the 20 hour incubation with ³H thymidine, one assay was harvested onto the glass fiber filters and transported to the cell harvester for harvesting immediately, while the other was transferred into wells of a 96-well flat-bottom plate and frozen. When both assays were complete, the cultures that had been transferred into flat-bottom plates and frozen were allowed to thaw at room temperature and then transported to the harvester for harvesting. After harvesting, the filters were dried, placed into scintillation fluid and counted on a β-scintillation counter as previously performed. Results for proliferative responses were expressed as mean cpm of triplicate stimulated minus unstimulated cultures.

Table 3.3 and Figure 3.3 show the respective results and kinetic curves following PHA stimulation. The results suggest that proliferative responses of the frozen/thawed cultures were similar (except for day 4) to proliferative responses of cultures that were harvested immediately. There was no result available for day 5 for freshly harvested cultures due to a technical problem.

The freeze/thaw process did not appear to have a dramatic effect on the proliferative responses, therefore, for reasons of scale and economy, it was decided to include this procedure in the method.

3.1.4 Determination of optimal harvesting time points of supernatant fluid for cytokine analysis

Heparinised blood was obtained from four individuals, which included two patients diagnosed with TB from the Ravensmead/Uitsig communities (1 child and 1 adult) and two Mantoux-positive individuals (both adults). One Mantoux-positive individual was a healthy laboratory volunteer (Man pos 1) and the other was an individual from the Uitsig community (Man pos 2). Blood was diluted 1:10 with RPMI and plated in multiple wells, into a 96-well round bottom plate. Samples were stimulated with PHA (10µg/ml) and PPD (3.3µg/ml) for 5

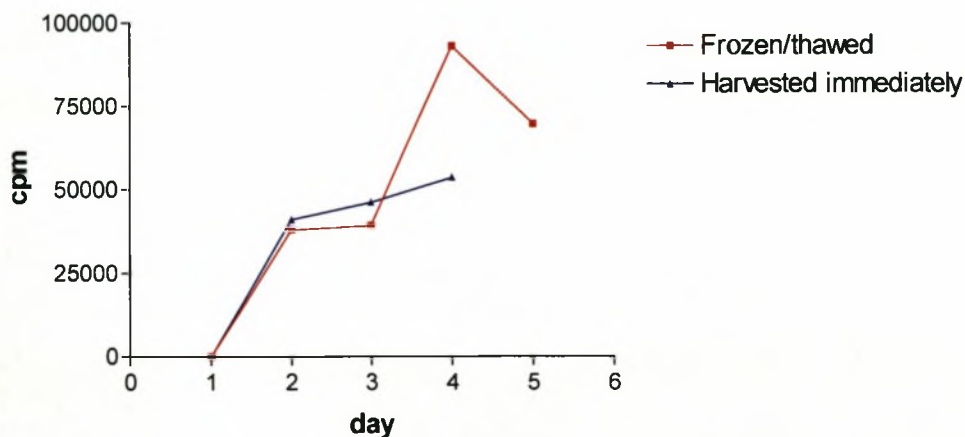
Table 3.3**PHA-induced proliferative responses of frozen/thawed cultures compared to freshly harvested cultures**

Results are expressed as mean cpm of stimulated cultures (triplicate measurements) minus mean cpm of unstimulated cultures (triplicate measurements)

Day	Freshly harvested cultures (cpm)	Frozen/thawed cultures (cpm)
1	38	141
2	41223	38225
3	46415	39473
4	53805	93302
5	Technical error	69911

Figure 3.3**Time course of PHA-stimulated proliferative responses of frozen/thawed cultures compared to freshly harvested cultures**

Results are expressed as mean cpm of stimulated cultures (triplicate measurements) minus mean cpm of unstimulated cultures (triplicate measurements)



and 7 days respectively (section 2.3.5). Blood incubated with medium alone served as a negative control. Each day the supernatant fluid of identical stimulants was harvested (section 2.3.5), pooled, mixed, aliquotted and frozen until assayed for cytokine levels by ELISA. The cytokines measured included IFN- γ , IL-2, TGF- β , IL-12, IL-10, TNF- α and IL-4. Results are expressed in pg/ml of cytokine (value for stimulated cultures minus value for unstimulated cultures).

Note: for comparison and reference, all cytokine data (tables and figures) are grouped together at the end of this section.

IFN- γ

Table 3.4 and Figure 3.4 show levels of IFN- γ production of PPD-stimulated cultures for all four individuals, over time. As seen in Table 3.4 and Figure 3.4, the general trend observed for the three adults was a steady increase of production of IFN- γ levels until a time point between day 3 and day 5, after which a plateau was reached. One Mantoux-positive subject (Man pos 1) demonstrated peak concentration levels on day 3 (10659pg/ml), a second Mantoux-positive subject (Man pos 2) on day 4 (26056pg/ml) and the adult with TB on day 5 (22763 pg/ml). The child produced consistently low levels of IFN- γ throughout, only reaching a maximum of 3658pg/ml on day 7.

PHA-induced IFN- γ levels for Man pos 1 and Man pos 2 peaked on day 2 (11290 pg/ml) and day 4 (28389 pg/ml) respectively, and then plateaued (Table 3.5 and Figure 3.5). The adult with TB demonstrated peak concentration levels on day 3 (13575pg/ml), remained consistent at day 4 (14325pg/ml), and declined on day 5. The child produced maximum levels of 5768 pg/ml IFN- γ on day 3, after which levels decreased.

Background counts were minimal for Man pos 1 and the child with TB throughout the time courses, but Man pos 2 and the adult with TB showed initial background counts in unstimulated cultures similar to stimulated cultures, when stimulation was not yet optimal. The counts in unstimulated cultures did not subsequently increase at later time points, as opposed to stimulated cultures, but remained at those levels.

IL-2

PPD-stimulated IL-2 production for all individuals was extremely variable over time (Table 3.6 and Figure 3.6). Generally, except for Man pos 1, levels were very low. Since IL-2 levels are very low, variations may possibly be artefacts or concentrations may be regarded as background values.

PHA-stimulated IL-2 production for all individuals was marginally higher than PPD-induced IL-2, but also variable over time (Table 3.7 and Figure 3.7). Man pos 1 already demonstrated maximal levels at day 1 (833pg/ml) after which the concentration of IL-2 showed a steady decline. Man pos 2 levels increased till day 3 (656pg/ml) and then diminished. IL-2 levels of the adult with TB demonstrated peak concentration levels between day 3 (344pg/ml) and day 4 (266pg/ml) and then declined, while the child with TB demonstrated peak concentration levels on day 2 (439pg/ml) with a subsequent decrease.

Background counts from unstimulated cultures were generally low, with the child producing slightly higher counts from unstimulated cultures.

TGF- β

PPD-stimulated TGF- β production for all individuals was variable over time (Table 3.8 and Figure 3.8). Man pos 1 demonstrated peak concentration levels on day 4 (1505pg/ml), which subsequently declined and then showed a second peak on day 7 (1589pg/ml). Man pos 2 demonstrated a similar trend to Man pos 1, but at a slightly lower concentration. The adult with TB produced the highest levels of TGF- β , increasing until day 6 (3638pg/ml) and declining to 1857pg/ml at day 7. The child with TB demonstrated fluctuating levels, showing a peak on day 2 (686pg/ml) and another at between day 4 (687pg/ml), which was sustained till day 6 (593pg/ml), and subsequently declined on day 7 (0pg/ml).

PHA-stimulated TGF- β production for all individuals was also variable over time (Table 3.9 and Figure 3.9). Levels for Man pos 1 increased steadily from day 1 (450pg/ml) to reach a peak on day 5 (2051pg/ml) while Man pos 2 already produced maximal levels on day 1 (1288pg/ml), declined till day 3 (0pg/ml) and then recovered to reach 362pg/ml at day 5. TGF- β levels in the adult with TB peaked between day 2 (1046pg/ml) and day 3 (1243pg/ml), whereafter it declined. TGF- β levels in the child with TB had a peak value of

3726pg/ml on day 2 which declined to day 4 (471pg/ml) and then showed a slight increase on day 5 (927pg/ml).

Background counts for unstimulated cultures were high for all subjects throughout both time courses.

IL-12

All individuals, except for the child had well-maintained levels of IL-12 throughout the 7-day period (Table 3.10 and Figure 3.10). Man pos 1 had the highest values of the adults, with levels already at 3906pg/ml on day 1. The levels peaked at between day 2 (5296pg/ml) and day 3 (5465pg/ml) and showed a second peak on day 5 (5637pg/ml), whereafter IL-12 concentration showed a slight decline. Man pos 2 and the adult with TB had very similar trends with both producing good levels at day 1 (2253 and 1450pg/ml respectively). Thereafter, levels declined slightly, with Man pos 2 demonstrating peak concentration levels at day 4 (3034pg/ml) and the adult with TB producing highest levels at day 5 (1989pg/ml). Both declined slightly till day 7. The child with TB had lower levels compared to the adults, peaking with a value of 971pg/ml on day 3 and remaining more or less at those levels for the rest of the time course.

The kinetics of PHA-stimulated IL-12 production for all individuals was similar (Table 3.11 and Figure 3.11). Man pos 1 showed a gradual increase in levels until reaching a peak at day 4 (2056pg/ml) and subsequently plateaued. Man pos 2 and the child with TB also showed a similar gradual increase in levels, which peaked at day 5 (1017 and 793pg/ml respectively). The adult with TB had maximal production of 656pg/ml on day 4, but levels declined slightly thereafter.

Background counts from unstimulated cultures were moderate for all subjects, except for Man pos 1, who produced none at all.

TNF- α

Generally, PPD-induced levels were very low for all subjects. The kinetics of PPD-stimulated TNF- α production for Man pos 1 and the child with TB were similar, with both showing initial maximal values of 128 and 40pg/ml respectively (Table 3.12 and Figure 3.12). Thereafter, values showed a steady decline throughout the rest of the 7-day period. The

values for the child were much lower than those of the adults. Man pos 2 had a peak value of 321pg/ml at day 2 and a second peak at day 6 with a value of 256 pg/ml. The adult with TB had a concentration of 269pg/ml on day 1, whereafter levels declined until day 3 (96pg/ml) and subsequently increased steadily to peak at day 7 with a value of 406pg/ml.

Man pos 1 and the adult with TB showed similar trends for the PHA-stimulated time courses (Table 3.13 and Figure 3.13). Both had gradual increases in concentration to reach peak values of 808 and 340pg/ml respectively, on day 3. Man pos 2 also had a similar trend, but peaked with a value of 762pg/ml on day 4. The child with TB already had maximal levels on day 1 (339pg/ml), where after levels declined for the rest of the 5-day period.

Background counts from unstimulated cultures were minimal for all subjects.

IL-10

The kinetics of PPD-stimulated IL-10 for all individuals was extremely variable (Table 3.14 and Figure 3.14). Man pos 1 showed maximal concentration at day 5 with a value of 402, while Man pos 2 already produced maximal levels at day 2 (1110pg/ml). The adult with TB produced high levels on day 1 (457pg/ml) which declined and subsequently increased again on day 7 (584pg/ml) while the child with TB had a maximum level on day 2 (376pg/ml) and another on day 6 (396pg/ml).

PHA-induced IL-10 was also variable for all individuals (Table 3.15 and Figure 3.15). Man pos 1 demonstrated a peak concentration value of 7037pg/ml on day 3, while Man pos 2 showed maximal concentration on day 2 (2868pg/ml) and then again on day 4 (3697pg/ml). The adult with TB produced consistently low amounts of IL-10 throughout the 5-day period, only reaching a maximal value of 620pg/ml on day 4. The child with TB showed maximal concentration of 2002pg/ml already at day 2, where after levels decreased and remained low throughout the rest of the time course.

Background counts from unstimulated cultures were moderate for all subjects.

IL-4

Levels of PPD-induced IL-4 were extremely low for all individuals throughout the 7-day period (Table 3.16 and Figure 3.16).

In general, levels of PHA-induced IL-4 were slightly higher than those found with PPD stimulation (Table 3.17 and Figure 3.17). Man pos 1 produced a high concentration of IL-4, showing maximal concentration on day 3 (238pg/ml), while Man pos 2 levels were much lower, peaking at day 2 (56pg/ml) and at day 4 (41pg/ml). The adult with TB had consistently low levels throughout while the child only produced a maximum of 35pg/ml on day 1.

Background counts from unstimulated cultures were minimal for all subjects.

Table 3.4**PPD-stimulated IFN- γ production of four subjects, over time**Results are expressed in pg/ml of IFN- γ

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	43	1858	1815	991	2624	1633	2348	1948	0	157	170	13
2	0	5552	5552	1136	12424	11288	960	3573	2613	115	395	280
3	47	10706	10659	1658	17280	15622	1298	7985	6687	97	492	395
4	67	9612	9545	1291	27347	26056	873	14573	13700	67	1052	985
5	67	11325	11258	1902	28591	26689	1135	23898	22763	80	1092	1012
6	0	9765	9765	1202	28313	27111	898	21910	21012	162	1917	1755
7	76	11325	11249	1713	28213	26500	1523	24998	23475	130	3787	3657

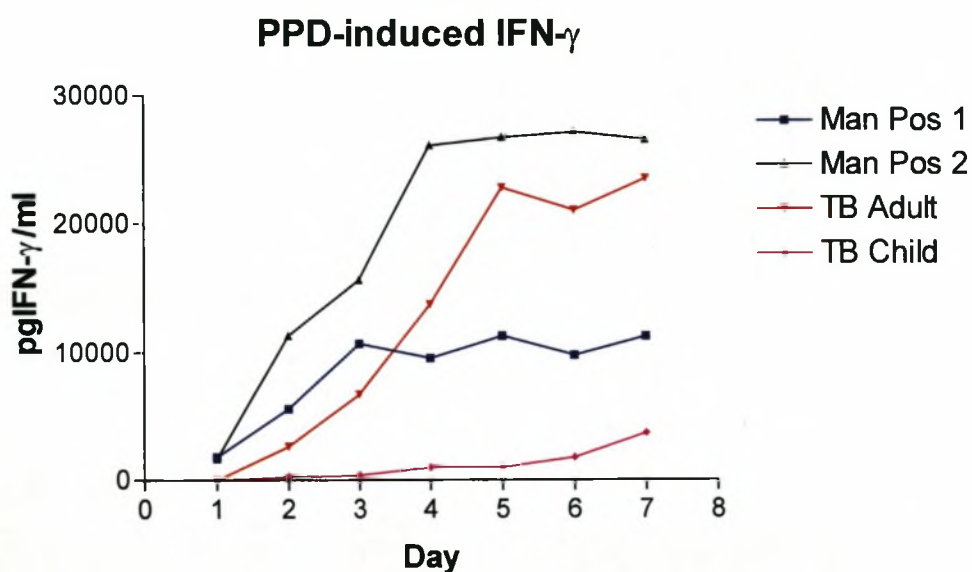
Figure 3.4**PPD-stimulated IFN- γ production of four subjects, over time**Results are expressed in pg/ml of IFN- γ (stimulated cultures minus unstimulated cultures).

Table 3.5**PHA-stimulated IFN- γ production of four subjects, over time**Results are expressed in pg/ml of IFN- γ

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	21	1313	1292	980	15680	14700	1535	1298	0	85	3415	3330
2	34	11324	11290	1113	20736	19622	948	1310	36	67	5720	5653
3	0	10359	10359	3024	28502	25478	610	14185	13575	247	6015	5768
4	267	11324	11057	1536	29924	28388	1435	15760	14325	152	1072	920
5	39	11325	11286	1336	29336	28000	1035	2385	1350	112	1350	1238

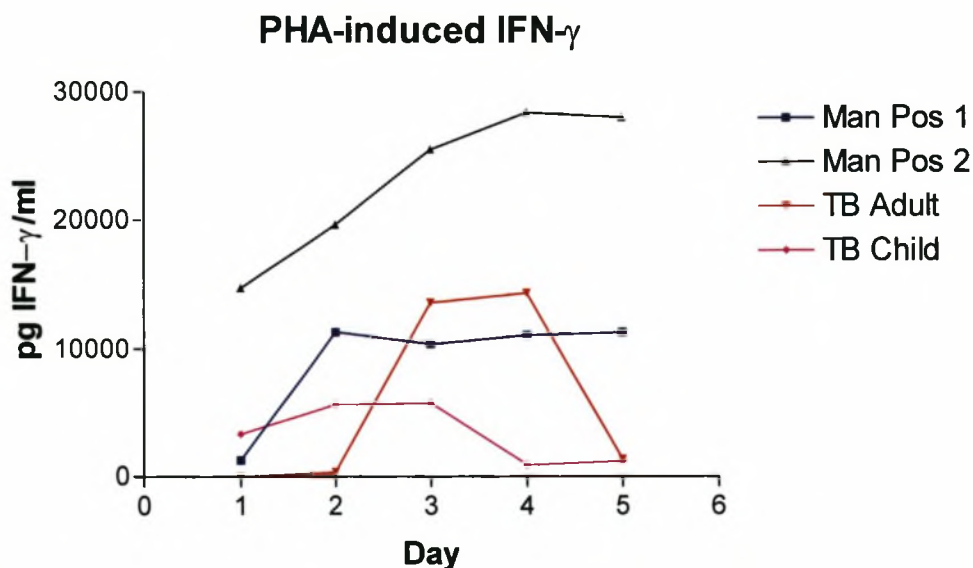
Figure 3.5**PHA-stimulated IFN- γ production of four subjects, over time**Results are expressed in pg/ml of IFN- γ (stimulated cultures minus unstimulated cultures).

Table 3.6

PPD-stimulated IL-2 production of four subjects, over time

Results are expressed in pg/ml of IL-2

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	16	414	398	0	29	29	0	0	0	76	55	0
2	0	162	162	100	20	0	0	102	102	12	112	100
3	0	77	77	0	89	89	0	48	48	35	63	28
4	0	12	12	14	0	0	0	40	40	20	192	172
5	0	14	14	0	0	0	99	163	64	43	41	0
6	0	373	373	52	0	0	0	0	0	4	102	98
7	0	22	22	10	70	60	0	0	0	76	60	0

Figure 3.6

PPD-stimulated IL-2 production of four subjects, over time

Results are expressed in pg/ml of IL-2 (stimulated cultures minus unstimulated cultures).

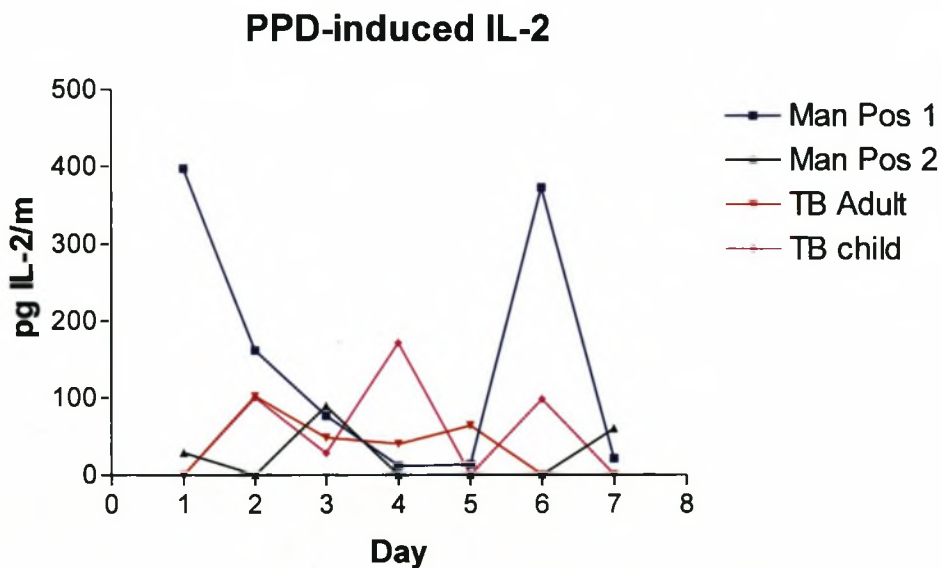


Table 3.7**PHA-stimulated IL-2 production of four subjects, over time**

Results are expressed in pg/ml of IL-2

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	0	833	833	51	260	209	0	0	0	19	293	274
2	25	800	775	0	312	312	0	9	9	19	458	439
3	0	475	475	115	771	656	0	344	344	0	283	283
4	0	205	205	0	333	333	0	266	266	48	37	0
5	0	339	339	113	122	10	15	0	0	8	138	130

Figure 3.7**PHA-stimulated IL-2 production of four subjects, over time**

Results are expressed in pg/ml of IL-2 (stimulated cultures minus unstimulated cultures).

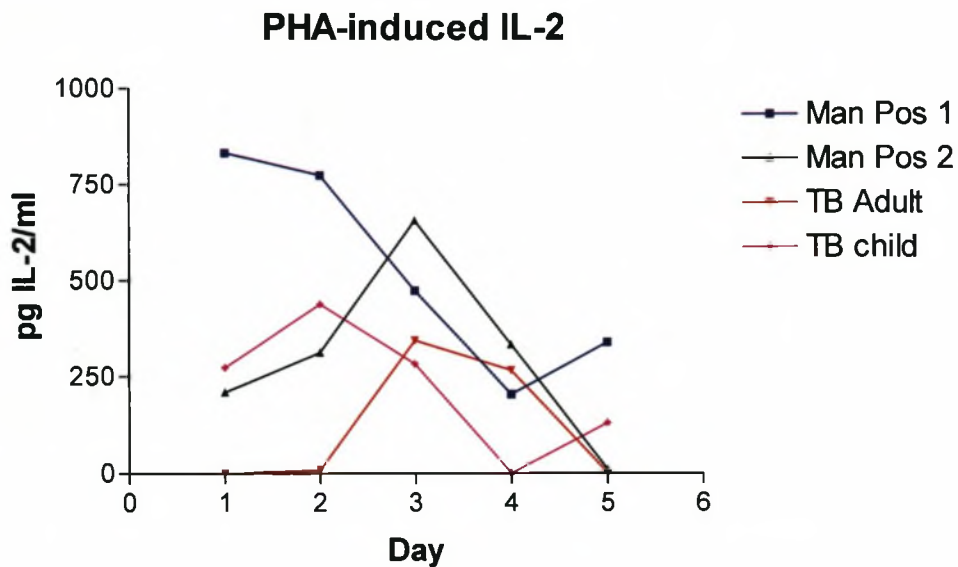


Table 3.8**PPD-stimulated production of TGF- β four subjects, over time**Results are expressed in pg/ml of TGF- β

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	519	591	72	2008	2200	192	5304	6263	959	2397	2582	185
2	346	1022	676	3066	3119	53	7083	7652	569	2935	3621	686
3	707	1843	1136	2773	3176	403	6601	7443	843	4075	3894	0
4	1202	2707	1505	2877	3287	410	6751	8045	1294	3855	4542	687
5	1360	1900	539	3376	2773	0	7744	10500	2756	4704	5257	553
6	1471	2261	790	2550	2539	0	7946	11584	3638	2216	2809	593
7	1159	2748	1589	2459	3757	1298	8770	10627	1857	2061	1214	0

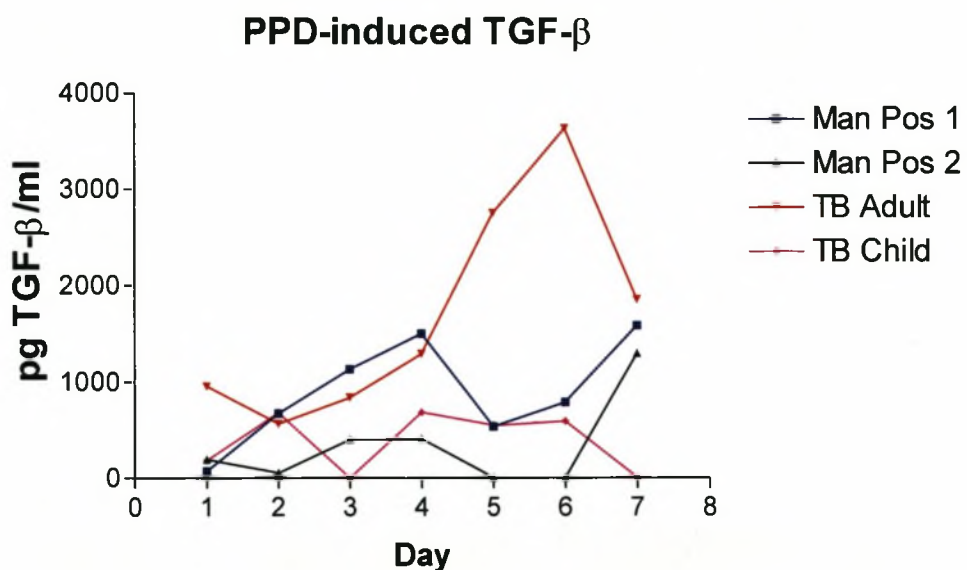
Figure 3.8**PPD-stimulated TGF- β production of four subjects, over time**Results are expressed in pg/ml of TGF- β (stimulated cultures minus unstimulated cultures).

Table 3.9**PHA-stimulated production of TGF- β four subjects, over time**Results are expressed in pg/ml of TGF- β

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	335	785	450	292	1580	1288	5646	5056	0	2284	3841	1557
2	420	988	568	2388	1915	0	5257	6303	1046	471	4198	3727
3	464	1471	1007	3157	2363	0	6538	7781	1243	2917	4242	1325
4	743	2144	1401	2431	2438	7	7414	7867	453	4051	4522	471
5	827	2878	2051	2652	3013	361	7836	6882	0	3645	4571	926

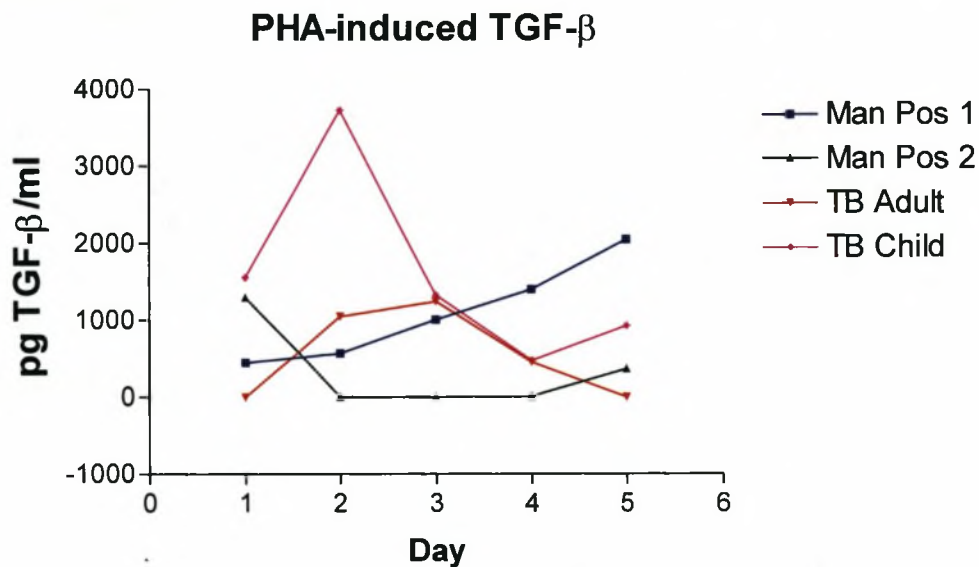
Figure 3.9**PHA-stimulated TGF- β production four subjects, over time**Results are expressed in pg/ml of TGF- β (stimulated cultures minus unstimulated cultures).

Table 3.10**PPD-stimulated IL-12 production of four subjects, over time**

Results are expressed in pg/ml of IL-12

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	0	3906	3906	2043	4296	2253	438	1888	1450	1181	924	0
2	0	5296	5296	1808	3646	1838	424	1763	1339	670	1374	704
3	0	5465	5465	1521	3471	1950	495	1334	839	849	1820	971
4	0	4602	4602	1933	4968	3035	370	1980	1610	727	1659	932
5	0	5637	5637	1849	4389	2540	495	2484	1989	649	1138	489
6	0	4668	4668	2514	5005	2491	449	2374	1925	431	1013	582
7	0	4846	4846	2539	3427	888	356	1406	1050	834	1702	868

Figure 3.10**PPD-stimulated IL-12 production of four subjects, over time**

Results are expressed in pg/ml of IL-12 (stimulated cultures minus unstimulated cultures)

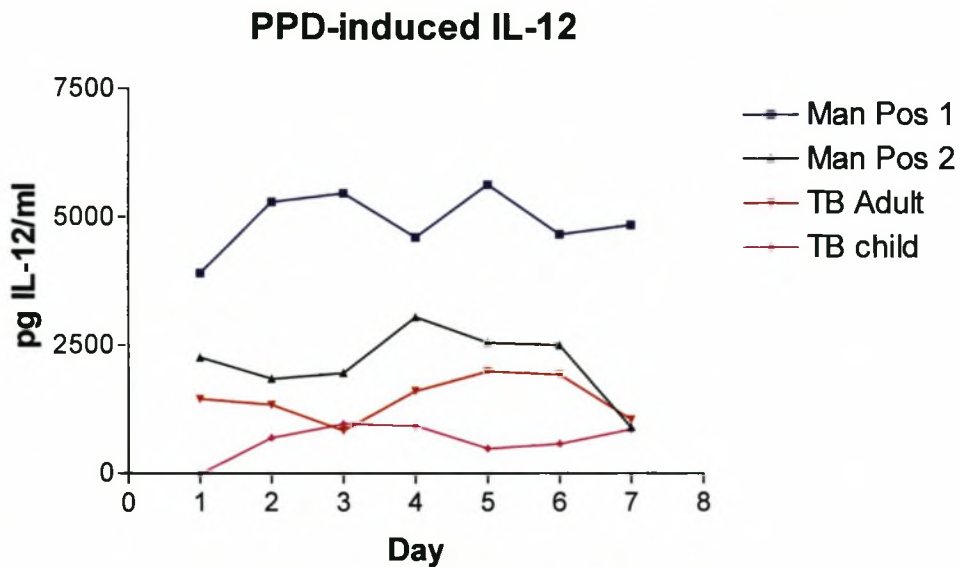


Table 3.11**PHA-stimulated IL-12 production of four subjects, over time**

Results are expressed in pg/ml of IL-12

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	0	753	753	1070	1242	172	386	505	119	945	645	0
2	0	984	984	740	1349	609	321	968	647	741	527	0
3	0	1759	1759	1059	1454	395	352	911	559	741	916	175
4	0	2056	2056	1010	1638	628	280	936	656	834	1131	296
5	0	2031	2031	1000	2017	1017	502	671	169	641	1434	793

Figure 3.11**PHA-stimulated IL-12 production of four subjects, over time**

Results are expressed in pg/ml of IL-2 (stimulated cultures minus unstimulated cultures).

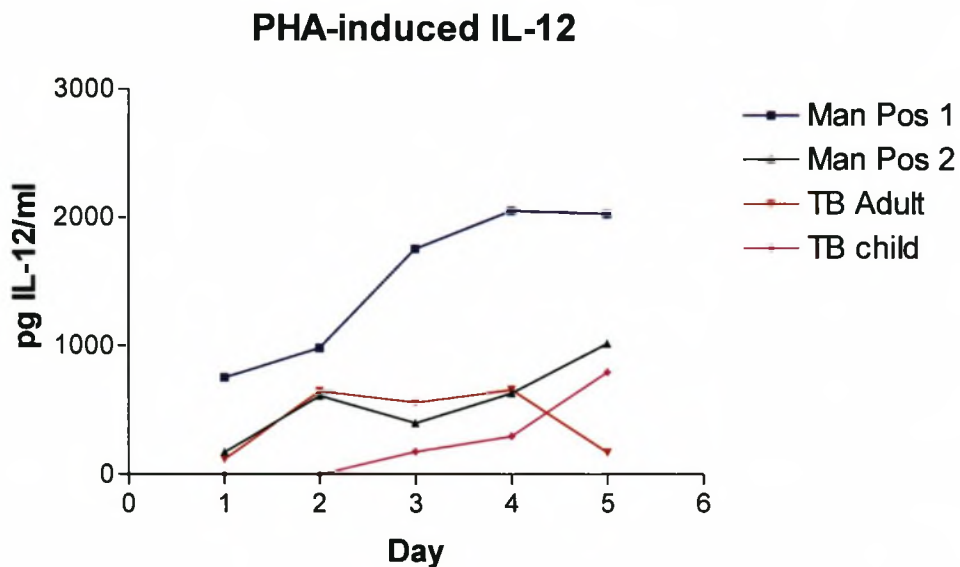


Table 3.12**PPD-stimulated TNF- α production of four subjects, over time**Results are expressed in pg/ml of TNF- α

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	0	128	128	11	115	104	21	290	269	45	85	40
2	0	81	81	23	344	321	0	152	152	7	13	6
3	0	92	92	25	151	126	0	96	96	0	14	14
4	0	76	76	19	151	132	0	176	176	0	3	3
5	0	85	85	0	234	234	0	181	181	0	0	0
6	0	75	75	0	256	256	0	254	254	0	6	6
7	14	80	66	0	122	122	0	406	406	0	16	16

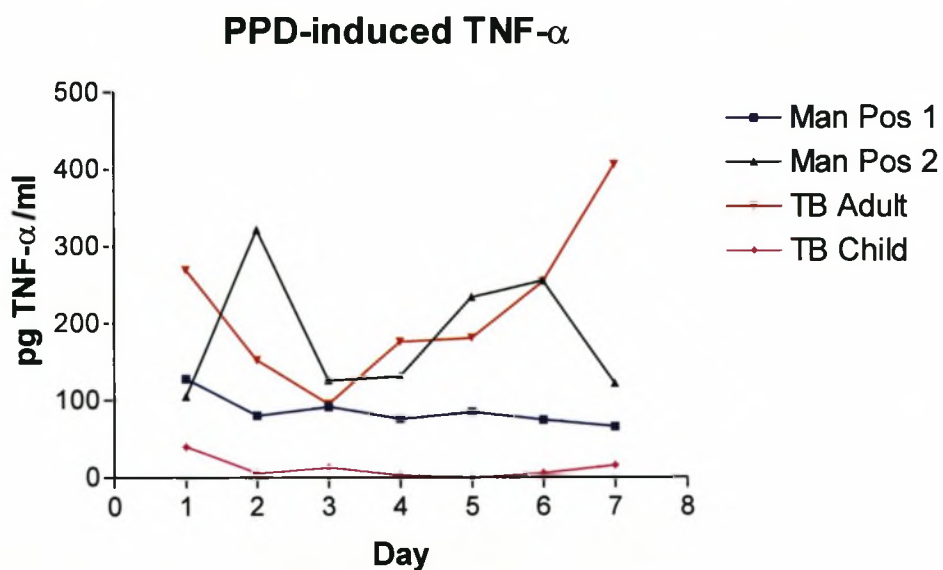
Figure 3.12**PPD-stimulated TNF- α production of four subjects, over time**Results are expressed in pg/ml of TNF- α (stimulated cultures minus unstimulated cultures).

Table 3.13**PHA-stimulated TNF- α production of four subjects, over time**Results are expressed in pg/ml of TNF- α

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	0	287	287	44	447	403	0	30	30	43	382	339
2	0	736	736	0	525	525	0	52	52	0	207	207
3	0	808	808	85	771	686	0	340	340	0	139	139
4	0	405	405	0	762	762	0	307	307	0	0	0
5	0	418	418	0	539	539	0	0	0	0	0	0

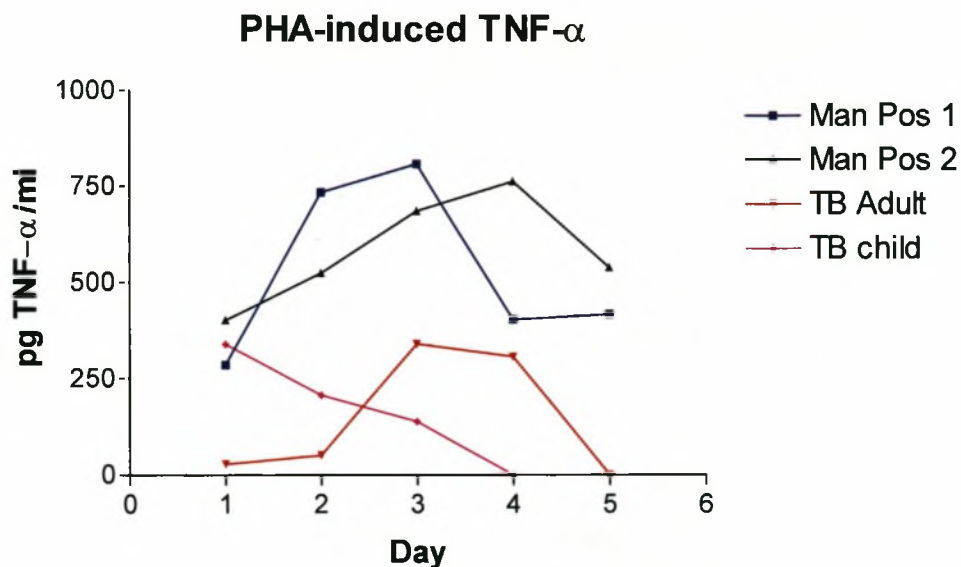
Figure 3.13**PHA-stimulated TNF- α production of four subjects, over time**Results are expressed in pg/ml of TNF- α (stimulated cultures minus unstimulated cultures).

Table 3.14**PPD-stimulated IL-10 production of four subjects, over time**

Results are expressed in pg/ml of IL-10

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	0	116	116	67	879	812	387	844	457	1010	1264	254
2	226	14	0	1134	2244	1110	228	269	41	716	1092	376
3	8	122	114	311	392	81	364	518	154	839	884	45
4	0	66	66	1010	717	0	206	328	122	746	800	54
5	0	402	402	118	0	0	866	504	0	922	870	0
6	142	0	0	929	336	0	378	527	149	386	782	396
7	0	134	134	205	330	125	418	1002	584	958	868	0

Figure 3.14**PPD-stimulated IL-10 production of four subjects, over time**

Results are expressed in pg/ml of IL-10 (stimulated cultures minus unstimulated cultures).

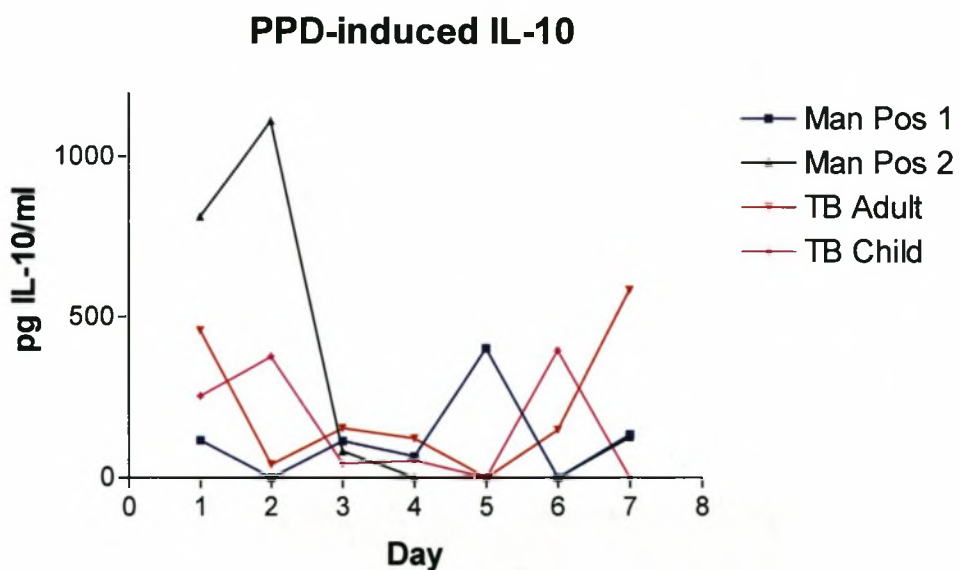


Table 3.15**PHA-stimulated IL-10 production of four subjects, over time**

Results are expressed in pg/ml of IL-10

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	0	1435	1435	1633	935	0	420	269	0	1187	2849	1662
2	462	4984	4522	349	3217	2868	31	106	75	966	2968	2002
3	0	6661	6661	1502	3354	1852	583	695	112	1769	1673	0
4	603	5631	5028	318	4015	3697	119	739	620	1633	1249	0
5	0	5200	5200	1122	2762	1640	370	370	0	944	978	34

Figure 3.15**PHA-stimulated IL-10 production of four subjects, over time**

Results are expressed in pg/ml of IL-10 (stimulated cultures minus unstimulated cultures).

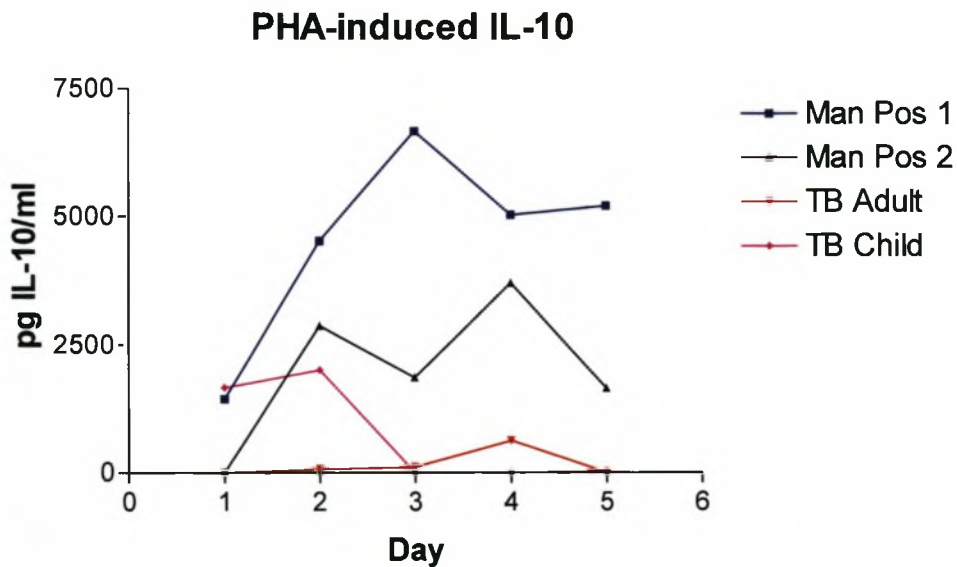


Table 3.16**PPD-stimulated IL-4 production of four subjects, over time**

Results are expressed in pg/ml of IL-4

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	0	0	0	8	24	16	0	0	0	57	29	0
2	0	0	0	18	13	0	0	0	0	49	53	4
3	0	0	0	15	28	13	0	0	0	55	42	0
4	0	7	7	11	4	0	0	0	0	36	49	13
5	3	0	0	7	20	13	0	0	0	37	28	0
6	0	5	5	21	12	0	0	0	0	30	67	37
7	11	0	0	14	32	18	0	0	0	48	36	0

Figure 3.16**PPD-stimulated IL-4 production of four subjects, over time**

Results are expressed in pg/ml of IL-4 (stimulated cultures minus unstimulated cultures).

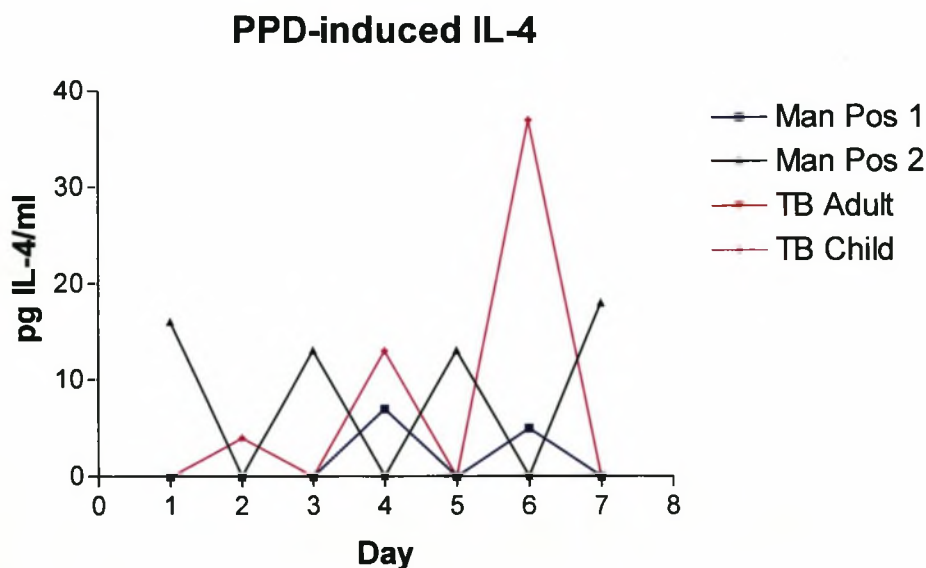


Table 3.17**PHA-stimulated IL-4 production of four subjects, over time**

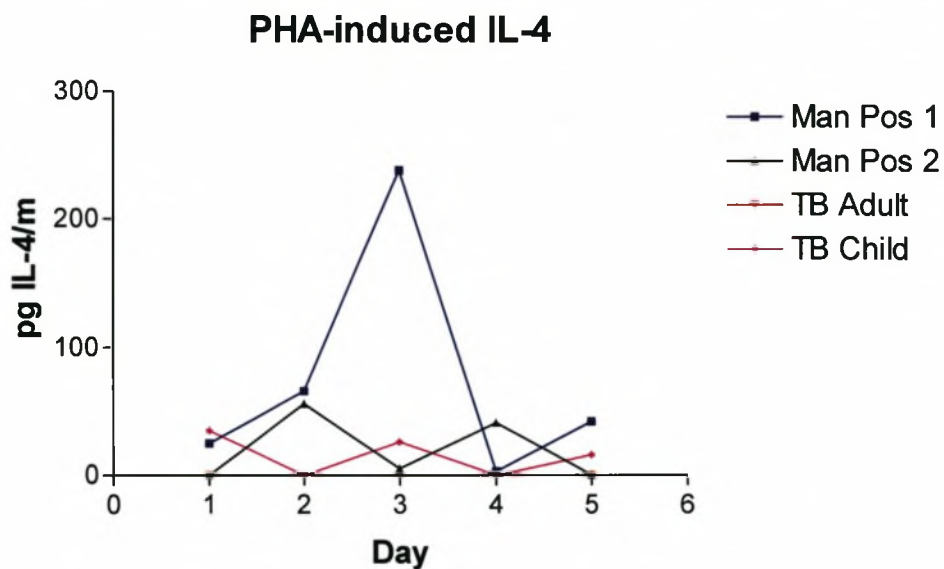
Results are expressed in pg/ml of IL-4

U - unstimulated; S - stimulated; S - U - stimulated minus unstimulated

Day	Man Pos 1			Man Pos 2			TB Adult			TB Child		
	U	S	S - U	U	S	S - U	U	S	S - U	U	S	S - U
1	2	27	25	36	28	0	0	0	0	36	71	35
2	1	67	66	30	86	56	0	0	0	54	44	0
3	0	238	238	37	42	5	0	0	0	38	64	26
4	32	35	3	29	70	41	0	0	0	46	37	0
5	0	42	42	48	33	0	0	0	0	44	60	16

Figure 3.17**PHA-stimulated IL-4 production of four subjects, over time**

Results are expressed in pg/ml of IL-4 (stimulated cultures minus unstimulated cultures).



After examination of all the time courses, it was decided to use the harvesting time points as summarised in Table 3.18.

Table 3.18

Harvesting time points of supernatant fluid for cytokine assays

Cytokine	PHA	PPD
IFN-γ	Day 4	Day 7
IL-2	Day 3	Day 3
IL-12	Day 4	Day 7
IL-10	Day 3	Day 7
TNF-α	Day 3	Day 7
TGF-β	Day 4	Day 7

3.1.5 Discussion

3.1.5.1 Reasons for using RPMI as culture medium

Proliferative responses using RPMI and KSLMS were comparable and it was decided that RPMI would be used as the culture medium for the present study. The main reason for this was that it is more commonly used by other investigators, which would allow the results found in this study to be compared more readily to those found in the literature.

3.1.5.2 Effect of the freeze/thaw process on proliferative responses

The freeze/thaw process did not appear to have a dramatic effect on proliferation responses. It was therefore decided to include this procedure in the method as it allowed many samples to be frozen, before being thawed and transported to the Microbiology laboratory for bulk harvesting. This modification proved to be a practical solution to the problem of the location of the harvester.

3.1.5.3 Optimal harvesting time points of SNF for cytokine analysis

The majority of investigators have traditionally used isolated PBMC for in-vitro analysis of proliferative and cytokine responses. It is therefore not always possible to compare optimum harvesting time points obtained in this study with those found in the literature. Furthermore, since some of the time courses were so variable among the four subjects, it was not always

possible to distinguish a clear optimum harvesting time point. Extreme person-to-person variation in cytokine production is well known. When this was the case, decisions on choices of harvesting times were based on whether the stimulant was a mitogen (PHA) or a specific antigen (PPD). Earlier time points (day 3 or day 4) were chosen for PHA stimulation, as the immune system does not require time for antigenic processing and presentation, whereas PPD does. Day 7 was therefore chosen as harvesting time point for PPD stimulation.

Although some of the cytokine time courses exhibit extreme variability, person-to-person variation as well as age-related differences is well recognised. The decision to harvest at one particular time point is perhaps an oversimplification, but is based on practicalities such as labour, time, blood-taking, cost, comparison, etc.

IFN- γ

Day 4 and day 7 were chosen as harvesting time points for IFN- γ analysis from PHA- and PPD-stimulated cultures respectively. The choice of optimum harvesting time points was reasonably simple as the all the time courses concurred (except for PHA-induced IFN- γ of the child), indicating these to be the optimum time points. PPD- and PHA-stimulated IFN- γ production in the child was decreased compared to that of the adults. It is well documented that IFN- γ production in neonates and young children is reduced compared to that of adults (Holt et al., 1995; Elsässer-Beile et al., 1995; reviewed by Smith et al., 1997).

These harvesting time points were in agreement with Kluter et al., (1995) and Elsässer-Beile et al., (1995), who also chose day 4 as the harvesting time point for PHA-stimulated cultures. The kinetics of PPD-induced IFN γ production compared well with those found by Elsässer-Beile et al (1991).

IL-2

It was decided to harvest supernatant fluid for IL-2 analysis from both PHA- and PPD-stimulated cultures on day 3. In general, this time point appeared to be optimal for PHA-induced IL-2, as can be seen by the mean concentration on that day. This is in agreement with a report by Elsässer-Beile et al., (1991) who found optimal PHA-induced IL-2 secretion at between day 2 and day 3. Elliot et al., (1999), however, harvested PPD-stimulated IL-2 on day 1. It is important to measure IL-2 levels at a reasonably early time point to avoid the

problem of receptor-mediated consumption. Therefore, day 3 was chosen for harvesting PPD-stimulated IL-2.

TGF- β

Day 4 and day 7 were chosen as harvesting time points for TGF- β analysis from PHA- and PPD-stimulated cultures respectively. The time courses for the four individuals were extremely variable and no clear optimum harvesting time point for either stimulant could be distinguished. The adult with TB produced the highest levels of PPD-induced TGF- β . TB patients have previously been shown to produce higher levels of TGF- β when compared to healthy Mantoux positive controls. An early time point, day 4, for PHA-stimulated cultures was chosen, as this stimulant is a mitogen which does not require time for antigen processing and presentation. A later time point, day 7, was chosen for PPD-stimulated cultures to allow sufficient time for antigen processing and presentation.

Background counts from unstimulated cultures were high in all individuals throughout both time courses. This phenomenon has previously been documented by Hirsch et al., (1996). In this study, a possible explanation could be that individuals from the Ravensmead/Uitsig community are continually exposed to *M.tuberculosis* and other environmental factors (for example, stress), which could result in their cells being primed, or activated in-vivo. When these cells are then put into culture, monocytes (MO) could spontaneously release TGF- β . This is possibly why the adult subjects from the Ravensmead/Uitsig community (Man pos 2 and adult TB subject), produce more background than the adult who is not (Man pos 1).

IL-12

Day 4 and day 7 were also chosen as harvesting time points for IL-12 analysis from PHA- and PPD-stimulated cultures respectively. Day 4 was chosen for PHA-stimulated cultures as all individuals showed good IL-12 responses at that time point. Day 7 was chosen for PPD-stimulated cultures as IL-12 levels were well maintained throughout the 7-day period. It is interesting to note the child's capacity to produce IL-12, (which is an important promoter of IFN- γ production), is low compared to that of the adults. This could also be an explanation for the low levels of IFN- γ produced by the child. Quantitation of differences in IL-12 production between children and adults is a controversial subject.

Except for Man Pos 1, background counts in unstimulated cultures were high. IL-12 is also produced by MO/MA and its presence in this study could be due to the same reasons as discussed for TGF- β . The fact that Man pos 1 had no background compared to the individuals from the Ravensmead/Uitsig communities lends support to this hypothesis.

TNF- α

Day 3 and day 7 were chosen as harvesting time points for TNF- α from PHA- and PPD-stimulated cultures respectively. Except for the child, day 3 showed high levels levels of TNF- α for all subjects. Van Crevel et al., (1999) also found a continual increase of TNF- α up to day 3. TNF- α appears to contribute to some of the pathology seen in advanced TB in adult patients (Filley et al., 1992; Tramontana et al., 1995). This pathology includes fever and wasting. Children however, mostly have the lymph node involvement and not much pathology. It is interesting to note that PPD-stimulated TNF- α in the child was very low compared to that of the other adults. The time course of PPD-stimulated cultures for the four individuals was variable and no clear optimal harvesting time point could be distinguished. Day 7 was chosen to allow sufficient time for antigen processing and presentation. Van Crevel et al., (1999) harvested PHA- and PPD-stimulated cultures for TNF- α on day 3 while Elsasser-Beile et al., (1995) harvested their PHA-stimulated cultures for TNF- α on day 4.

IL-10

Day 3 and day 7 were chosen as harvesting time points for IL-10 analysis from PHA- and PPD-stimulated cultures respectively. The trend for both PHA- and PPD-stimulated time courses was variable, therefore an earlier (day 3 for PHA) and later (day 7 for PPD) time point, was chosen.

Background counts in unstimulated cultures were generally high for both time courses. IL-10 is also produced by MO/MA and its presence in this study could be due to the same reasons as discussed for TGF- β . Once again, the fact that Man pos 1 had less background, in general, than the individuals from the Ravensmead/Uitsig communities also lends support to this hypothesis.

IL-4

IL-4 levels in PHA- and PPD-stimulated cultures were extremely low in all individuals, except for one Mantoux-positive individual, who showed an increased level of IL-4 production on day 3, following PHA stimulation. It was therefore decided to exclude the measurement of IL-4 in this study. Low IL-4 levels have also previously been documented in the literature (Petrovsky et al., 1995).

Technical problems with TGF- β and IL-12

During the initial “before therapy” study, it was discovered that the unstimulated background values for these cytokines in the group of study subjects recruited from the end of March until June 1999 had significantly higher values compared to the previously recruited patients. This phenomenon appeared to persist and was present in the follow up “after therapy” study as well. No differences could be found in any of the other cytokines and no changes in laboratory protocols or reagents had been made. Various measures were taken to investigate a possible source of contamination leading to the non-specific production of these cytokines. These included the checking of incubators, distilled water systems and laminar flow hoods for sources of contamination, changing to commercially-made RPMI as well as reinforcement of measures to prevent contamination. The possibility of LPS contamination was excluded by measuring the levels of TNF- α and IFN- γ in a large subgroup of unstimulated samples at various time points (before and after the discovery of the high background levels as well in the “after therapy” study). These values were all below detection levels of the assays (15 pg/ml). Unfortunately, no satisfying reason was found for the high backgrounds in these cytokines and it was thus decided to exclude the TGF- β and IL-12 values for all study subjects from both the “before” and “after therapy” studies.

3.2 Optimization of the Duoset ELISA system

3.2.1 Background

In our laboratory we previously used prepackaged ELISA kits for the measurement of IFN- γ , IL-2p40, TGF- β , IL-12p40, TNF- α , IL-10 and IL-4. Each kit contained a matched, fully optimized set of reagents as well as buffers and a coated plate. These kits were ready for use and did not require any development or optimization. However, the kits proved to be very costly as only forty samples (in duplicate) could be analysed from one kit. We therefore decided to convert to a Douset ELISA system which allowed analysis of 480 samples. This

system contained the basic components required to develop an immunoassay and included a capture antibody, a mass calibrated standard, a biotinylated secondary antibody, streptavidin horseradish peroxidase and a generic protocol to aid in the development of an optimized protocol.

3.2.2 Determination of optimal concentrations of the capture and secondary antibodies

There are many parameters which influence the results obtained in an ELISA. These include component quality, antibody concentrations, incubation times and temperatures, detection reagent quality and concentration and substrate type and quality. It was decided to commence the optimization procedure by determining the optimal concentrations of the capture and secondary antibodies. This was achieved by using the ELISA protocol (Section 2.3.6) to titrate the capture antibody against the secondary antibody. Although the basic ELISA protocol was followed, there were some modifications. These included dividing the 96-well plate into 2 equal components and titrating the capture antibody down the plate and the secondary antibody across the plate, in an identical fashion on both halves of the plate (see Figure 3.18). Furthermore, on one half of the plate, a constant concentration of sample (usually the highest concentration of the standard) was used. On the second half of the plate, no antigen was used. Initial experiments produced optical densities that were too low, but after increasing the incubation time of the secondary antibody and the substrate, the optical densities improved. After the ELISA was completed and the optical densities read, the concentrations which gave the best signal to noise ratio were chosen. The left side of the plate, which contained antigen, gave a “signal” resulting from each of the many antibody pair concentrations. The right side of the plate, with no antigen, gave the “noise” or the background value that could be expected for each of the antibody concentrations. The signal that produced the highest signal to noise ratio and which still gave an acceptable background (<0.3 optical density units), was chosen. Table 3.19 shows a typical example of the optical density results obtained when a capture antibody was titrated against a secondary antibody in the ELISA protocol.

Figure 3.18 Plate layout for the titration of the capture and secondary antibody

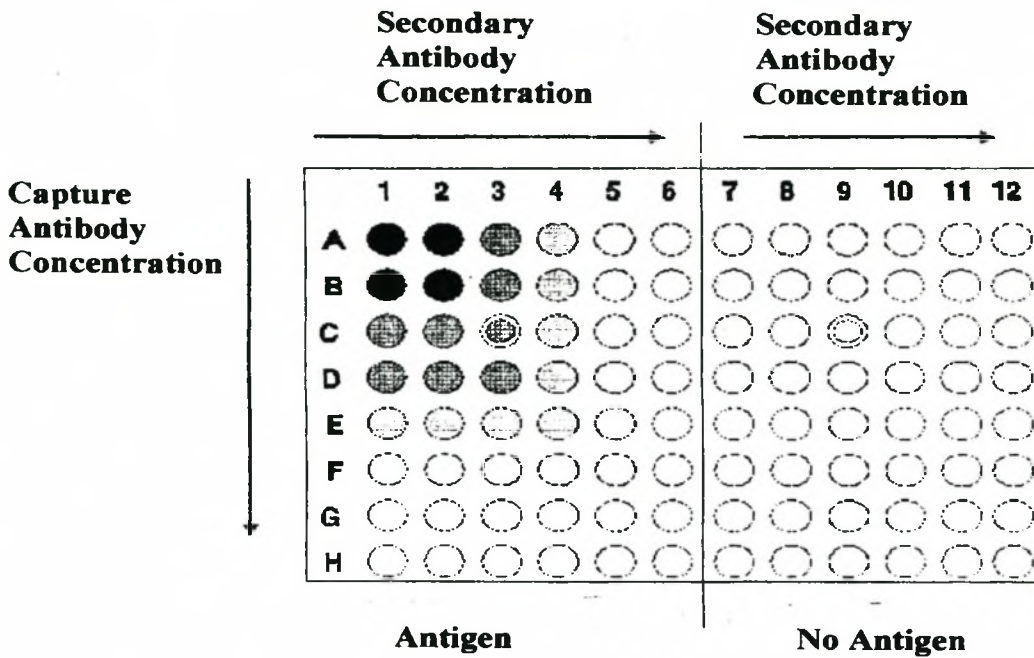


Table 3.19

Typical example of ELISA results obtained when an IFN- γ capture antibody was titrated against an IFN- γ secondary antibody using the ELISA protocol.

Results are expressed in optical density units

		Secondary Antibody ($\mu\text{g/ml}$)						Secondary Antibody ($\mu\text{g/ml}$)						
		5	2.5	1.25	0.62	0.31	0.15	5	2.5	1.25	0.62	0.31	0.15	
		1	2	3	4	5	6	7	8	9	10	11	12	
Capture Antibody ($\mu\text{g/ml}$)	4	A	1.422	1.433	1.429	1.380	1.176	0.879	0.292	0.278	0.199	0.189	0.138	0.112
	2	B	1.487	1.460	1.449	1.382	1.172	0.858	0.280	0.263	0.246	0.157	0.153	0.121
	1	C	1.444	1.433	1.416	1.354	1.129	0.841	0.306	0.251	0.196	0.151	0.148	0.110
	0.5	D	1.395	1.417	1.388	1.332	1.102	0.829	0.347	0.271	0.257	0.159	0.135	0.113
	0.25	E	1.418	1.392	1.375	1.299	1.095	0.758	0.369	0.268	0.223	0.154	0.138	0.108
	0.12	F	1.407	1.400	1.393	1.301	1.111	0.771	0.387	0.329	0.242	0.156	0.139	0.115
	0.06	G	1.406	1.382	1.368	1.296	1.116	0.811	0.437	0.299	0.230	0.155	0.135	0.110
	0.03	H	1.440	1.421	1.407	1.358	1.164	0.881	0.471	0.356	0.250	0.199	0.152	0.125

The highest signal to noise ratio was obtained from the concentrations in C3 and C9.

Table 3.20 shows the concentrations of the capture and secondary antibodies that gave the highest signal to noise ratio for each of the cytokines. These concentrations were thus used in all subsequent ELISAs.

Table 3.20

Capture and secondary antibody concentrations giving the highest signal to noise ratio for each of the cytokines.

Cytokine	Capture Antibody	Secondary Antibody
IFN- γ	1 μ g/ml	0.625 μ g/ml
IL-2	2.5 μ g/ml	1.25 μ g/ml
TGF- β	2 μ g/ml	300ng/ml
IL-12	2 μ g/ml	300ng/ml
TNF- α	2.0 μ g/ml	0.25 μ g/ml
IL-10	2 μ g/ml	250 μ g/ml
IL-4	2.0 μ g/ml	1.0 μ g/ml

3.2.3 Discussion

Although the antibodies in the Douset kits came with the a range of manufacturer's recommended concentrations, titrations of capture and secondary antibodies had to be performed in order to determine the optimal concentration. Results obtained from the titration experiments showed that the optimal concentrations fell within the recommended range and these were thus used for all subsequent ELISAs. Therefore, although these Duoset kits required initial optimization, they proved to be more economical than the prepackaged kits, which cost approximately R100 per sample as opposed to the Duoset kit, which cost approximately R8.75 per sample.

CHAPTER 4

IMMUNE RESPONSES OF PATIENTS (WITH A PAST HISTORY OF TB) AND HEALTHY SUBJECTS OF DIFFERENT AGES

4.1 Study population

Of the 99 subjects (TB patients and Healthy Mantoux positive individuals) studied in the initial “before therapy” study, 68 subjects could be followed up and re-studied in this “after therapy” study (approximately 1 year after initial investigations). Follow-up of 31 individuals was not possible for the following reasons: subjects moved out of the area or were untraceable (n = 21), therapy non-compliance (n = 6), development of TB disease in a Mantoux positive control (n = 1), received prophylactic therapy (n = 1), refusal to partake in study (n = 1) and unsuccessful blood sampling from 1 subject. The subjects who were successfully followed up in this study comprised TB patients, who had completed TB therapy (n = 27) and healthy Mantoux positive control subjects (n = 41). All adolescent and adult patients (n = 12) had sputum samples re-tested for presence of *M. tuberculosis* by ZN staining after completion of TB therapy, all of which were negative.

Because of the age-related risk of infection as well as the clinical spectrum of disease, the study population was stratified into 2 age groups (<12 years old and > 12 years old). The patients with a history of TB (successfully treated TB patients) were classified according to their clinical status at diagnosis, either primary TB (<12 years old; n = 15) or postprimary TB (>12 years old; n = 12). Table 4.1 shows the number of successfully treated TB patients and healthy Mantoux positive control subjects recruited in each age group as well as the male/female distribution. Table 4.2 shows the median (interquartile ranges) ages in years for each of the age groups. Table 4.3 shows the median (interquartile ranges) Mantoux sizes in millimetres (mm) for each of the age groups, which was performed at the initial “before therapy” study. The Mantoux test was not repeated in the “after therapy” study.

Table 4.1**Number and gender of patients with a history of TB and healthy controls per age group**

Age	Patients Sex (M/F)	Controls Sex (M/F)	Total Sex (M/F)
<12 yrs	15 (8/7)	22 (11/11)	37 (19/18)
>12 years	12 (5/7)	19 (6/13)	31 (11/20)
Total Sex (M/F)	27 (13/14)	41 (17/24)	68 (30/38)

M = male; F = female

Table 4.2**Median (interquartile ranges) ages in years for patients with a history of TB and healthy controls**

Age	n	Patients	n	Controls
<12 years	15	4.1 (2.6-9.5)	22	6.2 (4.0-9.4)
>12 years	12	19.2 (15.5-28.4)	19	17.1 (13.8-37.0)

Table 4.3**Median (interquartile ranges) of Mantoux sizes in millimetres (mm) for each of the age groups.**

Age	n	Patients	n	Controls
<12 years	14	16.6 (0.00-20.2)	22	18.4 (16.2-20.4)
>12 years	5	20.0 (19.0-21.0)	19	18.5 (15.7-28.0)

4.2 Lymphocyte proliferation studies

4.2.1 Proliferation after PHA stimulation

Day 4 PHA-stimulated lymphocyte proliferative responses from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

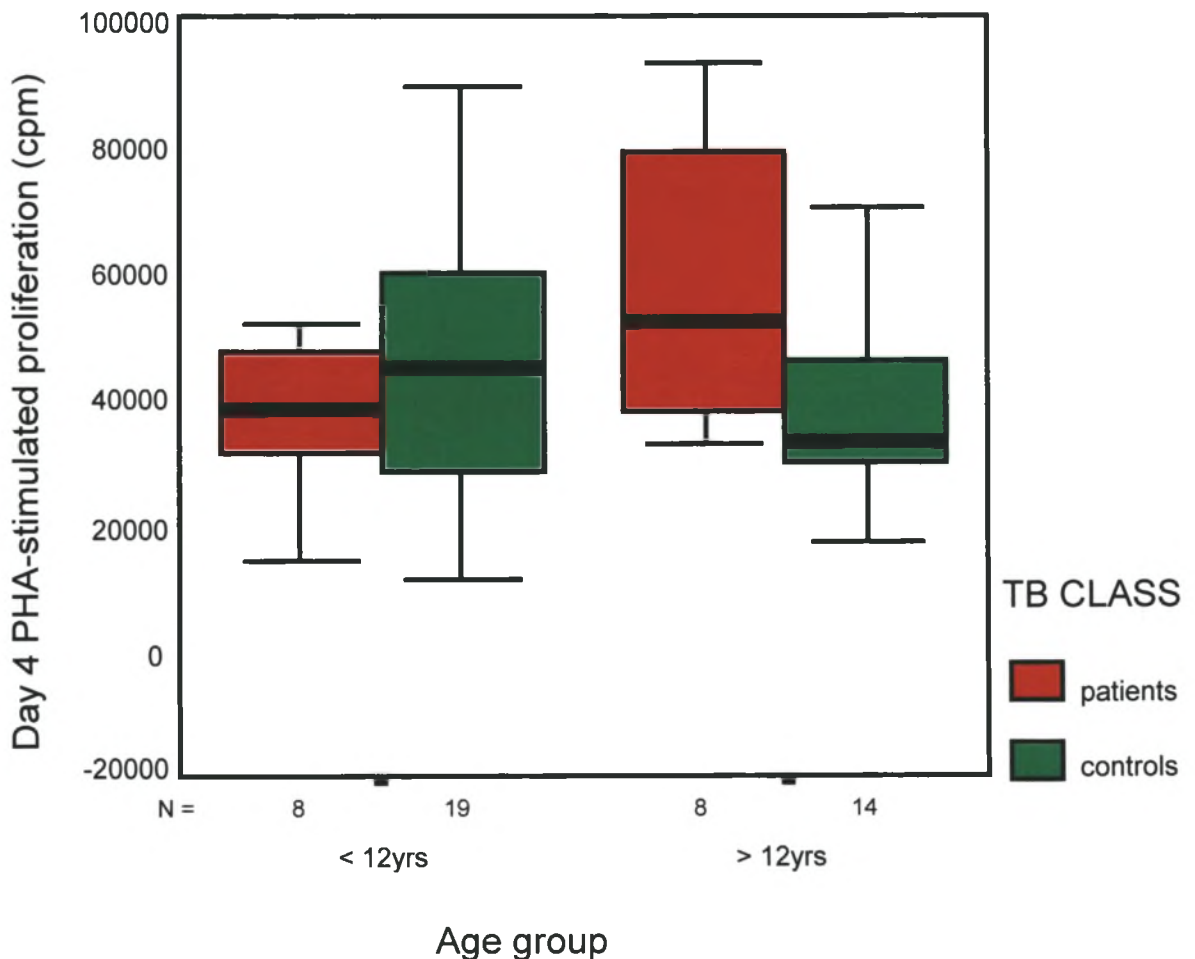


Table 4.4

Median (interquartile range: IQ) proliferative responses (cpm) of whole blood stimulated with PHA (10µg/ml) (after subtraction of values from unstimulated cultures) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p-value</i> ¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	8	37783 (30495-47897)	19	44612 (26607-60978)	.418
>12 years	8	51700 (36160-82881)	14	32869 (26816-49879)	.029
<i>P-value</i> ²		.065		.321	

*p-value*¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

*p-value*²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

PHA-stimulated proliferation (cpm) did not correlate in any way with age (yrs). Patients >12yrs differed significantly from controls >12yrs ($p = .029$) with enhanced proliferation observed. No other significant differences in PHA-induced proliferative responses were found between any of the groups.

4.2.2 Proliferation after PPD stimulation

Day 7 PPD-stimulated lymphocyte proliferative responses from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

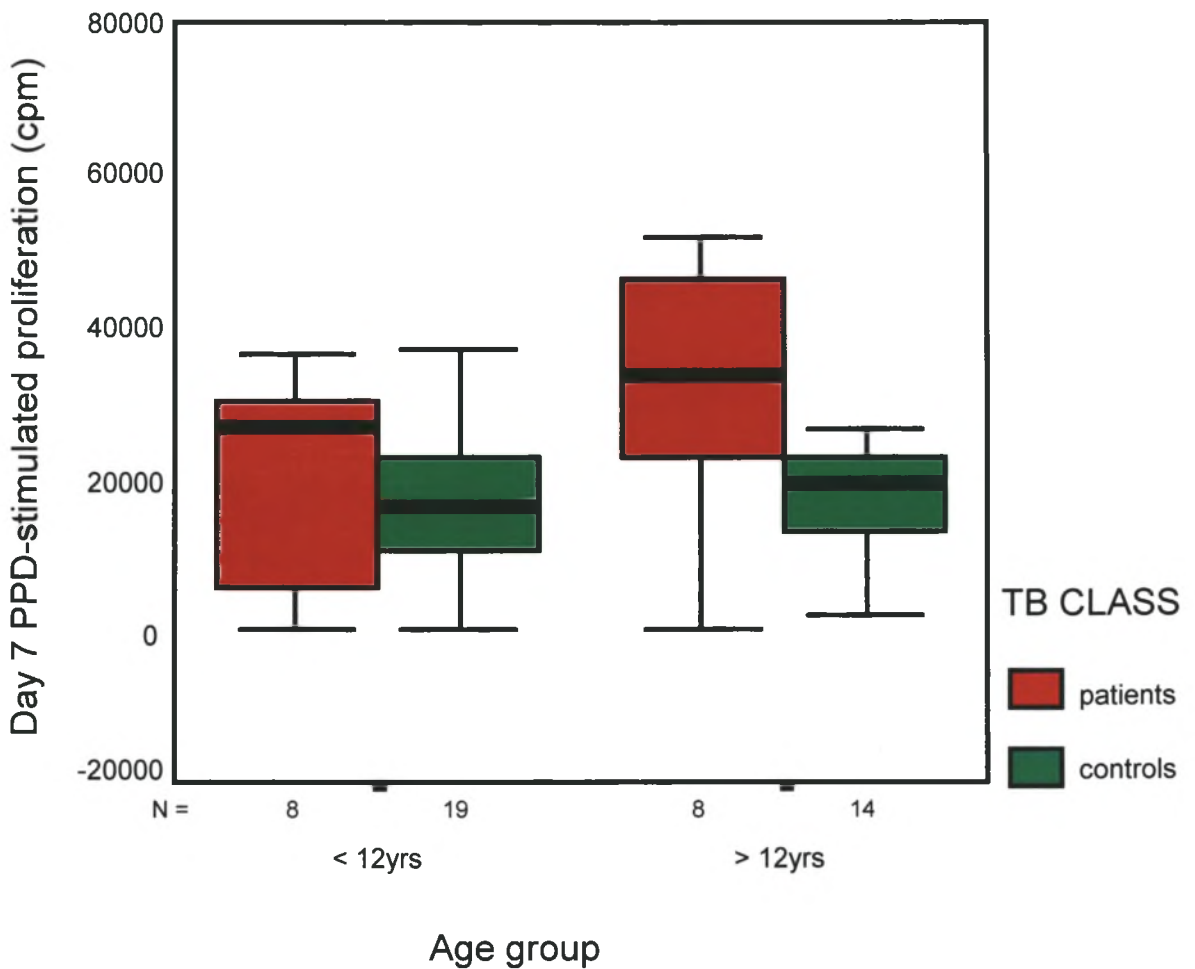


Table 4.5

Median (interquartile range: IQ) proliferative responses (cpm) of whole blood stimulated with PPD (3.3µg/ml) (after subtraction of values from unstimulated cultures) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p-value</i>¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	8	26981 (5001-31420)	19	16257 (10058-24460)	.515
>12 years	8	33973 (20668-49111)	14	19351 (10893-23372)	.020
<i>P-value</i>²	.161		.900		

*p-value*¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

*p-value*²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

PPD-stimulated proliferation (cpm) did not correlate in any way with age. Patients >12yrs differed significantly from controls >12yrs ($p = .020$) with enhanced proliferation observed. No other significant differences in PPD-induced proliferative responses were found between any of the groups.

4.3 Cytokine studies

4.3.1 IFN- γ production

4.3.1.1 IFN- γ production after PHA stimulation

Day 4 PHA-stimulated IFN- γ production from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles.

The lines extending from the box extend to upper and lower adjacent values.

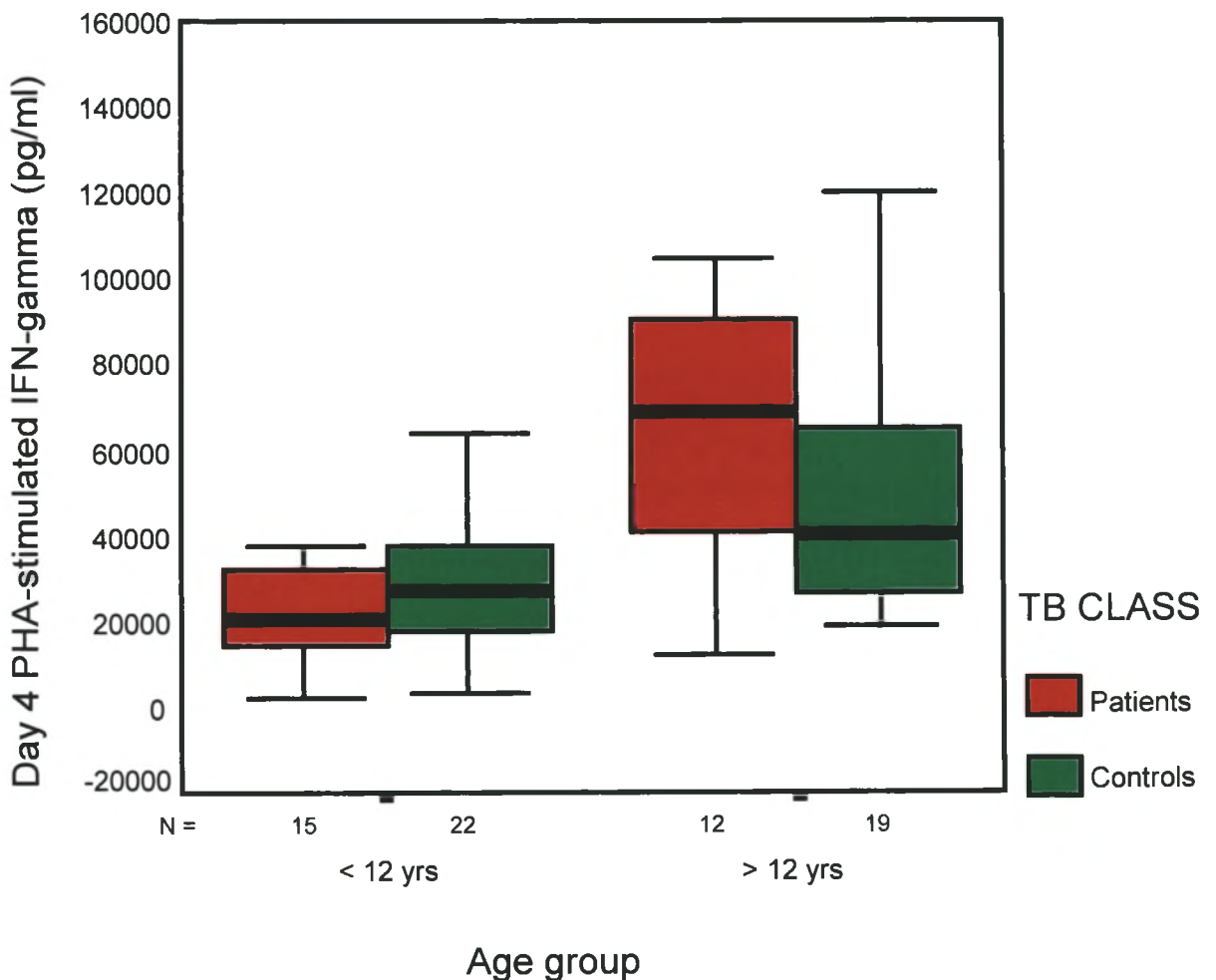


Table 4.6

Median (interquartile range: IQ) of IFN- γ in pg/ml after stimulation of whole blood with PHA (10 μ g/ml) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p</i>-value¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	15	19937 (13760-32102)	22	27114 (16488-37942)	0.417
>12 years	12	68351 (36537-91963)	19	40441 (25939-75349)	0.205
<i>P</i>-value²		.001		.044	

p-value¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

p-value²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

Age (yrs) correlated significantly with PHA-induced IFN- γ (pg/ml) within the whole study group (n = 68; $r = .603$, $p < .001$) as well as for all patients (n = 27; $r = .740$, $p < .001$) and all controls (n = 41; $r = .466$, $p = .002$). Patients, as well as controls <12 years, produced significantly less IFN- γ than their older counterparts ($p = .001$ and $p = .044$).

There was no significant correlation between values for PHA-stimulated IFN- γ and values for PHA-induced proliferation in any of the groups.

4.3.1.2 IFN- γ production after PPD stimulation

Day 7 PPD-stimulated IFN- γ production from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

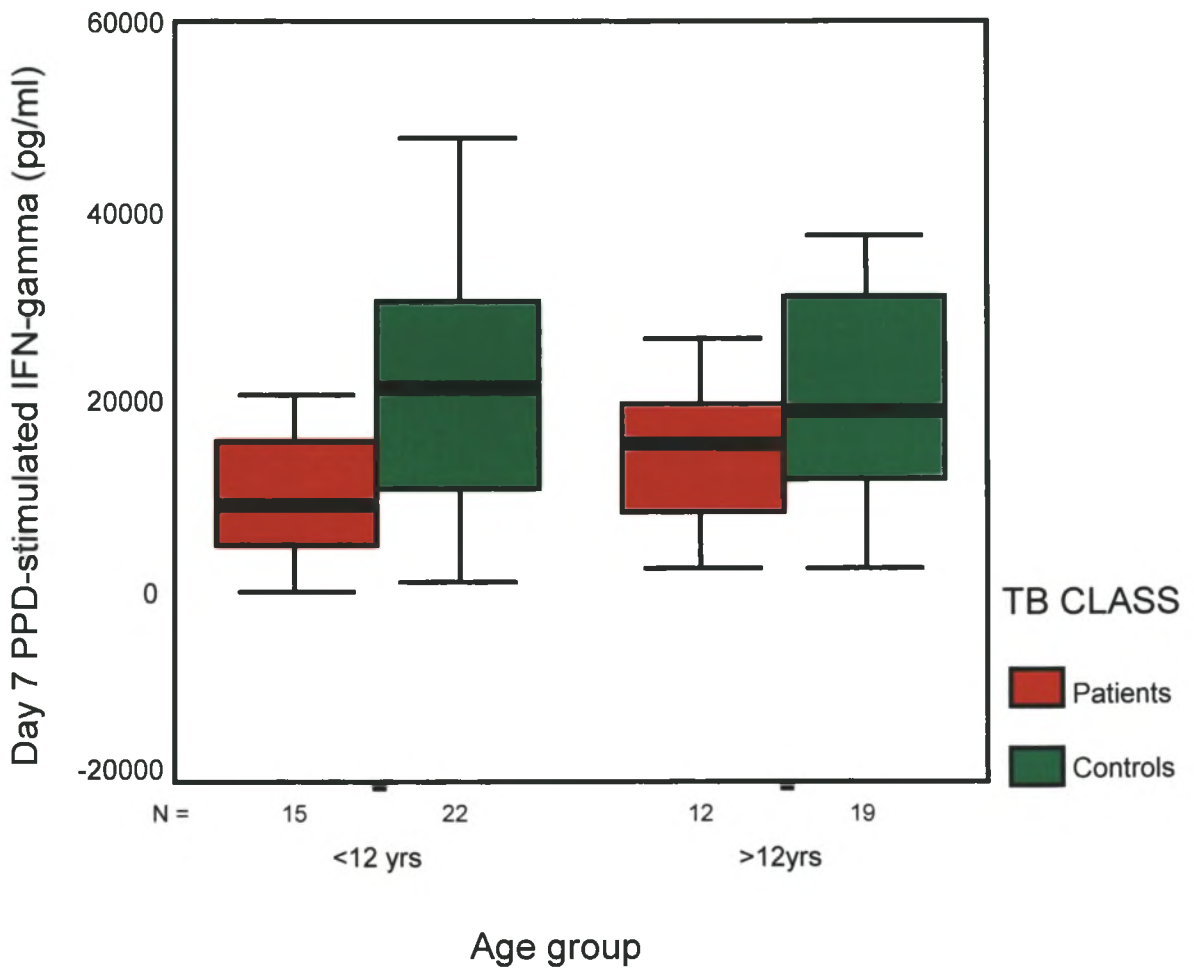


Table 4.7

Median (interquartile range: IQ) of IFN- γ in pg/ml after stimulation of whole blood with PPD (3.3 μ g/ml) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p-value</i>¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	15	9396 (3792-16779)	22	21239 (9902-30962)	0.024
>12 years	12	15364 (7566-20549)	19	19078 (11920-31038)	0.141
<i>P-value</i>²		.256		.896	

*p-value*¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

*p-value*²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

Age (yrs) did not correlate with PPD-induced IFN- γ production (pg/ml) for any of the groups. Patients <12 yrs produced significantly less IFN- γ than controls <12 yrs ($p = 0.024$).

There was no significant correlation between values for PPD-stimulated IFN- γ and values for PPD-induced proliferation when all samples were analysed ($n = 49$) or when values for all patients (<12yrs and >12yrs; $n = 16$) were analysed. When values all for controls (<12yrs and >12yrs; $n = 33$) were analysed, a positive and significant correlation with proliferation ($r = .410$, $p = .018$) was demonstrated.

4.3.2 IL-2 Production

4.3.2.1 IL-2 production after PHA stimulation

Day 3 PHA-stimulated IL-2 production from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

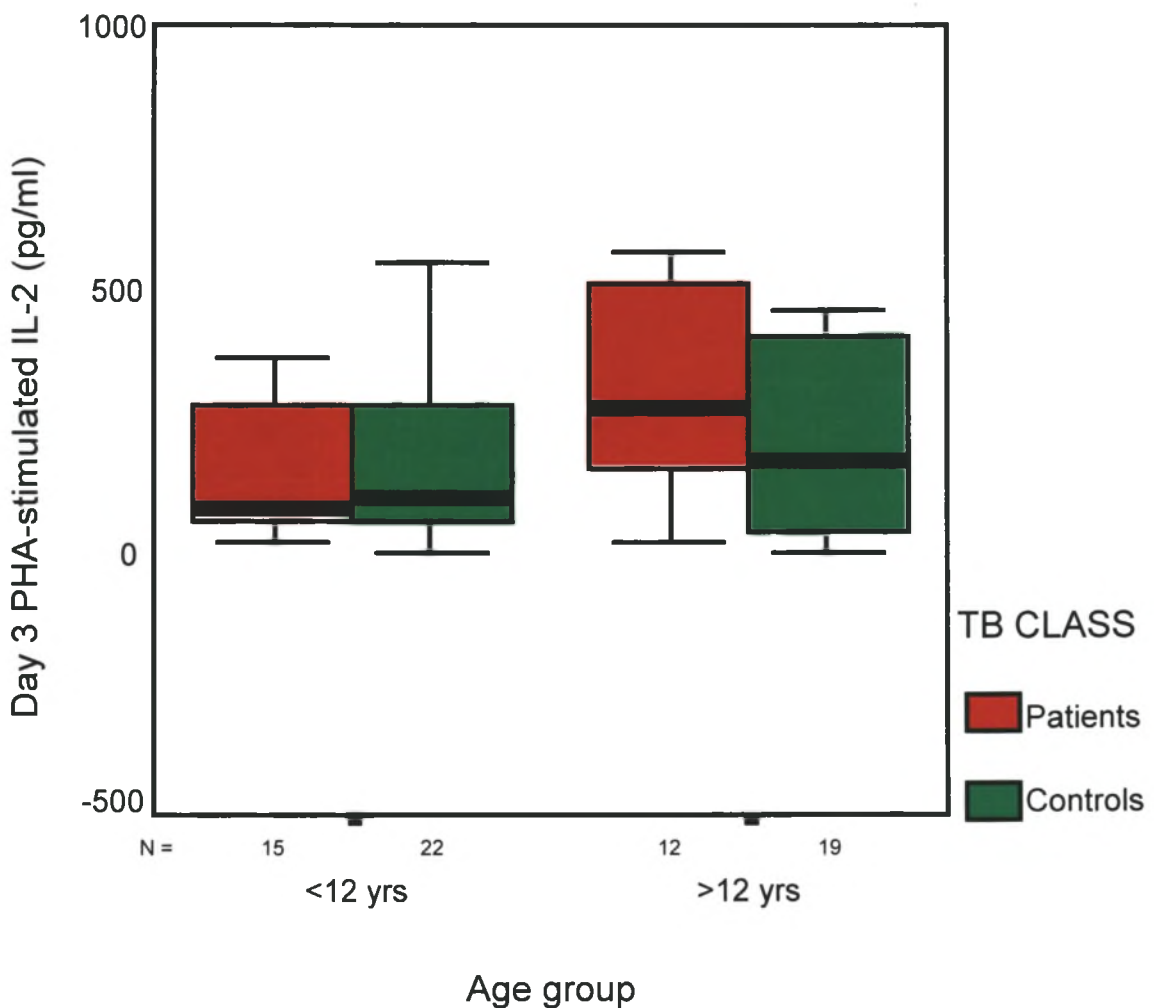


Table 4.8

Median (interquartile range: IQ) of IL-2 in pg/ml after stimulation of whole blood with PHA (10µg/ml) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p</i>-value¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	15	86 (61-305)	22	106 (49-282)	.939
>12 years	12	276 (157-513)	19	174 (40-436)	.287
<i>P</i>-value²		.103		.676	

p-value¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

p-value²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

Age (yrs) did not correlate with PHA-induced IL-2 production (pg/ml) in any of the study groups. Nor were there any other significant differences between any of the study groups.

When all samples were analysed (n = 68), PHA-induced IL-2 correlated positively with PHA-induced proliferation ($r = .444$, $p = .001$) and IFN- γ ($r = .518$, $p < .001$). When values for all controls were analysed (n = 41), all these correlations remained significant (proliferation: $r = .529$, $p = .002$; IFN- γ : $r = .464$, $p = .002$), but only for IFN- γ ($r = .579$, $p = .002$) when values for all patients were analysed (n = 27).

4.3.2.2 IL-2 production after PPD stimulation

Day 3 PPD-stimulated IL-2 production from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

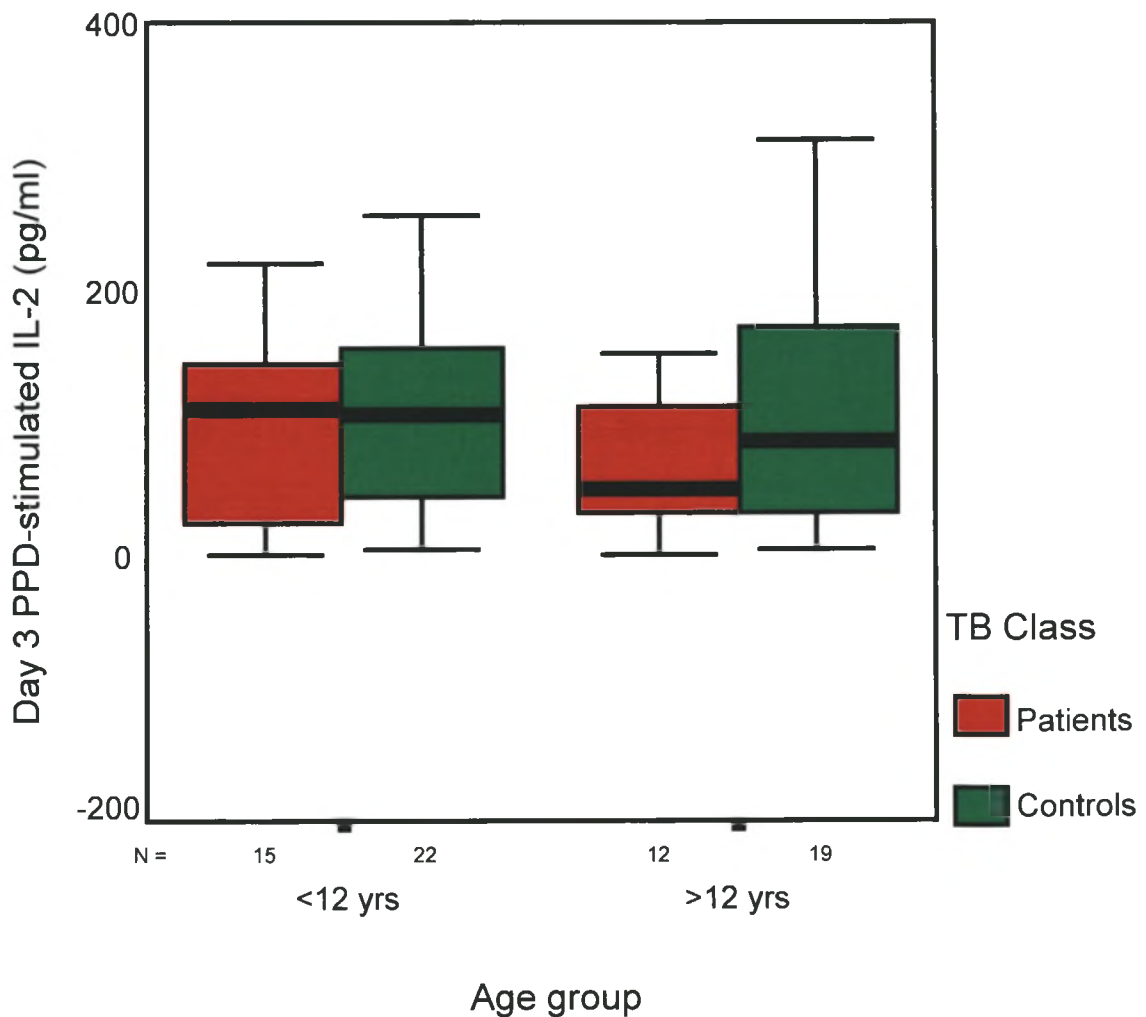


Table 4.9

Median (interquartile range: IQ) of IL-2 in pg/ml after stimulation of whole blood with PPD (3.3µg/ml) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p-value</i>¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	15	111 (21-152)	22	104 (45-162)	.915
>12 years	12	49 (30-120)	19	87 (30-176)	.287
<i>P-value</i>²		.399		.896	

*p-value*¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

*p-value*²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

Age (yrs) demonstrated a negative correlation with PPD-induced IL-2 (pg/ml), but this did not reach significance when the whole study group was analysed. This non-significant negative correlation was maintained for patients as well as controls.

PPD-induced IL-2 correlated positively with proliferation ($r = .561, p < .001$) and IFN- γ ($r = .505, p < .001$) after PPD stimulation, when all subjects were analysed ($n = 68$). This positive correlation was maintained when values for all controls ($n = 41$) were analysed (proliferation: $r = .737, p < .001$; IFN- γ : $r = .591, p < .001$) as well as when values for all patients ($n = 27$) were analysed (proliferation: $r = .509, p = .044$; IFN- γ : $r = .388, p = .046$).

4.3.3 TNF- α production

4.3.3.1 TNF- α production after PHA stimulation

Day 3 PHA-stimulated TNF- α production from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

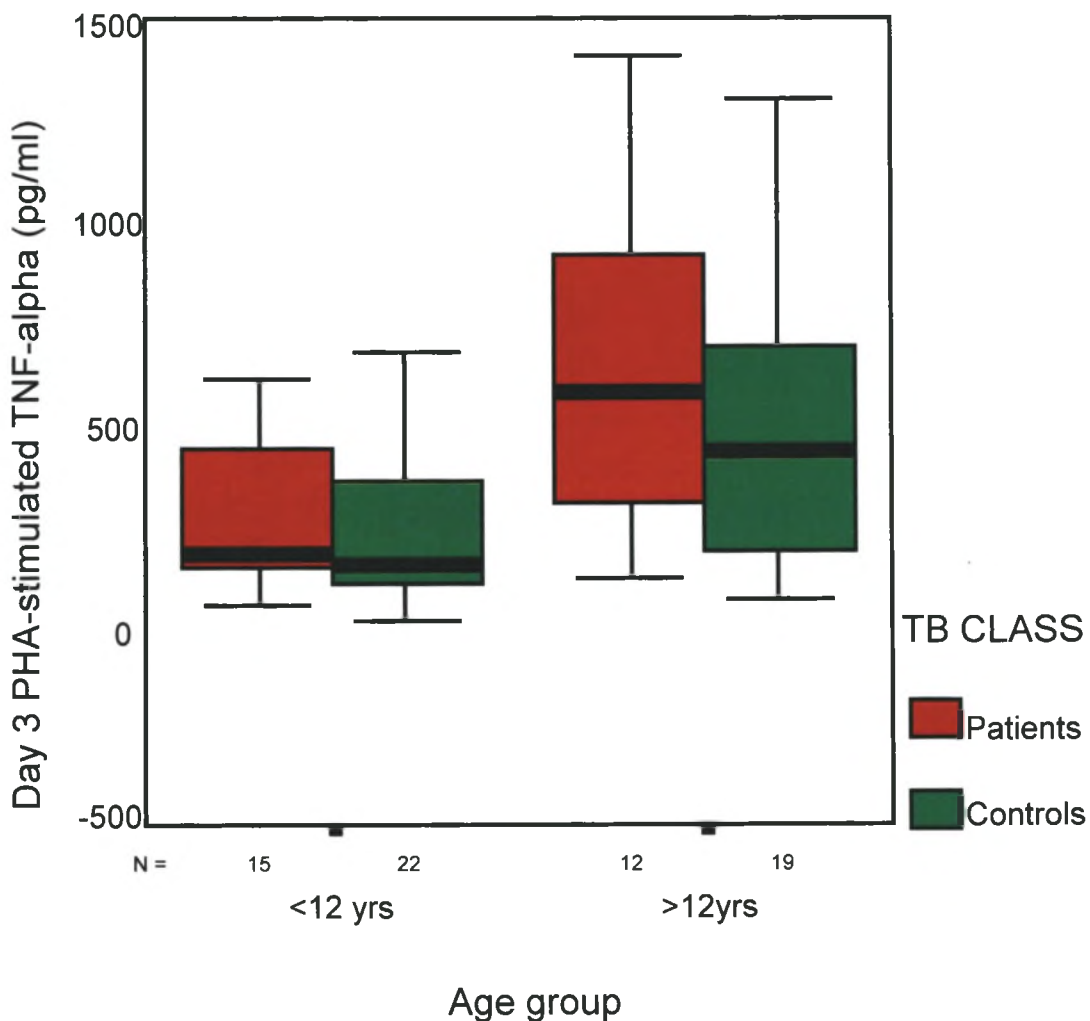


Table 4.10

Median (interquartile range: IQ) of TNF- α in pg/ml after stimulation of whole blood with PHA (10 μ g/ml) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p-value</i> ¹
Age group (years)	N	Median (IQ)	n	Median (IQ)	
<12 years	15	172 (141-433)	22	142 (94-353)	.319
>12 years	12	573 (286-961)	19	427 (167-684)	.236
<i>P-value</i> ²		.010		.032	

*p-value*¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

*p-value*²: Patients <12 years compared to patients > 12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

Age (yrs) correlated significantly with PHA-induced TNF- α (pg/ml) within the whole study group ($n = 68$; $r = .518$, $p < .001$) as well as for all patients ($n = 27$; $r = .571$, $p = .002$) and all controls ($n = 41$; $r = .483$, $p = .001$). Patients and controls (<12yrs) produced significantly less than their older (>12yrs) counterparts ($p = .010$ and $p = .032$ respectively).

PHA-induced TNF- α correlated positively with proliferation ($r = .295$, $p = .040$), IFN- γ ($r = .679$, $p < .001$) and IL-2 ($r = .599$, $p < .001$) when all samples were analysed ($n = 68$). When values for all patients were analysed ($n = 27$) the positive correlation was not maintained for proliferation, but was maintained for IFN- γ ($r = .681$, $p < .001$) and IL-2 ($r = .692$, $p < .001$). When values for all controls were analysed ($n = 41$) the positive correlation was also lost for proliferation, and was also maintained for IFN- γ ($r = .706$, $p < .001$) and IL-2 ($r = .547$, $p < .001$). PHA-induced TNF- α also demonstrated a positive and significant correlation with PHA-induced IL-10, but only in controls ($r = .369$, $p = .018$), and not patients.

4.3.3.2 TNF- α production after PPD stimulation

Day 7 PPD-stimulated TNF- α production from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

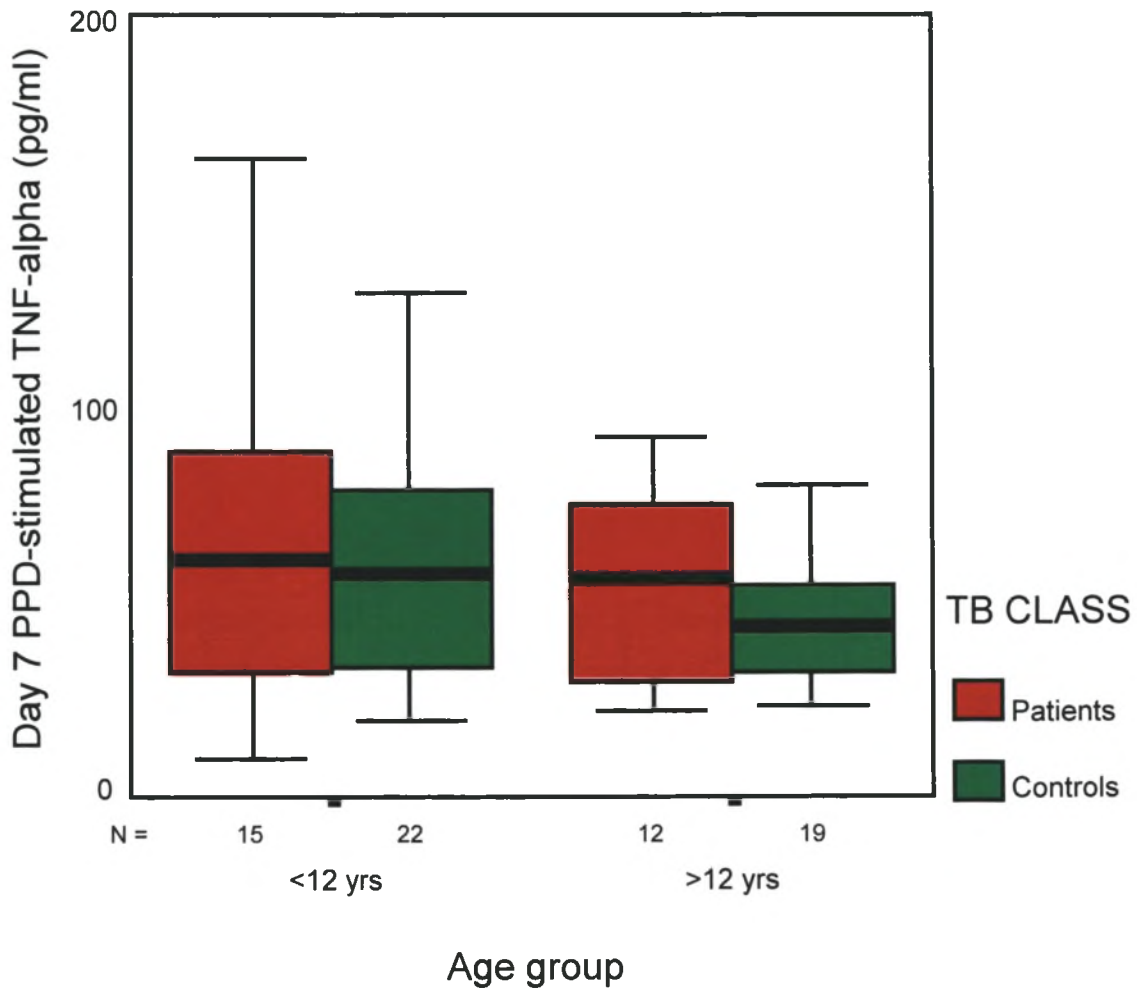


Table 4.11

Median (interquartile range: IQ) of TNF- α in pg/ml after stimulation of whole blood with PPD (3.3 μ g/ml) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		p-value¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	15	60 (31-90)	22	56 (33-80)	.939
>12 years	12	55 (27-74)	19	43 (29-55)	.306
p-value²		.755		.191	

p-value¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

p-value²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

Age did not correlate with PPD-induced TNF- α production in any way.

PPD-induced TNF- α showed a weak positive correlation with PPD-induced IFN- γ ($r = .288$, $p = .017$) when all samples were analysed ($n = 68$). When values for all patients were analysed ($n = 27$), the positive correlation was maintained ($r = .499$, $p = .008$) but was not maintained when values for all controls were analysed ($n = 41$).

4.3.4 IL-10 production

4.3.4.1 IL-10 production after PHA stimulation

Day 3 PHA-stimulated IL-10 production from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

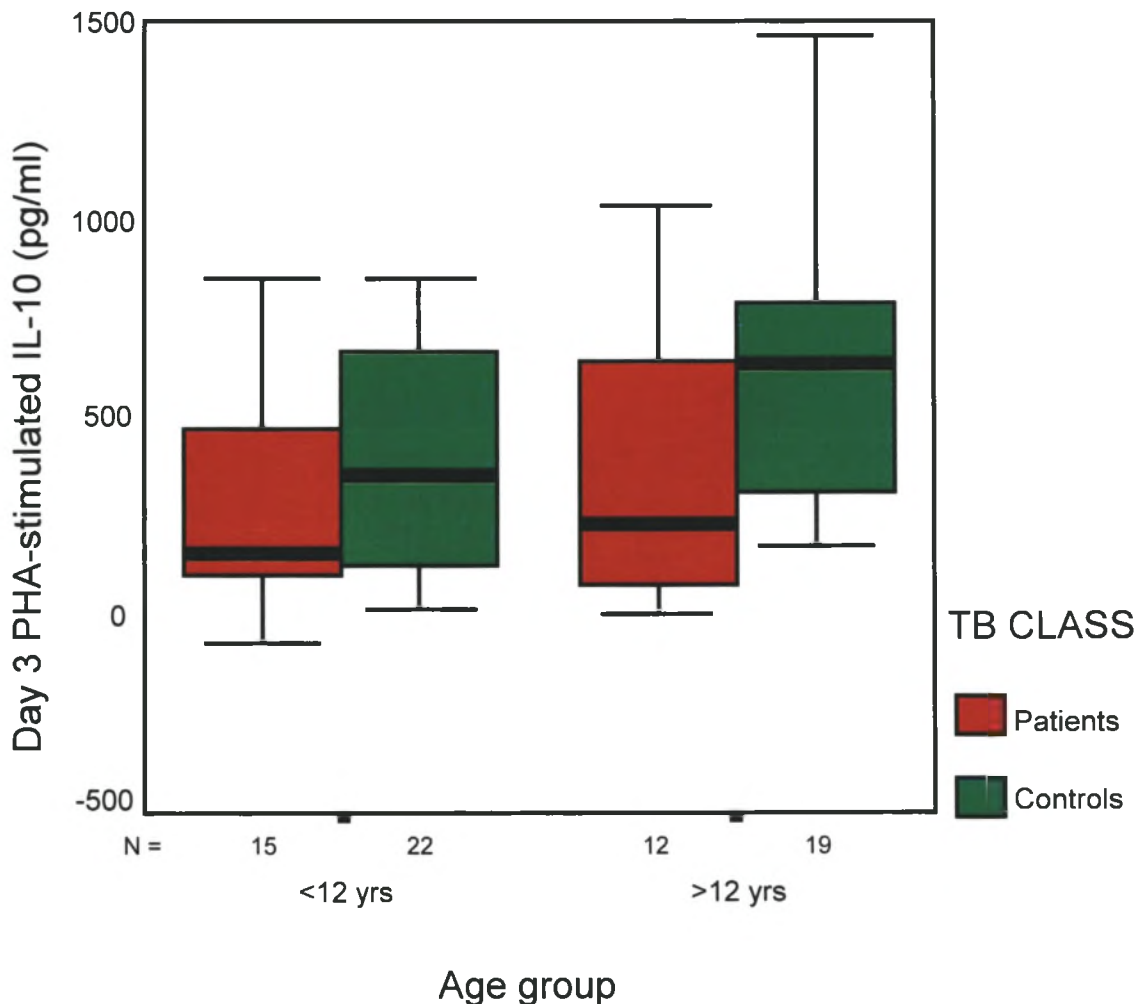


Table 4.12

Median (interquartile range: IQ) of IL-10 in pg/ml after stimulation of whole blood with PHA (10µg/ml) (after subtraction of values from unstimulated cultures) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p-value</i> ¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	15	159 (92-488)	22	352 (105-681)	.614
>12 years	12	227 (72-648)	19	631 (298-803)	.043
<i>P-value</i> ²		.905		.030	

*p-value*¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

*p-value*²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

Age (yrs) correlated significantly with PHA-induced IL-10 (pg/ml) within the whole study group (n = 68; $r = .354$, $p = .003$) as well as for all controls (n = 41; $r = .549$, $p < .001$) but not for all patients (n = 27). Controls <12 yrs old produced significantly less IL-10 than controls >12 yrs old ($p = .030$). In addition, patients >12yrs old produced significantly less IL-10 than controls >12yrs old ($p = .043$)

PHA-stimulated IL-10 correlated positively with PHA-stimulated IFN- γ ($r = .414$, $p < .001$) and IL-2 ($r = .308$, $p = .011$) when all samples were analysed (n = 68). When values for all controls were analysed (n = 41), correlations remained significant for IFN- γ ($r = .552$, $p < .001$) and IL-2 ($r = .451$, $p = .003$). When values for all patients were analysed (n = 27), these significant correlations were lost. PHA-induced IL-10 also correlated positively with TNF- α ($r = .369$, $p = .018$), but only when values for controls were analysed (n = 41).

4.3.4.2 IL-10 production after PPD stimulation

Day 7 PPD-stimulated IL-10 production from diluted whole blood from TB patients (with a history of TB) and healthy Mantoux positive controls according to age group. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

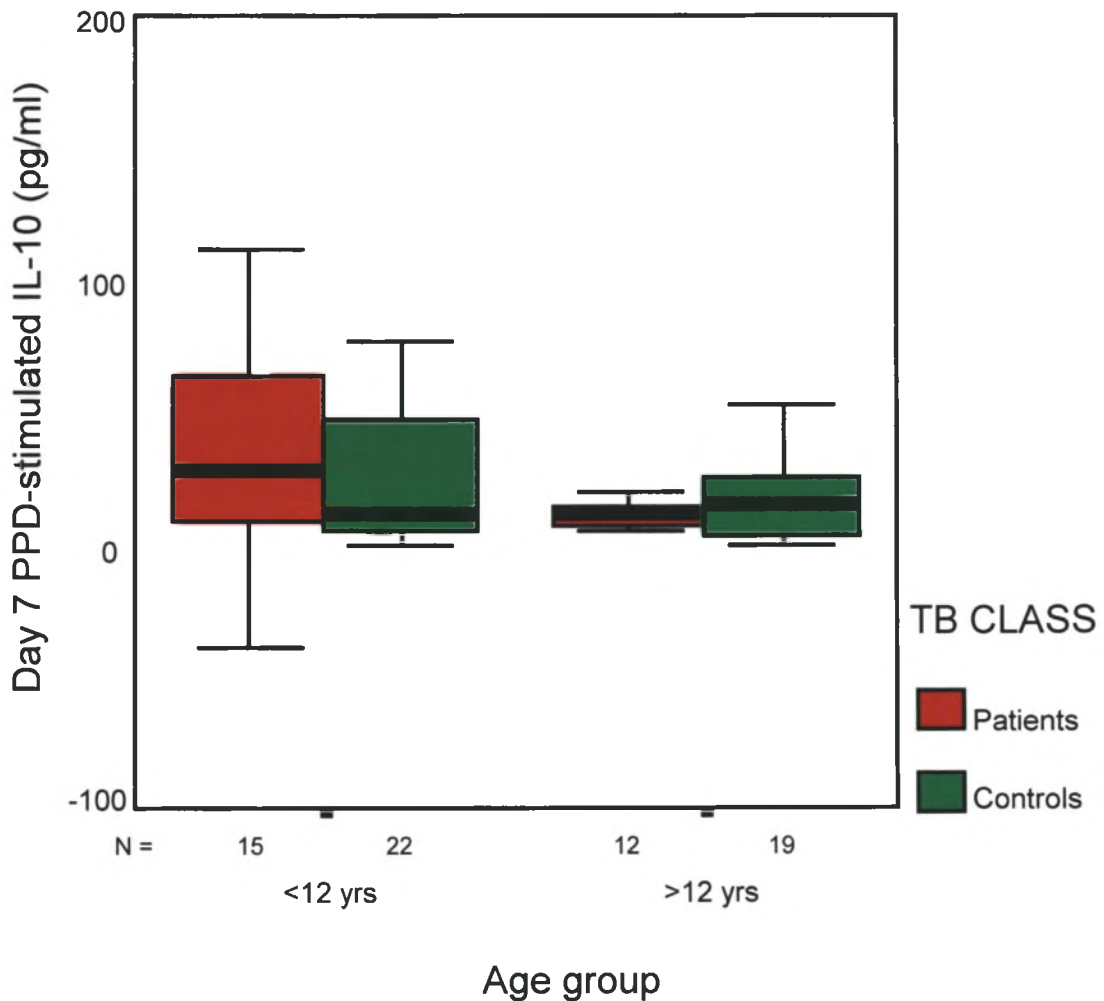


Table 4.13

Median (interquartile range: IQ) of IL-10 in pg/ml after stimulation of whole blood with PPD (3.3µg/ml) (after subtraction of values from unstimulated cultures) for patients with a history of TB and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs)

TB status	Patients		Controls		<i>p-value</i>¹
Age group (years)	n	Median (IQ)	n	Median (IQ)	
<12 years	15	28 (9-64)	22	11 (4-49)	.225
>12 years	12	10 (6-15)	19	15 (1-25)	.412
<i>P-value</i>²		.114		.565	

*p-value*¹: Patients <12 years compared to controls <12 years and patients > 12 years compared to controls >12 years (Mann-Whitney U test)

*p-value*²: Patients <12 years compared to patients >12 years and controls <12 years compared to controls >12 years (Mann-Whitney U test)

Age (yrs) showed a negative correlation with PPD-induced IL-10 (pg/ml) for all samples (n = 68). This correlation did not reach significance within the whole study group, but when patients and controls were analysed separately, the negative correlation demonstrated a weak negative significance for patients (n = 27; $r = -.415$, $p = .032$). Values were extremely low for all samples.

PPD-induced IL-10 did not correlate in any way with PPD-induced proliferation or any of the PPD-induced cytokines.

4.4 Discussion

4.4.1 Lymphocyte proliferation assays

This assay measures [^3H] thymidine uptake into the DNA of dividing cells and is a commonly used method for assessing cellular proliferation in response to a particular mitogen or a specific antigen. Mitogenic stimulation results in non-specific activation of multiple T cells and is generally used to assess maximal proliferative capacity not necessarily related to antigen exposure while specific antigenic stimulation will reflect prior exposure and sensitisation of the subject to that particular antigen.

Most studies in the literature have found that circulating neonatal T cells and adult T cells have similar proliferative responses to mitogens, bacterial superantigens or alloantigens (Clerici et al., 1993; Hayward et al., 1994). Our study was in agreement with this data, as age did not correlate in any way with PHA- or PPD-induced proliferative responses. Pollard et al., (1999) found no age-related differences in proliferative responses of PBMCs of children convalescing from meningococcal disease or controls less than 10 years of age (in response to meningococcal antigens), although there was a trend toward higher responses in children over 10 years of age. Similarly, Smart et al., (2001) found no age-related differences in staphylococcal enterotoxin B-stimulated PBMCs from normal healthy children as well as atopic children.

The positive correlation between PHA and PPD-induced proliferative responses and IL-2 supports the fact that this cytokine is a growth factor for T cells and is necessary for T cell proliferation.

Cured TB patients older than 12 years proliferated significantly more in response to PHA and PPD than their age-matched healthy controls. Enhanced proliferation in cured TB patients could possibly be due to an increased number of memory T cells which were still circulating in the peripheral blood. This would expand the total T cell pool, which could result in an increased proliferative response to PHA and PPD, compared to that of the age-matched controls. The PPD-induced proliferative responses demonstrated a significant positive correlation with IFN- γ (for controls only). The finding that there was no correlation between IFN- γ and PHA- induced proliferative responses demonstrates that stimulation with different

antigens can influence lymphocyte proliferation in relation to cytokine production. Ribera et al., (1990) report a significant positive correlation between PPD-induced proliferation results and IFN- γ production in healthy controls, but not TB patients. D'Andrea et al., (1986) also report a positive correlation between cytomegalovirus-stimulated proliferation values and IFN- γ production in healthy cytomegalovirus seropositive adults, while Surcel et al., (1994) have previously reported a discordant relationship between lymphocyte proliferation and IFN- γ , after stimulation with various *M.tuberculosis* proteins.

PHA-stimulated proliferative responses, but not PPD-stimulated proliferative responses, showed a weak positive correlation with TNF- α for all samples. This finding demonstrates that different stimulating agents can elicit quite distinct patterns of cytokine production.

The findings of this study indicate that the proliferation assay appears to correlate with IL-2 production, however it does not appear to correlate with age and therefore would probably not be useful in the assessment of age-related function.

4.4.2 Cytokine studies

4.4.2.1 IFN- γ production

As mentioned previously in the introductory chapters, IFN- γ is an important cytokine involved in host defence from *M.tuberculosis*. Young children, who are particularly susceptible to TB disease, have decreased production of IFN- γ (Holt et al., 1995; reviewed by Smith et al., 1997). The majority of reports in the literature on IFN- γ production in children have been performed on newborn and very young children. These reports have shown decreased PHA-induced IFN- γ production by cord blood lymphocytes compared to adults (Wakasugi et al., 1985; Frenkel et al., 1987; Vigano et al., 1999; Perenne-Ansart et al., 1995). Elssässer-Beile et al., (1995), using a whole blood assay, also report significantly lower PHA-induced IFN- γ production in children under 10 years of age, compared to adults. This study is in agreement, as it has shown a strong correlation with PHA-induced IFN- γ and age. Various factors are likely to be involved. Lack of exposure to antigen, as shown by a predominantly “naïve” T cell phenotype in neonatal peripheral blood (Hassan et al., 1996), is probably a crucial factor. Exaggerated cellular sensitivity to the suppressive effect of prostaglandin E,

produced endogenously in normal amounts, has also been shown to be involved (Wakasuki et al., 1985). In their study, Taylor and Bryson (1985), have demonstrated that functionally immature macrophages are primarily responsible for the impaired IFN- γ response by neonatal newborn cells.

The positive correlation with age was lost when PPD was used as a stimulant. Lilic et al., (1997) also did not find any significant difference in IFN- γ production in healthy children (mean age of 9 years) and adults (mean age of 32 years) after specific antigenic stimulation. It therefore seems likely that the nature of the antigen determines if there is a relationship between age and the amount of cytokine produced.

PPD-induced IFN- γ values for controls correlated significantly with proliferation. This has previously been reported (Ribera et al., 1990).

The close agreement with limited data from the literature suggests that the data obtained from these experiments is reasonably reliable. The findings in this study indicate that the production of PHA-induced, but not PPD-induced, IFN- γ was age-related and therefore it appears that the nature of the antigen determines if there is a relationship between age and the amount of cytokine produced.

4.4.2.2 IL-2 production

IL-2 acts as an autocrine and paracrine growth factor of T cells, helping to expand a pool of antigen-specific effector cells. It also increases the capacity of effector T cells to produce additional cytokines when they are reactivated by antigen (Smith, 1993). The production of IL-2 occurs early after T cell activation and this was confirmed by our kinetics experiments, which demonstrated maximal production on day 3 for both PHA- and PPD-stimulated cultures.

IL-2 production from neonates was found to be greater than or similar to that of adults (Clerici et al., 1993; Wilson et al., 1986; Miyawaki et al., 1985; Ehlers et al., 1991) or significantly decreased compared to adults (Splawski et al., 1991). Lilic et al., (1997) demonstrated significantly decreased production of mitogenic- and antigenic-induced IL-2 in PBMC of children (n = 10; mean age of 9 years) compared to that of adults (n = 10; mean age

of 32 years). Elsässer-Beile et al., (1995), using a whole blood system, also found decreased amounts of PHA-induced IL-2 in children, aged between 1 and 9 years old (n= 33) compared to adults, aged between 20 and 59 years old (n = 67). Our study showed no relationship between age and PHA- or PPD-stimulated IL-2 production.

PHA-and PPD-induced IL-2 both correlated positively with proliferation and IFN- γ . Lilic et al., (1997) also demonstrated a significant positive correlation between IFN- γ and IL-2 in healthy children, but not adults. This relationship is plausible as both of these cytokines are type 1 cytokines. This correlation is consistent with the fact that that IL-2 is required for proliferation and generation of cytokine-producing effectors and both cytokines are considered to be Th1 cytokines.

The findings of this study indicate that the production of IL-2 does not appear to be related to age.

4.4.2.3 TNF- α production

TNF- α is important in the formation of granulomas during *M.tuberculosis* disease, but it also has some detrimental effects on the host, such as cachexia and fever when produced in excess.

In children, TNF- α production is described as modestly reduced compared to adults (English et al., 1988; Seghaye et al., 1998). Elsässer-Beile et al., (1995), using a whole blood system, also demonstrated decreased PHA-induced TNF- α production in children less than 10 years of age, compared to adults. Our study was in concordance, demonstrating a positive correlation between age and PHA-induced TNF- α production. One of the few studies that used a specific antigen to induce TNF- α production in healthy children demonstrated that TNF- α production, after meningococcal antigen stimulation, was strongly age-dependent in control children but not in children after meningococcal infection (Pollard et al., 1999).

PHA- and PPD- induced TNF- α correlated positively with IFN- γ . This positive correlation could be due to the finding that the production of both of these cytokines was shown to be age-dependent after PHA stimulation. PHA-induced, but not PPD- induced TNF- α correlated

positively with IL-2. This finding once again demonstrates that different stimulating agents can elicit quite distinct patterns of cytokine production.

Generally, levels of TNF- α produced by PHA and PPD stimulation were very low in this whole blood system. Elsässer-Beile et al., (1995), also using a whole blood system, report median PHA-induced TNF- α values to be 340ng/ml and 920ng/ml in children and adults respectively. These values are markedly higher than the values found in this study. This difference can probably be ascribed to the fact that the final concentration of PHA in their study was 10mg/ml as opposed to 10 μ g/ml, in this study.

The findings in this study indicate that the production of TNF- α appears to be age-related (after PHA stimulation) and therefore, may be useful in the assessment of immune response function at various ages.

4.4.2.4 IL-10 production

IL-10 production in the human response to *M.tuberculosis* is considered to be anti-inflammatory as it appears to have a down-regulatory effect on IFN- γ and TNF- α production. This was demonstrated when recombinant human IL-10 was added to PPD-stimulated monocyte cell cultures from PPD skin test positive subjects (Othieno et al., 1999).

Decreased IL-10 production has been reported in cord blood mononuclear cells compared to PBMCs from adults (Chheda et al., 1996). Pollard et al., (1999) reported higher values in older children compared to younger children, but this difference was not significant. The production of IL-10 from chord whole blood cultures was found to be similar to that of adults (Seghayé et al., 1999). Other studies on IL-10 production in children with various diseases have been reported, but no conclusions on age-relationship have been mentioned. Our study found that PHA-stimulated IL-10 correlated positively with age.

PPD-induced IL-10 concentration was generally extremely low. This phenomenon was also found when immune responses of these subjects were examined in the “before therapy” study, approximately one year earlier. One report of PPD-induced IL-10 in whole blood in the literature could be located (Van Crevel et al., 1999). The authors found measurable amounts of PHA-induced, but not PPD-induced IL-10, in PPD skin-test positive as well as

PPD skin-test negative controls. The phenomenon of finding measurable PHA-induced, but not PPD-induced IL-10 in whole blood is intriguing and would be interesting to study in greater depth. Plasma components such as cortisol (Petrovsky et al., 1994), lipids (Ulevitch and Johnston, 1978) or soluble cytokine receptors (Dinarello, 1996) may all influence results.

PHA-induced IL-10 correlated positively with proliferation, IFN- γ and IL-2. The relationship between IFN- γ , IL-2 and IL-10 is plausible as IFN- γ and IL-2 are considered to be a Th1 cytokines, while both Th1 and Th2 phenotypes can secrete IL-10 in humans (Zhai et al 1999). This correlation perhaps reflects an age-related regulation between these cytokines. The production of IL-10 does not seem to have a down-regulatory effect on the production of IFN- γ in these conditions. A positive correlation was also found between PHA-induced TNF- α and PHA-induced IL-10 in control subjects. This correlation has previously been documented by Pollard et al., 1999, who suggest that release of IL-10 is involved in the regulation of TNF- α .

The findings of this study indicate that the production of PHA-induced IL-10 appears to be age-related.

4.5 Summary

In this section of the study, subjects with a past history of TB (successfully treated TB patients) and healthy Mantoux positive controls, of different ages, were investigated for their proliferative and cytokine responses to a mitogen (PHA) and a specific mycobacterial antigen (PPD).

Age was a significant variable for the following PHA-stimulated cytokines: IFN- γ , TNF- α and IL-10. Proliferation and IL-2 production after PHA stimulation did not demonstrate any relationship with age.

None of the PPD-induced proliferative or cytokine responses demonstrated any correlation with age.

CHAPTER 5

IMMUNE RESPONSES OF TB PATIENTS BEFORE TREATMENT (AT DIAGNOSIS) COMPARED TO IMMUNE RESPONSES AFTER TREATMENT

5.1 Study population

The study population comprised TB patients (n = 27) and apparently healthy Mantoux positive control subjects (n = 41). The TB patients had immune responses studied before commencement of TB therapy, at diagnosis, (referred to as the “before therapy” study from here on) and again after therapy, approximately 6 months after completion of treatment (referred to as the “after therapy” study from here on). None of the healthy controls received any treatment during this period and they had their immune responses tested at the same time intervals as the TB patients. For ease of reference, the data for healthy controls will therefore also be presented as “before therapy” and “after therapy” results, even though they did not receive any treatment. This chapter thus reports on the comparison of the results for patients and controls at the two time points. Table 5.1 shows the median (interquartile ranges) ages in years for patients and controls in the “before and after therapy” study.

Note: The “before therapy” study formed part of another study and was performed by another researcher.

Table 5.1

Median (interquartile ranges) ages in years for patients and controls “before therapy” and “after therapy”

	“Before therapy”		“After therapy”	
	n	Median (IQ)	n	Median (IQ)
pts <12 years	15	3.1 (1.6-7.9)	15	4.1 (2.6-9.5)
con <12 years	22	5.2 (3.0-8.4)	22	6.2 (4.0-9.4)
pts >12 years	12	18.2 (14.5-27.4)	12	19.2 (15.5-28.4)
con >12 years	19	16.1 (12.8-36.0)	19	17.1 (13.8-37.0)
Total	68	10.5 (4.3-16.6)	68	11.5 (5.3-17.6)

5.2 Lymphocyte proliferation studies

5.2.1 Proliferation after PHA stimulation

Day 4 PHA-stimulated lymphocyte proliferative responses from diluted whole blood from TB patients and healthy Mantoux positive controls according to age group, “before therapy” and “after therapy”. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

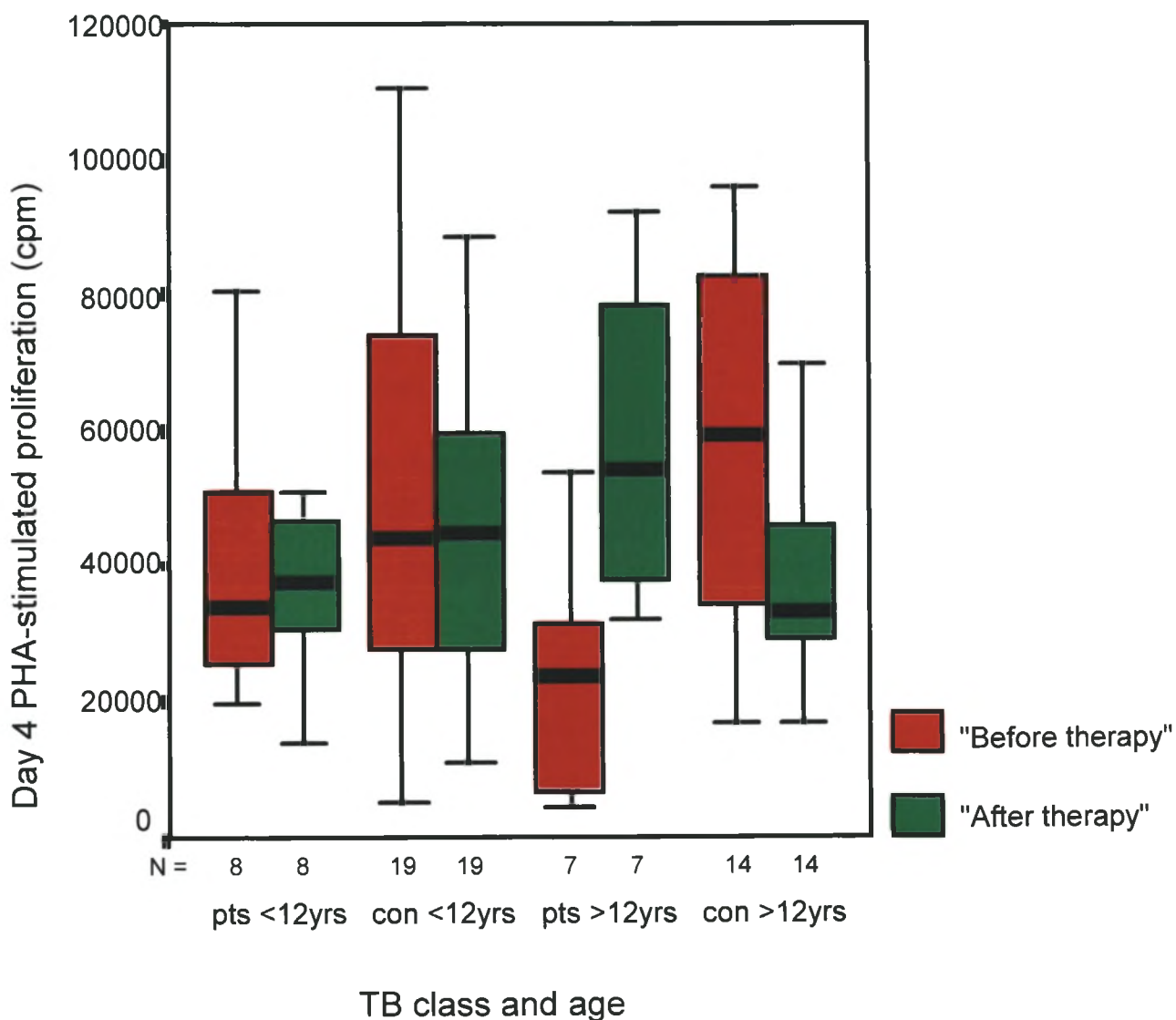


Table 5.2

Median (interquartile range: IQ) proliferative responses (cpm) of whole blood stimulated with PHA (10µg/ml) (after subtraction of values from unstimulated cultures) for TB patients and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs), “before therapy” (at TB diagnosis) and “after therapy” (approximately 1 year later)

	“Before therapy”			“After therapy”			<i>p</i> -value ²
	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	
pts <12 years	8	33535 (23191-54128)	.449	8	37783 (30495-47897)	.418	.674
con <12 years	19	43785 (23591-80297)		19	44612 (26607-60978)		.629
pts >12 years	7	23917 (5261-33325)	.010	7	54152 (34780-86918)	.038	.063
con >12 years	14	59157 (31718-85799)		14	32869 (26816-49879)		.074
Total	48	38154 (23672-75719)		48	40747 (29423-57592)		.580

p-value¹: Values of patients <12 yrs compared to values of controls <12 yrs and values of patients >12 yrs compared to values of controls >12 yrs (Mann-Whitney U test)

p-value²: Values of each group, “before therapy”, compared to values of each group, “after therapy” (Wilcoxin Ranks test)

PHA-induced proliferative responses of patients >12yrs, which were significantly lower than controls >12 yrs “before therapy”, showed a dramatic increase “after therapy”, but the difference fell just short of achieving statistical significance. Controls >12yrs had higher proliferation values “before therapy”, compared to “after therapy”, but the difference did not achieve statistical significance

5.2.2 Proliferation after PPD stimulation

Day 7 PPD-stimulated lymphocyte proliferative responses from diluted whole blood from TB patients and healthy Mantoux positive controls according to age group, “before therapy” and “after therapy”. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

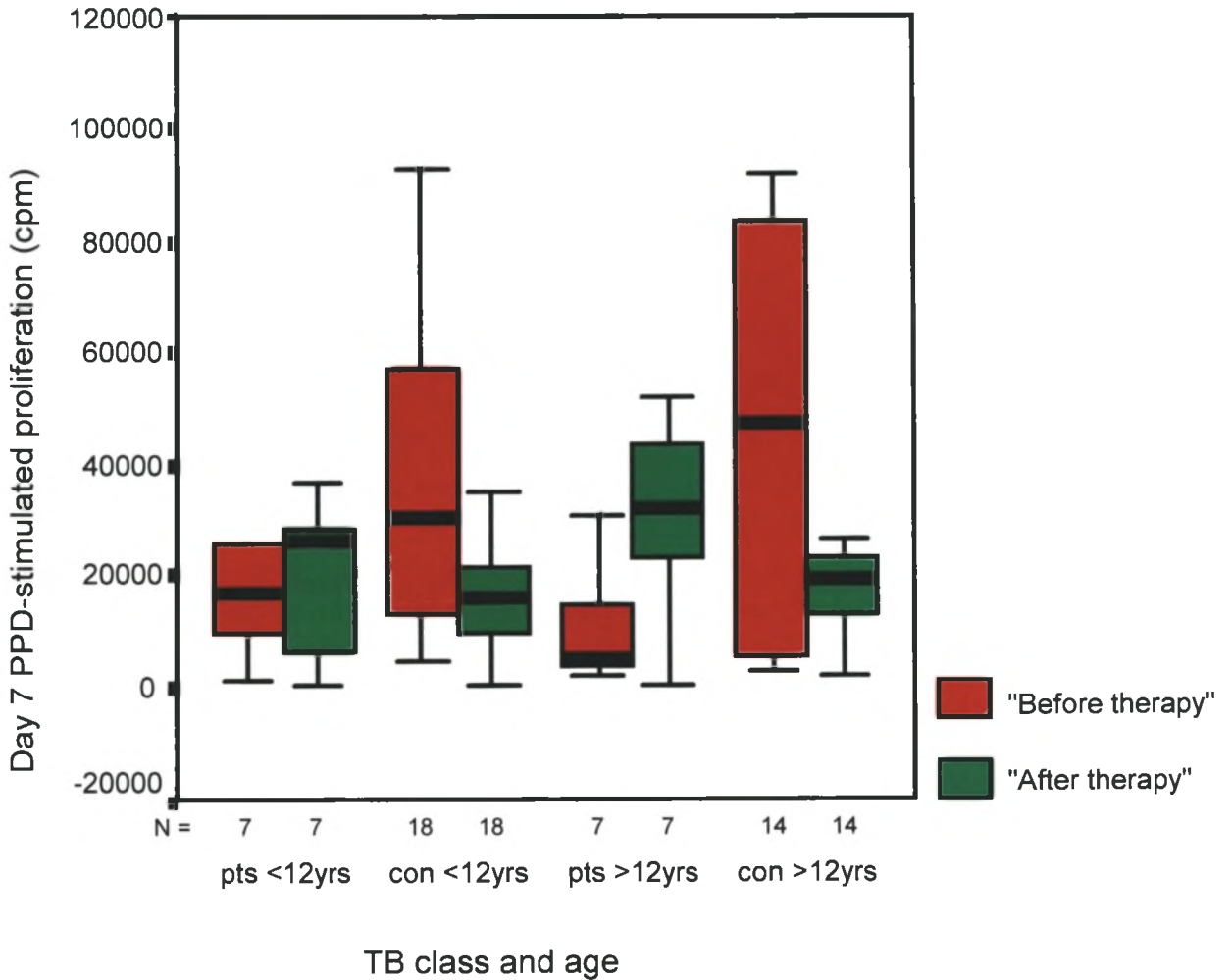


Table 5.3

Median (interquartile range: IQ) proliferative responses (cpm) of whole blood stimulated with PPD (3.3µg/ml) (after subtraction of values from unstimulated cultures) for TB patients and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs) “before therapy” (at TB diagnosis), and “after therapy” (approximately 1 year later)

	“Before therapy”			“After therapy”			<i>p</i> -value ²
	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	
pts <12 years	7	16587 (5070-26042)	.297	7	25833 (3962-28737)	.615	1.000
con <12 years	18	30640 (12562-58365)		18	15981 (9400-22492)		
pts >12 years	7	5370 (3035-16731)	.046	7	31788 (18114-51862)	.046	.028
con >12 years	14	47293 (5382-84145)		14	19351 (10893-23372)		
Total	46	19229 (7709-56460)		46	19155 (9573-26834)		.042

p-value¹: Values of patients <12 yrs compared to values of controls <12 yrs and values of patients >12 yrs compared to values of controls >12 yrs (Mann-Whitney U test)

p-value²: Values of each group, “before therapy”, compared to values of each group, “after therapy” (Wilcoxin Ranks test)

PPD-induced proliferative responses for TB patients > 12yrs were again significantly lower than controls “before therapy” ($p = .046$), and also demonstrated an increase “after therapy”, but this time the difference was statistically significant ($p = .028$). Values for controls <12 yrs as well as values for controls >12yrs were lower “after therapy” compared to “before therapy”, and this time the differences also achieved statistical significance ($p = .025$ and $p = .013$ respectively).

When the values of all controls ($n = 32$) were analysed, PPD-induced proliferation was significantly lower “after therapy”, compared to “before therapy” ($p = .001$). (data not shown on table).

5.3 Cytokine responses

5.3.1 IFN- γ production

5.3.1.1 IFN- γ production after PHA stimulation

Day 4 PHA-stimulated IFN- γ production from diluted whole blood from TB patients and healthy Mantoux positive controls according to age group, “before therapy” and “after therapy”. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

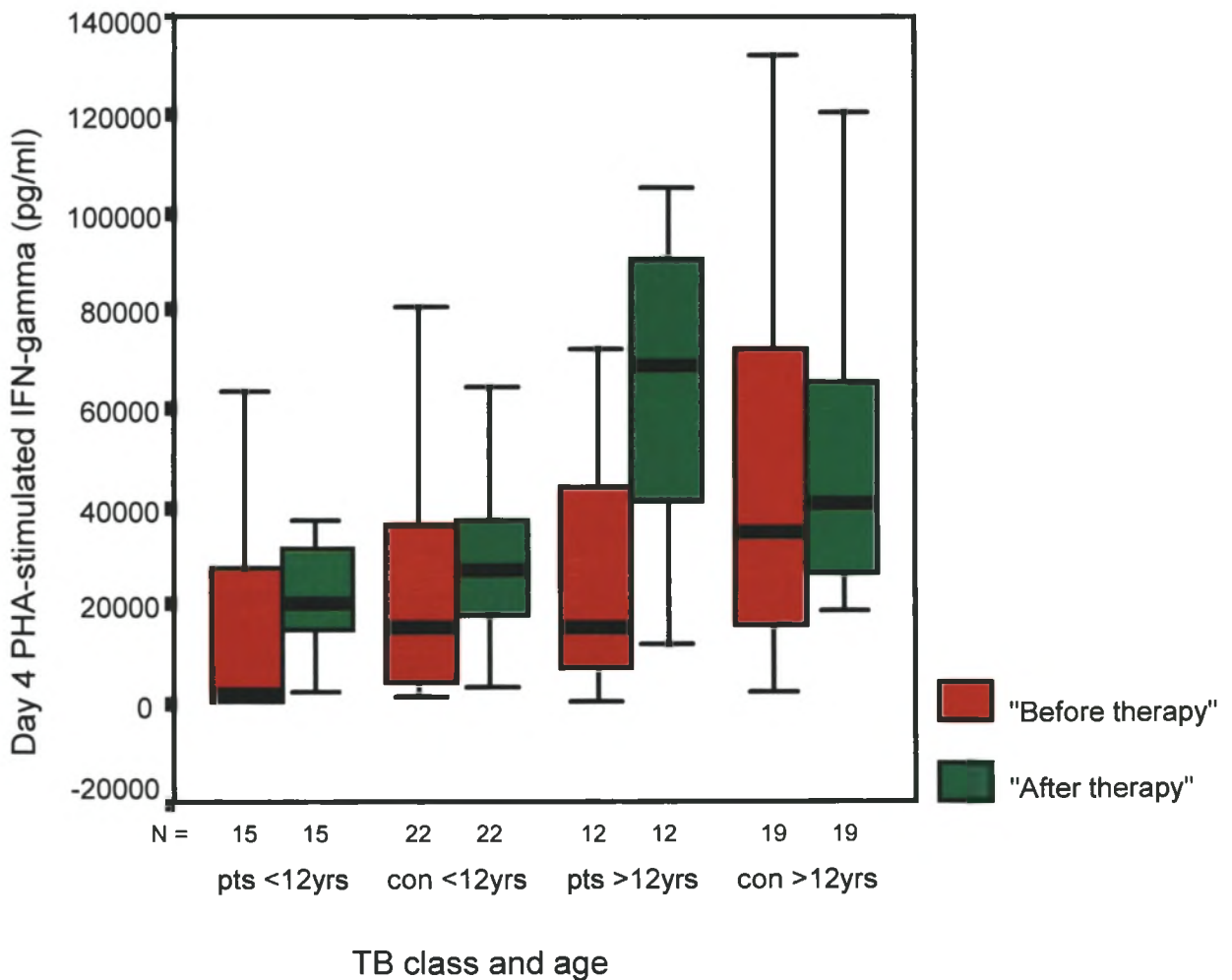


Table 5.4

Median (interquartile range: IQ) of IFN- γ in pg/ml after stimulation of whole blood with PHA (10 μ g/ml) for TB patients and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs) “before therapy” (at TB diagnosis), and “after therapy” (approximately 1 year later)

	“Before therapy”			“After therapy”			<i>p</i> -value ²
	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	
pts <12 years	15	2016 (560-40188)	.182	15	19937 (13760-32102)	.417	.173
con <12 years	22	15176 (4572-36115)		22	27114 (16488-37942)		.200
pts >12 years	12	15179 (5624-50375)	.027	12	68351 (36537-91963)	.205	.060
con >12 years	19	35045 (15450-74518)		19	40441 (25939-75349)		.687
Total	68	15502 (4449-56077)		68	31646 (20176-55921)		.018

p-value¹: Values of patients <12 yrs compared to values of controls <12 yrs and values of patients >12 yrs compared to values of controls >12 yrs (Mann-Whitney U test)

p-value²: Values of each group, “before therapy”, compared to values of each group, “after therapy” (Wilcoxin Ranks test)

Patients >12 yrs, who produced significantly less IFN- γ in response to PHA stimulation compared to controls >12 yrs, “before therapy” ($p = .027$), showed a dramatic increase “after therapy”, but the difference fell just short of achieving statistical significance.

When the values of all patients ($n = 27$) were analysed, PHA-induced IFN- γ production was significantly higher “after therapy”, compared to “before therapy” ($p = .025$). (data not shown on table).

PHA-induced IFN- γ values for all samples “after therapy” were also significantly higher than values for all samples “before therapy” ($n = 68$; $p = .018$).

5.3.1.2 IFN- γ production after PPD stimulation

Day 7 PPD-stimulated IFN- γ production from diluted whole blood from TB patients and healthy Mantoux positive controls according to age group, “before therapy” and “after therapy”. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

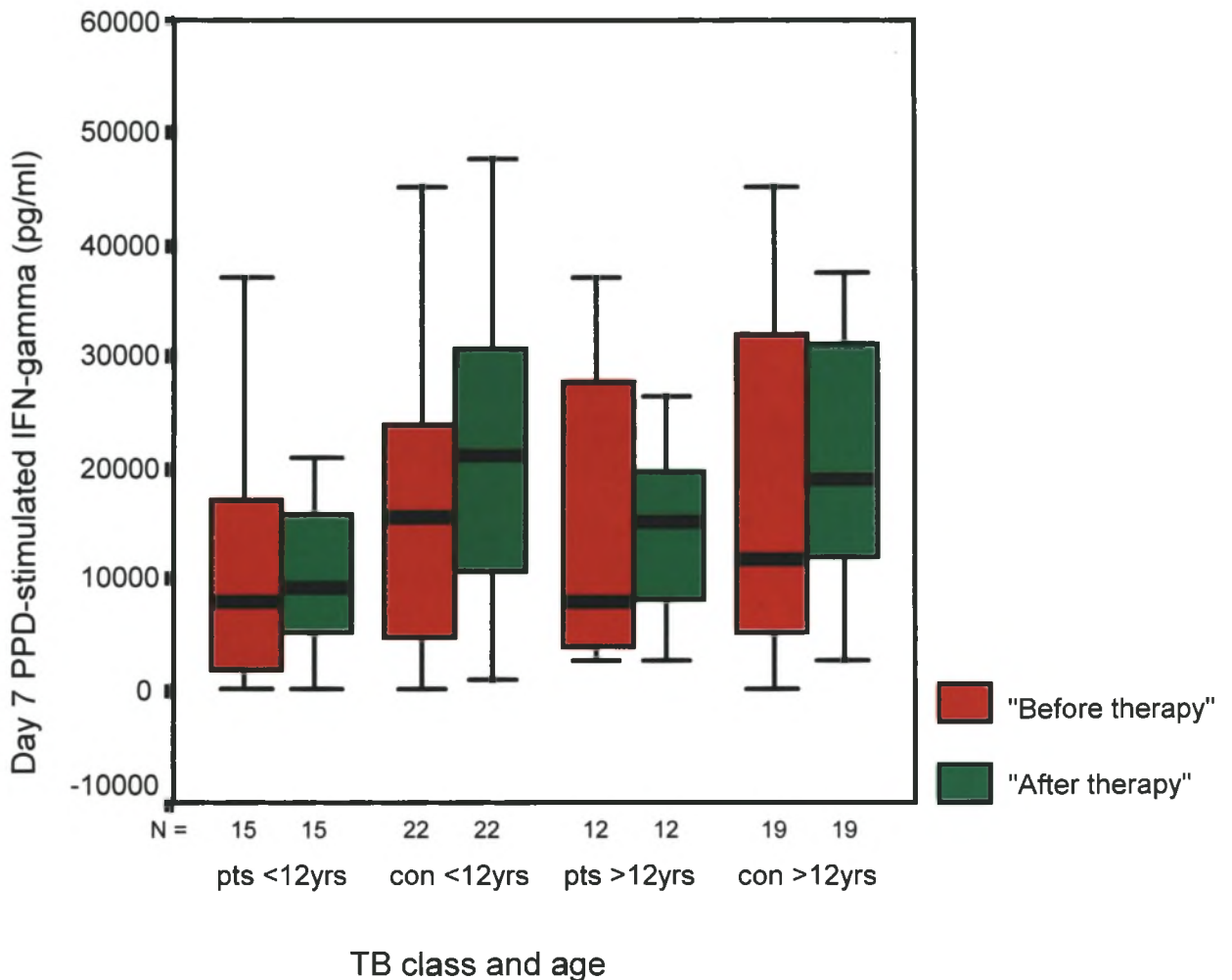


Table 5.5

Median (interquartile range: IQ) of IFN- γ in pg/ml after stimulation of whole blood with PPD (3.3 μ g/ml) for TB patients and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs) “before therapy” (at TB diagnosis), and “after therapy” (approximately 1 year later)

	“Before therapy”			“After therapy”			<i>p</i> -value ²
	n	Median (IQ)	<i>p</i> -value ¹	n	Median (IQ)	<i>p</i> -value ¹	
pts <12 years	15	8226 (1802-18465)	.258	15	9396 (3792-16779)	.024	1.000
con <12 years	22	15675 (4477-24291)		22	21239 (9902-30962)		.223
pts >12 years	12	8139 (3713-29360)	.845	12	15364 (7566-20249)	.141	.814
con >12 years	19	11968 (4758-33573)		19	19078 (11920-31038)		.445
Total	68	9623 (3935-28460)		68	16741 (8382-24947)		.279

p-value¹: Values of patients <12 yrs compared to values of controls <12 yrs and values of patients >12 yrs compared to values of controls >12 yrs (Mann-Whitney U test)

p-value²: Values of each group, “before therapy”, compared to values of each group, “after therapy” (Wilcoxin Ranks test)

Although patients <12yrs produced significantly less PPD-induced IFN- γ compared to controls < 12yrs “after therapy” ($p = .024$), no significant differences were found between any of the groups “before therapy” and “after therapy”.

5.3.2 IL-2 Production

5.3.2.1 IL-2 production after PHA stimulation

Day 3 PHA-stimulated IL-2 production from diluted whole blood from TB patients and healthy Mantoux positive controls according to age group, “before therapy” and “after therapy”. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

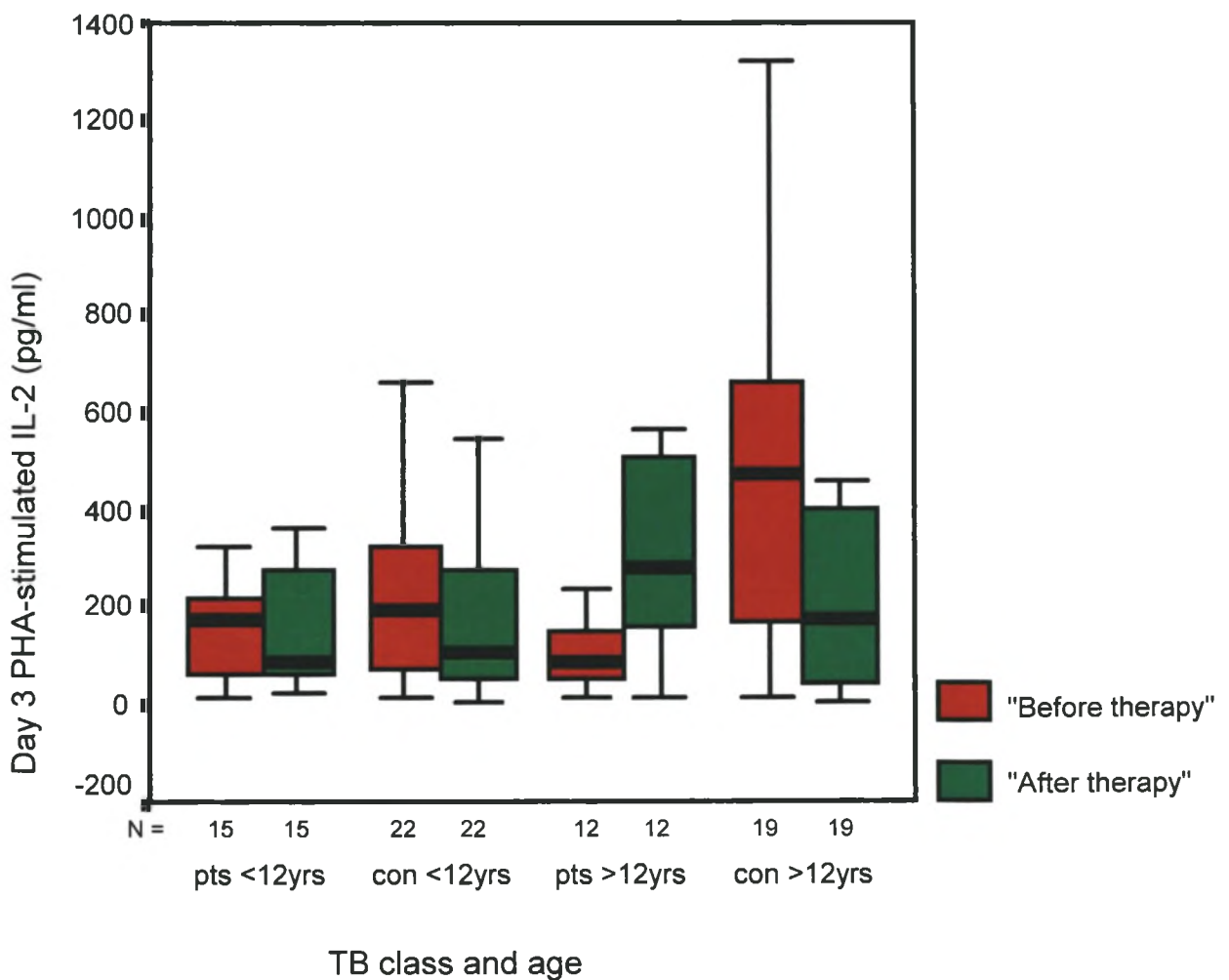


Table 5.6

Median (interquartile range: IQ) of IL-2 in pg/ml after stimulation of whole blood with PHA (10µg/ml) for TB patients and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs) “before therapy” (at TB diagnosis), and “after therapy” (approximately 1 year later)

	“Before therapy”			“After therapy”			<i>p</i> -value ²
	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	
pts <12 years	15	172 (44-220)	.246	15	86 (61-305)	.939	.691
con <12 years	22	194 (67-340)		22	106 (49-282)		
pts >12 years	12	88 (48-153)	.004	12	276 (157-513)	.287	.010
con >12 years	19	474 (136-660)		19	174 (40-436)		
Total	68	184 (68-325)		68	158 (61-322)		.869

p-value¹: Values of patients <12 yrs compared to values of controls <12 yrs and values of patients >12 yrs compared to values of controls >12 yrs (Mann-Whitney U test)

p-value²: Values of each group, “before therapy”, compared to values of each group, “after therapy” (Wilcoxin Ranks test)

Patients >12 yrs, who produced significantly less PHA-stimulated IL-2 compared to controls >12 yrs, “before therapy” ($p = .004$), subsequently produced significantly more IL-2 “after therapy” compared to before therapy ($p = .010$).

When the values of all patients ($n = 27$) were analysed, PHA-induced IL-2 production was significantly higher “after therapy”, compared to “before therapy” ($p = .029$). (data not shown on table).

No other significant differences between values “before therapy” and “after therapy” were found between any of the groups.

5.3.2.2 IL-2 production after PPD stimulation

Day 3 PPD-stimulated IL-2 production from diluted whole blood from TB patients and healthy Mantoux positive controls according to age group, “before therapy” and “after therapy”. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

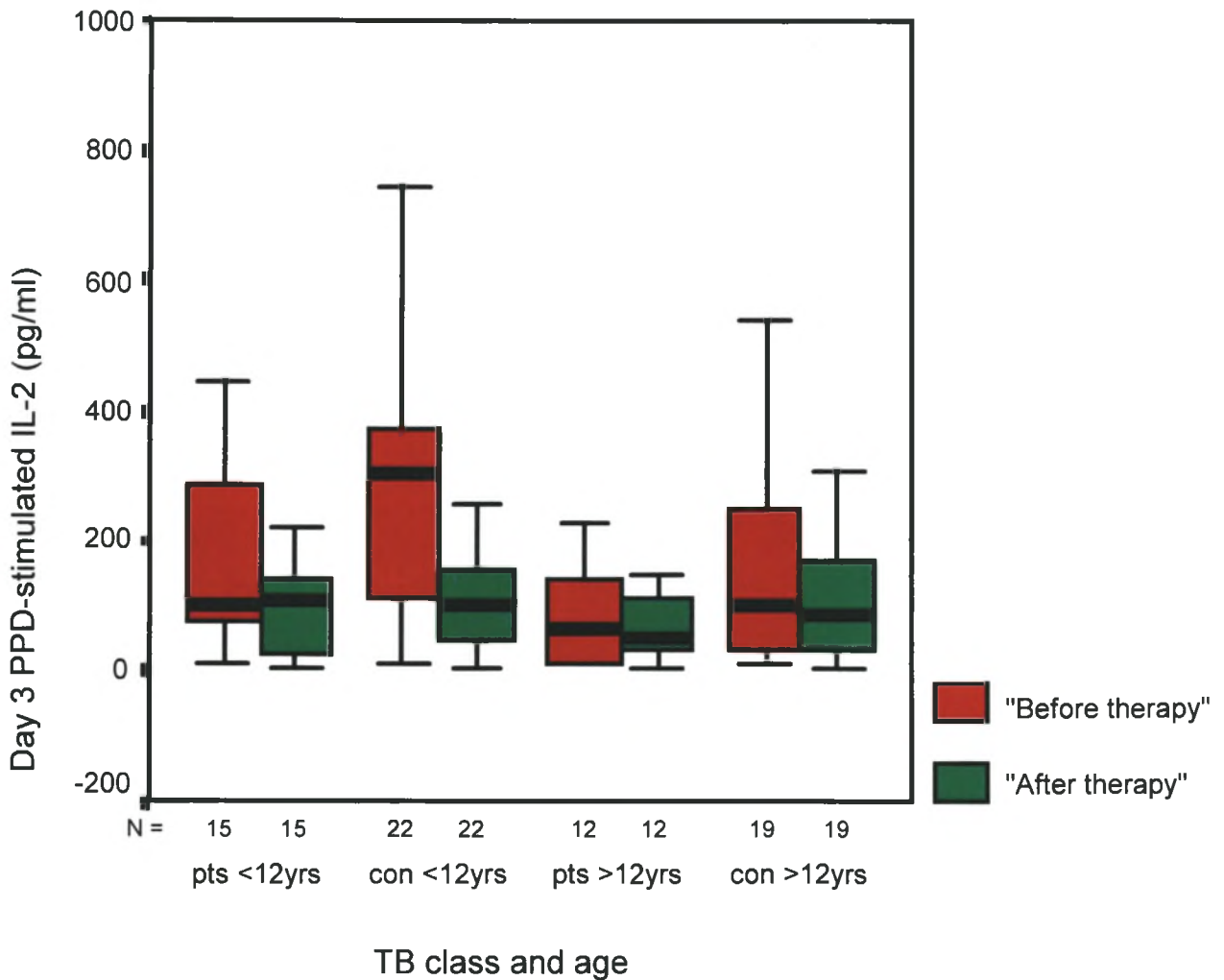


Table 5.7

Median (interquartile range: IQ) of IL-2 in pg/ml after stimulation of whole blood with PPD (3.3µg/ml) for TB patients and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs) “before therapy” (at TB diagnosis), and “after therapy” (approximately 1 year later)

	“ Before therapy”			“After therapy”			<i>p</i> -value ²
	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	<i>n</i>	Median (IQ)	<i>p</i> -value ¹	
pts <12 years	15	102 (71-371)	.191	15	111 (21-152)	.915	.031
con <12 years	22	305 (106-402)		22	104 (45-162)		
pts >12 years	12	65 (10-151)	.527	12	49 (30-120)	.287	.638
con >12 years	19	98 (26-267)		19	87 (30-176)		
Total	68	126 (47-327)		68	90 (36-152)		<.001

p-value¹: Values of patients <12 yrs compared to values of controls <12 yrs and values of patients >12 yrs compared to values of controls >12 yrs (Mann-Whitney U test)

p-value²: Values of each group, “before therapy”, compared to values of each group, “after therapy” (Wilcoxin Ranks test)

Generally, all PPD-induced IL-2 values were lower “after therapy” compared to “before therapy”, but only patients and controls < 12yrs produced significantly less IL-2 “after therapy” ($p = .031$ and $p < .001$ respectively) compared to “before therapy”.

When the values of all controls ($n = 41$) were analysed, PPD-induced IL-2 production was significantly lower “after therapy”, compared to “before therapy” ($p < .001$). (data not shown on table).

In addition, when values of all samples, were compared “before therapy” and “after therapy”, values “after therapy” were significantly less than values “before therapy” ($n = 68$; $p < .001$).

5.3.3 TNF- α production

5.3.3.1 TNF- α production after PHA stimulation

Day 3 PHA-stimulated TNF- α production from diluted whole blood from TB patients and healthy Mantoux positive controls according to age group, “before therapy” and “after therapy”. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

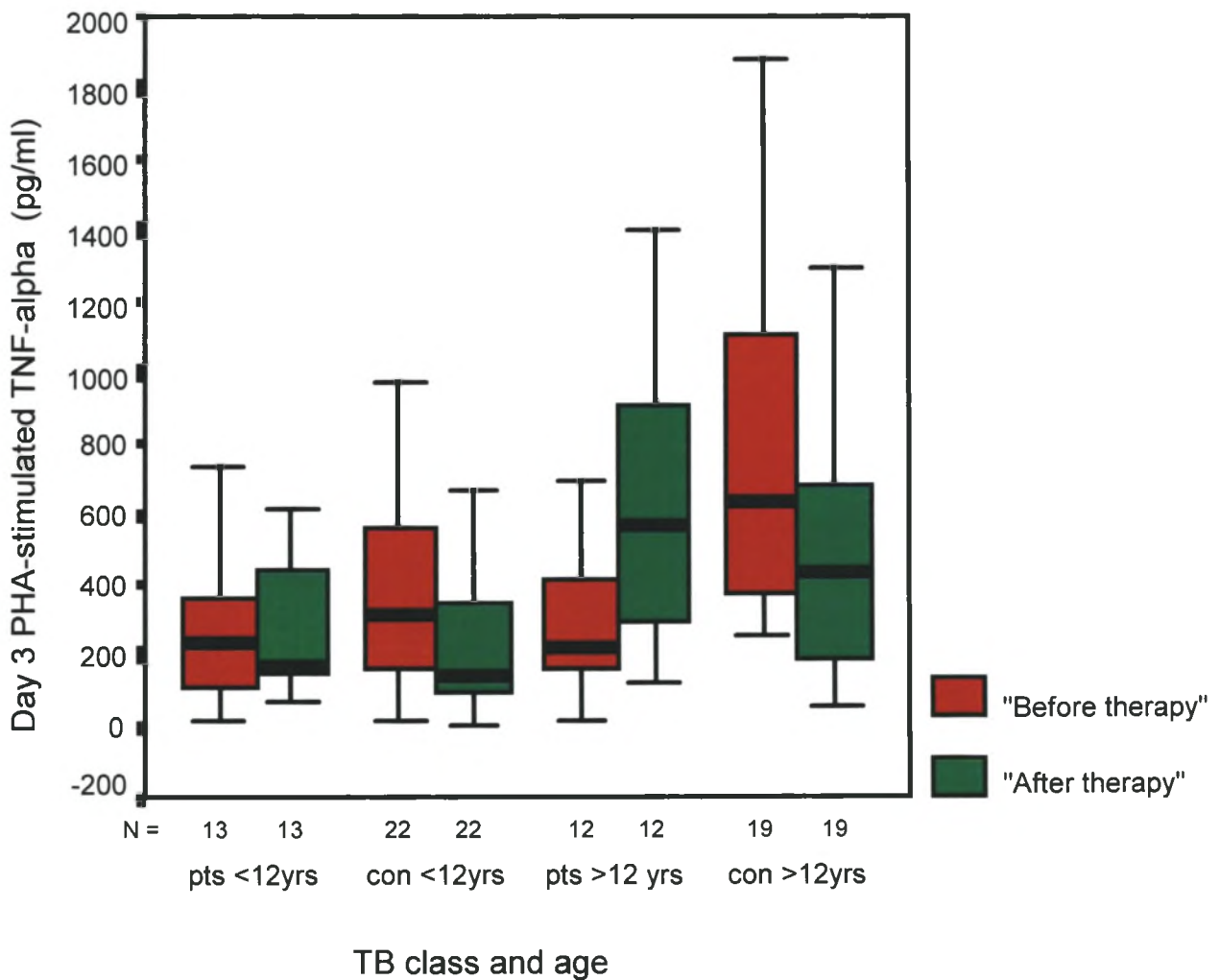


Table 5.8

Median (interquartile range: IQ) of TNF- α in pg/ml after stimulation of whole blood with PHA (10 μ g/ml) for TB patients and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs) “before therapy” (at TB diagnosis), and “after therapy” (approximately 1 year later)

	“Before therapy”			“After therapy”			
	n	Median (IQ)	<i>p</i> -value ¹	n	Median (IQ)	<i>p</i> -value ¹	
pts <12 years	13	234 (56-434)	.428	13	172 (141-468)	.271	.600
con <12 years	22	313 (150-575)		22	142 (94-353)		.014
pts >12 years	12	222 (156-420)	.001	12	573 (286-961)	.236	.004
con >12 years	19	632 (360-1110)		19	427 (167-684)		.004
Total	66	343 (194-625)		66	270 (136-569)		.095

p-value¹: Values of patients <12 yrs compared to values of controls <12 yrs and values of patients >12 yrs compared to values of controls >12 yrs (Mann-Whitney U test)

p-value²: Values of each group, “before therapy”, compared to values of each group, “after therapy” (Wilcoxin Ranks test)

“Before therapy”, patients >12yrs produced significantly less TNF- α in response to PHA than controls >12yrs ($p = .001$). These patients subsequently produced significantly more TNF- α “after therapy” compared to “before therapy” ($p = .004$). However, controls < 12yrs and > 12yrs produced significantly less TNF- α “after therapy” compared to “before therapy” ($p = .014$ and $p = .004$ respectively).

5.3.3.2 TNF- α production after PPD stimulation

Day 7 PPD-stimulated TNF- α production from diluted whole blood from TB patients and healthy Mantoux positive controls according to age group, “before therapy” and “after therapy”. Data are represented as box plots showing the median and extending to the 25th and 75th percentiles. The lines extending from the box extend to upper and lower adjacent values.

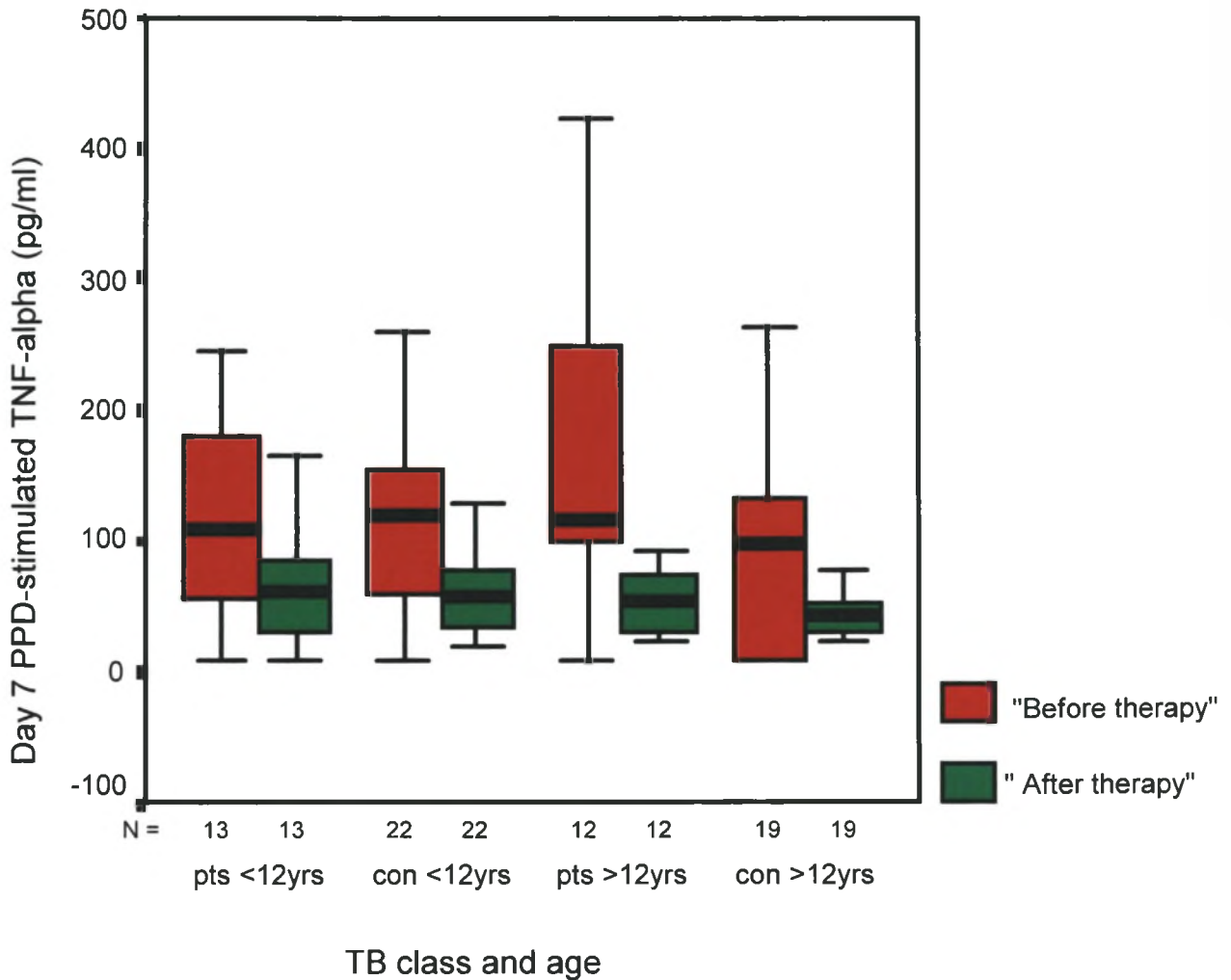


Table 5.9

Median (interquartile range: IQ) of TNF- α in pg/ml after stimulation of whole blood with PPD (3.3 μ g/ml) for TB patients and their age-related healthy Mantoux positive controls (<12 yrs or >12 yrs) “before therapy” (at TB diagnosis), and “after therapy” (approximately 1 year later)

	“Before therapy”			“After therapy”			<i>p</i> -value ²
	n	Median (IQ)	<i>p</i> -value ¹	n	Median (IQ)	<i>p</i> -value ¹	
pts <12 years	13	109 (34-207)	.699	13	60 (31-88)	1.000	.055
con <12 years	22	121 (46-154)		22	56 (33-80)		.006
pts >12 years	12	116 (95-255)	.101	12	55 (27-74)	.306	.006
con >12 years	19	97 (10-150)		19	43 (29-55)		.036
Total	66	109 (10-172)		66	52 (31-73)		<.001

p-value¹: Values of patients <12 yrs compared to values of controls <12 yrs and values of patients >12 yrs compared to values of controls >12 yrs (Mann-Whitney U test)

p-value²: Values of each group, “before therapy”, compared to values of each group, “after therapy” (Wilcoxin Ranks test)

Generally, all PPD-induced TNF- α was lower “after therapy” compared to “before therapy”. Values for all groups, “before therapy”, compared to “after therapy”, were significantly lower, except for patients < 12yrs, whose values fell just short of reaching statistical significance ($p = .055$).

When the values of all patients ($n = 25$) was analysed, PPD-induced TNF- α production was significantly lower “after therapy”, compared to “before therapy” ($p = .001$). When the values of controls ($n = 41$) was analysed, PPD-induced TNF- α production was also significantly lower “after therapy”, compared to “before therapy” ($p = .001$). (data not shown on the table)

In addition, values for all samples, “after therapy” were significantly lower than values for all samples, “before therapy” ($n = 66$; $p < .001$).

5.4 Discussion

5.4.1 Lymphocyte proliferation studies

As discussed previously, proliferation assays assess the proliferative capacity of T cells in response to a particular mitogen or antigen. The majority of studies in the literature report on immune responses of TB patients at diagnosis. Generally, these studies have reported lower proliferative responses in TB patients compared to healthy sensitised controls. Few studies have been performed in which these responses have been re-assessed after completion of TB therapy. Of the few that have been reported, most of them report restoration of proliferative responses in TB patients after therapy, compared to before therapy. Diele et al., (2000) report an increase in PPD-induced proliferative responses in 14 out of 16 children after TB therapy. Similarly, Onwubalili et al., (1985) report an increase in proliferative responses in response to PPD stimulation in 9 out of 9 adults while Torres et al., (1998), found an increase in proliferative responses after therapy in 4 out of 7 adults after stimulation with the 30kDa antigen of *M. tuberculosis*. A more recent report of Portales-Perez et al., (2002), using a non-radioactive proliferation assay, reported no significant differences in proliferation in response to mycobacterial antigens, at diagnosis of TB patients compared to healthy sensitised controls, nor after completion of TB therapy. They speculate that the use of a non-radioactive assay (that detects the number of viable cells) was not an efficient manner of detecting lymphocyte reactivity in human PBMC cultures.

In this study, values for PHA-induced proliferative responses for TB patients > 12yrs, which were significantly lower than controls “before therapy”, showed an increase “after therapy”, but the difference was just short of achieving statistical significance. PPD-induced proliferative responses for TB patients > 12yrs were also significantly lower than controls “before therapy”, and also demonstrated an increase “after therapy”, but this time the difference was statistically significant. Interestingly, values for controls <12 yrs and > 12yrs were significantly lower “after therapy” compared to “before therapy”, after PPD stimulation. This finding may be due to the fact that the “before therapy” assays were performed approximately a year earlier by a different investigator.

A possible reason for the restoration of proliferative responses in TB patients “after therapy” could be that antigen-responsive T cells, which were sequestered at the site of disease during active TB infection, could have recirculated into the bloodstream. Also, the levels of

immunosuppressive cytokines such as TGF- β and IL-10, which have been shown to be increased in TB patients with active disease, have been reported to be lower after TB therapy (Hirsch et al., 1999b; Garcia et al., 2002; Torres et al., 1998).

The findings in this study generally agree with those in the literature that proliferative responses of patients (in older children and adults in this study) are increased after TB therapy, compared to before therapy.

5.4.2 Cytokine studies

Note: IL 10 values “before therapy” could not be directly compared to levels “after therapy” as the Genzyme Duoset ELISA kits, used in the “before therapy” study, were not available anymore at the time when the “after therapy” study was performed.

5.4.2.1 IFN- γ production

The assessment of immune responses, specifically the measurement of cytokine production, in TB patients compared to healthy sensitised controls has often been previously reported. These studies have generally reported depressed production of IFN- γ in TB patients compared to controls. Very few studies have followed up these patients and re-assessed immune responses after TB therapy. Hirsch et al., (1999b) report that PPD-induced IFN- γ production from PBMCs of TB patients, which was depressed before TB therapy, showed a slight increase, but remained significantly lower than control values, one year after therapy. Turner et al., (2000) showed an increase in the production of IFN- γ after stimulation with *M. bovis* BCG after therapy, compared to values found before therapy. Verbon et al., (1999) measured serum cytokine levels in TB patients before, during and after TB therapy. They reported significantly higher levels of IFN- γ in TB patients compared to healthy sensitised controls, before therapy. These levels showed a decline during therapy and a further decline after therapy, when they were no longer significantly different to the healthy controls. Portales-Perez et al (2002), after PBMC stimulation with the 30kDa antigen of *M. tuberculosis*, did not find any significant differences between TB patients before and after TB therapy.

In this study, all values for PHA- and PPD-induced IFN- γ for patients as well as controls were generally increased “after therapy”, compared to “before therapy”. A possible

explanation for the increase in IFN- γ production in all subjects may be due to the increased capacity of the immune system to produce this cytokine as all subjects would be one year older when the “after therapy” study was performed and the immune system would be more “mature”.

The findings in this study do not conclusively show that IFN- γ concentrations in TB patients improve after therapy.

5.4.2.2 IL-2 production

Studies in the literature generally report a diminished production of Th1 cytokines (IFN- γ and IL-2) in newly diagnosed TB patients compared to healthy controls (Hirsch et al., 1995; Zhang et al., 1995; Torres et al., 1994). Very few studies have re-assessed immune responses after completion of TB therapy and studies reporting on IL-2 production in TB patients, after TB therapy, are very rare. One report, using a flow cytometric technique, did not find any PPD-induced IL-2 production from peripheral blood lymphocytes before or after therapy, although they did measure PPD-induced IL-2 in cells from bronchoalveolar lavage fluid. Hirsch et al., (1999a) measured *M. tuberculosis* -induced IL-2 responses in TB patients and healthy sensitized controls, before therapy and 6 months after therapy. They found significantly lower IL-2 values in patients compared to controls, before therapy. After therapy, IL-2 levels in patients increased to levels comparable to those of healthy controls.

Patients >12 years produced significantly more PHA-induced IL-2 “after therapy” compared to “before therapy”. In this study, values for PHA- and PPD-induced IL-2 were generally lower “after therapy” compared to “before therapy” (except for patients >12 years after PHA stimulation). Both patients as well as controls < 12 years produced significantly less PPD-induced IL-2 “after therapy” compared to “before therapy”.

The general depression of IL-2 values, “after therapy” compared to “before therapy”, is unusual and will be further commented on in the general discussion.

5.4.2.3 TNF- α production

Studies on TNF- α production in TB patients (before TB therapy) compared to healthy sensitised controls, have reported TNF- α to be increased in TB patients compared to controls

(Dlugovitzky et al., 2000), or have demonstrated no significant differences between TB patients and healthy controls (Portalez-Perez et al., 2002; Schwander et al., 1998; Hirsch et al., 1996). Only one of the latter studies reports on TNF- α production after TB therapy. They report that TB patients produced significantly more TNF- α after stimulation with the 30kDa antigen of *M.tuberculosis*, 8 months as well as 12 months after the commencement of therapy compared to before commencement of therapy.

Patients >12 years produced significantly more PHA-induced TNF- α “after therapy” compared to “before therapy”. Values for all other groups were lower “after therapy” compared to “before therapy” for PHA- and PPD-induced TNF- α . These differences were all significant in all groups except for patients <12yrs (which fell just short of reaching significance).

The general depression of TNF- α values, “after therapy” compared to “before therapy”, is unusual and will be further commented on in the general discussion.

5.5 Summary

In this section of the study, TB patients were investigated for their proliferative and cytokine responses to a mitogen (PHA) and a specific mycobacterial antigen (PPD) before and after commencement of TB therapy.

After PHA-stimulation, patients <12 years generally did not show any differences in responses before and after TB therapy, except for an increase in IFN- γ production. Patients >12yrs demonstrated improvement in all PHA-induced proliferative and cytokine responses “after therapy”, compared to “before therapy”.

After therapy, patients <12 years demonstrated decreased PPD-induced IL-2 and TNF- α production, compared to before therapy. Patients >12yrs demonstrated improvement in PPD-induced proliferation, but decreased production of TNF- α , after therapy, compared to before therapy.

Except for IFN- γ production, all proliferative and cytokine responses for controls (<12 years and >12 years) were generally lower after therapy, compared to before therapy.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The first aim of this study was to optimise a whole blood assay for the assessment of proliferative and cytokine responses in response to a mitogen (PHA) and a specific mycobacterial antigen (PPD). There are many advantages of whole blood assays and these have already been discussed in the introductory chapters. In addition, results of whole blood assays and that of conventionally used PBMCs have been shown to be comparable (Elsässer-Beile et al., 1995; Yaqoob et al., 1999). One may wonder how the results of whole blood assays can be directly compared to that of PBMCs when it is not known exactly how many white blood cells are present in each well of the culture plate in the whole blood assay. This issue is of even greater importance when comparing in-vitro immune responses between children and adults, as it is known that age-related differences in white blood cell counts per volume of whole blood exist. Elsässer-Beile et al., (1995) as well as Schölvinc (2001) in the initial “before therapy” part of this study normalised their whole blood assay cytokine results to the blood samples’ initial absolute amount of mononuclear cells and found results to be comparable.

In our laboratory, the whole blood assay proved to be a technically simple, non-laborious assay that, according to the literature, produces results that seem to correlate well with that of conventionally used PBMCs.

The major shortcoming of studies of this kind, where immune responses in TB patients are measured from peripheral blood or its components, is that it may not be representative of the actual events at the site of disease. It has previously been documented that different balances of cytokines within the same patient can be seen when bronchoalveolar lavage cells or pleural fluids are compared to cells from peripheral blood (Schwander et al., 1998; Barnes et al., 1993). It can thus be argued that the measurement of peripheral responses would not provide accurate information on the pathogenesis of TB disease in humans. However, in studies of this kind, it is more difficult to obtain bronchoalveolar or pleural fluids from patients. In addition, this study was performed approximately one year after TB diagnosis, by which time

antigen specific cells, that may have been sequestered at the site of disease, would have re-circulated into the periphery.

Another issue to be in mind is the inter- and intra-individual variations in cytokine measurements that are known to occur. One limitation of this study is that it did not address this issue, but Yaqoob et al., (1999), who also found significant inter-individual variation in cytokine production, demonstrated that the level of cytokine produced appeared to be characteristic of an individual.

Diurnal variations of levels of serum cytokine levels in healthy individuals are also known to occur (Petrovsky et al., 1995). All blood samples were taken between 9 and 12am and processed within 2-3- hours in order to minimise this variation.

The measurement of cytokines by ELISA is a standard assay and is usually reliable and reproducible and was thus also used for the measurement of cytokine concentrations in this study. Variations in laboratory conditions is an important factor that can influence the outcome of ELISA results and thus rigid quality control measures, such use the of internal reference controls and inter- and intra-assay studies, are essential. In this study, the cytokine standard served as the internal reference control and was also used for the inter-assay variation studies, which was always well within the required limits (<10% variation).

The second aim of the this study was to determine if there was a relationship between age and whole blood PHA- and PPD-induced proliferation and cytokine production in subjects of different ages. Neither PHA- and PPD-induced proliferation showed any correlation with age. Except for IL-2, age correlated positively with all PHA-stimulated cytokines, namely: IFN- γ , TNF- α and IL-10. This study is in agreement with reports in the literature as proliferation and IL-2 production have previously been reported to have no relationship with age (Lewis et al., 1996). In addition, IFN- γ and TNF- α have previously been reported to be lower in children compared to adults (reviewed by Smith et al., 1997). None of the cytokines induced by PPD showed any relationship with age. A possible reason for this finding could be that all subjects in this study had been exposed to *M.tuberculosis* and that in itself may have been sufficient to generate an in-vitro response, irrespective of age. These findings appear to indicate that the nature of the stimulant as well as previous exposure, determines if there is a relationship with

age. The establishment of normal reference ranges, for individual stimulants, as well as the use of age-matched controls in studies such as these, is thus essential. Further studies utilizing larger numbers of children and a broader panel of cytokines and stimulants would help to address these issues.

The third aim of this study was to compare immune responses of patients after treatment (“after therapy”) to data obtained at diagnosis (“before therapy”). In the literature, immune responses of TB patients have generally been reported to be depressed at the time of diagnosis, with subsequent improvement, after therapy.

In this study, there was a trend towards increased concentrations of PHA- and PPD-induced IFN- γ for all subjects (patients and controls) “after therapy”, compared to “before therapy”. An increase in IFN- γ production in TB patients after TB therapy has previously been documented in the literature (Turner et al., 2000) however, in this study, controls also demonstrated an increase in IFN- γ production. This finding could therefore possibly be due to a technical variation of the assays (the assays were performed a year apart and by different researchers) or due to maturation in the capacity of the immune system to produce IFN- γ (but this is less likely in subjects older than 12 years of age).

Patients >12yrs demonstrated improvement in all proliferative and cytokine responses (except for PPD-induced IL-2 and TNF- α) “after therapy”, compared to “before therapy”. These results are in good agreement with other studies showing a restoration of immune responses after drug treatment (Diele et al., 2000; Onwubalili et al., 1985; Turner et al., 2000; Wilkinson et al., 1998; Hirsch et al., 1996). The cause for the diminished immune response in untreated active TB can probably be ascribed to a number of factors, with compartmentalization at the site of disease (Barnes et al., 1989; Diele et al., 1999), production of immunosuppressive cytokines such as IL-10 or TGF- β (Hirsch et al., 1996) and defective antigen presentation (Pancholl et al., 1993) all contributing.

TNF- α and IL-2 values “after therapy” were all generally lower than values, “before therapy” (except for patients >12yrs). In addition, except for IFN- γ production, all proliferative and cytokine responses for controls (<12 years and >12 years) were generally lower after therapy, compared to before therapy. A major limitation of the study was that a different researcher

performed the “before therapy” study. In addition, a significant period of time (approximately one year) separated the two studies. These two factors could have been responsible for variations in laboratory conditions (for example, reagents and technical proficiency) that could have influenced the outcome of these results. Another possible explanation for the lower cytokine values found in the “after therapy” study compared to the “before therapy” study could be the fact that the DuoSet kits had been purchased well in advance and were very close their expiry dates when the “after therapy” study was performed and perhaps were not as sensitive as they were one year earlier (although this is not likely to be the case as the inter-assay variation of the internal reference control was always <10%). No satisfactory explanation for this phenomenon could be found. It can perhaps only be ascribed to some unknown variation in laboratory conditions. Recommendations for future longitudinal studies which measure the production of cytokines by ELISAs would be to try and purchase DuoSet kits which have a longer shelf life and that, where possible, the same person perform all ELISAs. Also, storage of samples so that all ELISAs are performed together would reduce any assay variability, provided the stability of the cytokine being measured is satisfactory.

Further studies, investigating these and additional immunologic parameters in TB patients of different ages, both before, as well as after TB therapy will provide important information regarding the pathogenesis of TB.

In conclusion:

1. The whole blood assay is a simple, non-laborious assay that, according to the literature, produces results that seem to correlate well with that of conventionally used PBMCs.
2. Age appears to be an important variable in the quantitative assessment of cellular immune responses when the mitogen, PHA is used as a stimulant.
3. Immune responses of older TB patients appear to improve after TB therapy, compared to before TB therapy.

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