The influence of age on the cellular immune response in patients with tuberculosis and healthy controls

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Declaration:

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

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Date

"…..Every age has a kind of universal genius,
which inclines those that live in it to do particular studies…"

J. Dryden 1633-1700

Abstract

Children and adults may differ in their immune function. An adequate function of the individual's immune system is crucial to the risk for development of tuberculosis (TB) after infection with *Mycobacterium tuberculosis (M.tb).* Epidemiological evidence suggests an age-related incidence of TB. Furthermore, the prevailing clinical expression of TB varies between age groups.

The aims of this study were to characterise the cellular immune response at different ages in TB patients and healthy individuals living in a region highly endemic for TB and to relate the findings to the clinical expression of TB in different age groups.

A total of 150 persons of different ages were included in this study: 50 TB patients, (identified on the basis of clinical, radiological and microbiological characteristics), 49 healthy Mantoux positive (≥15mm) and 51 healthy Mantoux negative (<15mm) subjects. All patients <12yrs were identified as having primary TB and postprimary TB was only diagnosed in patients ≥12yrs.

Haematologic indices were obtained from all the included subjects and found to be age-related. With the exception of the absolute lymphocyte counts, all indices were significantly different in TB patients when compared to healthy controls.

Whole blood was cultured and stimulated with PHA, PPD and ESAT-6 to measure lymphocyte proliferation and IFN-γ, TNF-α, IL-2 and IL-10 production in the supernatants of the cultures.

After stimulation with PHA, the production of IFN-γ, TNF-α and IL-10 as well as lymphocyte proliferation were all age-related.

After stimulation with PPD, age correlated positively with IFN-γ production in healthy Mantoux positive subjects < 12yrs. In the age groups <20 yrs, patients produced similar

amounts of IFN-γ when compared to healthy age-related Mantoux positive controls. TNF-α and IL-2 production were not different between patients and controls. In this whole blood system, measuring any of these cytokines on their own did not differentiate patients from controls at all ages.

The ratio of PPD stimulated IFN-γ to TNF-α production was significantly less in patients with primary TB and postprimary TB when compared to Mantoux positive controls, irrespective of age. These findings indicate that calculated ratios between several cytokines may be useful markers of disease at all ages.

ESAT-6 stimulated IFN-γ production did not result in any significant correlation with age, but was significantly less in healthy Mantoux positive subjects ≥12 yrs when compared to healthy Mantoux positive subjects <12 yrs and TB patients of all ages. This finding suggests that a positive immune response to ESAT-6 is indicative of recent immunological contact with *M.tb*.

Total IgE was measured in serum. In children <12 yrs these values correlated with age and were highest in healthy Mantoux positive controls, thereby not confirming any inverse correlation between IgE and TB.

Age should be recognised as a significant variable in quantitative measurements of cellular immune responses.

Opsomming

Die immuunsisteem van kinders en volwassenes kan verskillend wees. Die mate van immuniteit van 'n individu is deurslaggewend vir die risiko om tuberkulose (TB) na infeksie met die *Mycobacterium tuberculosis* (*M.tb*) te ontwikkel. Epidemiologiese bevindings suggereer dat die insidensie van TB ouderdomgebonde mag wees. Verder verskil die voorkomende kliniese beeld van TB ook tussen ouderdomsgroepe.

Die doelstellings van hierdie studie was om die sellulêre immuunrespons op verskillende ouderdomme by TB-pasiënte en gesonde individue wat in 'n streek met hoogs endemiese TB-insidensie woon te vergelyk. Die doel was ook om vas te stel hoe hierdie bevindings by die kliniese beeld van TB by verskillende ouderdomsgroepe inpas.

Daar is 150 persone van verskillende ouderdomme in hierdie studie ingesluit: 50 TB-pasiente (geïdentifiseer op grond van kliniese, radiologiese en mikrobiologiese karakteristieke), 49 gesonde Mantoux -positiewe (≥15mm) en 51 gesonde Mantoux-negatiewe (<15mm) persone. Alle pasiënte <12 jaar is gediagnoseer met primêre TB. Postprimêre TB is alleenlik gediagnoseer in pasiënte ≥12 jaar.

Daar is aangetoon dat hematologiese indekse van al die persone in hierdie studie ouderdomsverwant was. Daar was n beduidende verskil in alle indekse van TB-pasiënte in vergelyking met die gesonde kontroles met die uitsondering van die absolute limfosiet-tellings.

Kulture van volbloed is gedoen en gestimuleer met behulp van PHA, PPD en ESAT-6 om limfosiet-proliferasie, IFN-γ-, TNF-α-, IL-2- en IL-10-produksie in die supernatante van die kulture te meet.

Na stimulasie met PHA was die produksie van IFN-γ, TNF-α en IL-10, asook die limfosiet-proliferasie ouderdomsverwant

Na stimulasie met PPD het ouderdom positief gekorreleer met IFN-γ produksie in gesonde Mantoux-positiewe persone <12 jaar. In die ouderdomsgroep <20 jaar het pasiënte dieselfde hoeveelhede IFN-γ geproduseer as gesonde, ouderdomsverwante Mantoux-positiewe kontroles. Daar was geen verskil tussen die produksie van TNF-α en IL-2 tussen pasiënte en kontroles nie. In hierdie volbloed-sisteem het die meet van nie een van hierdie sitokiene op sigself 'n verskil getoon tussen pasiënte en kontroles van alle ouderdomme nie.

Die verhouding van PPD-gestimuleerde IFN-γ- tot TNF-α-produksie was betekenisvol minder in pasiënte met primêre TB en postprimêre TB in vergelyking met Mantoux-positiewe kontroles, ongeag ouderdom. Hierdie bevindings toon dat berekende verhoudings tussen verskillende sitokiene waardevolle merkers van 'n siektetoestand (TB) by alle ouderdomme kan wees.

ESAT-6 gestimuleerde IFN-γ-produksie het geen betekenisvolle korrelasie met ouderdom getoon nie. Daar was egter betekenisvol minder produksie in gesonde Mantoux-positiewe persone ≥12 jaar as in gesonde Mantoux-positiewe persone <12 jaar, asook in vergelyking met TB-pasiënte van alle ouderdomme. Hierdie bevinding kan daarop dui dat 'n positiewe immuun respons op ESAT-6 'n aanduiding van onlangse immunologiese kontak met *M.tb* is.

Totale IgE was in serum bepaal. In kinders <12 jaar het hierdie waardes gekorreleer met ouderdomme en die waardes was die hoogste in gesonde Mantoux-positiewe kontroles. Hierdeur is daar nie bevestig dat daar 'n omgekeerde korrelasie tussen IgE en TB is nie.

Ouderdom behoort dus as 'n belangrike veranderlike gesien te word in die kwantitatiewe meting van die sellulêre immuunrespons.

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List of abbreviations

APCs	Antigen Presenting Cells
cpm	counts per minute
CMI	Cell Mediated Immunity
CR	Chest Radiograph
BAL	bronchio-alveolar lavage
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme Linked Immunosorbent Assay
ESAT-6	Early Secreted Antigen Target-6
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-12	Interleukin-12
LAM	Lipoarabinomannan
Mantoux neg	Mantoux negative
Mantoux pos	Mantoux positive
MHC	*Major Histocompatibilty Complex*
M.tb	Mycobacterium tuberculosis
NK cells	Natural Killer cells
PBMCs	Peripheral Blood Mononuclear Cells
PPD	Purified Protein Derivate
PHA	Phytohaemaglutinin
TB	tuberculosis
TBM	tuberculous meningitis
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumor Necrosis Factor- alpha
WBC	White Blood Cell Count
yrs	years

Age and the development of the immune system

Introduction to the functioning of the immune system

The immune system keeps a balance between health and disease through its capacity to mount a response to a wide variety of stimuli, including foreign pathogens.

The functioning of the immune system can be outlined through several, not always mutually exclusive, pathways:

Innate *vs* adaptive immune response is a segregation to differentiate a usually non-specific response from a more specialized effector function. The key difference lies in the fact that genes encoding recognition receptors rearrange in cells of the adaptive immune system, thus allowing clonal differentiation and memory, while this does not happen in cells of the innate immune system.[1] Cells from the innate immune system (polymorphonuclear leucocytes, monocytes and macrophages) respond rapidly, are polyspecific and discriminate self from non-self. Antigen presenting cells (macrophages, dendritic cells, but also B-lymphocytes) present the adaptive immune system (T and B lymphocytes) with antigen in order to activate a specific, memory immune response, which evolves at a slower pace than the innate response.

The adjectives naive *vs* memory describe T-cells that respond to either antigens never encountered before or to antigens for which a memory has been formed through earlier encounter(s).

A cellular *vs* humoral immune response discriminates between a response largely mediated through direct cell-cell interaction from one that is primarily dependent on antibody production. The T-lymphocyte *vs* the B-lymphocyte lineage are the respective main players in these two forms of responses.

A type 1 *vs* a type 2 immune response is distinguished on the basis of the predominant cytokines (soluble proteins secreted by immunoregulatory cells) involved in a particular effector function. This discrimination was originally described in the mouse model[2] and subsequently proved to be of use in the understanding of human immune responses in a variety of diseases[3], including TB. Initially the distinction was thought to be applicable primarily to CD4+ T helper lymphocytes, but recent studies have shown that other cells of the immune system are also capable of committing themselves to producing mainly type 1 or type 2 cytokines. The most important factor in T cell development towards type 1 or type 2 is the nature of the cytokines that are present during initial T cell activation. By releasing specific cytokines the innate immune system directs the adaptive immune system towards a certain type of effector response.[4] Production of IL-12 leads towards a type 1 and IL-4 production towards a type 2 direction. Immune responses that are not clearly dominated by typical type 1 or type 2 cytokines are called type 0 responses. In general type 1 responses are primarily required for elimination of intracellular pathogens and type 2 responses for antibody mediated control of pathogens, but most often both types are needed for an optimal overall immune response.

Maturation of the immune system

Important for the context of this thesis is the notion that the immune system develops throughout life, from the early fetal stages to "old age". Normally, the basic requirements for development of immune competence are present at birth. However, full expansion of the capacities of the immune system, in resemblance to the central nervous system, requires learning. "Maturation" is a qualification used to describe this concept. Essential to the learning curve of the immune system is "exposure": only the actual encounter with a pathogen will provoke a response and memory for a subsequent encounter. Relating age to maturation of the immune system, requires some kind of time-scale. Since exposures do not necessarily follow each other in the same order in every individual or indeed in any given population, the general use of a time-scale may be too simplistic. Nevertheless epidemiological evidence from around the world suggests that age is an important

confounding factor in data on morbidity and mortality. Pabst[5], Wilson[6] and Holt[7], authors of key review articles on age-related immune competence, argue that research in this field will help to provide us with insight in mechanisms responsible for disease susceptibility and/or specific expression of disease at given ages.

Much on the development of the immune system has been learned from mouse models and one of the basic questions in the field of immunology has always been how the discrimination between self and non-self develops. At some stage(s) in the ontogeny of the immune system, the ability to recognize self and non-self is established and it has generally been assumed that this happens during intrauterine and neonatal life. In the 1950s McFarlane-Burnett proposed that developing, potentially self destructive T-lymphocytes, are removed from the thymus before they can mature. This process is called negative selection as opposed to positive selection, when self-MHC restricted lymphocytes are selected for survival. The classic experiments by Medawar and co-workers[8] showed that introduction of foreign tissues in neonatal mice, but not in older animals, induced tolerance to these tissues. Recently, a series of experiments[9, 10, 11] have shown that, at least in a mouse model, tolerance induction might also be dependent on the dose of a given antigen, the adjuvant in which the antigen is presented and the amount of co-stimulatory help from APCs for T-lymphocytes. These studies also suggested that the neonatal period is not exclusive in this respect and that, given the right conditions, tolerance can be learned at any time during life. The whole original evolutionary concept of self, non-self and tolerance induction, which has been a cornerstone in immunology for the last decades is currently widely debated by immunologists[12], who have not yet found a common ground.

The ontogeny of immunological milestones, in terms of time needed to acquire a certain competence, differs between animals[13] and experimental models may not reflect adequately the order and circumstances of events in humans. However, it is reasonable to assume that the timing of antigen presentation, dosage of antigen as well as the presenting mode of a given antigen have short and possibly long term effects on the immune response in humans as well. For example, infections in early life may protect against

atopy later in life[14] and non-conjugated vaccines for polysaccharide encapsulated bacteria do, in general, not induce a protective response if given before the age of two years.[15] The degree of endemicity (number of infective bites per year) in a given region dictates the main age specific causes of morbidity and mortality from malaria.[16] Natural infection with measles virus seems to confer a higher degree of protection against atopic disorders when compared to vaccination against measles.[17]

A seemingly wide ranging "physiologic immunodeficiency" with respect to handling of infections, has made the neonatal period a comparatively well researched childhood period (reviewed by Wilson[18]), both in experimental and human models. Cord blood (easily obtainable material) has been used in most studies on neonates. The notion that a healthy neonate comes into the world as an immunologically completely naive subject, previously protected in a sterile uterine environment by the mother's immune system, cannot be upheld anymore. For example, cord blood lymphocytes of children born from mothers residing in Kenya, where helminth infections and tuberculosis are prevalent, respond to helminths and mycobacterial antigens *in vitro* in much the same way as their mothers' cells and differently from North American controls.[19] The degree to which cord blood represents the actual immune competence of the newborn is not clearly established, inasmuch as perinatal stress and maternal factors may well influence the results of studies using cord blood.

In most of the literature, the immune system is regarded as competent around the age of five. Adolescence, a period in life where rapid changes in body functioning and composition take place, might be interesting from an immunological point of view. Adolescence is, however, difficult to model in any experimental setting. Some infectious diseases, like measles and varicella, if not encountered during early childhood, may cause serious morbidity in adolescents. Others, like schistosomiasis, often present during adolescence, while the infection has taken place at much earlier age. The incidence of autoimmune diseases rises during adolescence with different expressions of disease and preference for a particular sex. Hormones, increasingly known for their potential influence on immune processes, are probable role players. One study, focusing on immune function in 331 healthy adolescents aged 12-18yrs, found age- (WBC,

phagocytic and bactericidal activity of granulocytes), gender- (lymphocyte subtypes and bactericidal activity) and race- (WBC, lymphocyte subtypes, PWM responses) related differences in a set of enumerative and functional immune markers.[20]

Quantification of immune cells throughout age

One of the ways to quantify characteristics of the immune system is counting specific cell populations, by making use of the flow cytometry technique. In part, the reason for evaluating children of different ages with this method has been driven by the HIV-epidemic. In HIV-infected persons, absolute CD4+-lymphocyte counts relate to immune competence and normal values in children have been shown to differ from those in adults, depending on the age of the child. The numbers of subjects included in published reports on changes in lymphocyte subpopulations over age are not large[21, 22, 23] and most of the studies published thus far described only absolute numbers of lymphocytes, T cells (CD4, CD8), B cells (CD19) and NK cells (CD56) in relation to age. A graphic display of the changes over age is shown in the figure below.

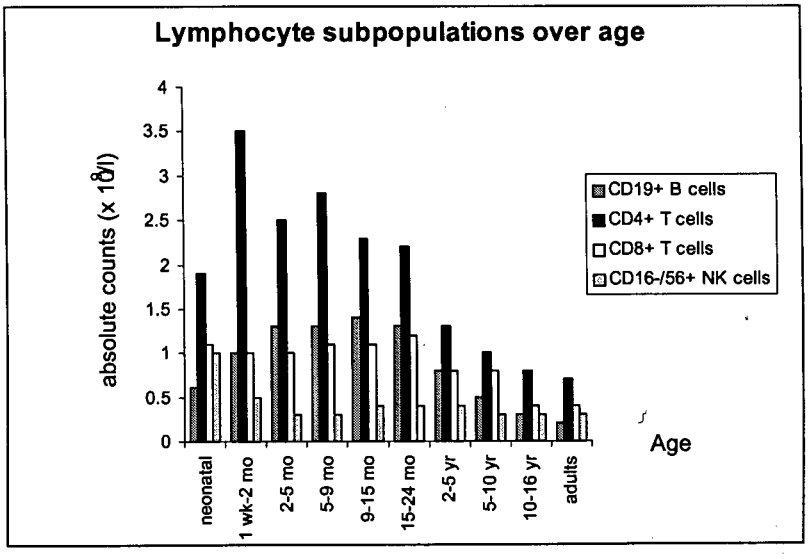


Fig : Changes of lumphocyte subpopulations over age (adapted from Comans-Bitter et al[21])

Although the age-related trends of change are similar, populations from various regions in the world are documented to differ with respect to absolute lymphocyte, CD4+ and CD8+ counts.[24, 25] These differences are possibly due to varying infectious pressures and/or genetic background in different parts of the world. Information on numbers and/or phenotypes of cells in relation to age from any body compartment other than blood is even sparser. One study found age-related changes in tonsillar lymphocytes similar to peripheral blood. Interestingly, this phenomenon was independent of a history of recurrent infections.[26] Although there is thus a demonstrable relation between age and the development of the immune system, how this relates to the functional aspects of the cellular immunity is not always obvious.

Immune competence throughout age

Diagnostics

Over the past decade or two, there has been an impressive expansion of the possibilities in diagnostic immunology. Isolated PBMCs, with or without stimulated culture conditions, have been widely used to measure cellular immune responses *in vitro*. Important information has been yielded from it. However, the procedure to isolate PBMCs may by itself cause alterations in their functioning, and, more importantly, the absence of autologous serum factors may limit interpretations with regard to *in vivo* conditions. Furthermore the methods used to measure cytokines are potential sources of variations: detecting protein or the corresponding mRNA, the nature of a stimulating agent and the presence of accessory cells in a culture system may all result in differing conclusions. The use of a whole blood culture system, whereby there is no need for cell separation and cells are cultured with all the autologous components of peripheral blood has been validated and used increasingly in recent years.[27, 28, 29, 30] Advantages of this method are a simpler way of processing blood, the possibility of using of smaller blood volumes and the probability of better approximation of *in vivo* conditions. A potential disadvantage is that subsets of cells cannot routinely be scrutinized for their functioning. Apart from the technical procedures used to evaluate immune competence, differences in study populations (age groups, gender, race and living environment) should be taken into

account when comparing study results. Notwithstanding these comparative limitations, a number of publications have, quantitatively and/or qualitatively, provided more detailed insight in immune competence of selected age groups.

Naive vs memory cells

One of the striking age related variations in T cell populations is the ratio of CD45RA+RO- (naive type) and CD45RA-RO+ (memory type) subsets in CD4+, CD8+ as well as γδ T-cells. Naive type cells are involved in the primary immune response after an initial encounter. Memory type cells are responsive to recall antigens and their clonal expansion is thought to be driven by environmental antigens; adult levels are generally attained between 15-20 years of age. The two cell types require different activation signals and may secrete other cytokines in response (reviewed by Akbar[31]). Additionally, the availability of co-stimulatory help and the dose and nature of antigens will likely prove to differ, as in mouse models.[32] Thus, age-related quantitative variations in naive- and memory- T- populations probably result in different qualitative effector functions.

Cytokines

IFN-γ

IFN-γ, produced mainly by T cells and NK cells, is regarded as the archetypal type 1 cytokine. Its major biologic effects are activation of macrophages with respect to intracellular killing of pathogens; upregulation of MHC class I and class II expression on macrophages; downregulation of type 2 immune responses and induction of isotype and class switching by B-cells to produce IgG1, IgG3 and IgE antibodies. It is generally accepted that the ability to produce adult-equivalent levels of IFN-γ is not attained before the age of 3-5 yrs.[7] One of the contributing factors to this phenomenon is the fact that naive T cells, if compared to memory T-cells, are deficient in producing this cytokine.[33] Evidence for other mechanisms has, however, been put forward: T-cell clones from cultured neonatal cells do not have the adult capacity to produce IFN-γ[34] and turnover rates from naive to memory cells are not the same in neonates and adults.[35] While young childrens' PBMCs may produce as much IL-2 upon stimulation with PHA, their capacity to produce equal amounts of IFN-γ is diminished compared to PBMCs from adults.[36]

TNF-α

TNF-α, produced by mononuclear phagocytes, T-cells and NK cells, is considered an important pro-inflammatory cytokine. When produced in excess, it is also well known for its systemic side effects like fever, cachexia and tissue destruction in a variety of diseases. Its production is counter-regulated by anti-inflammatory cytokines like IL-10[37]. Production upon mitogenic stimulation seems to be age related, with neonates producing less than older individuals.[18, 38] However under pathologic conditions, like bacterial meningitis[39] and malarial disease[40] young children are quite capable of producing high levels of TNF-α. Interestingly, a study on cellular responses to *N.meningitidis* antigens in patients and controls of different age groups, found stimulated TNF-α production to be age-related in controls (higher in older children), but not in patients after meningococcal infection.[41]

IL-2

IL-2 is produced by T lymphocytes upon activation and is a major growth factor for T cells, as well as B cells and NK cells. Its production is probably not age dependent under most circumstances although naive cells have a higher capacity to produce IL-2 than memory cells. Consequently one might expect a relatively high or at least equal production in young children.[42] On the other hand isolated neonatal T cells produce less IL-2 than those of adults.[18]

IL-12

IL-12 is produced by antigen presenting cells that have been exposed to various microbial antigens. Capable of bridging the innate and the acquired immune system, it can directly activate NK cell function (killing). Indirectly, via inducing IFN-γ production by NK cells and T lymphocytes, it promotes effective killing by phagocytic cells. As a strong promotor of type 1 immune reactions in general, it may also indirectly inhibit type 2 responses or even cause a switch from an established type 2 to a type 1 response.[43] No reports on age related IL-12 production have been published, however IL-12 seems to exert differential effects on cord blood naive CD4 cells when compared to adult naive

CD4 cells: it promotes the maturation of cord blood naive cells into IFN-γ as well as IL-4 producers.[44]

IL-10

IL-10 is produced predominantly by mononuclear phagocytes, but also by CD4+ T cells and B-cells. In mice, IL-10 is generally classified as a type 2 cytokine. Pathways for production in humans are somewhat different in that production is stimulated by IFN-γ as well as TNF-α and not solely regarded as a type 2 cytokine.[45] IL-10 has potent anti-inflammatory properties and can actively downregulate levels of IFN-γ, TNF-α and IL-12. On the other hand, IL-10 promotes B cell proliferation and differentiation, which accounts for its immuno-stimulatory properties. Studies of IL-10 production by cells from cord blood or young children were studied did not show a low production per se, but most of these studies were not designed to address a possible age relation.[46, 39] In Pollards' study of responses to *N.meninigitidis* antigens[41] an age related production of IL-10 (higher with increasing age) was suggested, but did not reach statistical significance.

TGF-β

TGF-β can be produced by virtually all cells, including immunologically active cells.[47] The three mammalian isoforms seem to have specific functions in vivo and need to be activated from their latent form in order to become biologically functional. TGF-β has counter-regulatory effects on proinflammatory cytokines. No studies with regard to age-related production have been published.

IL-4

IL-4, produced by T cells, mast cells and basophils, is one of the cytokines typically involved in type 2 immune reactions. It can stimulate B cells to synthesize IgE and act as a co-factor in T and B cell growth. Furthermore it can inhibit macrophage functions. IFN-γ is able to antagonize some of the effects of IL-4. Age related production upon stimulation with mitogens, lower at younger age, has been elegantly shown in a series of experiments by Tang et al.[48] They also concluded that the mechanisms responsible for age

related production may involve naive/memory subpopulations, as well as inhibitory other cellular or serum factors. However, Holt's group has shown that Il-4 production after stimulation with specific antigens may well differ in this respect. After stimulation with housedust-mite allergen, production of IL-4 was high in non-atopic children and declined with progressive age, but was low at birth with a subsequent rise to a sustained high level in atopic children.[49]

(Knowledge of the age-related production capacity of other cytokines, chemokines and growth stimulating factors as well as cytokine-receptors and co-stimulatory molecules, is either not available or will be addressed at a later stage in the text of this thesis)

Antigen presenting cells

APCs deserve special mention, since so little is known about their function at different ages and it has become increasingly clear that they are key players in the type of immune response that follows encounter with an antigen. In dendritic cells from the respiratory tract of neonatal mice, local hyporesponsiveness to stimulatory signals when compared to adult mice has been demonstrated.[50] Various researchers have combined cells from subjects of different age groups *in vitro*. Their experiments were mainly designed to clarify if (purified) T-cells were intrinsically incapable of a particular effector function. If it was found that with "adult help" (APCs from adults), T cells or leukocytes from younger subjects behaved similar to those from adult subjects, it was concluded that deficiencies of APCs from younger subjects were the probable cause of this phenomenon.[51, 52] Differences in expression of receptors on T cells and/or co-stimulatory molecules on APCs, which could explain poorer cross-talking between the cell populations have also been found in these studies. The mixing of cells from young children with those from adults is, however, not a natural i*n vivo* condition.

In future, when some of the mechanics involved in deficient antigen presentation in young children are unraveled, it will probably have implications for interventional strategies like vaccination. The, already mentioned, recent findings on tolerance induction in mice (dependency on dose, mode and co-stimulatory help)[9, 10, 11] may well prove to be of strategic importance for future research in this respect.

B cells

"The" immune response *in vivo* is determined by all cells involved and attention towards components other than the cellular arm of the immune system is entirely justified, but given the scope of this thesis beyond a detailed discussion. The reduced capacity before the age of two to produce a sustained and memorised antibody response to so called T-cell independent antigens (mostly bacterial polysaccharides) is, despite the name given to these antigens, at least, in part, likely to be due to insufficient help from T cells.[53] This illustrates that the cellular and humoral arms of the immune system are seldom totally independent of each other. Maturation of B cells follows, however, a different path than T cells.[54] The ability to respond with the production of antibodies of a certain class develops quite chronologically with age, reaching adult type levels only after the teenage years.[55] Furthermore the different phenotypic characteristics of the B-cell population at birth gradually evolve towards adult type over age with a reduction in $CD5^+$ B cells, the dominant polyreactive type of B-cell at birth.[56]. This maturational process has implications for the functionality of the antibodies produced: low-affinity/polyreactive at young age *vs* high affinity/specific at older age.[57]

The notion that immune responses at an early age (in mice and man) are more readily skewed towards type 2 has been around for some time. However, it has recently become increasingly clear that environmental factors, be they of infectious or allergic nature, may have considerable influence on the degree to which a skewing towards one or the other type of immune response develops from birth onwards.[14, 48] Age is probably an independent variable in this process, but the quantity and nature of environmental factors may prove to be just as instrumental.[57]

The layered evolution of the immune system

Much of the literature on the development of the immune system uses terms like "deficient" or "immature" when describing differences between young subjects compared to adult counterparts, thereby implying that adult immune functions might be superior. Herzenberger et al [58] propose that:

"...The evolution of the immune system is layered, whereby evolution sequentially gives rise to lymphocytes that are similar to their predecessors, but have either lost or gained functional capacities. Inasmuch as the evolutionary success of the latest layer in such a system depends on its ability to introduce a selective advantage, each new layer can be expected to increase the sophistication of the immune system with respect to its ability to efficiently protect the animal against challenges from its environment..."

The maturation of the individual's immune response throughout life reflects such a layered evolution whereby the system evolves from versatile to more efficient, which if given the multitude of immunologic challenges shortly after birth, is probably the most adequate from an evolutionary perspective.

Immunobiology of tuberculosis

Introduction

The sequence of possible events that occur within the host after encountering *M.tb* is the result of an active and complex interplay between host and the mycobacterium. Of central importance in these events are the host's immune response and the ability of *M.tb* to evade, stimulate or suppress that response. 90% of people infected with *M.tb* are, during their lifetime, able to limit the potential damage triggered by infection with *M.tb* and manage to keep a balance in favor of protection from disease. TB can be considered as a disease of the immune system, whereby the trigger and, in part, the ongoing stimulus for pathology is provided by *M.tb*. The cell mediated immune (CMI) response is universally regarded as the cardinal role player in any reaction to *M.tb* from the immune system. Macrophages, in their various stages of activation, and T-lymphocytes are considered the crucial cells. The functions of dendritic and NK-cells are probably important, but less well defined at the moment. Although a role for the humoral immune system, recently reviewed by Glatman et al[60], can certainly not be ruled out, the nature of such a role is at present ill defined and will not be considered in any detail.

Conflicting results between studies addressing the immune response in TB have been obtained. Differences in the models (animal or human in vitro studies), subjects under study (animals or humans in vivo) and techniques used, contribute largely to this problem. Knowledge gained of immune responses in humans forms the backbone of this overview, unless stated otherwise.

The pathogen: *Mycobacterium tuberculosis*

Tubercle bacilli are non-motile rods, measuring between 1 and 4μm in length and 0.3 to 0.6μm in diameter, considered Gram positive, although they stain poorly by the usual Gram stain procedure. After heating and dye fixation, they resist decolourisation by acid treatment (acid-fast bacilli). They are obligate aerobes that grow very slowly, with a

doubling time of about 20-24 hrs under optimal conditions. They posses a complex and lipid-rich cell envelope, which probably accounts for a number of its unusual characteristics: they resist chemical and physical stress and are impermeable to many antimicrobials.[61] Furthermore, *M.tb* has the potential to evade or counteract a number of immune attacks by the host relying on a strategy that enables its survival within the host cell in the phagosomal vacuole, from where it can secrete antigens into the cytoplasm. Lipoarabinomannan (LAM), a cell wall associated lipopolysaccharide produced in large amounts by *M.tb*, has been associated with active induction of cytokine transcription by macrophages.[62] Phagosome-lysosome fusion within the macrophage normally provides an (acidic) environment in which the intracellular killing of bacteria takes place. *M.tb* has the ability to block formation of a phagolysosome and produce NH4+, thus making the environment non-acidic. Recently it has been suggested that the virulence of a given *M.tb* strain may be related to its speed of replication in macrophages.[63, 64] *M.tb* has the capacity to survive in a "dormant" state within the macrophage for as long as the lifetime of the human host or become pathogenic many years after the original infection. The mechanisms by which the bacillus survives during this period of "latency" are still a mystery. Breakdown of this balanced system between immune response and pathogen causes "reactivation TB".

Macrophages

Macrophages act as the principal effectors of protective reactions of the host, but have, at the same time, the dual role of being the preferred biotype for *M.tb*. They serve as antigen presenting cells to T-lymphocytes and can, when activated by T cells, effectively kill *M.tb*. In their end stage of differentiation they form part of the granuloma as giant or epitheloid cells. *M.tb* can enter the macrophage via specific binding to a number of cell surface molecules (macrophage mannose receptor, complement receptors CR1, CR3 and CR4, signalling receptor CD14, and possibly some Fc receptors)[65]; phagocytosis follows subsequently. The signals for killing, mediated through cytokines and chemokines, are provided by lymphocytes as well as the macrophages themselves.

T-Lymphocytes

Of all the T-lymphocytes involved in host defense against *M.tb*, the CD4+ T-cells are probably the major inducers of protection. Mice depleted of CD4+ cells are unable to control mycobacterial infection, adoptive transfer of CD4+ cells from sensitized animals confers protection[66] and humans with diminished CD4+ cell counts have an increased susceptibility to tuberculous disease. CD4+ cells amplify the immune response by activating effector cells like macrophages and enabling them to kill *M.tb.* They also recruit additional immune cells to the site of disease and are prominently present in the lymphocytic layer of the granuloma. Peptide antigens that have been processed by phagocytic cells are presented in the context of MHC class II molecules to the α/β receptor on the surface of the CD4+ cell. Little is known about help from co-stimulatory molecules in activating the T-cells in TB, but one study in this field[67] reported down regulation of CTLA-4 expression in TB patients. Memory T cells proliferate and produce IFN-γ in response to *M.tb*[68] and are presumed to be predominant in local immune defences. Human naive T cells have hardly been studied specifically for their potential role in the immune response to *M.tb*, but one study designed to address this issue, found that upon priming with *M.tb* or a subunit antigen (Ag85B) these cells were able to produce IFN-γ. Interestingly this was not dependent only on IL-12 production, since IL-12 antibody in this (highly experimental) system did not eliminate all IFN-γ production.[69] Mice, deficient in functional CD8+ cells, are more susceptible to mycobacterial infection and show enhanced pathology in the lungs.[70] The role for CD8+ cells in human TB is less clearly defined. They are present at the site of disease, but in one study successful treatment resulted in decreasing numbers of CD8+ cells, while numbers of CD4+ cells rose in peripheral blood and BAL fluids from TB patients.[71] They do however seem to contribute to cytolitic activity of human *M.tb* infected cells[72] as well as IFN-γ production upon stimulation of PBMCs with *M.tb*.[73]

In much the same way the role of $\gamma\delta$ T-cells in human TB is inconclusive. Expansion of these cells in peripheral blood from healthy Mantoux positive individuals upon stimulation with mycobacterial antigens and decreased presence of $\gamma\delta$ T-cells in BALs from diseased patients relative to non-TB diseased individuals suggest a role in protection

from disease.[74] They are probably involved in the initial response to infection and are potent producers of IFN-γ after mycobacterial stimulation. In a recent review on the subject[75] a complementary role to CD4+ cells is suggested for γδ T-cells, primarily in and around maturing granulomas.

Cytokines

Knowledge of the dominant cytokines (type 1 vs 2) produced by particular cells involved in the immune response may lead to valuable insight in the pathogenesis of a variety of diseases. Over the past decade or so, the role of cytokines in mycobacterial infections, as in other infectious diseases, has been widely studied. Production of a type 1 cytokine profile by T-cells is crucial for protection from mycobacterial disease, as can be inferred from numerous animal and human studies. Whether a type 2 profile is linked to susceptibility to disease is less certain in humans as opposed to some animals.

IFN-γ

A central role for IFN-γ in protection from TB is generally assumed on the basis of animal and human data. As the main activating factor for murine macrophages to enhance mycobacterial killing, it has been well researched in mice models of TB and defined as being crucial to the host's defence. However, human macrophage cell lines are not able to kill mycobacteria as effectively *in vitro* upon activation with exogenous IFN-γ. An intermediate role for $1,25(OH)_2D_3$ in this respect has been shown.[76] Recently a likely mechanism by which $1,25(OH)_2D_3$ can enhance mycobacterial killing, namely by upgrading nitric oxide production, has been described.[77] IFN-γ, in conjunction with TNF-α, does have the capacity to stimulate $1,25(OH)_2D_3$ production by human macrophages *in vitro* through activation of 1-α hydroxylase, the enzyme that converts $25(OH)D_3$ to $1,25(OH)_2D_3$.[78] This link in the pathogenesis of human TB is further strengthened by the fact, that alveolar macrophages from TB patients, recovered by BAL, produce more $1,25(OH)_2D_3$ than control patients suffering from sarcoid.[79]

IFN-γ receptor deficiency is now a well established risk factor promoting disseminated mycobacterial disease in humans.[80, 81]

A number of studies of IFN-γ production by PBMCs from adult patients suffering from active TB, have found decreased levels if compared to healthy Mantoux positive controls[82,83], although this diminished production in the periphery has not always been a consistent finding.[84] However, at the site of infection, in pleural and BAL fluids of TB patients, the production of this cytokine seems abundant[85,86], suggesting compartementalisation of the immune response in TB. A potential negative effect of "too much" IFN-γ at the site of disease might be increased inflammation at the cost of tissue necrosis, but this phenomenon has been more widely attributed to TNF-α.

TNF-α

Human mononuclear cells and alveolar macrophages produce large quantities of TNF-α in response to stimulation with *M.tb* and specific mycobacterial antigens like LAM.[87] *In vitro,* addition of TNF-α to human and animal macrophages enhances mycobacterial killing. *In vivo*, at the site of infection, proper granuloma formation in mice seems, in part, dependent on adequate levels of TNF-α.[88]

Symptoms like fever, cachexia and extensive tissue damage in very ill TB patients have, on the other hand, also been attributed to excessive amounts of this particular cytokine.[89] In a rabbit model of TBM, high TNF-α levels are correlated with more extensive disease and clinical detoriation of the animals.[90] An additional argument for the role of TNF-α as a contributor to immunopathology relates to the finding that thalidomide, a selective inhibitor of TNF-α production by monocytes, seems to reverse some of the tissue necrosis in mice.[91]

IL-2

IL-2 is a growth factor for expanding antigen-reactive T-cells, thereby increasing concentrations of macrophage activating factors. In trials of mice with *M.bovis* infection, humans with lepromatous leprosy and humans with multidrug resistant TB who were treated with intra-dermal recombinant IL-2, an enhancement of the antimicrobial response

by a reduction in the bacillary load and improvement of clinical status was shown.[92, 93] Interestingly, in cattle infected with virulent and attenuated strains of *M.bovis*, only the virulent strains induced high levels of IL-2 (and IFN-γ) production by PBMCs .[94]

IL-12

IL-12, an important inducer of type 1 T cell responses, has been shown in increased concentrations in pleural fluids and lymph nodes of TB patients.[95] Cross regulatory interactions with IL-10 have been shown *in vitro* in PBMCs co-cultured with *M.tb Erdman.*[68] Poor granuloma formation in patients with disseminated atypical mycobacterial disease has been documented as a result of either a lack of functional IL-12R complexes, due to a mutation in the IL-12Rβ1 gene, or a mutation in the IL-12p40 gene. [96, 97]

IL-10

The anti-inflammatory cytokine IL-10 is produced in response to *M.tb in vitro* by murine and human macrophages and T cells. Furthermore PBMCs from adult TB patients produce higher levels of this cytokine than controls[98] and elevated serum levels of this cytokine in patients with active TB have been described.[99] Despite a down-regulating effect on inflammatory cytokines like IFN-γ and TNF-α, this might be of potential benefit at the site of disease in limiting tissue damage. IL-10 has been demonstrated at the site of pathology in a study of tuberculous pleural fluids[86], but not in a recent study where BAL fluids from patients with intrapulmonary TB were examined.[85]

TGF-β

TGF-β production is induced by *M.tb* and PPD in blood monocytes and TGF-β mRNA is expressed in epitheloid and Langhans cells of tuberculous granulomas.[100] Cell wall components from *M.tb* (LAM) and secreted mycobacterial proteins (30-kDa α antigen) are also able to stimulate TGF-β production by monocytes.[101] Higher values of this cytokine are measured in the supernatants of monocyte cultures from patients as compared to those from healthy tuberculin reactors. This might result in the suppression of peripheral T-cell responses in TB patients, by reducing IL-12Rβ1 and IL-12Rβ2

expression, a phenomenon reversible *in vitro* by addition of natural inhibitors of TGF-β to the culture system.[102] At the site of infection there may a potential beneficial effect of this protein as an antagonist and down regulator of TNF-α and thus in limiting immmunopathogenic effects. Furthermore TGF-β can promote fibrosis and angioneogenesis.[103]

A variety of other cytokines, chemokines and other soluble factors have been studied in relation to *M.tb* in a number of experimental models. Thus far information on their role in human TB is very limited. Of particular interest in this respect is the function of IL-4, a prominent type 2 cytokine in humans and animals and recognized down-regulator of IFN-γ production. In murine models of TB, lymphocytes from susceptible mice produce higher levels of IL-4 than resistant mice, although mice with disrupted genes for IL-4 display a normal resistance to infection with *M.tb*.[104] Nor has it been shown in any consistent way that elevated IL-4 production is related to disease in humans, perhaps in part due to technical constraints in measuring IL-4.[105] Elevated levels of IL-5, another type 2 cytokine produced by lymphocytes and of interest because of its role in helminthic infections, have recently been implicated in diminished protection from disease in humans.[106]

The granuloma

Granulomatous inflammation can be defined as a special variety of chronic inflammation in which cells of the mononuclear phagocyte system are predominant and take the form of macrophages, epithelioid cells and multinucleated giant cells. In addition a mixture of other cells, lymphocytes, plasma cells and fibroblasts are usually present.[107] A granuloma is usually regarded as a means by which the host defends itself through walling off a persistent exogenous or endogenous agent, hence containing it and possibly destroying it. Tissue damage during the active phase of inflammation and fibrosis during the healing process are frequent results. The tuberculous granuloma is classically characterised by the presence of central caseous necrosis in which acid-fast bacilli are present, although

neither a non-caseating granuloma nor the abcence of identified acid-fast bacilli exclude a tuberculous origin.

Developmental stages of granuloma formation have been best studied in the rabbit models of Lurie.[108] On the basis of these studies two, somewhat different, immune responses by the host are recognized. The cell mediated immunity (CMI) reaction is responsible for activating macrophages to kill ingested bacillli, through expansion of the population of specific T-cells and attraction of monocytes from the bloodstream into the lesion. The delayed type hypersensitivity (DTH) reaction kills bacilli-laden non-activated macrophages, thereby eliminating the intra-cellular environment so favorable for bacillary growth. DTH responses are accompanied by caseous tissue necrosis. A positive Mantoux skin test is regarded as a DTH reaction. Both responses, CMI and DTH, are in principal complementary and beneficial to the host, but have failed one another when the host is diseased. If failing, the DTH reaction in particular can cause important pathology within the host. The types of cytokines produced by the cells within the granuloma are thought to play an important role in its destiny. This has best been studied *in situ* in leprosy, where type 2 cytokines are predominant in lepromatous leprosy, which is the most severe presentation of this disease.[109] In tuberculoid leprosy, the more benign variant, type 1 cytokines dominate. Deficiencies in receptor genes for the type 1 cytokines IFN-γ and IL-12 are correlated with a spectrum of defective granuloma formation in individual patients with mycobacterial infections.[81] Steroids have been shown to influence granuloma formation and disease outcome in experimental models. Dehydroepiandrosterone (DHEAS) and cortisol (if given exogenously to mice infected with *M.tb*) differentially influence granulomatous pathology with DHEAS favoring a type 1 and cortisol a type 2 dominant cytokine profile. However outcome of disease seems to depend quite critically on the ratio of their concentrations and timing of administration. At appropriate dosages and intervals they may both have beneficial effects.[110]

Immunobiology of tuberculosis during childhood

Studies of the immune responses related to TB in children have historically been driven by the need for improvement of diagnostic tools and vaccination strategies. Antibody mediated responses have been the focus of many of these studies and have, unfortunately, yielded very little information in either of these areas. Very few studies involving children and their cellular immune responses in relation to TB have been published. Dieli et al[111] compared lymphocyte proliferation after stimulation of PBMCs with PPD and a peptide derived from the 38kDa *M.tb* protein (p38G) between children with TB and healthy Mantoux positive as well as Mantoux negative children. Stimulation with PPD resulted in similar proliferative responses by patients and Mantoux positive controls. p38G stimulation however in diminished responses by the patient group. This study was also designed to look at potential differences in αβ and γδ T cells enumeration's before and after stimulation; there were none before stimulation and patients and healthy controls did not differ in this respect after stimulation. Swaminathan et al[112] report on cytokine production by PBMCs from children with TB compared to Mantoux positive children without TB. IFN-γ production was diminished in the TB diseased group, whereas IL-12, IL-4 and IL-10 productions were similar in the two groups. In the Gambia, studies have been conducted in young infants to measure IFN-γ production after BCG vaccination.[113] It was found that PBMCs from vaccinated young infants are quite capable to produce this cytokine after stimulation with BCG *in vitro*. Donald et al[114] have found high levels of IFN-γ in cerebrospinal fluids of patients with TBM at diagnosis and persisting during 4 weeks on treatment whereas the latter is not a phenomenon commonly found in other types of bacterial meningitis.

Immunodiagnostics for TB

Immune-based diagnosis of TB has, for a long time, been impeded by the fact that there were no reliable methods to measure functional cellular immunity, while antibody-based technology proved to be neither sensitive, nor specific enough. Over the past decades, our improved understanding of the cellular immune system has resulted in an explosion of

techniques to measure cellular immune function, form gene to protein. The urgent need for better and faster diagnostic tools in TB as well as identifiable correlates of protection from TB, has recently driven the research into human cellular immune responses in TB. Results are not always consistent and sometimes confusing, often due to the practical constraints of measuring a response to a pathogen that causes disease in only 10% of infected persons during their life-time, while having a seemingly comfortable symbiotic host-parasite relationship in the other 90%. Furthermore the disease causing mechanisms are multi-factorial in origin, the major site of pathology is not easily accessible for diagnostic purposes and *M.tb* shares a substantial number of antigens with other, non-pathogenic, mycobacterial species. An extensive array of antigens, from mycobacterial wall components to culture filtrate proteins, has been used to probe the immune response in TB. PPD, prepared by precipitation of proteins from heat-killed *M.tb*, has been the most widely used, but is not specific for *M.tb*. ESAT-6, a short term culture filtrate protein of low molecular mass, is one of the interesting antigens for diagnostic purposes and has been quite extensively studied in different populations. Its gene is restricted to the tuberculosis complex, including *M.bovis* but importantly none of the BCG strains, and only three environmental mycobacteria (*M.kanasii, M.marinum and M.africanum*).[115] ESAT-6 stimulation induces IFN-γ production by PBMCs from TB patients, but not in healthy subjects that have been immunised with BCG. Identified contacts of TB patients have been to shown to have variable ESAT-6 responses[116], possibly related to their actual infection with *M.tb* as has been implied in another study[117].

Tuberculosis: age as a variable

Risk for infection

Although a range of animals can be infected with *M.tb* and become diseased, humans are the only known natural reservoir for *M.tb*. Transmission usually occurs through inhalation of infective particles from a diseased individual, who generates these in the air by coughing, sneezing or merely speaking. The risk of a person getting infected is dependent on a number of known and unknown factors. Infection occurs more readily, but not exclusively[118], from a source that is sputum-smear positive than from a smear-negative index case. The number of infective bacilli that are present in the air plays a role and consequently the space (size, ventilation) in which one is confined at the time of inhalation, as well as the amount of time spent in contact with the infective source.[119] Socioeconomic conditions like poverty, crowding and urbanization are linked to increased rates of infection. The virulence of the strain is most probably important, as can be postulated from reports on outbreaks.[120] Ethnicity may play a role as has been shown in a report on residents in nursing homes in Arkansas (US) when twice as many blacks as whites became infected[121], but not all studies confirm this[122]. The ability of a person to resist infection may also be dependent on immunological mechanisms, as can be assumed from Lurie's experimental rabbit model, in which he showed that natively resistant rabbits were less susceptible to the establishment of infection.[123] No human homologue for resistance to infection has been found to date.

Whether age as an independent variable influences the risk of getting infected with *M.tb* after exposure is uncertain. Certainly the prevalence of infection rises throughout childhood and adolescence in any given population[124], but most studies have not included enough very young children to make a valid statement concerning this matter. Some animal data suggest that newborns are more susceptible to infection.[125]

Mantoux Skin testing

The most sensitive diagnostic tool we have for identifying infected persons is the Mantoux skin test, whereby purified protein derivate (PPD, derived from *M.tb* culture filtrates) is injected intradermally. The size of the skin induration at the site of injection, caused by a delayed hypersensitivity reaction in the skin, is measured 72 hours after injection. The test becomes positive 6-8 weeks after infection. It is used for diagnostic purposes in individuals and as an epidemiologic tool to determine the prevalence of infection in populations.

However, despite it being our most sensitive tool for diagnosis of infection, it is an inadequate one. There are technical difficulties in performing the test that may raise uncertainty over some results. Prior BCG vaccination and exposure to environmental mycobacteria may both influence the size of the induration, thus diminishing the specificity of the test. An estimated 5% of people are anergic to the test and patients with TB can revert to a negative skin test during the acute phase of their illness. The positive and negative predictive values of the test are dependent on the prevalence of infection with *M.tb* in a given community.[126] Partly for this reason, the cut-off point that is regarded as a positive test result varies between countries and populations. Evidence for a recent infection in an individual is only provided by a recent skin test conversion from negative to positive and previous test results are not always available. Certain conditions, like AIDS, can render people non-responsive to PPD. In adult AIDS patients, this non-responsiveness is related to the number of CD4+ cells in peripheral blood.[127] A childhood infection like measles may also depress the magnitude of the induration for more than 10 weeks after the acute disease period.[128]

Age is an aspect to consider in the interpretation of Mantoux skin test results. Children have higher rates of anergy than older people do.[129, 130] Furthermore, the length of time after BCG vaccination has been shown to have an effect on the magnitude of the induration.[131] Under epidemic conditions a positive skin test in a person older than five, is not necessarily indicative of a recent infection, because of the high annual risk of

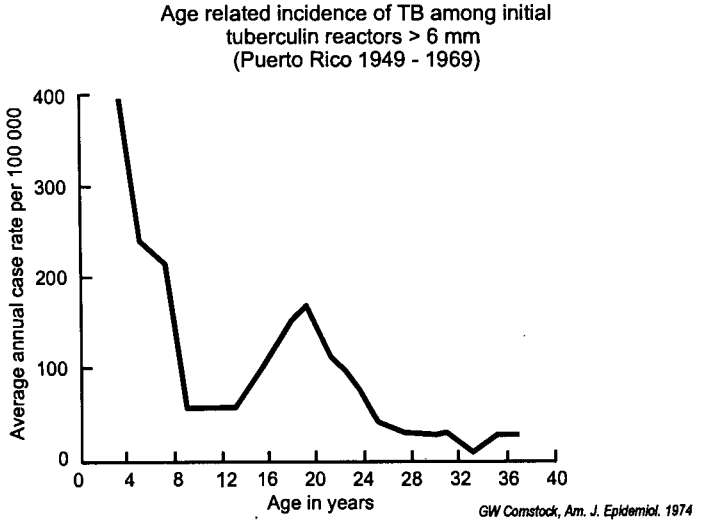
infection. The epidemiological context, including that of the country of origin of immigrant populations and their relevant risk of infection, has to be taken into consideration when results are being interpreted.

Risk for disease

The risk of becoming diseased after infection is also dependent on a number of known and probably a greater number of unknown factors. Time since infection is a risk factor, the risk being highest (45%) within two years of infection, while the lifetime risk of disease development after infection is estimated at 10%.[132] This is, however, dependent on the immune status of the infected individual, genetic factors, sex and the age at which one is infected. HIV infected persons have an approximate 10% per year risk of becoming diseased. However that risk varies with their immune status: the lower the CD4+ counts the higher the risk of tuberculous disease[133] and, interestingly, also the higher the chance of getting disseminated, rather than pulmonary types of TB.[134] The timing of infection with *M.tb* also matters: the risk for tuberculous disease is larger if the infection with *M.tb* occurs after HIV infection, rather than the other way around.[132] For other conditions influencing the immune system, including immunosuppressive drugs like corticosteroids, the increased risk for disease has been much less well quantified. Even without a known or demonstrable immunodeficiency, the host's immune response is still arguably the most important determinant of disease. But again socioeconomic circumstances are influential as well: known risk factors from epidemiological evidence are malnutrition and general poor health. Although speculation around certain vitamin and mineral deficiencies as contributors to the development of TB exists, unequivocal proof has not yet been provided for most of them. Vitamin D deficiency is possibly one such risk factor for which at least some *in vitro* data[77,135] support the epidemiological evidence of vitamin D deficiency (defined as low 25-hydroxyvitamin D3) contributing to the risk for tuberculous disease in parts of the world.[136] However, no *in vivo* study, controlled for seasonality, ethnicity and age, has had the power to show this in humans to date. Vitamin A deficiency may be common in patients with TB.[137] Recently a number of genetic factors have been identified in susceptibility to TB. Four NRAMP1 gene polymorphisms

have been linked to the development of tuberculous disease in the Gambian population.[138] In the same population a vitamin D receptor (VDR) polymorphism has been linked to disease.[139] The main problem with both these studies is the choice of the control population: blood bank donors without known tuberculin status. The function of the NRAMP gene in humans is ill defined, while polymorphisms in the murine NRAMP genes are related to risk of infection rather than disease[140] and the VDR polymorphisms in humans relate to bone densities rather than vitamin D status. Much easier to explain from a functional point of view, is, as already mentioned in the previous chapter on the immunobiology of TB, the vulnerability that persons with a partial or complete interferon-γ (IFN-γ) or IL-12 receptor deficiency have for diseases caused by mycobacterial species.[81]

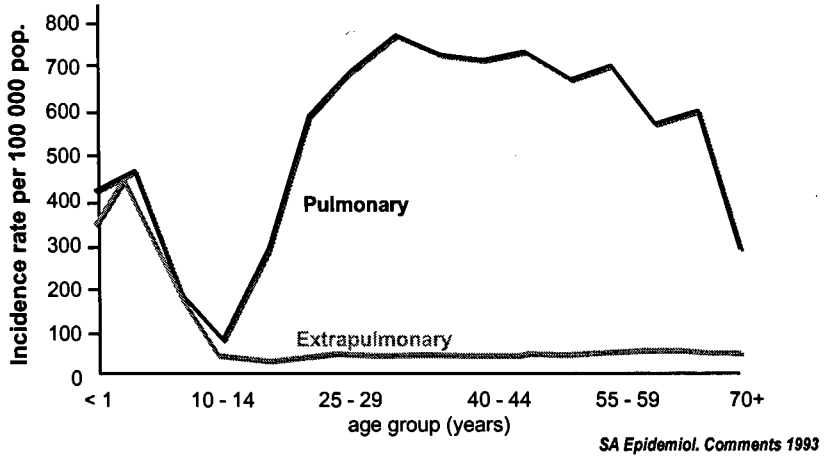
Age has long been recognised as an important determinant for the development of TB. In *Comstock's* large study of skin testing in Puerto Rican children the major risk factor for developing disease in tuberculin reactors was age.[141]



Children under 4 years of age had the highest rates of disease (and the most serious complications); a second peak was observed in persons of about 20 years of age. In any population children under the age of five have relatively high rates of disease after

infection, falling to a life time low between the ages of six to about twelve ("the golden age of TB"), only to rise again during adolescence. Depending on the prevalence of infection in a given population, the incidence of disease continues to rise, peaking at about 35 years (under epidemic conditions) or in old age (in low prevalence areas). The age-related incidence of disease in the Western Cape (SA)[142], an area with a very high prevalence of infection, is shown in the figure below:

TB incidence Western Cape, South Africa, 1989



The shape of this curve is similar to others from populations with a high incidence of TB and shows remarkable similarity to the age-mortality curves from the pre-chemotherapy era as described in the classical paper by WH Frost.[143] The prevalence of infection in any given population is arguably the single most important risk for age-related morbidity and hence the shape of the curve[144], but one cannot escape the notion that age may be an independent variable in the relative risk for development of disease after infection with *M.tb*.

Depending on the age period under study, sex differences are recognised. Boys are usually over represented in the very young children.[145, 146] During adolescence and early adulthood, the incidence of disease in most populations is highest in females.[147] After

about the age of twenty-five up to menopause, females consistently have lower rates of TB than males.

Tuberculous disease

The natural history of TB is intrinsically difficult to study, not in the least because of the lifelong risk of disease after infection and the variability of signs and symptoms. Furthermore, we have to depend largely on reports from the pre-chemotherapy era. To complicate matters not all authors have agreed on terminology for the different expressions of disease, something that still hampers present day communications on TB. A concise description of the pathology following infection with *M.tb* is given below, drawn from a large number of references of which the seminal book "The pathogenesis of tuberculosis" by Arnold Rich[148] and a more recent publication written by John McKinney et al[149] were the most influential.

The primary focus is the structural change in a tissue at the site of implantation of the tubercle bacillus. The primary complex is composed of the primary focus and the accompanying regional adenitis. Since the primary focus is nearly always located in the lungs, the primarily affected regional lymph nodes are usually also located intrathoracically. True congenital TB is one of the rare exceptions with a primary focus in the liver and the regional adenitis around the porta of the liver. Most authors also argue that a degree of lympho-haematogenous spread of bacilli takes place after establishment of infection. The subsequent fate of the primary complex varies greatly and is dependent to a large extent on the local and systemic immune response of the host. It is of importance to realize that the immune system of the great majority of people deals with an infection without any signs or symptoms other than a positive skin test conversion. The primary complex may vanish completely before or after calcification. However, in conventional thinking, we consider the host sensitized and consequently, at least partly, protected from complications of a possible reinfection. Primary TB is, by definition, any form of tuberculous disease that is the consequence of a primary infection, even if the

complication occurs years after the first encounter with the pathogen. Wallgren's timetable of primary untreated tuberculosis is still a useful tool to illustrate this[150].

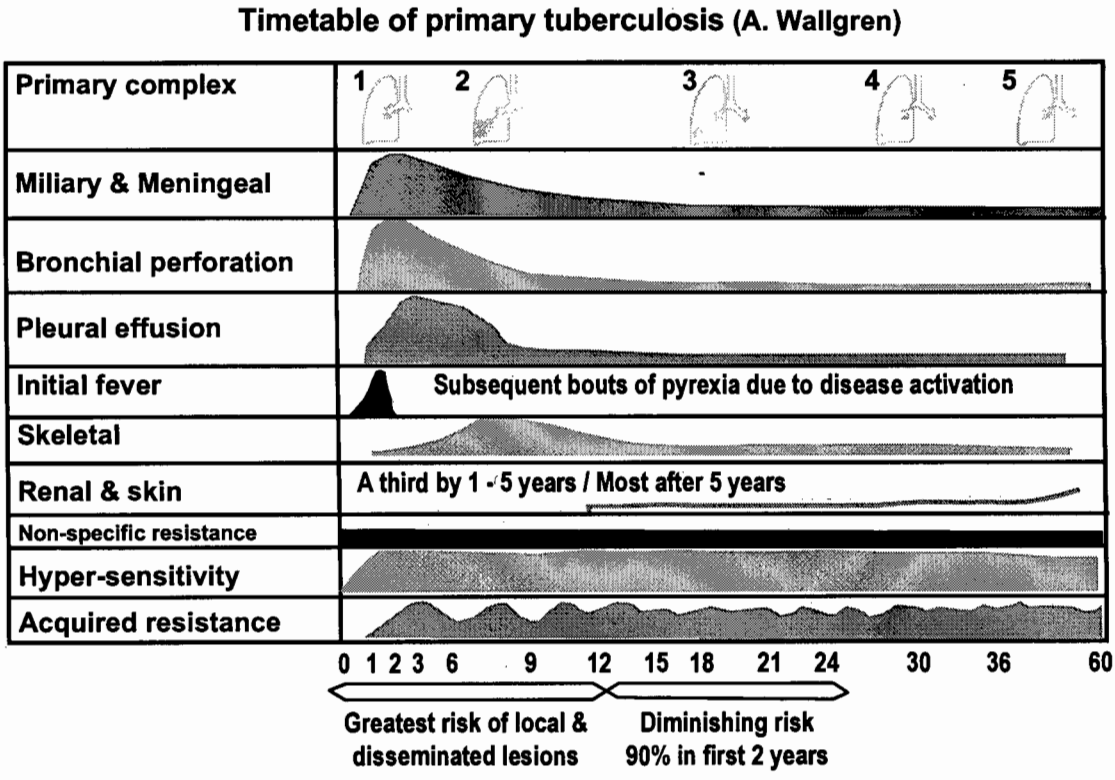


Fig: schematic representation of Wallgrens' timetable for primary tuberculosis

"Postprimary TB", "chronic pulmonary TB" or "adult-type TB" are synonyms for the type of TB that becomes the main presentation of disease in late adolescence and adulthood. It is characterized by more or less extensive lung parenchym involvement, most often in the upper lobes of the lung, with tissue necrosis and characteristically the development of cavities. There is little or no lymph node involvement. Cavities with central liquefaction and tissue necrosis offer favorable circumstances for the growth of *M.tb*, which is partly the reason why patients with this type of TB harbour many organisms. An open communication of a cavity with the airways gives the opportunity to cough up organisms and cause transmission of *M.tb*. It is generally assumed that patients with postprimary TB have been in contact with *M.tb* before, were immunologically sensitised to the bacillus and attained a certain degree of immunity to reinfection. There

are two pathways by which postprimary TB is thought to develop. Either by reactivation of dormant bacilli after a period of latency (endogenous reinfection) or by reinfection from a new contact (exogenous reinfection). In both instances the immune system of the patient has failed to protect from disease. What percentage of the patients with postprimary TB have been subject to reactivation or reinfection is open to research and debate, but is probably dependent on the epidemiological circumstances. In low prevalence areas reactivation is the most likely mechanism, in high prevalence areas reinfection is a more likely occurrence.[151, 152]

The age at which one becomes infected is consequently of great importance to the clinical presentation of disease. Primary tuberculosis is the commonest presentation of TB from early childhood to the teenage years. Most often the disease is a complication of enlarged hilar and paratracheal lymph nodes that may or may not cause collaps or hyperinflation of a lung segment and/or ulcerative erosion of the bronchi. (endo- or lympho-bronchial tuberculosis). Inflammatory ulceration of the primary focus by itself or aspiration of tuberculous material may cause further pathology in the lungs and in rare instances even cavitation might develop as a result. On the chest radiograph, differentiation of these different pathogeneses is not always easy. Extensive bronchopneumonia can occur as a result of widespread deposition of tuberculous material from whatever source. A pleural reaction may be seen during the course of these events, but large pleural effusions are thought to result from a different mechanism: the discharge of tuberculous material in the pleural space and a subsequent local hypersensitivity reaction. Large pleural effusions are very uncommon in early childhood, but become more frequent during adolescent years. The apparent age-related switch from primary to postprimary TB as the dominant form of clinical presentation during the adolescent years has been the topic of much speculation, but little research. The hypothesis, that hormones, as contributors to the growth spurt and sexual maturation of adolescents, might play a role in the changing pathology during this age period, has been put forward for quite some time. Recently, research in experimental models has revealed the influence of certain hormones on the course of TB and it might be apprpriate to investigate this in humans.[153]

Children are more prone to develop extrapulmonary complications than immunocompetent adults. Tuberculous meningitis (TBM) is in general an early complication after infection (90% within 1 year). For unknown reasons occurrence of TBM is especially prevalent in very young children, under the age of two years.[154]

Treatment

Although chemotherapeutic agents for TB have had major positive implications for the survival rates of patients, "cure" of TB was possible in the pre-chemotherapy era. Various regimens of sunlight, diet and supplements as well as more invasive procedures like injecting ergocalciferol, tuberculin or syphillis bacteriae have all been claimed as curative, at some point. With hindsight, a number of these therapies could have boosted the immune response of patients and may therefore have been beneficial.

There are however enough data to argue that pulmonary primary TB has a more favourable natural history than postprimary TB. The greater chance of surviving intrathoracic adenopathy, even with intrapulmonary complications, without important long term sequela has contributed to the notion that "childhood" primary TB is a relatively benign disease as opposed to the "adult" postprimary form. This does not hold true for the forms of primary TB, where haematogenous spread is the prime mode of bacterial dissemination, a more frequent event in young children than adults. Tuberculous meningitis in particular, is well known for its devastating implications for survival and permanent cerebral damage. Thus age is a factor to account for in evaluating cure rates and devising consequent therapeutic regimens.

Prophylaxis

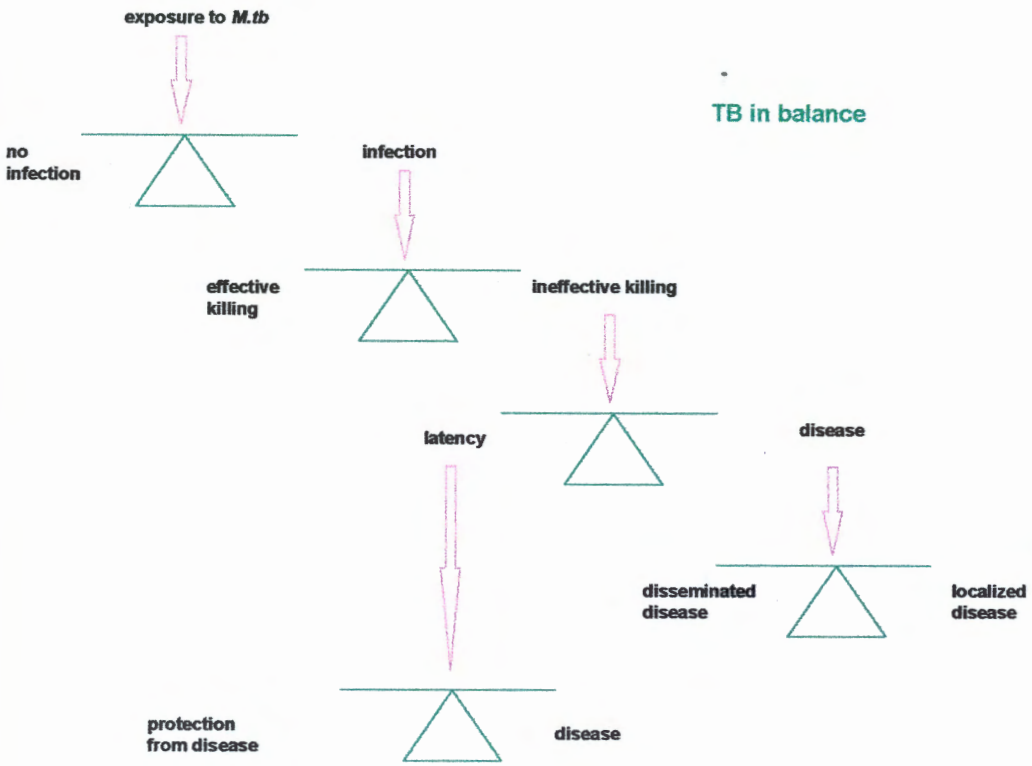
At present time, two measures for preventing TB are in use: vaccination and treatment of latent infection (= prophylactic chemotherapy).

Bacille Calmette-Guérin (BCG) is worldwide the most frequently given vaccine, despite being the least effective of the currently used range of childhood vaccines. Prevention of tuberculous meningitis during childhood and protection from leprosy after BCG vaccination has quite convincingly been shown in some, but not all, populations. Efficacy of BCG appears to be dependent on the population to which it is given, which in turn is probably in part determined by latitude and exposure to environmental mycobacteria in a given area.[155] Ethnicity and age at which the vaccine is given might be other factors, although not well researched in comparative studies. The large English MRC trial of vaccination in 14 year olds (conducted in the fifties with a follow-up of 7-10 years) did show a considerable protection from disease after vaccination with either BCG or vole bacillus at that age.[156a] The incidence of TB was less in the vaccinated group than in a control group already tuberculin test positive upon entry in the study and subsequently not randomised for vaccination.

Preventive chemotherapy after contact with an infective individual is regarded as beneficial for recent skin test converters of any age. In most countries where the TB incidence is high, this policy is implemented only for children under the age of five. Reasons for this are that the most serious complications after infection within the shortest period of time occur in this age group. Furthermore, it can be assumed that the contact was probably recent. However, one of the few large scale studies that compared preventive chemotherapy to no therapy in persons aged 0-24 years, known to be recently infected (within the previous year) and skin test positive, showed distinctive differences in age related morbidity as a result of preventive therapy. A more significant reduction in incidence of disease in the 10-24 yr old group as compared to the younger age groups was demonstrated.[156b]

Hypothesis: Age, immune-competence and tuberculosis

Exposure to *M.tb* may lead to a "balanced" life-long interplay between host and parasite.



Numerous factors may tip one of these balances at any point in life, but they all result in the "breakdown" of the hosts' immunological defence against the baccillus. As such, the immune competence of the host at a given time is arguably the most important determinant for the development of TB. "At a given time" remains one of the most intriguing, but very vague, issues in the pathogenesis of TB. With all the knowledge of TB that we have gained over the last century, we are still not able to answer the question: "who, when and where".

Tragically, the AIDS epidemic has created in many ways a window of opportunity to reinvestigate all angles of TB, from pathogenesis to treatment and prevention. AIDS has put into focus again the things we knew: that socio-economic circumstances and prevalence of infection as well as prompt treatment and proper treatment adherence are all important. We have learned in the mean time that CD4+ cells, quantitatively and qualitatively, are crucial in the immune response to *M.tb* as well as the clinical expression of TB and that timing of infection in relation to immune=competence of the host matters for outcome.[132,133,134].

As outlined in the previous chapters, children are known to differ from adults with respect to the functioning of their cellular immune system, not only quantitatively with respect to cell types, but, most probably, also qualitatively. An adequate cellular immune response is a crucial determinant to the risk for development of TB after infection with *M.tb*. Age-related differences in susceptibility to and clinical expression of TB are well recognised. It may thus be hypothesised that age-related immune competence may be a variable in the pathogenesis of tuberculosis.

Frost revisited:

After careful examination of the age-related TB mortality rates in the Massachusetts area over a period of 50 years (1880-1930), WH Frost noted in 1935: "... We are forced then to recognize, as at least highly probable, that the predominant factor in the up-and down movement of mortality along the age-scale is change in human resistance. And this is a complex of which we have very little knowledge except the plain fact that age and prior exposure bring no such immunity against tuberculosis as they establish against many of the acute infections..."[143]

Aims of this study

1. To characterise the cellular immune response in TB patients and healthy individuals of different ages living in a region highly endemic for TB.

2. To relate the results to the clinical expression of TB at different ages

To achieve these objectives a cross-sectional study was designed, whereby the cellular immune response of included study subjects was investigated by culturing their whole blood and measuring lymphocyte proliferation and cytokine levels after stimulation with a mitogen (PHA) and defined mycobacterial antigens (PPD and ESAT-6).

Materials and methods

Description of study subjects

Demographics

This study is set in the Western Cape Province of South Africa, where the case load of TB was around 1000/100.000 inhabitants in the 1985-1994 period.[157]
All the subjects in this study were of "coloured" origin, people who form a distinct anthropological entity of mixed Khoi-San, African, Malay and European origin, living predominantly in the Western Cape Province. Although from variable socioeconomic backgrounds, none of the study subjects were from the affluent sectors of South African society.

Recruitment

The subjects of this study were recruited from May 1998 - November 1999 from:
- The local authority health clinics in the City of Tygerberg (with permission from Dr I Toms, Head of Health Services, City of Tygerberg)
- The paediatric wards of the Tygerberg Hospital (with permission from Prof. PB Hesseling, Head of the Department of Paediatrics and Child Health)
- The paediatric surgical ward of Tygerberg Hospital, prior to routine surgery for non-infectious diseases (with permission from Prof. SW Moore, Head of the Department of Paediatric Surgery, Tygerberg Hospital).

Consent and ethical approval

Informed written consent was obtained from every subject 14 years and older or his/her caretaker when the participant was younger than 14 years. *(Copies of the consent form*

and patient information sheet are on pp 175 -176) All subjects volunteered to participate in this study and did not receive any compensation for participation.

HIV-test counseling was given to each subject and his/her caretaker.

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

Inclusion criteria

Subjects of three different categories were included in the study:

I. Patients with tuberculosis (TB)

TB patients were recruited before initiation of treatment. No patient was known with any disease other than TB or on treatment with corticosteroids at the time of recruitment.

After diagnosis of TB, the patients were fully treated with combination therapy under direct supervision (DOTs) in accordance with the South African National TB Control Programme.[158]

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.tb* from 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).

. A diagnosis of tuberculous meningitis on the basis of clinical findings, cerebrospinal fluid analysis and CT scan of the brain.

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

(The method used for Mantoux skin testing is described under methods of investigation)

. A person with a Mantoux skin test induration ≥15mm and no recent history or clinical symptoms of any disease.

. A chest radiograph (CR) was evaluated upon entry in the study to exclude radiological signs of tuberculous disease for all persons under the age of 12 yrs.

. In accordance with the South African National TB Control Programme, all healthy Mantoux pos children under the age of five received supervised prophylactic chemotherapy (INH + rifampicin for a period of three months for children < 2 yrs, INH for 6 months for children 2-5 yrs)

III. Healthy control subjects, who had a negative tuberculin skin test (Mantoux)

. A person with a Mantoux skin test induration <15mm and no a recent history or clinical symptoms of any disease.

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 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 months' period after giving birth if breastfeeding
- known immune deficiency or a positive HIV-1/HIV-2 test

- signs of any overt infection other than TB
- fever (>38°C) and/or malnutrition, as measured by weight under the 3rd percentile for age for healthy Mantoux pos and neg subjects

Methods of investigation

Mantoux skin testing was done with 0.1ml (5TU) Japanese freeze dried tuberculin, reconstituted shortly before use and injected intradermally on the volar aspect of the forearm by an experienced health care worker. The transverse diameter of induration was read 48-72 hours afterwards, with the ballpoint method, by an experienced health care worker. The Mantoux skin test was considered positive if the transverse diameter of induration was 15mm or more and negative if this measurement was less than 15mm. We used a 15mm skin induration as a cutoff value for to discriminate Mantoux negative from Mantoux positive subjects in accordance with the South African Tuberculosis Control Programme.[158]

All the subjects underwent Mantoux skin testing, except the sputum-smear positive patients who were older than 20 years. Most adult TB patients in this region exhibit a strongly positive Mantoux reaction with considerable discomfort (itching, blistering and ulcerating). For ethical reasons it was therefore felt unjust to skin test the adult patients. Furthermore when a Tine skin test (used as a screening test in some health clinics) had been done and was grade II-IV (n = 4), a skin test was not redone with the Mantoux method.

Chest radiographs (PA and lateral, CR) were read by a team of 3 experienced clinicians and classified as:

- Primary TB if there was hilar or paratracheal adenopathy with or without lung-parenchym involvement.
- Postprimary TB if infiltrating abnormalities in the lung-parenchym mainly affected the upper lobes, with or without cavities.

- Pleural effusion if uni- or bilateral pleural effusion without lung-parenchym involvement was seen.
- No TB if there were no abnormalities seen.

Microbiological confirmation of primary intrathoracic TB is notoriously difficult, due to the problems in obtaining sputum from young children and the relatively low numbers of bacilli these children harbour. Even modern techniques like PCR on bronchial lavage (BAL) specimens[159, 160] have not proven to be more sensitive than the "best" we currently have: culture of gastric aspirates taken in the morning before feeding and initiation of therapy (around 40% sensitivity in children diagnosed with TB[161]). A large proportion of the young patients was recruited from the local health clinics. We felt it was not justified to subject them to the (good) practice of obtaining proper early morning gastric aspirates. They would have had to be admitted to a hospital and there is no justification for this in a region where health care resources are very scarce. Our set criteria for identifying a patient as having primary intrathoracic TB are internationally recognised and every care has been taken to apply them to each patient. Since hilar adenopathy due to TB can have a relatively benign prognosis with few symptoms, we did justify a CR of all the healthy positive Mantoux controls under 12 yrs of age to exclude the possibility of asymptomatic TB in these children.

Blood was taken from each subject, between 9.00 and 12.00am for a whole blood assay (5 ml in a sodium heparin tube) and kept at room temperature. Furthermore a routine HB and WBC count with cell differentiation was done on each subject and serum was taken.

Serology for HIV-1/HIV-2 was done on all but 3 subjects, with the use of a MEIA (AxSym®, Abbott). None of the subjects included in this study tested positive for HIV-1/HIV-2. The 3 subjects who were not tested for HIV-1/HIV-2 did not have any risk factors for HIV infection.

Full blood cell counts were done at the Department of Haematology, Tygerberg Hospital, using routine laboratory methods.

Total serum IgE levels were measured with a radioimmune assay (Pharmacia, Uppsala, Sweden) at the Department of Chemical Pathology, Tygerberg Hospital.

Description of laboratory methods for whole blood assays

Whole blood cultures

Reagents

All solutions were made and handled under laminar flow conditions.

1. RPMI-1640 cell culture medium (SIGMA, C/N: R 1383). The solution was prepared according to the manufacturers instruction with the addition of 100mg PenicillinG Sodium/Streptomycin Sulphate, (Highveld Biologicals LTD, C/N:2276), 10 mL extra L-glutamine (200mM; Highveld Biologicals LTD, C/N:2342) and 174µL β-mercapto ethanol, at pH 7.2 before filter strerilisation. The medium was kept at 4°C.

2. PBS (Phosphate Buffered Saline) was made from 8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, dissolved in 1L destilled H_2O and filter sterilised.

3. PHA-P (Phytohemaglutinin-Sigma, C/N: L-8754, stored at 4°C). 1 mg PHA-P was dissolved in 1 mL of PBS (1mg/mL) and filter sterilized, aliquoted in 100µL fractions and freezed at -20°C before use. Aliquots were thawed shortly before use and diluted in RPMI to obtain a stock solution at a concentration of 100µg/ml.

4.PPD (Purified Protein Derivate, kindly provided by Prof. S Ress, Dept of Medicine-Clinical Immunology, Groote Schuur Hospital, Cape Town at a concentration of 10mg/mL) was stored 4°C. Shortly before use, a dilution was made with RPMI to obtain a stock solution at a concentration of 33µg/mL.

5. ESAT-6 (kindly provided by Dr P Andersen, Staten Seruminstitut, Copenhagen, Denmark) was frozen at -20°C in aliquots of 20µl (0.5mg/mL). Aliquots were thawed shortly before use and diluted with RPMI to obtain a stock solution at a concentration of 10µg/mL.

6. Methyl-^{3}H Thymidine (Amersham, C/N: TRK120-1mCi). Shortly before use, a dilution was made with RPMI to obtain a stock solution at a concentration of 25µCi/mL.

Culture conditions

All sodium heparinised blood samples were processed within a maximum of 2-3 hours following venous puncture. All processing took place under laminar flow conditions

Blood samples were diluted 1:10 in RPMI medium. 180µl of diluted sample was plated per well in a U-bottom 96-well plate.

20µL of stock solution was added to appropriate wells to obtain the following final concentration of stimulants in the wells:

- PHA: 10µg/mL
- PPD: 3.3µg/mL
- ESAT-6: 1µg/mL

20 wells were incubated with 20µL of RPMI to obtain non-stimulated, negative control wells.

The plate was incubated at 37°C in 5% CO_2 (NAPCO, water jacketted CO_2 incubator)

Pulsing of lymphocytes with Methyl-^{3}H Thymidine (20µL, final concentration 2.5µCi) was done, in triplicate wells, on the following days:

Day 3 for PHA stimulated wells

Day 6 for PPD and ESAT-6 stimulated wells

Triplicate negative control wells were pulsed on each of these days as well.
The total pulsing time was 20 hours for each well.
Collection of pulsed wells, after mixing, was done on:

Day 4 for PHA stimulated wells

Day 7 for PPD and ESAT-6 stimulated wells

The contents of the wells were transferred to 96-well flat-bottomed plates and immediately frozen at -20°C for future assaying of lymphocyte proliferation.

On days 3, 4 and 7 after proliferation, supernatants in the other wells were removed from the plates. Supernatants from wells cultured with identical stimulants were pooled, mixed and aliquoted in Eppendorf tubes after which they were immediately frozen in -20°C for cytokine measurements at a later time.

Lymphocyte proliferation assays

The 96-well flat bottomed plates were defrosted shortly before cell harvesting. Harvesting was done unto glass fibered filter paper with the help of a Titerteck Cell Harvester. Filter discs were placed, after drying, into scintillation vials with 4.5 ml of scintillation fluid (Insta-Gel Plus-Biotechnik, C/N: 6013399). Cell counting was done in a β-counter (Beckmann LS6500, multiple scintillation counter).
Proliferative responses are given as the mean of counts per minute (cpm) measurements of triplicate stimulated wells after subtraction of the mean cpm measurements of triplicate negative control wells.

Cytokine measurements

Reagents and materials used for the ELISAs:

- Coating plates: MaxiSorp microtiter 96-welled plates (Nunc^{TM} Brand Products C/N:439454)

- Coating solutions: IFN-γ, TNF-α and IL-2: 0.1M Carbonate buffer, pH 9.5
  IL-10: PBS, pH 7.5

- Blocking buffer: PBS with 1% (IFN-γ, IL-2, TNF-α, IL-10) Bovine Serum Albumin (BSA, Sigma Chemical Co, USA, C/N: A6003)

- Wash buffer: PBS with 0.05% Tween-20

- Reagent diluents: PBS with 1% BSA (IL-10) and 0.05% Tween-20 (IFN-γ, TNF-α and IL-2)

- Detection enzyme: HRP-conjugated streptavidin (DAKO A/S, Denmark, C/N: P0546) for IL-10

- Substrate: tetramethylbenzidine (TMB) and hydrogen peroxide (TMB Microwell Peroxidase Substrate System, Kirkegaard & Perry Laboratories INC, C/N: 50-76-00)

- Stop solution: 2N H_2SO_4

Cytokine concentrations of the supernatants from the whole blood cell cultures were measured utilizing the antibody sandwich ELISA principle, for which commercially available kits and reagents were used.

IFN-γ was measured with a DuoSet for human IFN-γ (Genzyme Diagnostics, C/N: 80-3932-00), IL-2 with a DuoSet for human IL-2 (Genzyme Diagnostics, C/N: 80-3606-00) and TNF-α with a DuoSet for human TNF-α (Genzyme Diagnostics, C/N:80-3933-00). For IL-10, the capture antibody was a monoclonal rat anti-human IL-10 (Genzyme Diagnostics, C/N: 80-4315-01), the secondary antibody a biotinylated anti-human IL-10 (Genzyme Diagnostics, C/N: 80-5037-01) and the standard a lyophilized recombinant human IL-10 (Genzyme Diagnostics, C/N: 2261-01)

General principle of the ELISAs:

ELISAs were performed according to the manufacturers' instructions. Reagents and materials, the general principle and specific conditions and dilutions are briefly described. A cytokine specific antibody (capture antibody) in coating solution was bound to a microtiter plate by overnight coating at 2-8^{o}C.

The following day, plates were washed with wash buffer to remove unbound antibody and blocked with a blocking buffer for 2 hours at 37^{o}C. Twofold serial dilutions of recombinant cytokines were used for each standard curve. After removing the coating buffer, supernatant samples (diluted in RPMI), dilutions of standards and blanks were added in duplicate on each plate to form a solid phase bound complex. After incubation plates were decanted and washed several times.

The secondary antibody was added to the wells and incubated for 1-2 hours, where after decanting and washing of plates. If the secondary antibody was directly bound to the detecting enzyme (HRP-streptavidin), the substrate solution was added after decanting and washing procedures. If the secondary antibody was a biotinylated one, an additional incubation was performed with HRP-conjugated streptavidin prior to decanting, washing and addition of substrate.

After 10-15 minutes incubation of the substrate solution, at room temperature, the reaction was stopped with the stop solution.

The optical densities (OD) of the substrates in the wells were then immediately read by microtiter plate reader (Reader 510 Microwell system, Organon Teknika) at 450nM wavelength.

The values of the mean cytokine concentrations of the samples were calculated from the standard curves on each plate using the Prism Graphpad software program.

Standardisation of the assays was obtained after pilot studies, done twice on the same volunteers.

Time courses for the different cytokines were evaluated to obtain the optimal sample day for the various cytokines.

Production of IL-10 in unstimulated (negative control) supernatant samples was found not to be negligible. Consequently the values for this particular cytokine were obtained by subtracting the value of negative control samples from the values of the stimulated supernatant samples.

For the specific ELISA conditions and dilutions of each of the cytokines measured: see table

Table: specific conditions and dilutions of the ELISAs per cytokine measured

cytokine	sample day	sample dilution	standardcurve dilution
IFN-γ			15.6-1000pg/mL
. PHA	4	children <12 yrs: 1:20 all others: 1:40	
. PPD	4 and 7	children <12 yrs: 1:20 all others: 1:40	
. ESAT-6	7	1:5	
IL-2			15.6-1000pg/mL
. PHA	3	1:2	
. PPD	3	1:2	
TNF-α			15.6-1000pg/mL
. PHA	3	1:10	
. PPD	7	1:5	
IL-10			15.6-1000pg/mL
. PHA	3	1:5	
. PPD	3 and 7	1:2	

If the measured values were higher than the highest concentration of the standard, repeat assaying was done, using lower concentrations of the supernatant sample. If the values were lower than the lowest concentration of the standard, an assigned value was 10pg/ml was given *(see methods of analysis)*.

Methods of analysis

Stratification

On the basis of the age-related incidence of TB in the Western Cape (*fig p 34*), subjects included in this study were stratified along age into four "***age groups***":

- 0-5 yrs (age 5 yrs not included))
- 5-12 yrs (age 5yrs included, age 12 yrs not included)
- 12-20 yrs (age 12 yrs included, age 20 yrs not included)
- 20 yrs and older (age 20 yrs included)

Furthermore subjects were classified on the basis of three categories of "***TB status***", according to their status as:

- patient
- Mantoux positive ("***Mantoux pos***")
- Mantoux negative ("***Mantoux neg***")

Consequently twelve different "***study groups***" were recognised.

The "***whole study population***" refers to all the subjects included in the study. When "***patients***" *vs* "***healthy***" subjects are compared, "healthy" subjects include the Mantoux pos *and* Mantoux neg subjects. "***Age***" in years, obtained after calculation from date of birth to date of venous puncture in up to two decimals, has been used as a continuous variable

Limitation of the assays

For all the ELISA assays to measure cytokine production, the lowest concentration on each plated standard curve was 15 pg/ml. Because of the sensitivity of these assays, data on cytokine production below the lowest point of a standard curve for a given assay plate can not be regarded as discriminative. Therefore every value below the lowest point of the standard curve has been given an "assigned value" of 10 pg/ml.

Statistical analysis

In this study non-mathematically transformed data were used for analyses with non-parametric statistical methods. There were low numbers of subjects (minimum 8, maximum 21) in each of the recognised twelve study groups, which necessitated non-parametrical statistical tests. Furthermore non-normal distribution of data in many subgroups, which could not all be normalised by mathematical transformation, prompted the use of non-parametrical tests. Differences among several independent groups were assessed with the Kruskall-Wallis One-Way ANOVA by Ranks test. Differences between two independent groups were assessed with the Wilcoxon-Mann-Whitney *U* test. Associations between continuous variable were tested using Spearman Rank-Order Correlation Coefficient.

Statistics were computed throughout using SPSS (version 9.0) software.

Introduction to results section

This study produced more than 3,700 different data points. To present them in a sensible and readable format has proved to be a challenging task on its own. I have made choices in this respect bearing in mind that:

- Restriction to the main study objectives helps to focus on issues of importance
- Consistency in presentation of data makes a thesis like this easier to read
- Repetitiveness of facts and numbers in a text are unattractive to read

Restriction to study objectives

The aim of this study was to characterise the cellular immune response of TB patients and healthy individuals of different ages. To this end study subjects were stratified along age and healthy control subjects according to their Mantoux skin test response (pos or neg) which served as a correlate for infection with *M.tb*. Furthermore the results were aimed to be related to clinical expression of disease at different ages.

The most important question was thus:

1. Do the data show age-related differences in immunological parameters?

However, analyses should also be done on a number of issues related to the main focus of this study:

2. Do patients of various ages behave differently from healthy controls in mitogen induced responses and do they behave differently after PPD stimulation if compared to healthy Mantoux pos controls?

As can be concluded from the presented data, healthy Mantoux neg subjects did not respond to PPD stimulation with any appreciable quantity of measurements.

3.Does clinical expression of disease make a difference?

Patients with primary and postprimary TB were included in this study and they were compared with age-related healthy controls.

Graphically, the data are presented by boxplots. This format of display allows the clearest overview of all the data in relation to age and TB status and describes best the non-parametric statistical methods that have been used throughout the analyses. Horizontal bars represent median values, boxes interquartile ranges (representing 50% of the values) and whiskers the highest and lowest values, excluding the outliers. Because non-parametrical tests are not very sensitive to outlying variables, the outliers have been omitted from the graphs for optical reasons.

Numerically, the data are described in tables. The interquartiles here are the mathematical 25^{th} and the 75^{th} percentages of all the values per study group. Although they compare in most instances to the interquartile ranges in the boxplots, they are not the same.

The boxplots are more representative of the non-parametric statistical tests that were used, the tables better show the actual figures from a mathematical perspective. This may cause some confusion when one compares the two forms of display and is most obvious in cases where there were a number of outliers or skewed distributions.

Theoretically, one could assume that there should not be any differences between Mantoux pos and Mantoux neg healthy controls in haematologic values or PHA induced responses by age group. Analyses of these measurements showed that there were indeed no significant differences, although the boxplots might sometimes suggest otherwise. Therefore, in the analyses on these particular measurements, patients are compared to all the healthy controls per age group *(p-value^1)* and age groups are compared by their status as patient or healthy control *(p-value^2)*.

PPD induced responses were very low to negligible in Mantoux neg healthy controls. They are represented in the figures and tables for completeness, but it made no sense to include them in any statistical analysis. Patients are compared to Mantoux pos healthy controls per age group *(p-value^1)* and age groups are compared by their status as patient or healthy Mantoux pos controls *(p-value^2)*.

Correlations between the different measurements, including age in years, are important in this study and are presented in table format as a separate chapter "tables on correlations" *(pp 68-74)*, whereby only the significant correlations are given.

The individual chapters of the results' section start with a graphical display of the data, followed by a table and a short text describing some results that cannot be inferred directly from the graph and/or table. The chapters in the results' section end with a summary on the key findings and a brief discussion.

Every attempt has been made to streamline the chapters in the same format; where this is not the case the reason for the deviations is explained.

Although it may be the custom in scientific writing to include all data in the actual text and not only in the tables, I found that doing so made the text indigestible. Because of the extensive use of graphs and tables to display the numerical facts, I have omitted most of them largely from the text, including only those that were important and not represented in any table. With respect to the correlations between the different measurements: The exact p-values and correlation coefficients (ρ) are all specified in the designated chapter mentioned above and the relevant page numbers *(p.68-74)* noted throughout the text.

Clinical characteristics of the study subjects

In- and exclusion of recruited study subjects

A total of 168 subjects were recruited for the study. Of these 18 were excluded from further analysis for the following reasons:

- 5 patients were, according to clinic files, found to have had previous TB treatment or chemoprophylaxis
- 2 patients and 1 control subject who were HIV positive on ELISA testing
- 1 patient had second thoughts on giving consent for HIV testing and was thus not included, nor tested for HIV
- 1 patient was diagnosed as not having TB but Crohn's disease at follow up
- 1 patient had documented complaints of chronic cough, weight loss and CR abnormalities, but repeatedly negative sputum smears for *M.tb* without a conclusive diagnosis over the three years prior to inclusion; she was excluded on the grounds that she might have an underlying chronic disease of the lungs
- 1 patient with concomitant diagnoses of Kwashiorkor and rota-virus infection at time of venous puncture
- 5 subjects, initially recruited as patients but on reviewing the evidence from the data did not meet sufficient criteria to be classified as such
- 1 control subject on whom we were unable to get the result of the Mantoux skin test

Consequently, a total number of 150 subjects were included in this study.

Numbers and sex of study subjects per age group and TB status

Table: Numbers and sex of subjects per age group and TB status included in the study

TB status	Patients Sex (M/F)	Mantoux pos Sex (M/F)	Mântoux neg Sex (M/F)	total
Age group (yrs)				
0-5	14 (5/9)	14 (6/8)	12 (7/5)	**40 (18/22)**
5-12	8 (4/4)	14 (8/6)	10 (5/5)	**32 (17/15)**
12-20	20 (8/12)	12 (3/9)	21 (4/17)	**53 (15/38)**
> 20	8 (3/5)	9 (1/8)	8 (0/8)	**25 (4/21)**
total	**50 (20/30)**	**49 (18/31)**	**51 (16/35)**	**150 (54/96)**

The larger number of female subjects in the study population was due to their overrepresentation in the healthy controls from age groups 12-20 yrs and > 20 yrs.

Age per age group and TB status of study subjects

Table: Median (interquartile range) age (yrs) of included subjects per age group and TB status

TB status	Patients		Mantoux pos		Mantoux neg		Total		p-value*
Age group (yrs)	n	Median (SD)	n	Median (SD)	n	Median (SD)	n	Mean (SD)	
0-5	14	1.8 (1.3-2.6)	14	3,2 (2.4-4.2)	12	2.9 (1.5-4.0)	40	2.4 (1.5-3.4)	**.031**
5-12	8	7.7 (5.6-8.8)	14	9.0 (6.6-10.8)	10	8.2 (6.9-9.2)	32	8.8 (6.7-10.0)	.338
12-20	20	17.2 (14.2-18.8)	12	14.6 (12.4-17.1)	21	14.6 (12.8-16.7)	53	15 (13.3-17.6)	**.015**
> 20	8	33.3 (23.8-41.6)	9	36.7 (26.3-42.6)	8	43.5 (30-53.3)	25	36.5 (26.8-43.6)	.287

**p-value: patients compared to healthy Mantoux pos and Mantoux neg controls by age group (Kruskal-Wallis One-Way ANOVA by Ranks test)*

Patients, aged 0-5 yrs were significantly younger than healthy controls of this age group:

p = .014 *(Mann Whitney U Test)*

Patients, aged 12-20 yrs were significantly older than healthy controls of this age group:

p = .004 *(Mann Whitney U Test)*

Mantoux size of study subjects by age group and TB status

Table: Median (interquartile range: IQ) Mantoux size (mm) by age group. TB status and totals

TB status	patients		Mantoux pos		p-value[1]	Mantoux neg	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	12'	16.5 (10.3-20)	13*	19.2 (16.5-22)	.131	12	0 (0-2.6)
5-12	6"	16.8 (0-23.5)	14	17.8 (16.3-20.7)	.458	10	0 (0-3.4)
12-20	16'''	19.5 (16.5-24.1)	12	18.3 (16-26.3)	.470	21	0 (0-0)
> 20	#	#	8**	26.5 (18.3-32)	nv	8	0 (0-0.9)
Total	34	18.5 (15-20.9)	47	18.8 (16.4-22)	.236	51	0 (0-1.8)
p-value[2]	.140		.173			.648	

p-value[1]: Patients compared to healthy Mantoux pos subjects per age group and totals (Mann- Whitney U test)
p-value[2]: Different age groups per TB status compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Missing values of Mantoux skin induration in mm:

 # : patients >20 yrs with smear positive TB were not Mantoux skin tested

' : 1 patient had a Tine skin test grade III and was not re-tested with the Mantoux method
1 patient did not show up on the day scheduled for Mantoux test reading

" : 1 patient had a Tine grade II skin test and was not re-tested with the Mantoux method
1 patient had a Tine grade II skin test, ulcerating, and was not re-tested with the Mantoux method

''': 1 patient did not show up on the day scheduled for for Mantoux test reading
3 patients with smear positive TB were not Mantoux tested

** : 1 control subject had a Tine grade IV skin test*

***: 1 definitely Mantoux pos control subject, whose exact measurement in mm induration was not recorded*

Age (yrs) correlated significantly with Mantoux size (mm) when patients and Mantoux pos subjects were analysed as one group ($p = .006$), but not when the group of patients and the group of Mantoux pos subjects were analysed separately.

Mantoux size (mm) had a significant correlation with cell proliferation after stimulation with PPD (cpm) in the whole study population ($p < .0001$), but not when analysed per TB status.

Mantoux size (mm) had a significant correlation with PPD stimulated cytokine production (pg/ml) for IFN-γ ($p < .0001$), IL-2 ($p < .0001$) and TNF-α ($p < .0001$) in the whole study population. These correlations were not significant when analysed in the Mantoux pos group separately. In the patient group only the correlation with IFN-γ remained significant ($p = .029$).

Characteristics of study patients

Table: Characteristics of TB patients per age group

Age group (yrs)	0-5	5-12	12-20	>20
Principal diagnosis				
Intrathoracic TB	11	6	19	8
TBM	3			
Abdominal TB		1		
Erythema nodosum		1	1	
CR findings				
Hilar adenopathy	11	8[§]		
Infiltrates/cavities			17	8
Pleural effusion			1	
Atypical findings	2*			
No abnormalities	1**		2[#]	
Microbiology				
Sputum sm +		1	17	8
Sputum cult+	1		1	
Other cult +	4		1	
Known contact with TB				
Yes	10	3	10	1
No	3	2	8	7
No information	1	3	2	

TBM: Tuberculous Meningitis, CR: Chest Radiograph, sm+: smear positive, cult+: culture positive

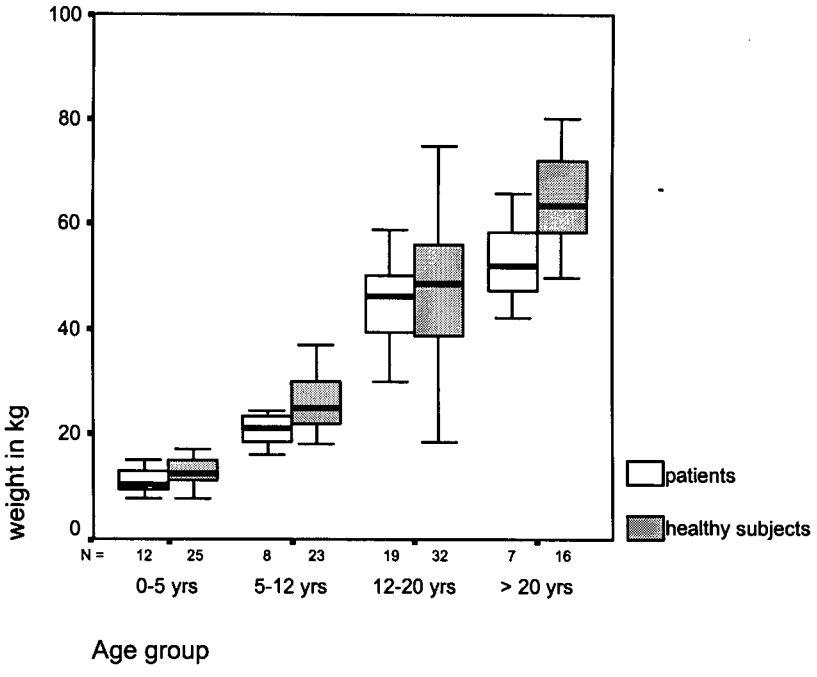
* : *1 patient with a pneumatocele and infiltrate behind the heart on the CR and positive CSF culture*
1 patient with an infiltrate on the CR and weight loss, a known TB contact and a gastric aspirate positive for M.tb on culture

**: *1 patient without abnormalities on the CR, presenting with clinical, cerebrospinal fluid and cranial CTscan findings compatible with TBM*

§ : *1 patient with hilar adenopathy on the CR and culture positive abdominal TB*
1 patient with hilar adenopathy on the CR and erythema nodosum

: *1 patient with a calcification on the CR, presenting with erythema nodosum*
1 patient without abnormalities on the CR, presenting with chronic cough and smear positive sputum

Weights of study subjects



Age (yrs) obviously correlated with weight in kilograms (n =142, $\rho = .921$, $p < .0001$).

Comparing weight between patients and healthy controls by age group gave *(Mann Whitney U test)* the following statistics:

Age group 0-5 yrs:	p = .071
Age group 5-12 yrs:	p = .020
Age group 12-20 yrs:	p = .365
Age group >20 yrs:	p = .018

Discussion on the clinical characteristics of the subjects included in the study

There was an overrepresentation of female healthy controls: it proved to be much more difficult to recruit healthy male subjects than healthy female subjects, because the males were more reluctant to have a venous puncture and participate in the study. That this occurred specifically in the two older age groups is a pity, because sex-related differences in disease notifications from the age of puberty are well recognised.[162]

The significant differences in age within the age groups of 0-5 yrs (patients younger than healthy controls) and 12 -20 yrs (patients older than the healthy controls) should be kept in mind when interpreting all the results. The comparisons of the recorded weights deserve mentioning in view of this. The very strong correlation between age and weight implies that in these two particular age groups, where no significant differences in weight were found between patients and healthy controls, this finding might well have been due to differences in age in both groups. However, stratifying the whole study population by any tighter age ranges would have produced study groups with insufficient numbers to perform adequate statistical analyses on.

Our cutoff value of 15mm skin test induration is a high cutoff value compared to the custom in many other regions in the world, where 10mm skin induration is often taken as the discriminative value. This value makes it highly unlikely that positive Mantoux reactions are solely based on previous BCG vaccination.[163] The reported BCG vaccination coverage in the Western Cape has been more than 90% over the past decades. BCG is given directly after birth or soon thereafter. The vaccination method consists of spreading the BCG preparation (Tokyo 172) on the skin of the upper arm and subsequently injecting it percutaneously with the help of the "Japanese Gun". According to the South African guidelines, the vaccination should be repeated if there is no scar visible after 6 weeks. Serious doubts about the technique and follow-up of these guidelines have, however, been raised. The gun tool is not really developed for the arms of neonates and a local study often found tools to be faulty.[164] Especially in the older age

groups, the absence of a visible scar does not necessarily imply lack of a BCG vaccination at birth and scars tend to wane over time, specifically when BCG is given in the neonatal period.[165] Vaccination and record keeping practices have undoubtedly changed over the past 45 yrs and in a study of this type, which includes subjects from 0 to 44 yrs age-range, there is an obvious difficulty in stratifying subjects along BCG vaccination status.

Although any presentation of TB was regarded as an inclusion criterion to participate in the study as a patient, none of the patients under the age of 12 had a postprimary (chronic pulmonary) form of disease. This divide largely reflects the local clinical epidemiology, although exceptions on this age-related pattern of clinical presentation are certainly seen in our study area. At the start of the study, it was hoped that a specific group of TBM patients could be recruited, since the incidence of TBM is high in the Western Cape. However, during the study period, only 3 patients with TBM proved to be eligible for participation, despite considerable effort to recruit them. The main obstacle was the fact that most patients had, appropriately, received their first doses of steroids before admission to Tygerberg Hospital and this would probably have affected the results of the cytokine responses.

All subjects: significant correlations between PHA stimulated cytokine production (pg/ml) and lymphocyte proliferation (cpm), absolute numbers of white blood cells (x10^9/l) and age (yrs)

	IFN-γ (PHA)	TNF-α (PHA)	IL-2 (PHA)	IL-10 (PHA)	prol (PHA)	WBC (x10^9/l)	Lymph (x10^9/l)	Neutr (x10^9/l)	Mono (x10^9/l)	Age (yrs)
IFN-γ (PHA)	*ρ* *p*									
TNF-α (PHA)	*.632* *< .0001*									
IL-2 (PHA)	*.496* *<. 0001*	*.658* *< .0001*								
IL-10 (PHA)	*.307* *< .0001*	*.333* *< .0001*	*.340* *< .0001*							
prol (PHA)		*.323* *<,0001*	*.380* *< .0001*	*.408* *< .0001*						
WBC (x10^9/l)	*- .424* *< .0001*	*-.386* *< .0001*	*- .302* *< .0001*	*- .286* *.001*						
Lymph (x10^9/l)					*.318* *<.,0001*	*.461* *< .0001*				
Neutr (x10^9/l)	*-,315* *< .0001*	*-.317* *< .0001*	*-.307* *< .0001*	*-.257* *.002*	*- .399* *< .0001*	*.773* *< .0001*				
Mono (x10^9/l)	*- .289* *< .0001*	*-.,267* *.001*	*-.368* *< .0001*	*-.,208* *.014*		*.622* *<. 0001*	*.267* *.001*	*.501* *<.0001*		
Age (yrs)	*.436* *< .0001*	*.314* *<.0001*		*.213* *.010*	*- .173* *.043*	*- .370* *< .0001*	*-.,571* *< .0001*		*-.197* *.018*	

Patients: significant correlations between PHA stimulated cytokine production (pg/ml) and lymphocyte proliferation (cpm), absolute numbers of white blood cells (x10^9/l) and age (yrs)

	IFN-γ (PHA)	TNF-α (PHA)	IL-2 (PHA)	IL-10 (PHA)	prol (PHA)	WBC (x10^9/l)	Lymph (x10^9/l)	Neutr (x10^9/l)	Mono (x10^9/l)	Age (yrs)
IFN-γ (PHA)	*ρ* *p*									
TNF-α (PHA)	*.725* *<.0001*									
IL-2 (PHA)	*.570* *< .0001*	*.647* *< .0001*								
IL-10 (PHA)	*.355* *.013*	*.385* *.008*	*.380* *.008*							
prol (PHA)		*.327* *.034*	*.437* *.003*	*.360* *.018*						
WBC (x10^9/l)	*-.440* *.002*	*-.477* *.001*	*-.326* *.022*							
Lymph (x10^9/l)					*.373* *.015*					
Neutr (x10^9/l)	*-.416* *.003*	*-.489* *.001*	*-.413* *.004*		*-.642* *< .0001*	*.801* *< .0001*				
Mono (x10^9/l)	*-.294* *.045*		*-.343* *.018*			*.678* *< .0001*		*.627* *< .0001*		
Age (yrs)						*-.340* *.017*	*- .649* *< .0001*			

Healthy Mantoux positive and negative subjects: significant correlations between PHA stimulated cytokine production (pg/ml) and lymphocyte proliferation (cpm), absolute numbers of white blood cells ($x10^9$/l) and age (yrs)

	IFN-γ (PHA)	TNF-α (PHA)	IL-2 (PHA)	IL-10 (PHA)	prol (PHA)	WBC ($x10^9$/l)	Lymph ($x10^9$/l)	Neutr ($x10^9$/l)	Mono ($x10^9$/l)	Age (yrs)
IFN-γ (PHA)	*ρ* *p*									
TNF-α (PHA)	*.584* *< .0001*									
IL-2 (PHA)	*.429* *< .0001*	*.640* *< .0001*								
IL-10 (PHA)	*.258* *.010*	*.269* *.010*	*.265* *.008*							
prol (PHA)		*-.313* *.002*	*.314* *002*	*.321* *.002*						
WBC ($x10^9$/l)	*-.421* *< .0001*		*-239* *.019*	*-.278* *.006*						
Lymph ($x10^9$/l)					*.315* *.002*	*.587* *< .0001*				
Neutr ($x10^9$/l)	*-.262* *.010*					*.741* *< .0001*				
Mono ($x10^9$/l)	*-.282* *.005*	*-.206* *.045*	*-.310* *.002*			*.542* *< .0001*	*.430* *< .0001*	*.290* *.004*		
Age (yrs)	*.511* *< .0001*	*.448* *< .0001*	*.241* *.016*	*.326* *.001*		*.437* *< .0001*	*- .490* *< .0001*		*-.316* *.002*	

All subjects: significant correlations between PPD or ESAT-6 stimulated cytokine production (pg/ml) and lymphocyte proliferation (cpm), absolute numbers of white blood cells, age (yrs) and Mantoux skin test (mm induration)

	IFN-γ (PPD)	IFN-γ (E-6)	TNF-α (PPD)	IL-2 (PPD)	IL-10 (PPD)	prol (PPD)	prol (E-6)	WBC ($x10^9$/l)	Lymph ($x10^9$/l)	Neutr ($x10^9$/l)	Mono ($x10^9$/l)	Age (yrs)	Mant (mm)
IFN-γ (PPD)	*ρ* *p*												
IFN-γ (E-6)	*.694* *< .0001*												
TNF-α (PPD)	*.637* *< .0001*												
IL-2 (PPD)	*.543* *< .0001*		*.510* *< .0001*										
IL-10 (PPD)	*.119* *.020*												
prol (PPD)	*.584* *< .0001*		*.363* *< .0001*	*.698* *< .0001*									
prol (E-6)	*.274* *.006*	*.395* *< .0001*				*.396* *< .0001*							
WBC ($x10^9$/l)					*.187* *.047*								
Lymph ($x10^9$/l)													
Neutr ($x10^9$/l)						*-.207* *.018*		*.773* *< .0001*					
Mono ($x10^9$/l)						*-.206* *.020*		*.622* *< .0001*					
Age (yrs)				*-.244* *.003*				*-.370* *< .0001*	*-.571* *< .0001*		*-.197* *.018*		
Mant (mm)	*-.571* *< .0001*	*.360* *< .0001*	*.481* *< .0001*	*.427* *< .0001*		*.527* *< .0001*	*.306* *.004*						

Patients and Mantoux positive healthy subjects: significant correlations between PPD or ESAT-6 stimulated cytokine production (pg/ml) and lymphocyte proliferation (cpm), absolute numbers of white blood cells, age (yrs) and Mantoux skin test (mm induration)

	IFN-γ (PPD)	IFN-γ (E-6)	TNF-α (PPD)	IL-2 (PPD)	IL-10 (PPD)	prol (PPD)	prol (E-6)	WBC ($x10^9$/l)	Lymph ($x10^9$/l)	Neutr ($x10^9$/l)	Mono ($x10^9$/l)	Age (yrs)	Mant (mm)
IFN-γ (PPD)	*ρ* *p*												
IFN-γ (E-6)	*.655* *< .0001*												
TNF-α (PPD)	*.516* *< .0001*												
IL-2 (PPD)	*.363* *< .0001*												
IL-10 (PPD)				*-.205* *.044*									
prol (PPD)	*.363* *< .0001*			*.660* *< .0001*	*- .356* *.002*								
prol (E-6)		*.346* *.012*				*.314* *.013*							
WBC ($x10^9$/l)	*- .281* *.005*					*-.332* *.002*							
Lymph ($x10^9$/l)				*.222* *.030*				*.346* *.001*					
Neutr ($x10^9$/l)	*-.311* *002*			*-.315* *.002*		*-.507* *< .0001*		*.791* *< .0001*					
Mono ($x10^9$/l)	*-.284* *.005*			*- .340* *.001*		*- .522* *< .0001*		*.663* *< .0001*		*.618* *< .0001*			
Age (yrs)				*-.290* *.004*				*-.330* *.001*	*-.556* *<.0001*				
Mant (mm)												*.300* *.006*	

Patients: significant correlations between PPD or ESAT-6 stimulated cytokine production (pg/ml) and lymphocyte proliferation (cpm), absolute numbers of white blood cells, age (yrs) and Mantoux skin test (mm induration)

	IFN-γ (PPD)	IFN-γ (E-6)	TNF-α (PPD)	IL-2 (PPD)	IL-10 (PPD)	prol (PPD)	prol (E-6)	WBC ($x10^9$/l)	Lymph ($x10^9$/l)	Neutr ($x10^9$/l)	Mono ($x10^9$/l)	Age (yrs)	Mant (mm)
IFN-γ (PPD)	*ρ* *p*												
IFN-γ (E-6)	*.601* *< .0001*												
TNF-α (PPD)	*.502* *< .0001*												
IL-2 (PPD)	*.315* *.026*												
IL-10 (PPD)													
prol (PPD)				*.531* *< .0001*									
prol (E-6)		*.419* *.021*				*.636* *< .0001*							
WBC ($x10^9$/l)													
Lymph ($x10^9$/l)								*.415* *.003*					
Neutr ($x10^9$/l)	*-.292* *.044*					*.322* *.038*		*.801* *< .0001*		*.627* *< .0001*			
Mono ($x10^9$/l)				*-.435* *.002*		*-.483* *.001*		*.678* *< .0001*					
Age (yrs)				*-.393* *.005*			*-.364* *.037*	*-.340* *.017*	*-.649* *< .0001*				
Mant (mm)	*.374* *.029*												

Healthy Mantoux positive subjects: significant correlations between PPD or ESAT-6 stimulated cytokine production (pg/ml) and lymphocyte proliferation (cpm), absolute numbers of white blood cells, age (yrs) and Mantoux skin test (mm induration)

	IFN-γ (PPD)	IFN-γ (E-6)	TNF-α (PPD)	IL-2 (PPD)	IL-10 (PPD)	prol (PPD)	prol (E-6)	WBC ($x10^9$/l)	Lymph ($x10^9$/l)	Neutr ($x10^9$/l)	Mono ($x10^9$/l)	Age (yrs)	Mant (mm)
IFN-γ (PPD)	*ρ* *p*												
IFN-γ (E-6)	*.718* *< .0001*												
TNF-α (PPD)	*.608* *< .0001*												
IL-2 (PPD)	*.299* *.037*		*.443* *.001*										
IL-10 (PPD)	*.327* *.048*												
prol (PPD)	*.466* *.001*		*.368* *.012*	*.713* *< .0001*									
prol (E-6)						*.408* *.048*							
WBC ($x10^9$/l)	*-.377* *.008*												
Lymph ($x10^9$/l)					*.345* *.039*			*.384* *.007*					
Neutr ($x10^9$/l)	*.331* *.021*					*-.320* *.032*		*.732* *< .0001*					
Mono ($x10^9$/l)								*.604* *< 0001*		*.364* *.012*			
Age (yrs)								*-.397* *.005*	*-.340* *.018*				
Mant (mm)													

Haematological Findings

Haemoglobin

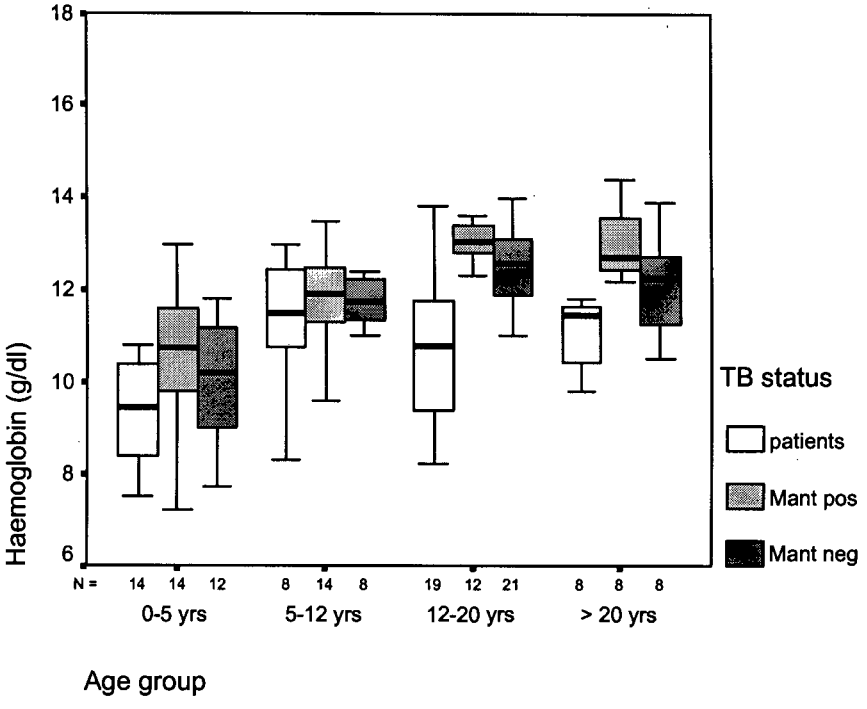


Table: Median (interquartile range: IQ) Hb (g/dl) of patients and healthy subjects per age group and totals

TB status	patients		healthy subjects (Mantoux pos and neg)		p-value[1]	total	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	14	9.5 (8.9-10.4)	26	10.7 (9.5-11.6)	**.012**	40	10,3 (9.1-10.9)
5-12	8	11.5 (10.7-12.5)	22	11.9 (11.3-12.5)	.504	30	11.8 (11.2-12.4)
12-20	19	10.7 (9.2-12)	33	12.8 (12.1-13.3)	**< .0001**	52	12.3 (10.9-13.1)
> 20	8	11.5 (10.4-11.7)	16	12.6 (12.1-13.4)	**.019**	24	12.3 (11.4-12.9)
total	49	10.6 (9.3-11.5)	97	12.0 (10.9-12.8)	**<.0001**	146	11.5 (10.4-12.6)
p-value[2]	**.004**		**< .0001**			**< .0001**	

p-value[1]: Patients compared to healthy subjects (Mantoux pos and neg) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) correlated significantly with Hb levels (g/dl), when analysed for the whole study population ($\rho = .429$, $p < .0001$). This correlation remained significant when investigated for patients ($\rho = .390$, $p = .006$) and controls ($\rho = .565$, $p < .0001$) separately.

Analysed per age group, the patients groups had significantly lower Hb values than control groups, with the exception of the patients from the 5-12 yrs age group, who had similar levels of Hb when compared to their age-related healthy controls.

White blood cell counts

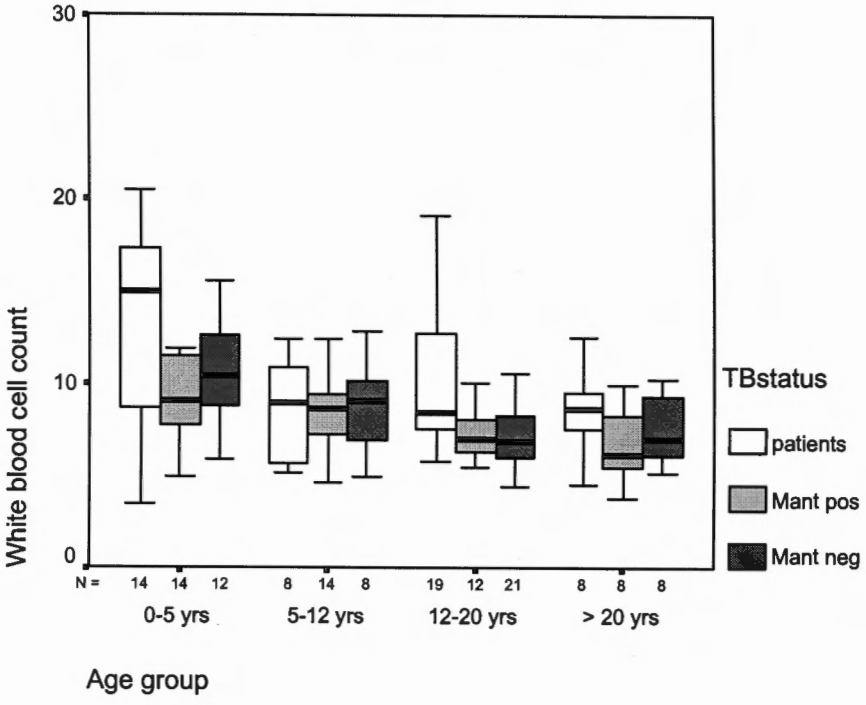


Table: Median (interquartile range:IQ) WBC (x10^9/l) of patients and healthy subjects per age group and totals

TB status	patients		Healthy subjects (Mantoux pos and neg)		p-value[1]	total	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	14	15 (8.7-17.4)	26	9.9 (7.9-12.3)	**.059**	40	10.3 (8.3-15)
5-12	8	8.9 (5.5-11.7)	22	11.8 (11.3-12.4)	.980	30	8.7 (6.6-9.6)
12-20	19	8.4 (7.5-12.9)	33	6.9 (6.1-8.3)	**.004**	52	7.6 (6.3-9.2)
> 20	8	8.6 (7-9.8)	16	6.9 (5.5-9.4)	.214	24	7.1 (5.6-9.3)
total	49	9.1 (7.6-13.1)	97	8.0 (6.4-9.9)	**.003**	146	8.5 (6.6-10.2)
p-value[2]	**.096**		**< .0001**			**< .0001**	

p-value[1]: Patients compared to healthy subjects (Mantoux pos and neg) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) had a significantly inverse correlation with the absolute amount of WBCs (x10^9/l), when analysed for the whole study population *(p. 68)*. The correlation remained significant when investigated for patients *(p. 69)* and controls *(p. 70)* separately.

With the exception of the 5-12 yrs age group, patients had higher absolute counts of WBCs than healthy controls, but this phenomenon did not always reached significance for the individual age groups.

Neutrophils

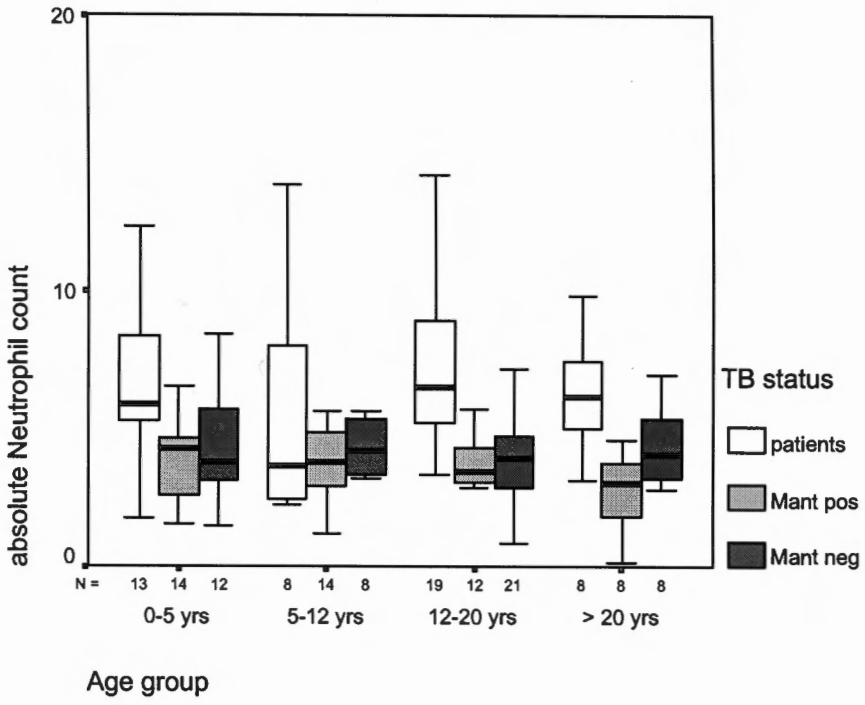


Table: Median (interquartile range:IQ) absolute neutrophil count ($x10^9/l$) of patients and healthy subjects per age group and totals

TB status	**patients**		**healthy subjects (Mantoux pos and neg)**		**p-value[1]**	**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)	-	n	Median (IQ)
0-5	13	5.9 (4.9-8.7)	26	4.1 (3.0-5.8)	**.021**	39	4.5 (3.2-6.2)
5-12	8	3.7 (2.4-8.0)	22	3.8 (3.1-5.3)	.982	30	3.8 (3-5.3)
12-20	19	6.5 (5.1-10)	33	3.5 (2.9-4.8)	**< .0001**	52	4.2 (3.1-6.5)
> 20	8	6.1 (4.9-7.7)	16	3.4 (2.9-4.7)	**.004**	24	4.2 (3-5.9)
total	48	6.1 (4-8.1)	97	3.7 (2.9-4.9)	**< .0001**	145	4.2 (3.1-5.9)
p-value[2]	.555		.686			.634	

p-value[1]: Patients compared to healthy subjects (Mantoux positive and negative) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) was not significantly correlated with the absolute number of neutrophils ($x10^9/l$) when analysed for the whole or any of the individual study groups *(p. 68-70)*.

Per age group, the absolute amount of neutrophils was significantly higher in patients than controls, except in the 5-12 yrs age group.

Lymphocytes

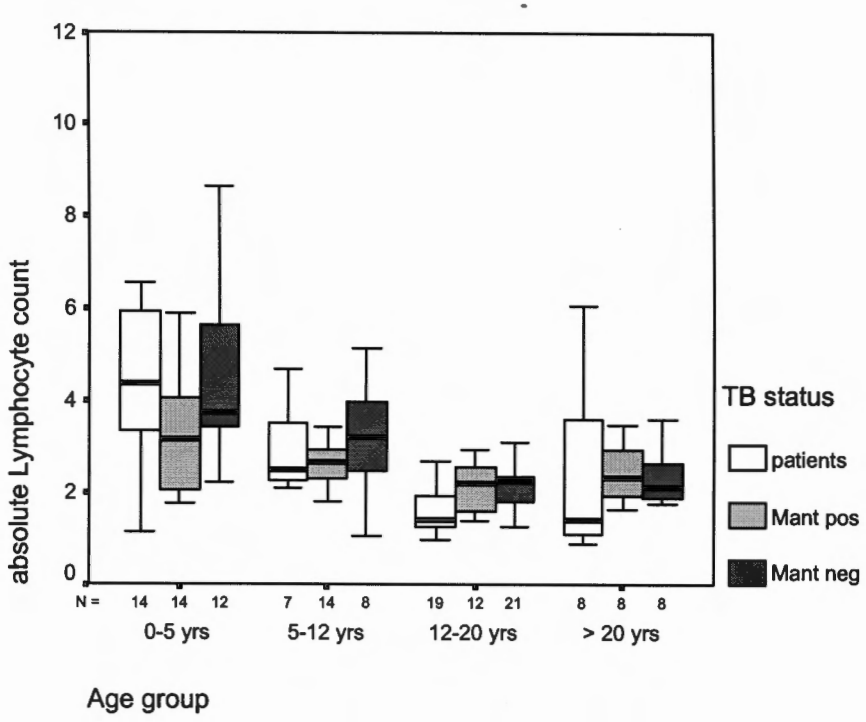


Table: Median (interquartile range:IQ) absolute lymphocyte count ($x10^9$/)l of patients and healthy subjects per age group and totals

TB status	**patients**		**healthy subjects (Mantoux pos and neg)**		**p-value[1]**	**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	14	4.4 (3.3-5.9)	26	3.5 (2.4-4.8)	.162	40	3.8 (2.9-5.1)
5-12	7	2.5 (2.3-3.9)	22	2.8 (2.3-3.4)	.980	29	2.8 (2.3-3.4)
12-20	19	1.4 (1.2-2)	33	2.2 (1.7-2.4)	**.001**	52	2,0 (1.4-2.3)
> 20	8	1.4 (1.1-4.2)	16	2.3 (1.9-2.8)	.172	24	2.1 (1.7-2.8)
total	49	2.2 (1.3-3.9)	97	2.4 (2.0-3.4)	.192	145	2.4 (1.8-3.4)
p-value[2]	**< .0001**		**< .0001**			**< .0001**	

p-value[1]: Patients compared to healthy subjects (Mantoux positive and negative) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) had a significant inverse correlation with the absolute amount of lymphocytes ($x10^9$/l) when analysed for the whole population under study *(p. 68)* as well as for the patients *(p. 69)* and healthy control groups *(p. 70)* separately.

Patients and controls, in general, did not differ significantly in their absolute amounts of white blood cells, with the exception of the 12-20 yrs age group, where patients had significantly lower counts than healthy controls.

Monocytes

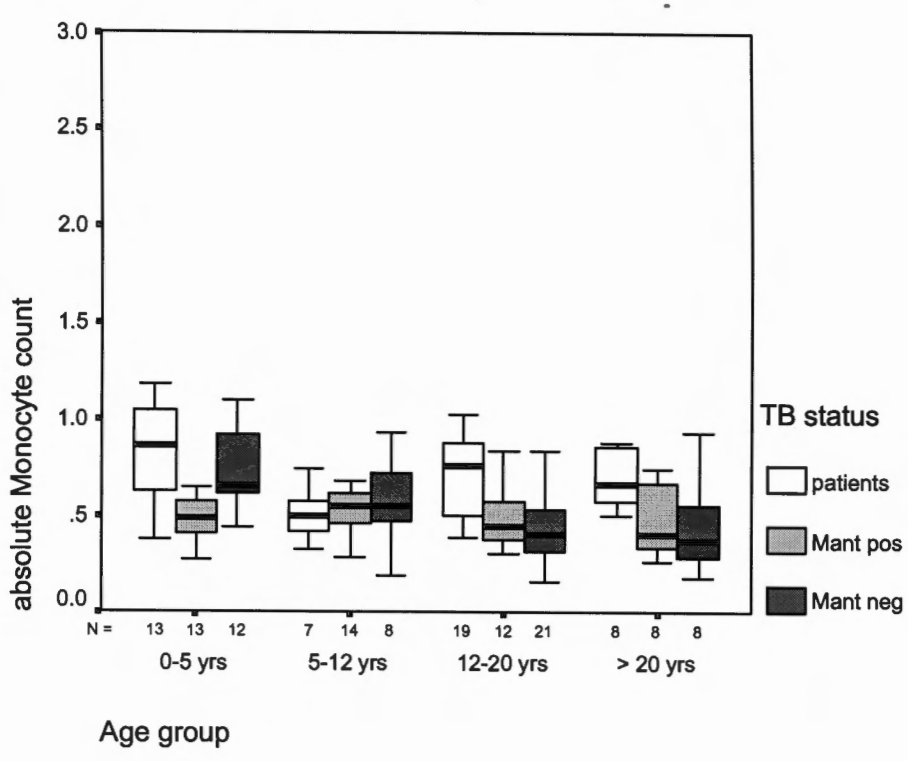


Table: Median (interquartile range) absolute monocyte count (x10^9/l) of patients and healthy subjects per age group and totals

TB status	patients		healthy subjects (Mantoux pos and neg)		p-value[1]	total	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	13	0.86 (0.62-1.07)	25	0.61 (0.46-0.8)	**.030**	38	0.63 (0.49-0.92)
5-12	7	0.5 (0.37-0.6)	22	0.55 (0.46-0.63)	.500	29	0.54 (0.46-0.61)
12-20	19	0.76 (0.5-0.89)	33	0.42 (0.33-0.58)	**< .0001**	52	0.5 (0.37-0.71)
> 20	8	0.69 (0.55-0.86)	16	0.41 (0.31-0.63)	**.016**	24	0.51 (0.33-0.73)
total	47	0.68 (0.5-0.88)	96	0.50 (0.37-0.63)	**< .0001**	143	0.55 (0.4-0.72)
p-value[2]	**.040**		**.011**			**.022**	

p-value[1]: Patients compared to healthy subjects (Mantoux pos and neg) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) had a weak, but significant correlation with the absolute amount of monocytes (x10^9/l), when analysed for the whole study population *(p. 68),* but in the healthy controls this correlation between was stronger *(p. 70)*.

Per age group, patients had significantly higher amounts of monocytes than controls, except in the 5-12 yrs group.

Discussion on the haematologic findings

In the context of this study an important observation with respect to the haematological parameters, is the fact that there are significant correlations between age in years and the haemoglobin levels (positive correlation), the absolute white blood cell count (negative correlation) and the absolute lymphocyte count (negative correlation). An inverse correlation between age and the absolute monocyte count was also found in the healthy controls. Given the fact that all published normal reference values of these parameters have age-ranges these findings are not surprising by themselves. However, within the perspective of this study it is important to note that these age-related differences are maintained irrespective of tuberculous disease. To what degree this finding has implications for the interpretation of other results from this study will be discussed later.

When patients were compared to healthy controls significant differences between the two groups were found for Hb, WBC, absolute neutrophil counts and absolute monocyte counts, but notably not for the absolute lymphocyte count. Most studies on haematological abnormalities in relation to TB have looked at adults and are reviewed in a publication by Knox-Macaulay[166]. Data on haematologic abnormalities in children with TB are scarce. One recently published study from our department on this subject[167] compared data from a group of hospitalised children with TB to a similar control group consisting of children admitted suspected to have TB, but subsequently found to have other diseases. The objective of this study was to investigate if haematological findings could discriminate between "TB or not TB" and it was concluded that they could not.

Low Hb, neutrophilia, monocytosis and lymphocytosis or lymphopenia are all seen in adult patients with TB.[166] The anaemia in TB is generally regarded as an anaemia of chronic disease. Although the red cell indices and iron status related measurements in serum (serum iron, total iron binding capacity, percentage saturation, ferritin and transferrin) might suggest iron deficiency, most of the patients do not have an anaemia secondary to iron-deficient erythropoiesis.[168] Disturbances in iron metabolism, inappropriate low serum erythropoietin levels and raised cytokine levels in the circulation

(specifically TNF-α) have been assumed as possible contributors to the low Hb levels in TB patients.[166,168,169] The degree of anaemia seems to be related to severity of disease and is most pronounced in disseminated disease. There were 4 patients with disseminated disease included in this study. Of these, three children had TBM, none of whom were anaemic. One patient with disseminated abdominal TB, did have a low Hb of 8.3 g/dl for which she received a blood transfusion.

Our results on the various white blood cells in paediatric TB patients are in accordance with published data on adults. It seems peculiar that lymphocytes, the only circulating white blood cells well recognised to be of importance in TB pathology are the only cells not to differ significantly in patients from healthy controls. Lymphopenia, lymphocytosis and normal lymphocyte counts in TB have all been described to the degree that no consistent statement can be made on this subject. However neutrophilia and monocytosis seem to be more constant findings. Both cell types are ill defined in the pathogenesis of TB, although they are both active phagocytic cell types, present in granulomas and possible producers of a variety of cytokines.

The main reason for doing full blood counts on all the included study subjects was to evaluate them in the light of the results from the stimulated cell proliferative and cytokine responses in whole blood. Any conclusions on specificity or sensitivity of the haematologic findings with respect to a diagnosis of TB or severity of illness cannot be made. The number of study subjects is relatively small and, as Wessels' study[167] has shown, the findings are not specific for tuberculous disease. One can conclude however, that, except for the 5-12 age group, TB patients do differ significantly from age related healthy controls for most haematological data. That the only age group where no haematological differences at all were found between patients and controls was "the golden age of TB" is a curious finding, but in view of the small number of subjects within this age group, not even speculative reasoning seems to be justified.

Lymphocyte proliferation assays

Lymphocyte proliferation after stimulation with PHA

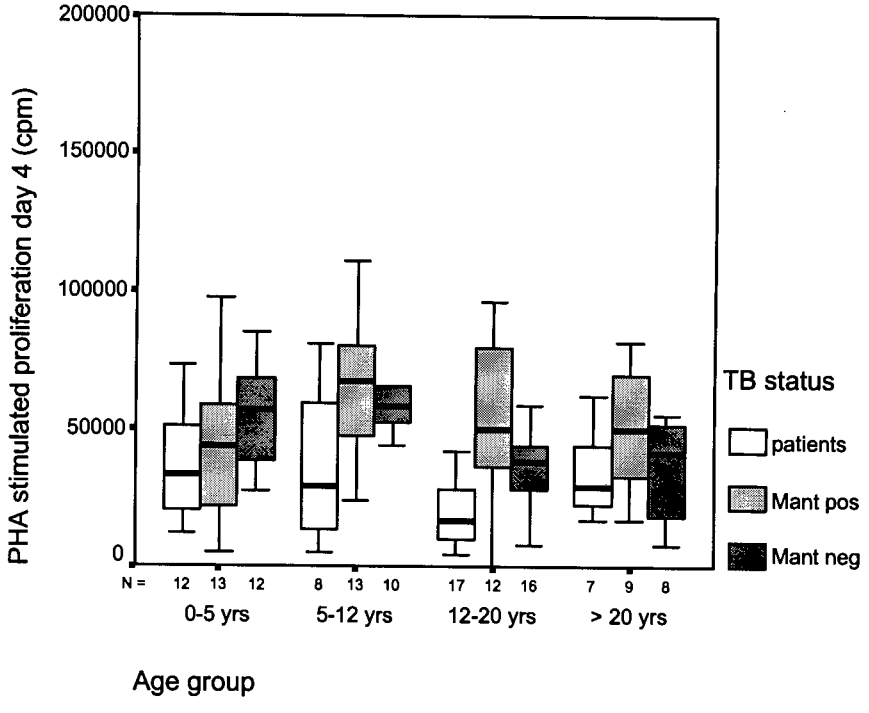


Table: Median (interquartile range: IQ) lymphocyte proliferative response (cpm) of whole blood stimulated with PHA (10μg/ml) on day 4 (after subtraction of unstimulated proliferation on day 4) of patients and healthy subjects by age group and totals

TB status	**patients**		**healthy subjects (Mantoux pos and neg)**		**p-value[1]**	**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)	-	n	Median (IQ)
0-5	12	33855 (20363-59274)	25	46110 (35173-61694)	.151	37	43859 (29367-60924)
5-12	8	30104 (11906-68650)	23	62073 (47821-80452)	**.026**	31	56104 (32108-79630)
12-20	17	16356 (9182-29456)	28	40458 (28787-58692)	**<.0001**	45	31595 (14770-44035)
>20	7	30677 (20857-57031)	17	43612 (21936-68469)	.383	24	40660 (21731-58861)
total	44	26952 (13245-42277)	93	46699 (34030-65842)	**<.0001**	137	40710 (23795-60943)
p-value[2]	**.034**		**.047**			**.005**	

p-value[1]: Patients compared to healthy subjects (Mantoux positive and negative) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

When analysing the whole study population, there was a weak, just significant, inverse correlation between age (yrs) and PHA stimulated lymphocyte proliferation (cpm) *(p. 68)*. The correlation became stronger and positive for the subjects under 12 years of age: $p = .039$ for all subjects under 12 yrs ($n = 68$, $\rho = .250$) and $p = .021$ for the healthy subjects under 12 yrs of age ($n = 45$, $\rho = .451$). No significant correlations were found between age and PHA induced lymphocyte proliferation in subjects older than 12 yrs.

Although PHA stimulated lymphocyte proliferation was overall significantly less in the patients than in the controls, this phenomenon only reached significance in the individual 5-12 yrs and 12-20 yrs age groups.

PHA stimulated lymphocyte proliferation (cpm) showed a significant positive correlation ($p < .0001$) with the absolute amount of lymphocytes ($x10^9/l$) *(p. 68)*.

Correlations between lymphocyte proliferation (cpm) and cytokine production (pg/ml) after stimulation with PHA were significant for IL-2, TNF-α and IL-10 *(p. 68)*. However, any significant correlation between lymphocyte proliferation and IFN-γ production was notably absent.

Lymphocyte proliferation after stimulation with PPD

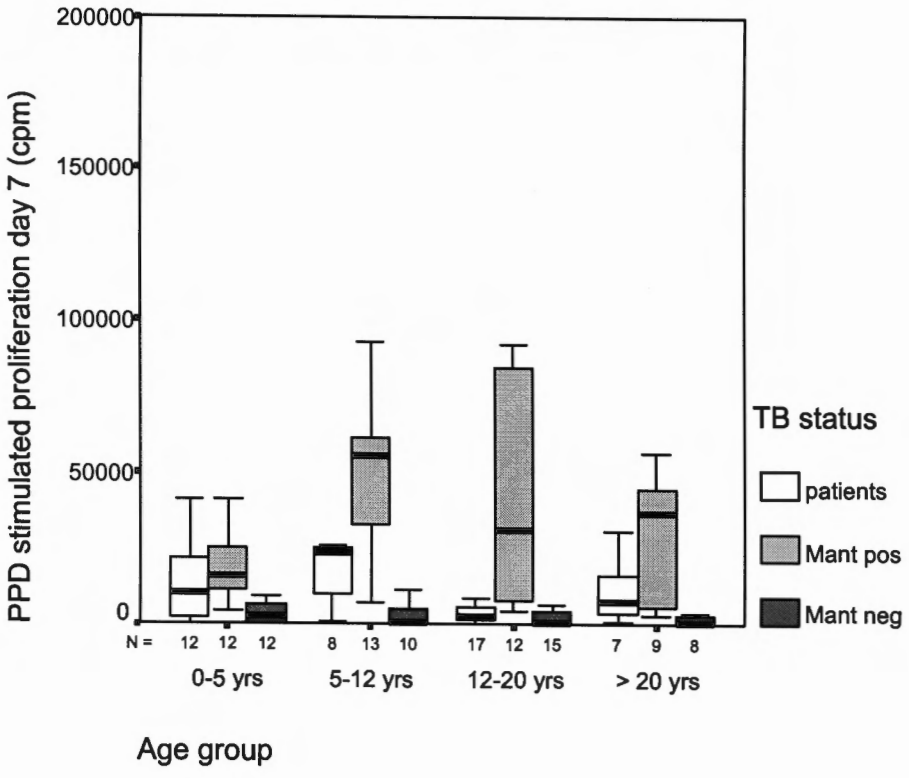


Table: Median (interquartile range: IQ) lymphocyte proliferative response (cpm) after stimulation of whole blood with PPD (3.3μg/ml) on day 7(after subtraction of unstimulated proliferation on day 7) by age group and TB status

TB status	**patients**		**Mantoux pos**			**Mantoux neg**		**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)	**p-value[1]**	n	Median (IQ)	n	Median (IQ)
0-5	12	12605 (1946-26126)	12	16025 (11114-30022)	.198	12	2672 (175-7264)	37	8795 (2405-20729)
5-12	8	23825 (8061-32276)	13	56132 (32794-70071)	**.020**	10	572 (127-8733)	28	23098 (3055-56378)
12-20	17	2447 (1401-7799)	12	36638 (7098-85196)	**< .0001**	15	491 (82-6360)	44	3913 (1119-15120)
> 20	7	8984 (2747-23350)	8	37063 (5146-54675)	.142	9	734 (482-5379)	23	5253 (1483-31695)
Total	44	5346 (1810-17661)	46	32824 (12082-57636)	**< .0001**	45	668 (141-5232)	132	6707 (1383-24387)
p-value[2]	**.047**		.062			.712		.069	

p-value[1]: Patients compared to healthy Mantoux positive subjects per age group and totals (Mann- Whitney U test)

p-value[2]: Different age groups per TB status and totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

PPD stimulated proliferative lymphocyte responses (cpm) did not correlate with age (yrs) in the whole population under study, but the correlation was strong in the healthy Mantoux pos subjects younger than 12 years ($n = 25$, $\rho = .649$, $p < .0001$).

Upon stimulation with PPD, lymphocyte proliferation in the patients was significantly less than in Mantoux pos healthy subjects, but when analysed per age group this only reached significance in the 5-12 yrs and 12-20 yrs groups. Patients younger than 12 yrs proliferated significantly better than patients older than 12 yrs ($p = .030$), while there was no such difference between Mantoux pos subjects < 12 yrs when compared to Mantoux pos subjects > 12 yrs.

There was no correlation between PPD induced lymphocyte proliferation (cpm) and the absolute amount of lymphocytes ($x10^9/l$).

There was a very strong and positive correlation between PPD stimulated lymphocyte proliferation (cpm) and IL-2 production (pg/ml) in patients *(p. 73)* and Mantoux pos subjects alike *(p. 74)*. In healthy Mantoux pos subjects, but not in patients, the correlations between PPD stimulated lymphocyte proliferation and IFN-γ production as well as TNF-α production were positive and significant *(p.74)*.

Lymphocyte proliferation after stimulation with ESAT-6

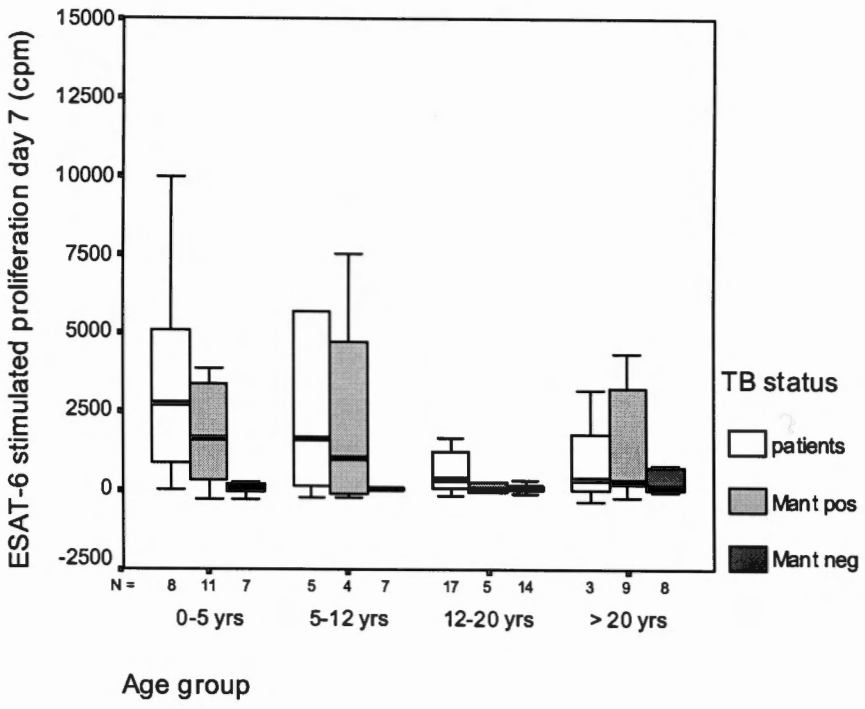


Table: Median (interquartile range: IQ) lymphocyte proliferative response(cpm) after stimulation of whole blood with ESAT-6 (1 μg/ml) on day 7 (after subtraction of unstimulated proliferation on day 7) of subjects per age group, TB status and totals

TB status	**patients**		**Mantoux pos**		**p-value[1]**	**Mantoux neg**		**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)	n	Median (IQ)
0-5	8	2722 (828-5906)	11	1661 (175-6692)	.657	7	87 (-76-198)	26	942 (112-3486)
5-12	5	2611 (-31-10865)	4	1495 nv*	.730	7	36 (14-333)	16	77 (18-2931)
12-20	17	404 (41-1351)	5	65 (-275-1514)	.249	14	86 (10-153)	36	108 (15-704)
> 20	3	1064 nv*	9	449 (82-3999)	.727	8	93 (-13-806)	20	272 (-13-1244)
Total	33	790 (83-3131)	29	607 (58-3197)	.762	36	57 (-10-157)	98	178 (17-1504)
p-value[2]	.247		.293			.807		.157	

p-value[1]: Patients compared to healthy Mantoux positive subjects per age group and totals (Mann- Whitney U test)

p-value[2]: Different age groups per TB status and totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

**nv: interquartile ranges are given as non valid (nv) if the number of subjects is less than 5*

There was a weak, inverse correlation between age (yrs) and ESAT-6 stimulated lymphocyte proliferation (cpm) in the patient group *(p. 73)*.

There was a positive and significant, but weak, correlation between lymphocyte proliferation (cpm) and IFN- γ production (pg/ml) after ESAT-6 stimulation in the patient and Mantoux pos group combined *(p. 74)*.

Because of the small numbers of subjects in some of study groups and large variations in values, any other statistical analysis is rather meaningless and probably invalid.

Discussion on lymphocyte proliferative assays

Lymphocyte proliferation assays are used to measure the blastogenesis of lymphocytes, a basic requirement of their immunologic function, in response to defined antigens. The system relies on the incorporation of a radioactive nucleoside (^{3}H-Thymidine) into the cellular DNA of dividing lymphocytes during chromosomal replication. By its nature, it is an *in vitro* method that assesses antigen responsiveness rather than functional quality of the immune response. The technique can be used for the purpose of measuring antigen responsiveness on its own and, in relation to cytokine measurements from harvested supernatants of stimulated cell cultures, as a positive control measurement. It is however a rather laborious technique for larger scale studies especially if no automated cell counter is available.

There are various ways in which lymphocyte proliferative responses are expressed in the literature: as a stimulation index (stimulated response/unstimulated response) or as a net value (stimulated response-unstimulated response).[170] When proliferative responses from this study were analysed by stimulation index, the results did not differ in any important way from the analysis by net value, as presented in this thesis. When the data were analysed by "normalising" for the absolute lymphocyte count (proliferative response-unstimulated response/absolute lymphocyte count at time of venous puncture) the comparisons of results by study group were also similar to the ones shown in the preceding graphs and tables on lymphocyte proliferative responses.

The fact that the lymphocyte proliferative responses after stimulation with PHA and PPD correlated well with IL-2 production signifies the importance of IL-2 as a growth factor for T-cells and, in a way, validates the outcomes of our proliferation studies.

Overall, there was a decreased lymphocyte poliferative response in patients when compared to Mantoux pos subjects and this has been found by others.[171, 172]

The different relation between lymphocyte proliferation and IFN-γ after stimulation with PHA (significant correlation) or PPD (no correlation), indicates that the dynamics of lymphocyte proliferation in relation to cytokine production depend on the nature of the antigen. Others have found a similar discordant relationship between PPD stimulated lymphocyte proliferative response and IFN-γ production.[84]

These findings indicate that antigen responsiveness (as measured by lymphocyte proliferation) is not necessarily the correlate for a functional antigen response by the active T cell repertoire (as measured by cytokine production). The function of lymphocyte proliferation assays as "a" positive control measurement for cytokine responses is probably not entirely justified. I doubt if the technique is of any use in a non-research setting, considering its time-consuming nature and need for expensive and fractious equipment.

IFN-γ Production

IFN-γ production after stimulation with PHA

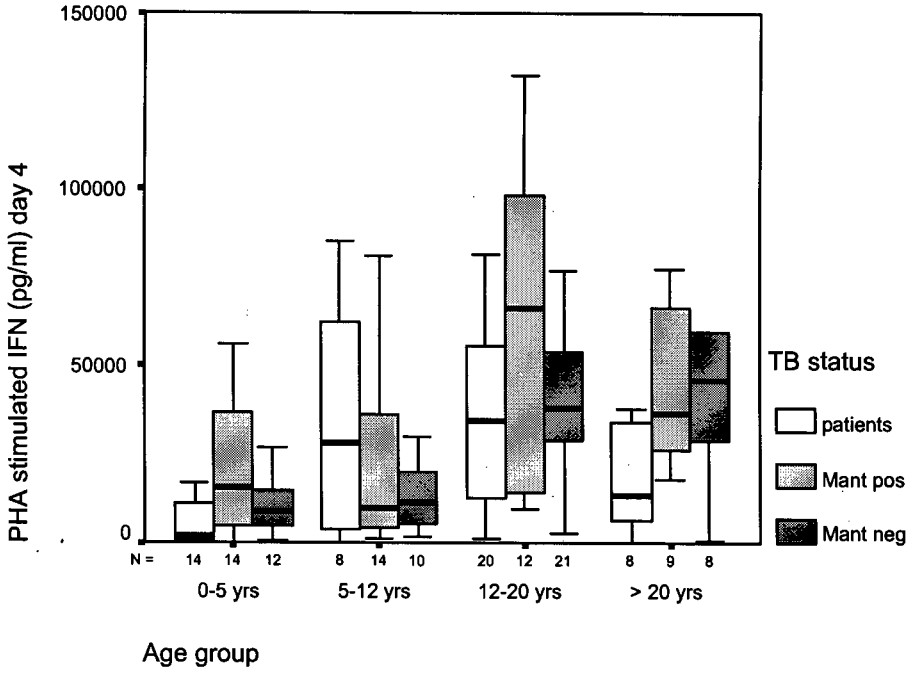


Table: Median (interquartile range:IQ) of IFN-γ *production (pg/ml) on day 4 after stimulation of whole blood with PHA (10μg/ml) in patients, healthy controls and totals*

TB status	**patients**		**healthy subjects (Mantoux pos and neg)**		**p-value[1]**	**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	14	1789 (543-11841)	26	10719 (4512-29751)	**.021**	40	9576 (1456-16986)
5-12	8	34011 (3027-65977)	24	10232 (4534-27213)	.454	32	11670 (4534-36438)
12-20	20	34284 (17570-69261)	33	39052 (17570-69261)	.349	53	38079 (16027-61355)
> 20	8	14211 (5745-44792)	17	41262 (25086-65635)	**.043**	25	35197 (16971-61355)
total	50	15203 (2077-48273)	100	26616 (9575-53566)	.086	150	18371 (5659-52175)
p-value[2]	**.011**		**< .0001**			**< .0001**	

p-value[1]: Patients compared to healthy subjects (Mantoux pos and neg) per age group and total (Mann- Whitney U test)

p-value[2]: All age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) correlated significantly with PHA stimulated IFN-γ production (pg/ml) within the whole study group *(p. 68)*. This correlation was stronger when the healthy subjects were analysed separately. In the patient group the correlation between age and IFN-γ production (pg/ml) remained significant only when analysed for the all patients younger than 20yrs ($\rho = .484$, $p = .001$) due to the diminished production of IFN-γ in the age group > 20 yrs. The 0-5 yrs age group (patients and healthy controls) produced significantly less IFN-γ after stimulation with PHA than all the older age groups.

Patients from the youngest and oldest age groups produced significantly less IFN-γ upon stimulation with PHA than their age related healthy controls.

IFN-γ production showed a significant inverse correlation with the absolute counts of white blood cells, monocytes and neutrophils, but notably not in any way (positive or negative) with the absolute counts of lymphocytes *(p. 68)*.

PHA stimulated IFN-γ production correlated significantly with PHA stimulated IL-2, TNF-α and IL-10 production *(p. 68)*. These correlations remained significant when analysed for patients and healthy subjects separately.

IFN-γ production after stimulation with PPD

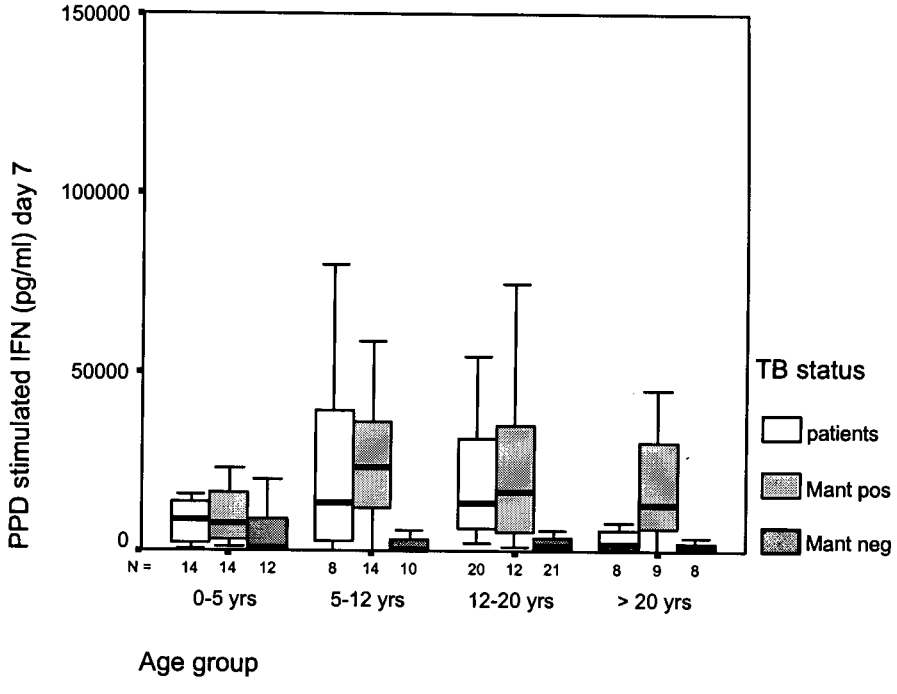


Table: Median (interquartile range: IQ) IFN-γproduction (pg/ml) on day 7 after stimulation of whole blood with PPD (3.3μg/ml) of subjects per age group, TB status and totals

TB status	**patients**		**Mantoux pos**		**p-value[1]**	**Mantoux neg**		**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)	n	Median (IQ)
0-5	14	8853 (1747-13620)	14	7460 (2946-16999)	.769	12	1200 (10-9771)	40	7178 (1265-13544)
5-12	8	15905 (2571-46187)	14	23693 (11243-36421)	.707	10	383 (10-4574)	32	9772 (985-29907)
12-20	20	15937 (5790-31991)	12	16923 (5196-36563)	1.00	21	1447 (10-4152)	53	5734 (1589-23824)
> 20	8	2417 (408-9566)	9	15608 (5507-33409)	**.034**	8	1022 (10-2146)	25	2978 (10-12151)
Total	50	8139 (2843-23412)	49	15355 (4762-30010)	.208	51	1328 (10-3599)	150	5627 (1301-17723)
p-value[2]	**.025**		.222			.765		.235	

p-value[1]: Patients compared to healthy Mantoux positive subjects per age group and totals (Mann- Whitney U test)

p-value[2]: Different age groups per TB status and totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age in years did not correlate with PPD stimulated IFN-γ production for the whole study population. However, in patients and Mantoux positives up to 12 yrs of age a significant correlation between age and PPD stimulated IFN-γ production ($n = 50$, $\rho = .406$, $p = .003$) did exist. In the group of Mantoux positives this correlation was even stronger ($n = 28$, $\rho = .604$, $p = .001$). The healthy Mantoux positive controls 0-5 yrs old produced significantly less IFN-γ than the older healthy Mantoux positive subjects ($p = .001$).

Patients of the oldest age group (> 20 yrs) produced significantly less IFN-γ than their Mantoux positive controls ($p = .034$) contrary to patients from the three other, younger

age groups who did not produce significantly lower amounts of IFN-γ if compared to age-related Mantoux positive controls.

There were significantly inverse correlations between IFN-γ production with the absolute amounts of neutrophils and monocytes, but not with absolute amounts of lymphocytes *(p. 72)*.

PPD stimulated IFN-γ production correlated significantly with PPD stimulated IL-2, TNF-α and IL-10 production *(p. 72)*. These correlations remained significant with IL-2 and TNF-α, but not with IL-10, when analysed for patients and healthy subjects separately *(p. 72 and p. 74 respectively)*.

IFN-γ production after stimulation with PPD compared to Mantoux skin testing

The Mantoux skin test is an imperfect tool to determine "status of infection". Currently, efforts to develop an alternative *in vitro* method are based on IFN-γ responses to mycobacterial antigens.[173,174] Here, I have used our data to assess the use of PPD-induced IFN-γ levels for its potential in identifying *M.tb.* infected people of different ages.

All the study subjects, patients and healthy controls, were stratified along their positive or negative Mantoux status with 15mm skin induration as the cut-off value. The two groups were compared for their PPD induced IFN-γ responses. Furthermore, the correlation between Mantoux size (mm) and IFN-γ level (pg/ml) was determined in the Mantoux positive subjects by age group.

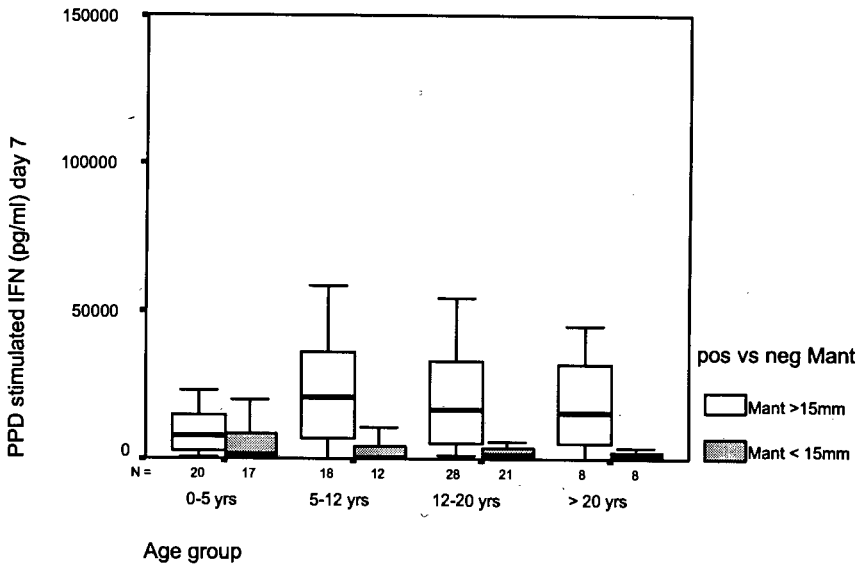


Table: Median (interquartile range: IQ) PPD stimulated (3.3μg/ml) IFN-γ production (pg/ml) of study subjects stratified along Mantoux positive (>15mm) or Mantoux negative (<15mm), by age group

Age group (yrs)	Mantoux pos (≥15mm) study subjects		Mantoux neg (<15mm) study subjects		p- *
	n	Median (IQ)	n	Median (IQ)	
0-5	20	7796 (2688-16274)	17	1533 (199-8899)	**.033**
5-12	18	21948 (6230-36421)	12	383 (10-5837)	**<.0001**
12-20	28	16923 (5269-33491)	21	1447 (10-4152)	**<.0001**
>20	8	19823 (4975-35277)	8	1022 (10-2473)	**.006**
Total	74	13250 (4975-31190)	58	1383 (10-4962)	**<.0001**

**: (Mann Whitney U test)*

Table: Correlation coefficients (ρ) plus significance values (p) between IFN-γ production (pg/ml) after PPD stimulation(3.3μg/ml) and Mantoux skin test induration (mm) by age group.

Age group (yrs)	n	ρ*	p*
0-5	37	.397	**.015**
5-12	30	.561	**.001**
12-20	49	.583	**<.0001**
>20	16	.813	**<.0001**
Total	132	.577	**<.0001**

**: Spearmann Rank-Order Correlation test*

The graph and tables show that there were significant differences in IFN-γ production between Mantoux positives and Mantoux negative persons in each age group. Furthermore the correlation between the magnitudes of IFN-γ production (pg/ml) and size of Mantoux skin test induration (mm) per age group were also good (relative exception: the 0-5 yr age group) and significant. It should be remembered here that we did not perform Mantoux skin testing on the patients older than 20 years of age and they are therefore not included in these analyses.

Validating a new test requires comparison with a "gold standard". The only "standard" we have at the moment for discriminating infected *vs* non-infected persons is the Mantoux skin test and calculations for sensitivity, specificity and positive predictive values come from the following 2x2 table.

Table: 2x2 table of IFN-γ responders and IFN-γ non-responders after PPD stimulation x Mantoux pos and neg subjects from study subjects; 10pg/ml IFN-γ is used as the cut-off value to discriminate non-responders from responders

	IFN-γ responders (≥10pg/ml)	**IFN-γ non-responders (<10pg/ml)**	Total
Mantoux positive (>15mm)	72	2	74
Mantoux negative (<15mm)	38	20	58
total	110	22	132

Sensitivity, i.e. the percentage of people that have a positive PPD/IFN-γ response of whom we know they have a positive Mantoux: 72/74 = 97% (3% false negatives)

Specificity, i.e. the percentage of people that have a negative PPD/IFN-γ response of whom we know they have a negative Mantoux: 20/58 = 90% (10% false positives)

Positive predictive value, i.e. the proportion of all PPD/IFN-γ responders who are truly Mantoux positive: 72/110 = 65%

Negative predictive value, i.e. the proportion of truly Mantoux negatives out of all PPD/IFN-γ non-responders: 20/22 = 91%

For the sake of the argument, calculations the other way around, with a positive IFN-γ response as the "gold standard" would give the following values:

Sensitivity, i.e. the percentage of people that have a positive Mantoux of whom we know they are PPD/ IFN-γ responders: 65% (35% false negatives)

Specificity, i.e. the percentage of people that have a negative Mantoux of whom we know they are PPD/IFN-γ non-responders: 90% (10% false positives)

Positive predictive value, i.e. the proportion of all Mantoux positives who are truly PPD/IFN-γ responders: 97%

Negative predictive value, i.e. the proportion of truly PPD/IFN-γ non-responders who are Mantoux negative: 34%

The 50 patients in this study were classified as having TB on the basis of clinical (including radiological and microbiological) parameters and not on Mantoux skin test induration. Of the patients 3 out of 50 (= 6%) were PPD/IFN-γ non-responders. Of the patients on whom we had a Mantoux skin test 7 out of 27 (= 26%) were Mantoux negative and 1 out of 27 (= 3%) was a PPD/IFN-γ non-responder.

IFN-γ production after stimulation with ESAT-6

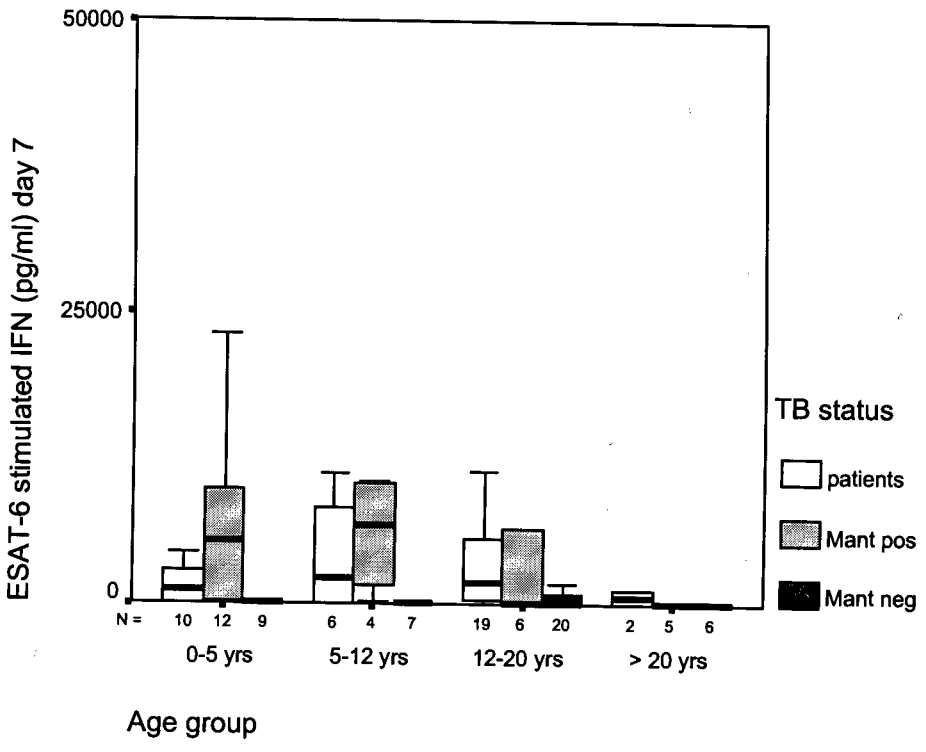


Table: Median (interquartile range: IQ) IFN-γ production in pg/ml on day 7 after stimulation of whole blood with ESAT-6 (3.3μg/ml) of subjects per age group, TB status and totals

TB status	**patients**		**Mantoux pos**		**p-value[1]**	**Mantoux neg**		**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)	n	Median (IQ)
0-5	10	1285 10-3743	12	5987 136-12291	.254	9	10 10-109	31	943 10-6811
5-12	6	2370 10-9993	4	8340 nv*	.476	7	10 10-10	17	32 10-7584
12-20	19	1916 264-7818	6	10 10-48555	.176	20	115 10-935	45	355 10-2852
> 20	2	856 nv*	5	57 10-854	.571	6	10 10-18	13	10 10-176
Total	37	1416 214-4313	27	1140 10-9170	.437	42	10 10-244		215 (10-2819)
p-value[2]	.216		**.005**					.066	

p-value[1]: Patients compared to healthy Mantoux positive subjects per age group and totals (Mann- Whitney U test)

p-value[2]: Different age groups per TB status and totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

**nv: interquartile ranges not valid because number of subjects in the study group too small*

Age did not correlate in any way with IFN-γ production (pg/ml) after ESAT-6 stimulation.

IFN-γ production after stimulation with PPD and ESAT-6 were positively and significantly correlated in patients as well as healthy Mantoux positive controls *(p. 73-74)*. However, the paucity of numbers, notably in the two oldest control groups, may cause some misinterpretation..

Discussion on IFN-γ production

The subjects included in this study produced IFN-γ in abundant quantities. So much so that I have repeatedly rechecked if we had not miscalculated by a factor 10, but that was not the case. A large subgroup of subjects (around 50%, mainly consisting of the very high producers) have been checked for unstimulated background levels and these values were all below the detection limit of the assay (<15pg/ml).

Stimulation with the mitogen PHA resulted in different age-specific responses when compared to stimulation with antigens PPD or ESAT-6. The strongly positive correlation between age in years and PHA stimulated IFN-γ production was lost when PPD or ESAT-6 were used as a stimulant. However, when the Mantoux positive healthy study groups were analysed, there was still a rather strong correlation between age in years and PPD stimulated IFN-γ production in the healthy Mantoux positives under 12 years of age. Furthermore, the 0-5 yrs age group produced significantly less IFN-γ compared to the older age groups after stimulation with both PHA and PPD. For a discussion and the possible implications of these findings I refer to the general discussion.

Mitogen induced IFN-γ responses from patients may be affected if compared to healthy controls, as shown in the data for the 0-5 and >20yrs age groups, but the other data show that this finding is not a general rule. One can only speculate on the reasons for this discrepancy. A possible explanation would be that patients of some age groups were in a compromised physical condition, whereby the comparison of weights between sick and healthy persons may serve as a proxy for physical condition. However, although the patients in the oldest age group were significantly lighter than their age-related healthy controls, this comparison did not reach significance in the 0-5yrs group where the patients were moreover younger than their age-related controls. Furthermore, the patients from the 5-12yrs age group were significantly lighter than their age related controls, but produced equal amounts of IFN-γ upon stimulation with PHA. Another speculation could evolve around "severity of disease", but we have no unified and standardised

classification at our disposal to compare patients with primary as well as postprimary by degree of disease severity.

In Mantoux-negative subjects, stimulation with *M.tb* related antigens like PPD and ESAT-6, does not result in appreciable amounts of IFN-γ, compared to Mantoux positive subjects. There have been a number of studies suggesting that quantified PPD induced IFN-γ production might be a valid and more sensitive substitute for Mantoux skin testing.[173,174] The problem with these studies, as with this one, is that there is no "gold standard" for identifying *M.tb* infected individuals other than the Mantoux test. Consequently the Mantoux test, for which a substitute is wanted, is being used as the gold standard for validation of a possible substitute. Methodologically flawed as this may be, it is hard to come up with another study design for this particular question. This study was not designed to examine if PPD induced IFN-γ responses may replace Mantoux skin testing, but speculations on this issue are interesting enough in view of the results. Furthermore, the published studies on this subject have all been on adults and our data are therefore the first from a group of children. It should be noted that although the tables on p-values and correlation coefficients for the different age groups show very satisfactory and significant values, they are slightly misleading. Because there were relatively few subjects with Mantoux skin test indurations between 0 and 14.9 mm (11%), the significance of the correlations is, in part, caused by the fact that most of the skin indurations had extreme values of 0mm (32%) or >15mm (56%).

In the evaluation of an individual patient, there may well be an added value of measuring IFN-γ after PPD stimulation, since the Mantoux test was more frequently negative in these subjects than the IFN-γ response (26% *vs* 6%).

For larger scale studies, especially in low incidence countries, the calculated positive predictive values are not good enough. Higher cut off values for PPD induced IFN-γ responders does improve the positive predictive value, be it at the cost of lower sensitivity and specificity.

We only obtained the ESAT-6 antigen when the study was already underway, which is why we have relatively few data in some study groups on ESAT-6 stimulated IFN-γ

production. Any statistical analysis in view of the results is, in my view not valid in this section, but an analysis of these data from the perspective of disease expression later in this thesis proved interesting.

Because IFN-γ is such a crucial cytokine in mycobacterial infection and quite extensively studied in adults, but rarely in children, our results deserve a more elaborate discussion than that given in this chapter, for which I refer to the general discussion at the end of the thesis.

TNF-α production

TNF-α production after stimulation PHA

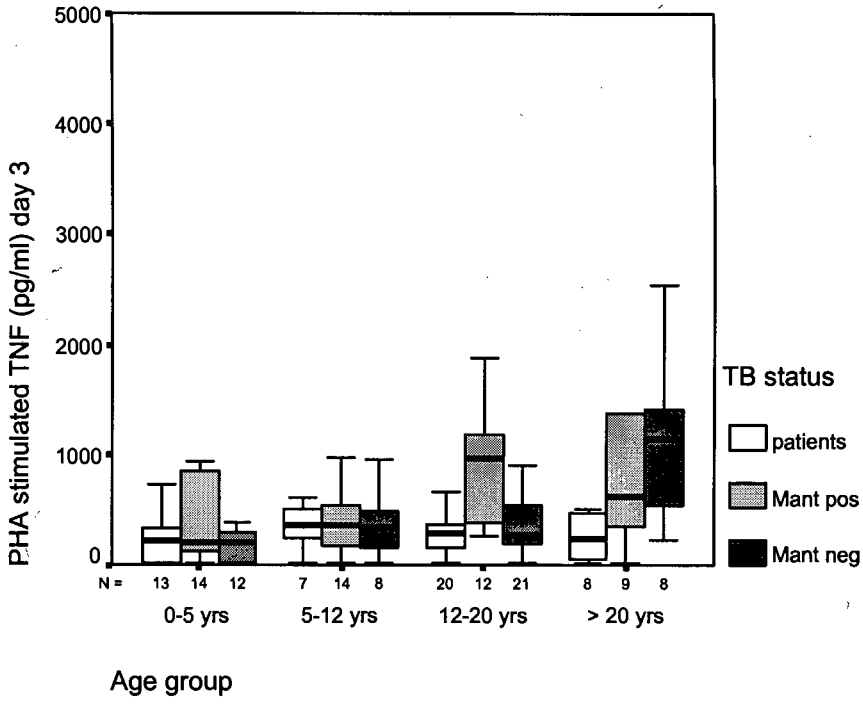


Table: Median (interquartile range) of TNF-α production (pg/m)l after stimulation of whole blood with PHA (10μg/ml) in patients, healthy controls and totals

TB status	patients		healthy subjects (Mantoux pos and neg)		p-value[1]	total	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	13	215 (10-421)	26	199 (25-342)	.670	39	200 (10-331)
5-12	7	397 (212-547)	22	356 (170-532)	.980	29	361 (196-510)
12-20	20	297 (157-386)	33	370 (228-908)	**.045**	53	334 (215-642)
> 20	8	242 (50-613)	17	1016 (331-1442)	**.023**	25	508 (242-1351)
total	48	262 (45-421)	98	343 (192-820)	.020	146	310 (164-563)
p-value[2]	.556		**< .0001**			**< .0001**	

p-value[1]: Patients compared to healthy subjects (Mantoux positive and negative) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) had a significant correlation with PHA stimulated TNF-α production (pg/ml), in the healthy controls *(p. 70)*, but not in the patients.

In the two oldest age groups, patients produced significantly less PHA stimulated TNF-α than healthy controls.

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PHA stimulated TNF-α production (pg/ml) did not show any significant correlation with the total amount of lymphocytes in any group, but a significant inverse relation with total amounts of neutrophils and monocytes *(p. 68)*. Correlations with other cytokines produced after stimulation with PHA were positive and significant for IFN-γ, IL-2, and IL-10 *(p. 68)*.

TNF-α production after stimulation PPD

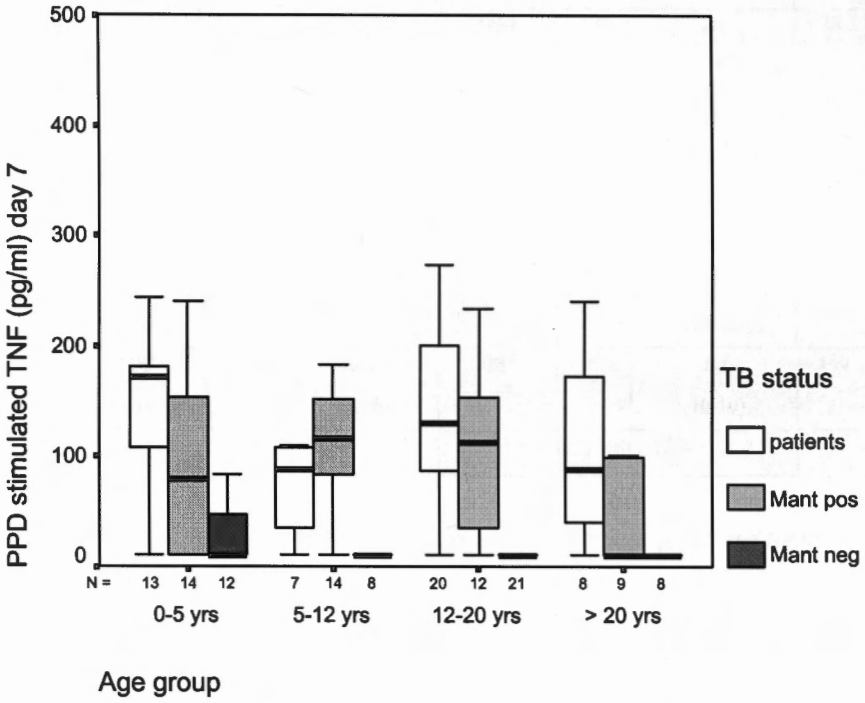


Table: Median (interquartile range: IQ) TNF-α production (pg/ml) after stimulation of whole blood with PPD (3.3μg/ml) of subjects per age group, TB status and totals

TB status	**patients**		**Mantoux pos**		**p-value[1]**	**Mantoux neg**		**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)	n	Median (IQ)
0-5	13	172 (90-227)	14	86 (10-187)	.094	12	10 (10-85)	39	85 (10-173)
5-12	7	92 (16-226)	14	116 (80-155)	.255	8	10 (10-18)	29	83 (10-140)
12-20	20	132 (85-215)	12	113 (28-159)	.346	21	10 (10-57)	53	91 (10-136)
> 20	8	90 (32-262)	9	10 (10-101)	.370	8	10 (10-22)	25	10 (10-101)
Total	48	109 (64-184)	49	97 (10-150)	.088	49	10 (10-10)	146	81 (10-135)
p-value[2]	.330		.294			.794		.088	

p-value[1]: Patients compared to healthy Mantoux positive subjects per age group and totals (Mann- Whitney U test)

p-value[2]: Different age groups per TB status and totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

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

On the whole, patients tended to produce more TNF-α upon stimulation with PPD, but this did not reach significance.

There were no significant correlations with any of the absolute amounts white blood cells and TNF-α production after stimulation with PPD.

PPD stimulated IFN-γ production correlated with TNF-α production in patients and healthy Mantoux pos controls *(p. 73-74)*. However IL-2 correlated with TNF-α only in the healthy controls *(p. 74)*, but not in the patients. The correlation between IL-10 and TNF-α was weak and only just significant in the healthy controls *(p. 74)*.

Discussion on TNF-α production

Although the quantities in pg/ml of TNF-α that were produced were rather small, the virtual absence of any production after stimulation with PPD in the healthy Mantoux negative group is an indicator that analyses of differences are valid. However, neither PHA nor PPD seem very good stimulants of TNF-α production in this whole blood model. Nor did PHA stimulation result in very large differences in the quantities of TNF-α when compared to PPD stimulation, in contrast to some of the other cytokines we measured (IFN-γ and IL-10). TNF-α was measured in all the non-stimulated samples of day 3 and day 7 and these values were below the minimal detection value (15pg/ml) of the assay; thus unstimulated background levels did not influence the results. It may be that soluble TNF receptors, known to be present in serum of adults with active TB before treatment[175], have influenced the results in this whole blood system.

The positive correlation between age in years and PHA stimulated TNF-α production in healthy controls is perhaps in itself not a remarkable finding. Cord blood cells have been shown as deficient producers of TNF-α, partly because of their reduced sensitivity to IFN-γ.[176] Elsässer-Beile et al[177] found significant differences in PHA induced TNF-α production of whole blood in relation to age with fewer numbers of children than we included. This latter study is the only one in this field where comparable methods to ours have been used. One study into age related TNF-α production after stimulation with PHA, using PBMCs, found higher values in children than adults, however the age range for their group of children was rather wide (0.6-16.5 yrs)[178] Few other studies have reported on healthy children's TNF-α responses to antigenic stimulation, one exception being Pollard's study[41], who found age related values in controls (higher in older children), but not in patients after meningococcal infection upon stimulation with menigococcal antigens. For a cytokine that is implicated is so many severe pathologic conditions, it is perhaps surprising that few normal age-related reference data exist.

The correlation between age and PHA stimulated TNF-α production is lost upon stimulation with PPD in all the study groups. Apparently the type of antigen is a determinant for a relation between age and TNF-α production.

Why patients from the groups older than 12 years were affected in PHA, but not in PPD stimulated production of TNF-α when compared to age-related controls is unclear. However, what is perhaps more interesting in this context, is the fact that healthy controls of all ages produced relatively larger amounts of TNF-α upon stimulation with PHA compared to stimulation with PPD, while this was not the case in all patient groups. This suggests that it is easier to induce TNF-α production with PPD in patients than in healthy controls.

Clinical and experimental data in animals and humans suggest a dual role for TNF-α in the immune regulation of TB. On the one hand its beneficial contribution to granuloma formation[179] but on the other hand its detrimental effect leading to cachexia and fever[89] are well recognised consequences that are most probably dependent on its relative production in various stages of the immune response to *M.tb*. In the context of this study, the phenomenon is further explored in the chapter on cytokine responses in relation to expression of disease.

IL-2 production

IL-2 production after stimulation with PHA

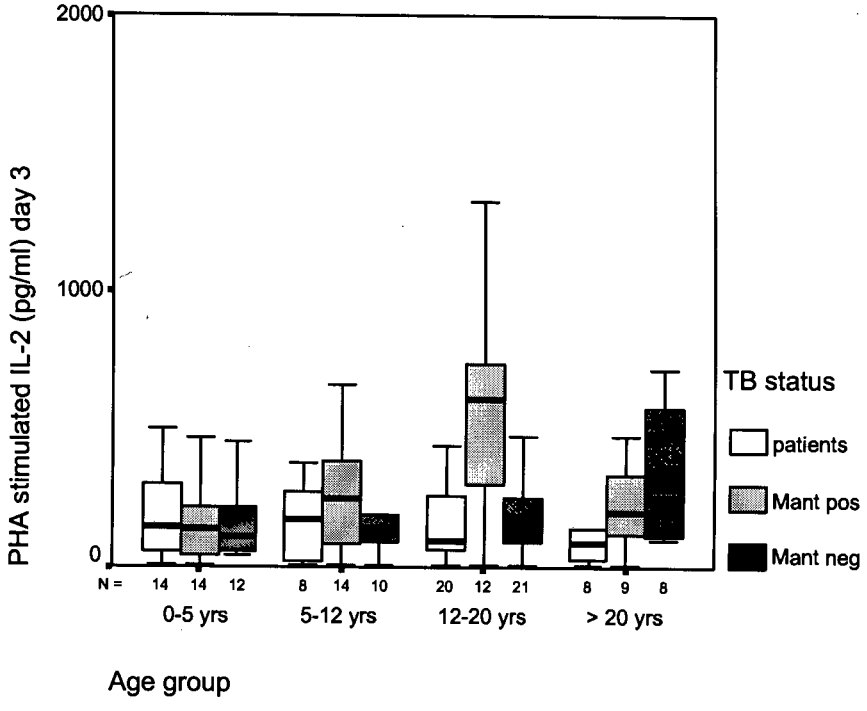


Table: Median (interquartile range:IQ) of IL-2 production (pg/ml) after stimulation of whole blood with PHA (10μg/ml) in patients, healthy controls and totals

TB status	patients		healthy subjects (Mantoux pos and neg)		p-value[1]	total	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	14	155 (56-331)	26	127 (49-225)	.747	40	127 (49-235)
5-12	8	187 (17-331)	24	178 (85-349)	.404	32	178 (76-328)
12-20	20	92 (62-300)	33	236 (99-642)	**.028**	53	195 (78-543)
> 20	8	107 (22-162)	17	226 (112-438)	.110	25	139 (81-381)
total	50	120 (46-263)	100	193 (87-387)	**.020**	150	157 (68-337)
p-value[2]	.841		.217			.321	

p-value[1]: Patients compared to healthy subjects (Mantoux positive and negative) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) had a weak correlation with PHA stimulated IL-2 (pg/ml) production in the healthy subjects *(p. 70)*, but not in the patients.

On the a whole, the patient group produced less IL-2 than the healthy control group, but after stratification by age group this difference proved only to be significant in the 12-20 years group.

PHA stimulated IL-2 production was not related to the absolute lymphocyte counts. Correlations were positive and significant between PHA stimulated IL-2 and IFN-γ, TNF-α and IL-10 *(p. 68)*.

IL-2 production after stimulation with PPD

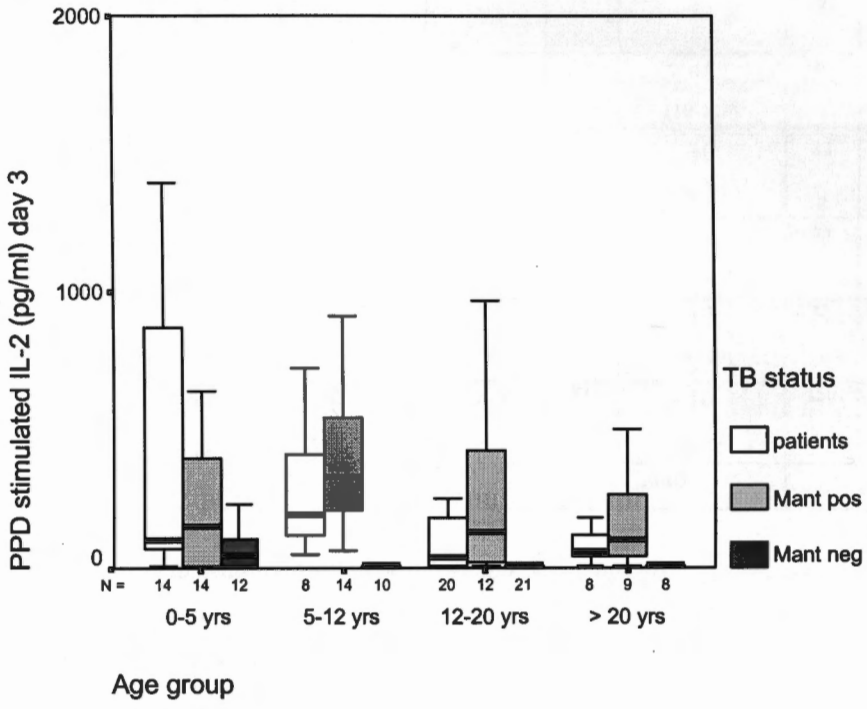


Table: Median (interquartile range: IQ) IL-2 production (pg/ml) on day 3 after stimulation of whole blood with PPD (3.3μg/ml) of subjects per age group, TB status and totals

TB status	patients		Mantoux pos		p-value[1]	Mantoux neg		total	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)	n	Median (IQ)
0-5	14	102 (68-941)	14	153 (10-451)	.804	12	44 (10-138)	40	102 (32-404)
5-12	8	194 (106-481)	14	337 (209-670)	.238	10	10 (10-10)	32	189 (10-371)
12-20	20	35 (10-224)	12	133 (16-549)	.209	21	10 (10-38)	53	10 (10-135)
> 20	8	60 (37-161)	9	128 (40-452)	.423	8	10 (10-40)	25	47 (10-175)
Total	50	79 (33-24)	49	213 (58-469)	.055	51	10 (10-42)	150	60 (10-253)
p-value[2]	.015		.167			.040		.009	

p-value[1]: Patients compared to healthy Mantoux positive subjects per age group and totals (Mann- Whitney U test)

p-value[2]: Different age groups per TB status and totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) had a significant inverse correlation with IL-2 production (pg/ml) after stimulation with PPD in the patients *(p. 72)*, but not in the healthy Mantoux pos controls, in which group however, the correlation with age tended to be negative.

The difference in PPD stimulated IL-2 production between patients and Mantoux positive healthy controls was nearly significant with median values in each age group lower in patients than controls. Patients < 12 yrs produced significantly more IL-2 than patients > 12 yrs (p = .001), while there was no difference between the healthy controls < 12 yrs *vs* > 12yrs.

IL-2 production did not correlate significantly with absolute lymphocyte counts in the patient group, nor in healthy Mantoux pos group, but there was a weak correlation between IL-2 and lymphocytes when these two groups were analysed together *(p. 72)*. Correlations with the other PPD stimulated cytokines were significant for IFN-γ, TNF-α (in Mantoux pos controls *(p. 74)*. However, in the patients IL-2 production did not have a significant correlation with TNF-α.

Discussion on IL-2 production

As with the stimulated TNF-α production the quantities of stimulated IL-2 in pg/ml were not very large. There were, however, a few very distinct and significant differences between groups. As a growth factor for lymphocytes, rapid IL-2 production is probably needed for specific cytokine responses. Indeed IL-2 production was maximal early in the time course of our cell cultures and we therefore harvested on day 3 for both PHA and PPD stimulated cultures, while PPD stimulated IFN-γ and TNF-α levels were maximal on day 7.

Since it is well recognised that naïve T cells invariably produce only IL-2 upon stimulation and the younger the child the more naïve T-cells are demonstrated, one would expect higher IL-2 production at younger age. However there is evidence that naive T-cells[35] as well as antigen presenting[180] cells from young children and adults differ in that respect and this might explain the fact that increased IL-2 production has not been demonstrated in any consistant way in studies involving T cells from children. Diminished capacity to produce IL-2 in cord-blood and neonates has been described[32], as well as equal production by cord blood and children of different ages[42] or lower production by the younger children in a small study of children aged 2-15 yrs.[181]

The level of IL-2 production is likely to depend on previous antigen exposure. However, in the patients we found a negative correlation between age in years and PPD stimulated IL-2 levels. The relatively high level of IL-2 in patients < 12 yrs might explain some of the comparatively good production of IFN-γ in patients of the 0-5 and 5-12 yrs age groups.

IL-10 production

IL-10 production after stimulation with PHA

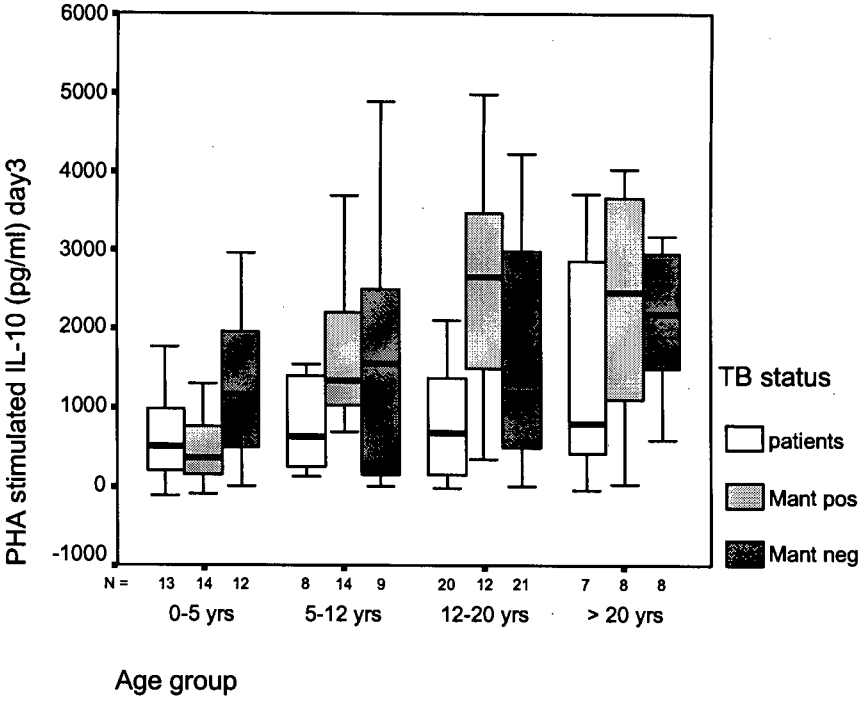


Table: Median (interquartile range: IQ) IL-10 production (pg/ml) after stimulation of whole blood with PHA (10μg/ml) on day 3 (after subtraction of unstimulated IL-10 production on day 3) of patients and healthy subjects by age group and totals

TB status	**patients**		**healthy subjects (Mantoux pos and neg)**		**p-value[1]**	**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	13	518 (142-1658)	26	734 (199-1488)	.848	39	610 (203-1408)
5-12	8	641 (235-1769)	23	1478 (959-2933)	.110	31	1187 (658-2301)
12-20	20	713 (136-1758)	33	1598 (619-3303)	**.009**	53	1217 (388-2981)
> 20	7	1298 (316-3200)	16	2432 (1344-3195)	.341	23	2402 (677-3136)
total	48	597 (161-1715)	98	1392 (580-2751)	**.004**	146	1095 (319-2485)
p-value[2]	.827		**.002**			**.005**	

p-value[1]: Patients compared to healthy subjects (Mantoux positive and negative) per age group and total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

There was a significant positive correlation between age (yrs) and PHA stimulated IL-10 production (pg/ml) in the healthy subjects *(p. 70)*, but not in the patient group when analysed separately. Furthermore the subjects of the youngest age group produced significantly less IL-10 upon stimulation with PHA than all the other age groups (in the healthy subjects: p = .013 for 0-5yrs *vs* 5-12yrs; p = .002 for 0-5yrs *vs* 12-20 yrs; p = .001 for 0-5 yrs *vs* > 20 yrs). This was not due to differences in unstimulated values between any of the study groups.

On the whole, patients produced less than healthy controls, but after stratification for age this was not significant for any of the individual age groups, except for the 12-20 years.

There were weak, but significant correlations with the absolute amounts of neutrophils and monocytes within the whole study population *(p. 68)*, but they did not hold when patients and healthy subjects were analysed separately. No correlation with the absolute amount of lymphocytes was found.

Correlations between PHA stimulated IL-10 and other cytokines were significant for: IFN-γ, IL-2 and TNF-α *(p. 68)*.

IL-10 production after stimulation with PPD

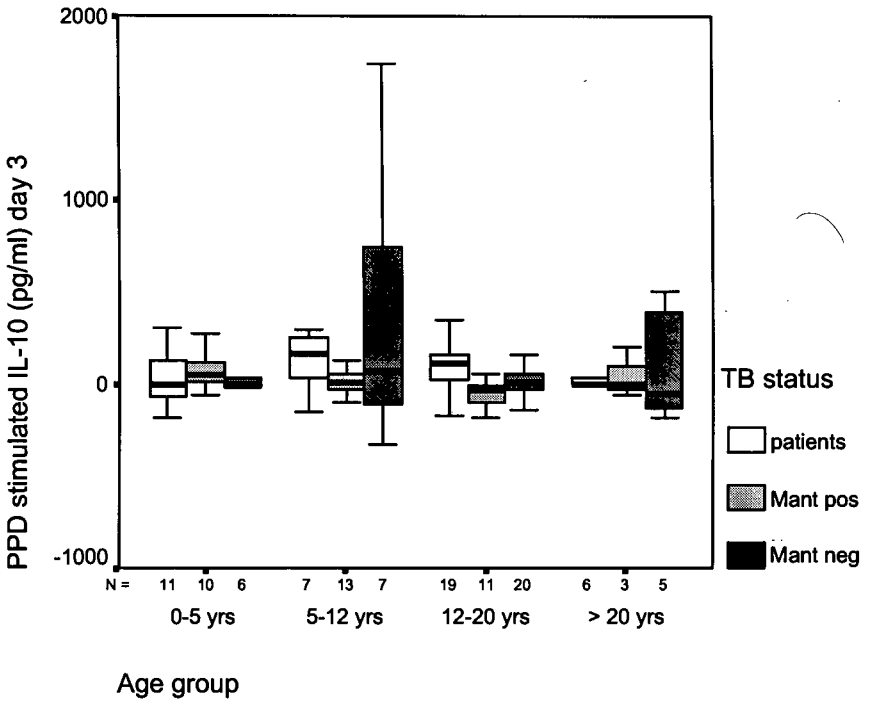


Table: Median (interquartile range:IQ) IL-10 production (pg/ml) after stimulation of whole blood with PPD (3.3μg/ml) on day 3 (after subtraction of unstimulated production on day 3) per age group, TB status and totals.

TB status	**patients**		**Mantoux pos**		**p-value[1]**	**Mantoux neg**		**total**	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)	n	Median (IQ)
0-5	11	-6 (-66/131)	10	61 (0/196)	.426	6	2 (-39/123)	27	10 (-60/125)
5-12	7	182 (9/295)	13	11 (-36/83)	.088	7	68 (-131/1538)	27	26 (-41/146)
12-20	20	114 (8/169)	12	-28 (-110/19)	**.004**	20	10 (-30/58)	50	14 (-34/118)
> 20	6	0 (0/256)	3	49 (-60/197)	.572	5	55 (-150/487)	14	0 (-57/317)
Total	43	75 (0/168)	37	2 (-62/86)	**.032**	38	3 (-59/69)	118	12 (-40/123)
p-value[2]	.206		.126			.963			

p-value[1]: Patients compared to healthy Mantoux positive subjects per age group and totals (Mann- Whitney U test)

p-value[2]: Different age groups per TB status and totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

PPD was a poor stimulant for IL-10 production in most groups. Negative values were the result of higher unstimulated than stimulated background values.

If analysed by responder (PPD stimulated IL-10 production > unstimulated IL-10 production) to non-responder (PPD stimulated IL-10 production < unstimulated IL-10 production) status, the following counts were obtained:

	patients	Mantoux pos	Mantoux neg	Total
Responder	26	18	19	63
Non-responder	17	19	19	55
total	43	37	38	118

In all age groups there were as many responders as non-responders in the Mantoux negative subjects.

Age did not correlate with IL-10 production after stimulation with PPD in any of the subgroups.

The patient group seemed to produce significantly more IL-10 than Mantoux pos healthy subjects, but the 12-20 yrs age group was the only age group where this was of any real significance.

There were no significant correlations between IL-10 production and any of absolute counts of white blood cells, nor with any of the other PPD stimulated cytokines.

Discussion on IL-10 production

IL-10 is often regarded as a cytokine that is produced late in the immune response to a pathogen.[182] After stimulation with the mitogen PHA, we found in the pilot studies that day 3 was the optimum time point of IL-10 production. Production after PPD stimulation started at day 3 and remained detectable, albeit at lower levels, on day 7. I have made some mistakes with the ELISAs for IL-10, which resulted in fewer data available for analysis from day 7 than from day 3. In addition the values from day 7 were in general even lower and I have therefore discarded the day 7 values from the results.

The very distinct and significant age-related difference in PHA stimulated IL-10 production between the subjects younger than 5 years and the older subjects, is interesting and, as far as I know, not previously described. IL-10 production has however been found defective in cord blood mononuclear cells when compared to mononuclear cells from adults.[183] Stimulated IL-10 production furthermore has been measured in young children with various diseases,[49, 184] but conclusions on an age-relation per se are rarely mentioned in these publications, with the exception of Pollards' study[41] who found a non-significant trend to higher production at older age. The correlation with PHA induced IL-10 levels was significant, for IFN-γ, TNF-α and IL-2 in our study and one can therefore hypothesise age-related regulation between these cytokines, but not necessarily age-related contra regulation.

The importance of IL-10 in the human immune response to *M.tb* has been demonstrated in a number of studies that, however, differed greatly in their methodology to highlight this issue. IL-10 has a down-regulating effect on IFN-γ and TNF-α production and is therefore regarded as anti-inflammatory. Two studies have proved the point that IFN-γ or TNF-α production can be down-regulated by adding recombinant IL-10 to monocyte cell cultures stimulated with mycobacterial antigens.[68, 185] One important study in this field[98] found higher levels of IL-10 in the supernatants of PBMCs stimulated with mycobacterial antigens from patients if compared to healthy household contacts, however without any differences in mRNA expression of IL-10 between these two groups. No difference in IL-

10 mRNA expression was found between patients and healthy controls in a study by Zhang et al[82], who, contrary to Torres et al[98], could not demonstrate a difference in IL-10 production by PBMCs either.

In our study IL-10 production after stimulation with PPD on day 3 was very low in all the study groups. The table on the responders and non-responders serves to illustrate the point that there were as many responders in the Mantoux negative as in the Mantoux positive group. In comparison to the other cytokines we measured after stimulation with PPD, this finding raises the question if PPD stimulation was at all specific. It should perhaps rather be regarded as a non-specific stimulant for some IL-10 production in 50% (healthy Mantoux pos and neg subjects)-60% (patients) of the whole population under study. The only study I could find where PPD was used as a stimulant of whole blood and IL-10 measured afterwards, was by van Crevel et al[30] who studied healthy skin test positive and negative individuals, but no patient group. They could not measure any IL-10 production either after stimulation with PPD, while they could measure IL-10 after stimulation of whole blood with PHA or LPS. On the basis of these arguments, I have decided to regard the results on the PPD stimulated IL-10 production as non-specific in our study, thereby disregarding the fact that there was a significant difference between patients and Mantoux pos controls, especially in the 12-20 yrs age group. Why PHA and LPS, but not PPD, seem to be effective inducers of IL-10 in whole blood cultures is an intriguing issue, that would be interesting to study further in some detail. Plasma factors are likely candidates, since the phenomenon seems not to be apparent in studies with PBMCs. The fact that IL-10 can be measured in serum from TB patients, and in significantly higher amounts than in healthy Mantoux positive controls[99] indicates that, with respect to TB, serum IL-10 has some role *in vivo*.

Serum IgE values

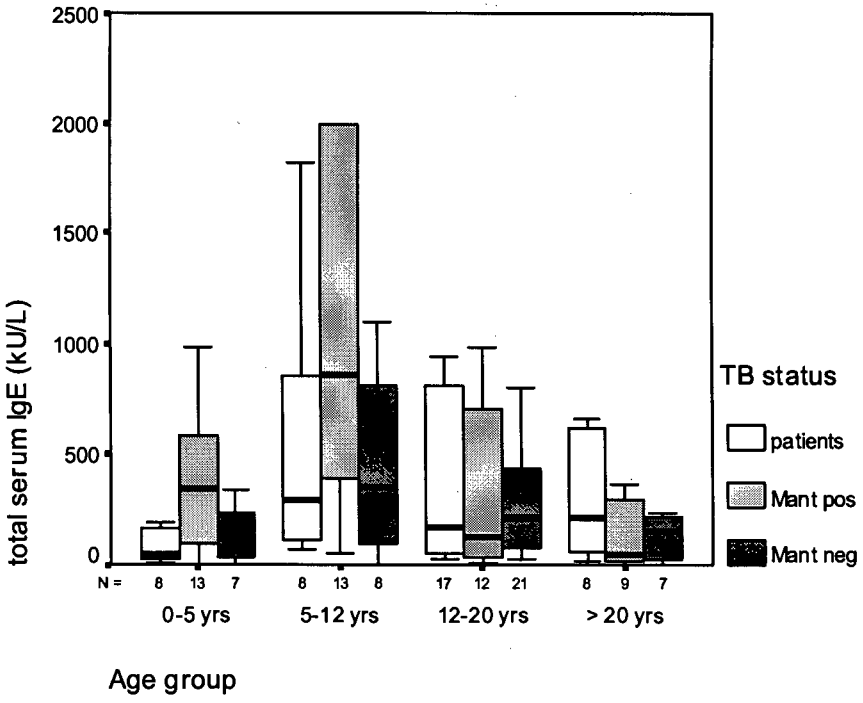


Table: Median (interquartile range:IQ) of serum IgE (in KU/l) levels in patients, healthy controls and totals

TB status	patients		healthy subjects (Mantoux pos and neg)		p-value[1]	total	
Age group (yrs)	n	Median (IQ)	n	Median (IQ)		n	Median (IQ)
0-5	8	44 (20-299)	20	247 (68-606)	.150	28	144 (43-477)
5-12	8	310 (101-1045)	21	627 (252-1882)	.301	29	529 (200-1076)
12-20	17	191 (51-910)	33	254 (62-530)	.690	50	164 (52-788)
> 20	8	251 (56-1005)	16	124 (16-303)	.214	24	145 (20-350)
total	41	157 (47-781)	90	239 (63-690)	.794	131	217 (52-758)
p-value[2]	.216		**.004**			**.006**	

p-value[1]: Patients compared to healthy subjects (Mantoux positive and negative) per age group or total (Mann- Whitney U test)

p-value[2]: Age groups of patients, healthy subjects or totals compared (Kruskal-Wallis One-Way ANOVA by Ranks test)

Age (yrs) had no correlation with total serum IgE levels (KU/L), although the various age groups had significantly different levels of serum IgE.

There were no significant differences in serum IgE levels between patients and healthy controls

Serum IgE levels (KU/L) did not correlate with any of the produced cytokines (pg/ml) that we measured, after stimulation of whole blood with PHA or PPD.

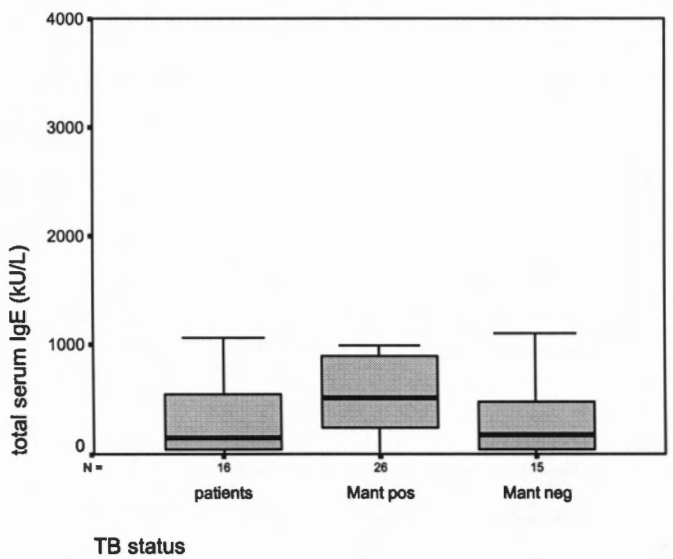
Serum IgE levels in children under 12 years of age

As can be inferred from the boxplot graph on total serum IgE levels in all age groups, there were more distinct differences in serum IgE levels in the subjects under 12 years of age than in the older subjects, which is why the children < 12 yrs will be analysed in some more detail.

Under the age of 12 years, there was a significant correlation between age in years and serum IgE levels ($n = 57$, $\rho = .444$, $p = .001$), when all the subjects of this age were taken into account.

IgE was the only measurement where, at least in the children under 12 yrs of age, there were differences between both patients and healthy Mantoux pos controls (significant) as well as between Mantoux pos and Mantoux neg healthy controls (nearly significant).

Graph: Total serum IgE (KU/l) in children under 12 yrs of age by TB status



The corresponding p-values *(Mann-Whitney U test)* of this analysis were:

. difference in serum IgE levels (KU/L) of patients *vs* Mantoux pos: $p = .028$
. difference in serum IgE levels (KU/L) of patients *vs* Mantoux neg: $p = .906$
. difference in serum IgE levels (KU/L) of Mantoux pos *vs* Mantoux neg: $p = .055$

There were no correlations between serum IgE levels (KU/L) and produced cytokines (pg/ml) that we measured, after stimulation with PHA or PPD in children under 12 years of age.

There was no correlation between IgE levels (KU/L) and Mantoux size (mm) in the patients < 12 yrs. There was however a, just significant, correlation between IgE levels (KU/L) and Mantoux size (mm) in the group of healthy controls, Mantoux pos and neg combined ($n = 40$, $\rho = .321$, $p = .044$).

Discussion on total serum IgE measurements

We measured serum IgE levels in our study subjects to obtain a "proxy" or surrogate marker for type 2, as opposed to a type 1, immune activity. In general, it is acknowledged that a type 2 immune response is driven by type 2 cytokines like IL-4, Il-5, Il-6, IL-13 and IL-10, resulting in a dominant humoral immune response. Cross-regulatory interactions between IL-4, IL-13 and IFN-γ are well described.[186] Increased levels of serum IgE are associated with high Il-4 and/or IL-13 production and IL-4 seems an essential factor for IgE synthesis[187]. We attempted to measure IL-4 in the supernatants of the stimulated whole blood cultures, but the levels of this cytokine were mostly below the detection levels of the assays. This may have been due to technical constraints in measuring IL-4[105] and/or a real absence of IL-4 derived biological activity in the human immune response to TB. A good number of groups studying the human immune-pathogenesis of TB have failed to demonstrate IL-4 by a variety of techniques, although others were able to show an increased frequency of IL-4 producing cells in TB patients.[84,188]

Total IgE levels in serum may be a reflection of polyclonal, non-specific IgE and/or antigen specific IgE. High serum IgE levels are associated with a number of pathologic conditions amongst which atopy and helminthic infections are the most common. In both these conditions high IgE levels may be found with or without elevated antigen specific IgE.

In recent years, there have been a number of reports and opinionating articles supporting the hypothesis that helminthic infections, which are so very prevalent in resource-poor tropical regions of the world, contribute to the rapid spread of diseases like TB and AIDS in these populations.[189] Helminths may drive the hosts' immune response towards high polyclonal serum IgE levels as well as elevated helminthic specific IgE. Complicated cross-talk with all elements of the immune system is the result, reviewed in an instructive paper by Maizels et al[190]. Furthermore this cross-regulation is not only helminth species specific, but also dependent on the stage of the parasite's life cycle and the organ of the

host in which the worm resides. Within this interplay, genetic factors are probably important, illustrated by the fact that in most populations "wormy people" are identified, i.e. persons who seem to have a predisposition favoring recurrent high burdens of helminthic infestations, despite treatment and preventive measures. High IgE may be an indication of past or present helminthic infection.[191] As such IgE is not only an indicator of infection but may be an expression of protection from infection as well.[192] Changes in mean worm burden typically follow a convex curve with the age of peak intensity in the host depending on life expectancy of the parasite, intensity of transmission and behavioral factors of the host.[190]

Age–prevalence curves of *Ascaris lumbricoides* infections rise steadily with age from young childhood to a peak in early teenage years and tail off thereafter. In our study, there was a significant correlation between age in years and total serum IgE levels in children younger than 12 years as well as a convex curve with age thereafter, which may very well be a reflection of endemic infection with *Ascaris lumbricoides* in the region. According to a 1993 study from the area of our study, *Ascaris lumbricoides* and *Trichuris trichuria* infections were already prevalent in 54% of crèche attendees (children aged 12-69 months), despite the availability of free anti-helminthic treatment.[193] *Ascaris lumbricoides* worms are of interest to our IgE data, because of their propensity to induce high serum IgE levels and perhaps, on a speculative note, because of their transient life-stage in the lungs. We have previously published a study where we showed that a group of adolescent TB patients, at time of diagnosis, did not have different levels of total serum IgE as compared to a group of Mantoux positive healthy controls.[194] Our study presented here confirms this finding for that particular age group (12-20 yrs). However, our findings from the children younger than 12 years are different. There were significant differences in IgE between patients and both healthy Mantoux- positive and negative controls. It would be attractive to hypothesise that an ongoing type 1 response against *M.tb*, as can be postulated a TB patient might exhibit despite it being not entirely effective against TB, lowers the total serum IgE. Even more speculative, but interesting, would then be the assumption that children infected with *Ascaris lumbricoides* have a lowered local pulmonary defense against infection with *M.tb*, caused by the migration

phase of *Ascaris lumbricoides* through the lungs. The infection with *M.tb* would then lead to a positive Mantoux skin test, but not necessarily disease in these children. However, none of the other data from this study can support these postulates.

Atopy and TB have been "linked" by a number of authors, in particular Shirakawa et al[195] who, in a much quoted study, argue that a decrease in exposure to *M.tb* (as measured by mm of Mantoux skin induration) is related to an increase in atopic conditions in Japanese school children. Fascinating as the retrospectively obtained data in this report are, the authors do not consider in their discussion that atopic conditions may be instrumental in diminished DTH skin reactions and thus the cause rather than the effect of the proposed association. In atopic persons high serum IgE levels are a reflection of the atopic condition and, with exceptions, elevated allergen- specific IgE levels indicators of clinical allergy for a particular antigen. Clinical allergy may, however, also be present without demonstrable elevated (specific) serum IgE. Although we are readily inclined to make the link "IgE-worms" when debating IgE levels in people from tropical climates, we often tend to forget that in these climates TB is an important disease of the urbanised poor, who live under shallow conditions in settlements favorable to allergens like house-dust mite. Indeed, allergic conditions are rising in the cities of Africa and frequently encountered in the Cape Town area, where a high and rising prevalence of house dust mites in mattresses (coupled to skin-prick test sensitivity) and asthma has been shown.[196] None of the children included in our study were known to have any clinically important manifestations of asthma or eczema, but if the high IgE levels in the healthy Mantoux positive children were related to atopic conditions, our data argue against those found in the Japanese study.

In the considerations of a possible inverse relation between "infections" and atopy, the type 1/type 2 paradigm may not prove to be very helpful in any way. High polyclonal IgE levels, caused by infections with helminths may saturate Fcε receptors on mast cells and in this way competitively inhibit binding of other allergen-specific IgE.[197] Children with *Schistosoma haematobium* infections had lower skin test reactivity to house dust mite in a recent study from Gabon and this finding correlated with high schistosome-specific

antigen mediated IL-10 responses.[198] These findings prompted Holt[199] in an invited commentary to cite the authors of a 1976 published study on IgE levels: *"....atopic disease is the price paid by some members of the white community for their relative freedom from diseases to viruses, bacteria and helminths..."*.

Thus, in the light of the reasoning on an "inverse" relation between IgE and TB, the findings of our study do not support any such hypotheses. However, we could not obtain IgE on all our study subjects, nor did we measure specific IgE to any allergen or examine stools to determine worm load. Furthermore, this is a relatively small study, so any definitive conclusions in this respect cannot be drawn.

One conclusion is very much warranted in my opinion, namely that future studies into the relation of infectious diseases and changing IgE levels by any cause, should take into account age factors and the dynamics of the prevalent regional helminthic infections and/or atopic conditions in the population under study.

Age-related expression of disease

Introduction

The aims of this study were to analyse the cellular immune response in healthy individuals and persons with TB at different ages and to relate the findings to clinical expression of disease at different ages.

Two distinct expressions of disease after infection with *M.tb* are generally recognised:
1. "Primary TB", which is a direct consequence of an infection with *M.tb*, occurring in immunologically naive persons who have not been previously sensitized with *M.tb*. The most common clinical phenotype of primary TB is hilar adenopathy, with or without lung parenchymal involvement as a consequence of bronchial obstruction or seeding of *M.tb* through eroded bronchi. Any form of disseminated disease is also regarded as a clinical expression of primary TB.[148]
2. "Postprimary TB" whereby, after a variable period of latency, endogenous or exogenous re-infection results in a disease principally of the lung parenchym, mostly affecting the upper lobes and classically with cavity formation.

To be able to classify patients as having primary or postprimary TB, their clinical characteristics have to be evaluated. Usually, the mode of presentation, which includes the findings on the CR, is sufficient ground to make a distinction between primary and postprimary TB, along the above described criteria.

The model of the following analysis was, in part, inspired by a number of findings in the preceding chapters of results:

- Mantoux negative healthy subjects of different ages did not produce appreciable amounts of cytokines in response to PPD or ESAT-6 when compared to Mantoux positive healthy subjects.
- Although there were significant differences between patients and Mantoux positive healthy subjects in certain age groups and a number of interesting trends, no single cytokine result stood out statistically as highly significant in any of the four age

groups individually. This may have been entirely the consequence of the relatively small numbers of subjects per group and the need to use non-parametrical tests throughout the analyses. The only way to single this factor out is to study larger groups of subjects.

Furthermore, the suggestion has been made by Ellner et al[200] that a profile, i.e. ratios between different cytokines, made up from cytokine responses to mycobacterial antigens might be more indicative in distinguishing patients from controls of all ages.

Methods

All the patients and Mantoux positive healthy controls of this study were eligible for this analysis. The Mantoux negative healthy controls were excluded, since they did not have any quantity of mycobacterial specific responses that were worth comparing to the other two groups.

Patients were stratified as having primary or postprimary disease on the basis of the following characteristics:

Primary TB:	Hilar adenopathy with or without lung parenchymal involvement. Disseminated disease, with or without abnormalities on the CR.
Posprimary TB:	Infiltrating abnormalities of the lung parenchym with or without cavities in the presence of a positive smear for acid-fast bacilli and/or positive sputum culture for *M.tb*.

The subjects' individual results on lymphocyte proliferation and cytokine production, obtained from the original study, were used for the analyses.

Non-parametrical statistical methods, as described before, were used.

Results

Clinical characteristics of included subjects

22 patients could be classified as having primary TB and 25 patients could be classified as having postprimary TB and their diagnostic characteristics are presented in the following table.

Table: Diagnostic characteristics of patients with primary or postprimary TB

	Primary TB	Postprimary TB
Principal presentation		
IntrathoracicTB	17	25
TBM	3	
Abdominal TB	1	
Erythema nodosum	1	
CR findings		
Hilar adenopathy	19	
Infiltrates/cavities		25
Pleural effusion		
Atypical findings	2*	
No abnormalities	1**	
Microbiology		
Sputum sm +	1	25
Sputum cult+	1	
Other cult +	4	

TBM: Tuberculous Meningitis
CR: Chest Radiograph

* : *1 patient with a pneumatocele and infiltrate behind the hart on the CR and culture positive TBM*
1 patient with an infitrate on the CR, loss of weight, a known contact and a gastric aspirate positive for M.tb on culture

**: *1 patient without abnormalities on the CR, presenting with clinical, cerebrospinal fluid and cranial CT scan findings compatible with TBM*

Three patients from the original study were excluded from the analysis. One, because he presented with a pleural effusion and a positive smear for TB, but was subsequently lost

to follow up and from his CR at presentation, it could not be established with certainty if the effusion was accompanying primary or postprimary TB. One was excluded because he had symptoms of chronic cough and a positive sputum smear for acid-fast bacilli, but no abnormalities on his CR. One, because she presented with erythema nodosum and only a calcification on the CR without a known contact with an index case. Erythema nodosum, mostly regarded as a hypersensitivity reaction to *M.tb*, is a well-known presentation of TB, but I could not classify her in any of the two patients groups.

All the patients with primary TB turned out to be younger than 12 years of age, all the patients with postprimary disease were older than 12 years.
The Mantoux positive healthy controls were stratified along age, < 12yrs or > 12yrs, and thus four study groups, displayed in the following table were formed.

Table: Median (Interquartile range: IQ) age in years and Mantoux skin test (mm) of the patients with primary TB or postprimary TB and their age-related healthy (<12yrs or >12yrs) Mantoux pos controls

	n	Age in years (IQ)	n	Mantoux in mm (IQ)
Primary TB	22	2.71 (1.5-6.6)	18	16.6 (8.7-20)
Healthy <12yrs	28	4.96 (3.1-9.5)	27	18 (16.3-21.3)
Postprimary TB	25	18.5 (15.9-24)	14	19.5 (15.9-32.5)
Healthy >12yrs	21	18.27 (14-35.5)	20	18.9 (16.8-28.1)

There was a significant difference in age (yrs) between the group of patients with primary TB and the group of age-related healthy controls < 12 yrs ($p = .002$).

PPD stimulated lymphocyte proliferation

Table: Median (interquartile range: IQ) lymphocyte proliferative response (cpm) after stimulation of whole blood with PPD (3.3μg/ml) on day 7 (after subtraction of unstimulated proliferation on day 7) from patients with primary TB or postprimary TB compared to age-related healthy Mantoux positive controls

	n	Median cpm (IQ)	p*
Primary TB	20	16202 (2529-26015)	
Healthy <12yrs	25	32705 (13673-60250)	**.008**
Postprimary TB	22	3950 (1873-12950)	
Healthy >12yrs	21	37063 (5263-57517)	**< .0001**

**p:* *patients compared to age-related healthy controls (Mann Whitney U test)*

Patients with primary and postprimary TB had significantly lower lympho-proliferative responses compared to their age-related controls. Patients with primary TB tended to have higher responses than patients with postprimary TB, but this was not a significant finding ($p = .056$). There was no difference in lymphocyte proliferation between the two healthy control groups.

Age (yrs) had a strong positive correlation with PPD stimulated lymphocyte proliferation in the healthy Mantoux pos controls < 12 yrs ($\rho = .649$, $p < .0001$) and this correlation was weaker, just significant, but inverse in the healthy Mantoux pos controls > 12 yrs ($\rho = -.461$; $p = .036$). There were no significant correlations between age and lympho-proliferation in any of the patient groups

PPD stimulated cytokine responses

PPD stimulated IFN-γ, TNF-α and IL-2 responses of whole blood in the 4 study groups were as follows:

Table: IFN-γ, TNF-α and IL-2 production (interquartile ranges: IQ) after PPD stimulation (3.3μg/ml) stimulation of whole blood from patients with primary TB or postprimary TB compared to age-related healthy Mantoux pos controls

	n	IFN-γ (pg/ml) (IQ)	P*	n	TNF-α (pg/ml) (IQ)	P*	n	IL-2 (pg/ml) (IQ)	P*
Primary TB	22	8938 (1855-17479)		20	121 (60-187)		22	174 (71-649)	.584
Healthy <12 yrs	28	14435 (4047-25945)	.296	28	109 (28-153)	.350	28	308 (115-491)	
Postprimary TB	25	7948 (3176-30248)		25	113 (80-211)		25	42 (10-142)	
Healthy >12 yrs	21	16075 (5487-33837)	.213	21	97 (10-146)	.077	21	128 (30-465)	.054

** p-values: patients compared to age-related healthy controls (Mann-Whitney U test)*

There was no single cytokine that significantly differentiated patients from age-related healthy controls, although the difference in IL-2 production between patients with postprimary disease and their age-related healthy controls was nearly significant. Differences in IL-2 production were significant when patients with primary TB were compared to patients with postprimary TB ($p < .0001$).

In the healthy control group < 12 yrs, age (yrs) had a strong correlation with IFN-γ ($\rho = .604$, $p = .001$) and a nearly significant correlation with IL-2 ($\rho = .341$, $p = .052$).

Ratios between PPD stimulated cytokine levels

Ratios of cytokine levels were calculated by dividing the measurements (in pg/ml) of two single cytokines in response to PPD from each included subject. Such a calculation should approximate the balance between the two cytokines in the culture supernatants after stimulation with PPD, whereby it must be remembered that the IL-2 levels were measured on day 3 after culture with PPD, while IFN-γ and TNF-α were measured on day 7.

Table: Ratios of cytokine levels after stimulation of whole blood with PPD (3.3μg/ml) from patients with primary or postprimary TB compared to healthy age-related Mantoux positive controls

	n	ratio IFN-γ/ TNF-α (IQ)	P*	n	ratio IFN-γ/ IL-2	P*	n	ratio IL-2/TNF-α (IQ)	P*
Primary TB	20	66 (38-195)		22	22 (10-103)		20	3.09 (.91-6.40)	
Healthy <12 yrs	28	191 (108-325)	**.023**	28	28 (18-240)	.211	28	2.53 (1.16-9.24)	.917
Postprimary TB	25	88 (29-217)		25	175 (64-786)		25	.40 (.13-1.00)	
Healthy >12 yrs	21	266 (157-475)	**.011**	21	158 (30-312)	.316	21	2.64 (.91-5.78)	**.001**

**: p-values: patients compared to age-related healthy controls (Mann-Whitney U test)*

Irrespective of age or expression of disease, patients had lower IFN-γ/ TNF-α ratios than their age-related healthy controls. Furthermore patients with postprimary TB had a significantly lower ratio of IL-2/TNF-α when compared to age-related healthy controls. If patients with primary TB were compared to patients with postprimary TB the differences in IFN-γ/ IL-2 ratios ($p = .002$), as well as the differences in IL-2/TNF-α ratios ($p = .001$) were significant.

Of the calculated ratios, IFN-γ/ IL-2 had a significant inverse correlation with age (yrs) in patients with postprimary TB ($\rho = -.647$, $p < .0001$). None of the calculated ratios had a significant correlation with age in any of the other 3 groups.

There were three children with TBM in the group of patients with primary TB. Two of these did not have any outlying values for the ratios between the different cytokines presented in the preceding table. One of them did have an abnormal low IFN-γ/ TNF-α ratio of 32, while her IFN-γ/ IL-2 (11) and IL-2/TNF-α (3) ratios fell within the 25th percentiles of the values from the other patients with primary TB. Her IFN-γ response to PPD (530 pg/ml) was well below the normal range for her age. The same was true for her PPD stimulated TNF-α (18 pg/ml) and her IL-2 (50 pg/ml) production, which explains why she had normal ratios for IFN-γ/ IL-2 and IL-2/TNF-α. Unfortunately her lymphocyte proliferation values are not available due to a technical problem. Her IFN-γ response to PHA (1331 pg/ml) was, however, normal for her age. It must be said that this patient had far more extensive involvement of her brain with residual neurological damage at follow-up compared to the other two patients with TBM.

This example does illustrate the fact that calculated ratios need to be interpreted only after examination of the individual cytokine responses. "Little" divided by "little" results in "little" and cytokine ratios of Mantoux negative healthy controls were thus also significantly lower than those of Mantoux positive healthy controls.

ESAT-6 stimulated IFN-γ production

The relatively small amount of subjects we could analyse for their ESAT-6 induced IFN-γ production (due to the later acquisition of ESAT-6) prevented a meaningful analysis by age group and TB status in the earlier analysis on ESAT-6 stimulated IFN-γ responses. Pooling of study subjects by phenotype of disease enabled an analysis that was more worthwile.

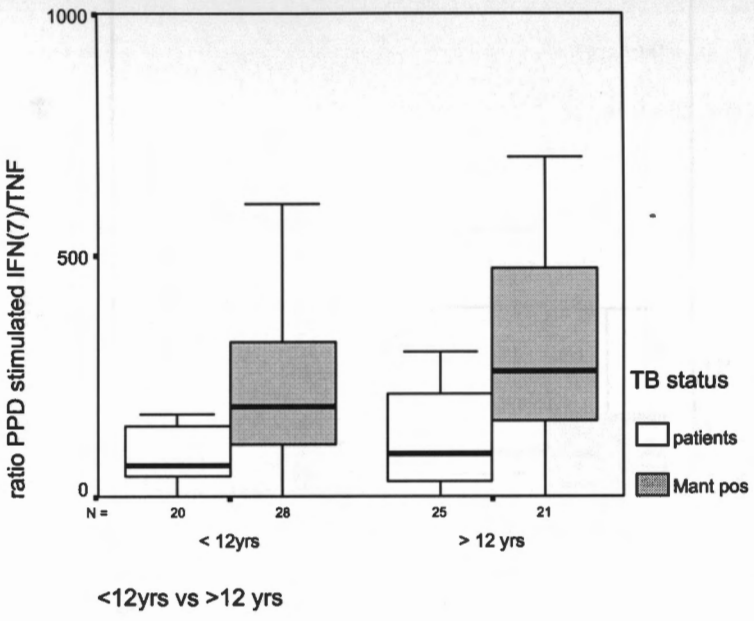
Table: Median IFN-γ production (interquartile range:IQ) on day 7 after stimulation with ESAT-6 (1 μg/ml) in patients with primary TB or postprimary TB compared to age-related healthy Mantoux pos controls.

	n	IFN- γ (pg/ml) (IQ)	P*
primary TB	16	1518 (10-4849)	.166
healthy subjects <12 yrs	16	5897 (193-10431)	
postprimary TB	18	1069 (217-6955)	**.025**
healthy subjects >12 yrs	11	10 (10-3018)	

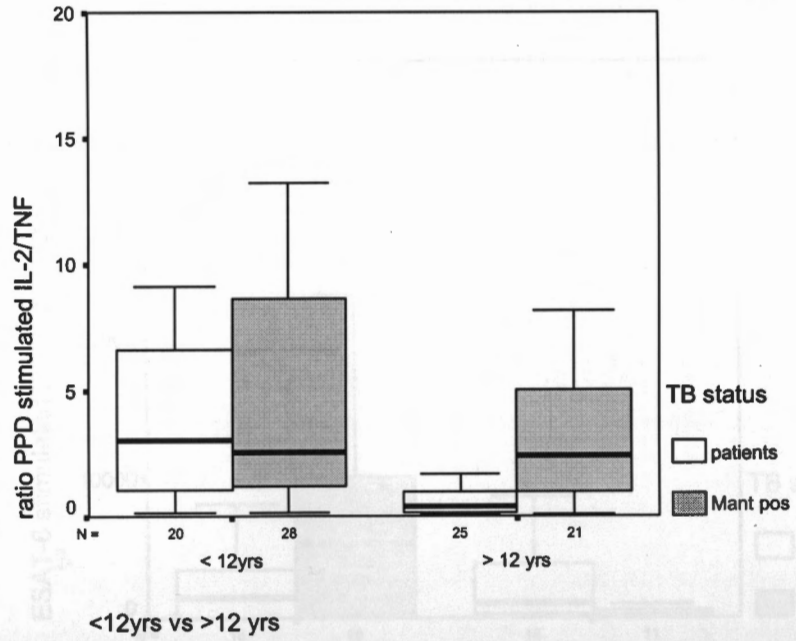
**: p-values: patients compared to age-related healthy controls (Mann-Whitney U test)*

While patients with primary TB did not produce significantly different amounts of IFN-γ after stimulation with ESAT-6 when compared to their age-related controls, patients with postprimary TB produced significantly more than their age-related controls. Furthermore, healthy Mantoux pos subjects < 12 yrs produced significantly more IFN-γ than healthy Mantoux pos subjects > 12 yrs ($p = .028$).

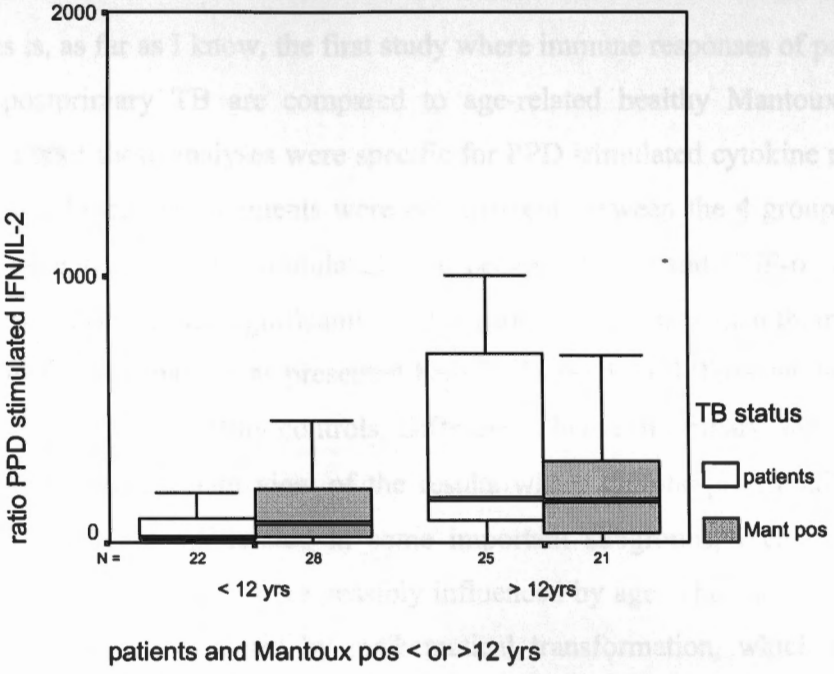
Graphic displays of the findings on cytokine ratios



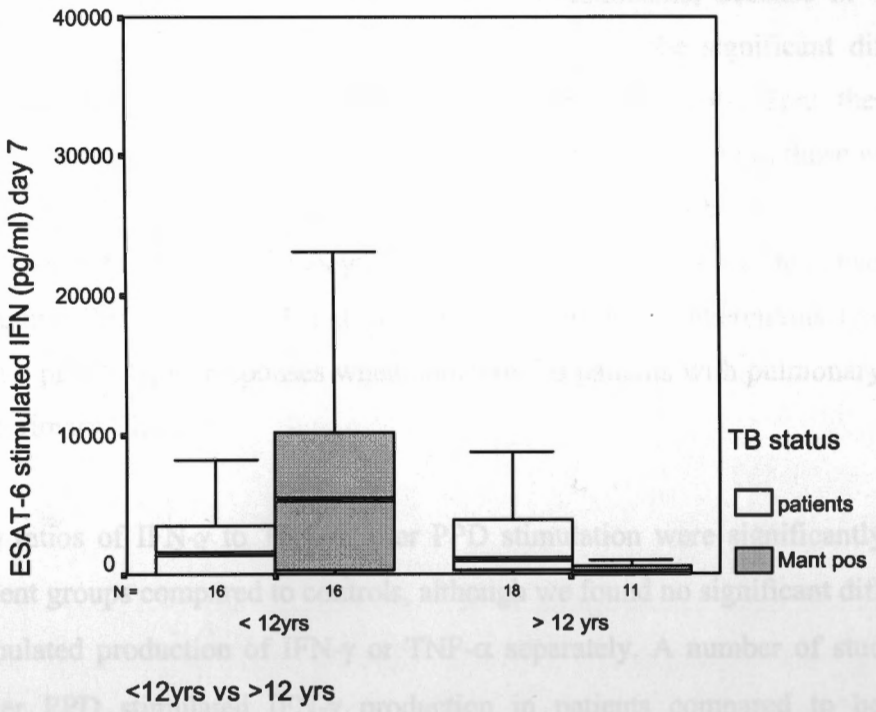
<12yrs vs >12 yrs



<12yrs vs >12 yrs



Graphic dysplay of IFN-γ production after stimulation with ESAT-6



Discussion on age related expression of disease

This is, as far as I know, the first study where immune responses of patients with primary or postprimary TB are compared to age-related healthy Mantoux positive controls. Results of these analyses were specific for PPD stimulated cytokine responses. Ratios of PHA induced measurements were not different between the 4 groups under study, with the exception of PHA stimulated ratio between IFN-γ and TNF-α, where patients with postprimary TB had significantly higher (not lower) values than their age-related healthy controls. The analysis as presented here, is focused on differences between patients and their age-related healthy controls. Differences between primary and postprimary disease are interesting, but in view of the results where lympho-proliferative, IFN-γ and IL-2 responses were age-related in some important subgroups, I cannot exclude that any significant differences were possibly influenced by age. The values of the measurements could not be normalised by mathematical transformation, which makes a regression analysis difficult to defend.

Although the lympho-proliferative responses differentiated both patient groups from their age-related healthy controls, this finding is questionable, because of the strong age-related values in the healthy controls < 12 yrs and the significant difference in age between the patients with primary TB and these controls. That these proliferative responses tended to be higher in patients with primary TB than in those with postprimary TB is notable. Wilkinson et al.[172] studied adult patients with various clinical phenotypes of disease for their lymphocyte responsiveness to a set of mycobacterial antigens, including PPD, and found that patients with peripheral tuberculous lymphadenitis had higher proliferative responses when compared to patients with pulmonary TB (78% with postprimary TB).

The ratios of IFN-γ to TNF-α after PPD stimulation were significantly lower in both patient groups compared to controls, although we found no significant differences in PPD stimulated production of IFN-γ or TNF-α separately. A number of studies have found lower PPD stimulated IFN-γ production in patients compared to healthy Mantoux

positive controls, but the finding has not been very consistent.[188, 200] This apparent discrepancy of results from different studies may be due to different techniques for measuring IFN-γ, distinct sites for sampling mononuclear cells, varying patient characteristics or any combination of these factors. With respect to TNF-α there have been equally conflicting results[87], although patients tend to produce more than healthy controls. The revealing calculation of the IFN-γ to TNF-α ratio suggests that, whilst this ratio is lower in healthy controls following infection with *M.tb*, patients suffer from the immunopathological effects of a different balance between these two (pro-) inflammatory cytokines.

Another key finding is a striking difference in the positive IFN-γ responses to ESAT-6 of healthy Mantoux positive children under twelve years of age as compared to lower such responses of the older healthy subjects. Both groups of patients responded well to ESAT-6. ESAT-6, as a *M.tb* specific antigen has, until now, only been evaluated in adults using PBMCs. Its promise as a more specific antigen than PPD in the immune diagnosis of TB[115] is confirmed in this analysis for subjects older than 12 years. However, in the age group where primary TB is far more common, stimulation with ESAT-6 did not help in discriminating patients from healthy Mantoux positive controls in this study. For young children with a positive Mantoux skin test, it is reasonable to assume that first infection with *M.tb* is of relatively recent date. As the annual risk for infection is 3.4% in the area where this study was done *(N Beyers, submitted for publication)*, a considerable time may have elapsed since first infection with *M.tb* in our older controls. Recently, a study showed that IFN-γ production after ESAT-6 stimulation decreased during antituberculous chemotherapy[201], implying that reactivity may wane. Our data suggest that a positive IFN-γ response to ESAT-6 is indicative of recent active immunological contact with *M.tb*, but not necessarily of disease, since every healthy control under the age of twelve had a normal CR.

Recombinant IL-2 has (rIL-2) been used in some settings as an additional therapeutic agent to anti-tuberculous therapy[93], whereby an enhancement of the antimicrobial response was observed (decreased sputum bacillary loads). The basis for this strategy has

been the observation that patients with lepromatous leprosy showed microbiological improvement when treated with intradermal rIL-2.[92]

Patients with postprimary TB in our study produced less IL-2 than age-related healthy controls, they furthermore produced less IL-2 than patients with primary TB. Patients with postprimary TB are likely to have much higher bacillary loads compared to patients with primary TB. Although highly speculative, it is an intriguing thought that the relatively good production of IL-2 upon PPD stimulation in young patients reflects their relative capacity to limit bacterial growth. The differences between patients with primary TB and those with postprimary TB with respect to the IFN-γ/ IL-2 ratio and the IL-2/TNF-α ratio are by themselves interesting, but possibly solely dependent on the age-related difference in IFN-γ and IL-2 production. They need confirmation from studies in adolescents and adults of different ages with different clinical phenotypes of TB.

In conclusion, in a whole blood model, the ratio of IFN-γ to TNF-α in response to PPD differentiates active disease from asymptomatic infection, regardless of age. Furthermore, our results suggest that a positive IFN-γ response to ESAT-6 is indicative of recent infection and therefore does not discriminate between infection with *M.tb* and disease in young children.

Technical problems during the study

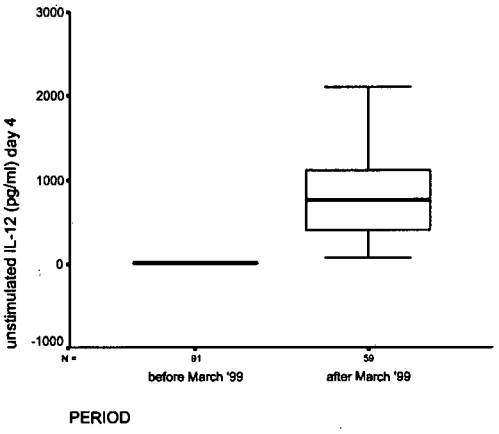
IL-12p40 and TGF-β

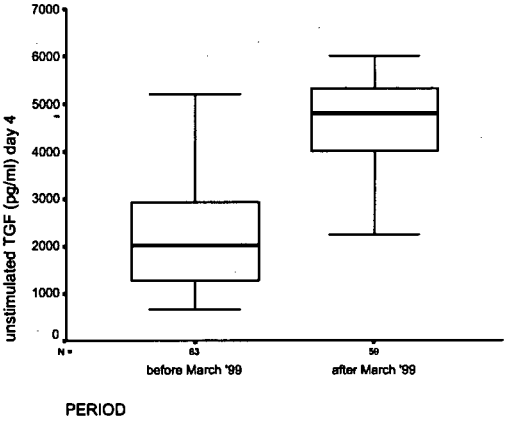
This study was set up to report on IL-12 and TGF-β levels after stimulation with PHA and PPD in the same way as on the other cytokines. There have, however, been a number of technical problems with the measurements of IL-12 and TGF-β.

Halfway through the study the biotechtechnical company Genzyme Diagnostics merged with R&D Systems. Although from the start of the study, we had purchased enough kits to perform all the analyses on the other cytokines with DuoSet kits from Genzyme Diagnostics for the whole study, we did not have enough kits for IL-12 and TGF-β to do all the samples. Genzyme Diagnostics was not able to supply us with anymore IL-12 or TGF-β kits at the time of the merger and, despite a concerted effort, no other research group could be found that might be able to provide us with any of their spare kits. Subsequently we had to wait until R&D Systems was able to deliver IL-12p40 and TGF-β kits, by which time the initially envisaged total number of study subjects had been recruited.

Since we found in the pilot studies that the background levels of these two cytokines were not always below detection levels of the assays, unstimulated background levels of IL-12 and TGF were routinely measured in all the study subjects.
When we received the kits from R&D Diagnostics and measured the IL-12p40 values, it became clear that in the group of study subjects included from end of March 1999 until June 1999, the background levels of IL-12p40 were significantly higher than those from the previous group. Although there had been no changes in the lab routines and no differences could be found in any of the other cytokines, I decided to reopen the study and recruit new subjects in order to substitute those recruited from March '99 until June '99. Unfortunately, I found that the newly recruited subjects, from the period June 1999 until November 1999, again had high unstimulated background levels of IL-12p40.
(See graphs)

Graphs: Unstimulated levels of IL-12p40 and TGF-β on day 4 of study subjects included before and after March 1999





The primary measures taken to investigate and prevent possible sources of contamination upon the initial finding of high background values were:

. All the incubators, the destilled water systems and hoods were checked for sources of contamination and nothing was found.

. RPMI, which was previously made from powdered extract, diluted in destilled water and sterilised, was now purchased in sterilised solution from the manufacturer.

. Measures to prevent contamination were reinforced.

The subjects that had to be newly recruited in June 1999 were predominantly patients between 5-12yrs (n=3) and 12-20yrs (n=9), Mantoux positive controls of 0-5 yrs (n=4) and Mantoux negative controls of 12-20 yrs (n=9). In total, the 59 subjects recruited as from the end of March 1999 until November 1999 were:

-1 patient, 9 Mantoux positive subjects and 1 Mantoux negative subject aged 5-12yrs

-6 patients and 3 Mantoux positive subjects aged 5-12 years

-17 patients, 2 Mantoux positive subjects and 19 Mantoux negative subjects aged 12-20 yrs

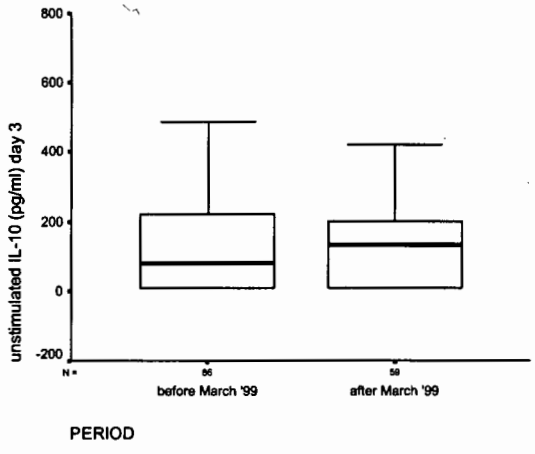
-1 patient aged over 20 years

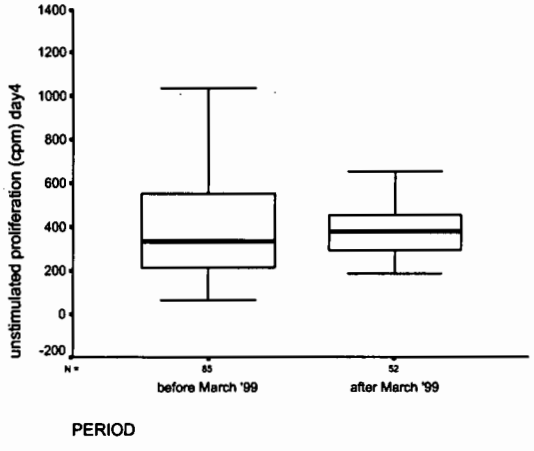
This was such a diverse group, that it is very difficult to come up with any reasonable explanation that the high background values might have been "natural", but this possibility cannot be rejected completely. Nevertheless I have decided that the IL-12p40 and TGF-β results we obtained were not to be trusted completely and therefore they have not been included in the results from this study.

Other cytokines by period of inclusion

From the start of the study unstimulated data were also routinely obtained for cell proliferation and IL-10 levels, because they were equally found to be potentially higher than the detection limits of the assays in the pilot studies. As can be inferred from the graphs below there were no differences in these values before and after March 1999. The boxplot of the background cell proliferative responses on day 7 has exactly the same outlook as the one displayed for day 4 and is omitted.

Graphs: Unstimulated IL-10 (pg/ml) on day 3 and unstimulated lymphocyte proliferation (cpm) on day 4 before and after March 1999

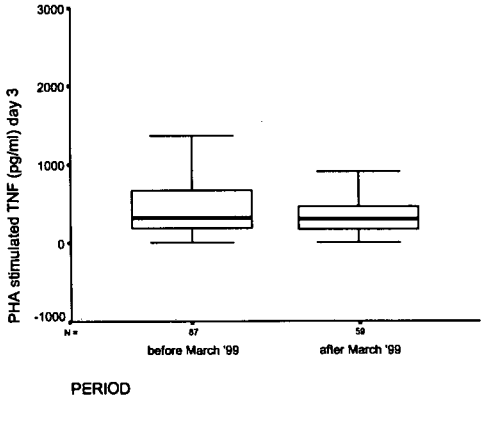


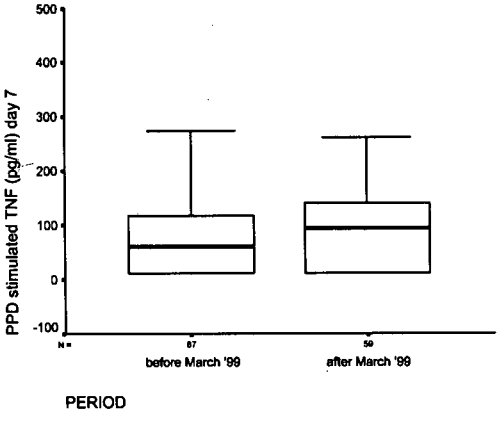


We thought that LPS could be responsible for the high background values of IL-12p40. LPS is a strong stimulant for both IL-12p40 and its downstream effector cytokine TNF-α. The background levels of TNF-α in all the unstimulated supernatant samples from the whole study population were subsequently measured, but they were all below detection level (15pg/ml) of the assay. Equally, a large subgroup of unstimulated supernatants from before March 1999 and all the unstimulated samples from after March 1999 were checked for unstimulated IFN-γ levels and these were also negative. Because our storage system for samples was such that we alliquoted supernatants in small volumes, we could check these background levels of TNF-α and IFN-γ from samples that had not previously been thawed, so any loss of cytokine activity due to repeated freezing and thawing in these samples was unlikely.

The values of TNF-α after stimulation with PHA or PPD did not differ between the two periods as shown in the following graphs

Graphs: Display of TNF-α levels (pg/ml) after stimulation with PHA on day 4 and PPD on day 7 before and after March 1999





Although no satisfactory reason has been found for the high unstimulated background values of IL-12p40 and TGF-β, I have found no reason to disregard either the results of the other cytokines or those of the lympho-proliferative cell responses.

General discussion

The definite question in a final discussion must surely be: "SO WHAT....?".
The appropriate answer would probably start with: "that is a good question.....!" and then the researcher will put forward the likely advantages of the methodology used, summarise the interesting findings, shed some favourable light over the possible implications and propose that further research in this field is needed. And that is exactly what I am going to do.

We used a whole blood technique to investigate cellular immune responses from TB patients and healthy subjects in an area highly endemic for TB. As already mentioned in one of the introductory chapters, there are clear advantages in the use of this method if compared to the use of PBMCs: lesser volumes of blood are required and there is no need for separation of cells from autologous serum. One of the difficulties in interpreting results from whole blood culture methods is the fact that there are no fixed amounts of white blood cells in the wells on the culture plates. When comparing children to adults *in vitro* this phenomenon is further accentuated by the age- related differences in white blood cell counts per volume of blood. In one important validation study for the use of whole blood *vs* PBMCs in people of different ages[177], the authors "normalised" their cytokine results to the blood samples' initial absolute amount of mononuclear cells. Although in that specific study, because of its aim for validation, such an exercise was necessary, one may argue that the use of the initial amount of white blood cells is not correct. White blood cells proliferate upon stimulation and thus the number of cells after proliferation might be a better denominator. I have done the calculations both with the initial amount of lymphocytes as well as the amount of lymphocytes after proliferation, but either way the results were comparable or, sometimes, more significant. (I took absolute amounts of lymphocytes rather than the absolute amounts of white blood cells, because pulsing with Methyl- ^{3}H Thymidine only incorporates lymphocytes.) However I chose not to present the results in this way,

because I feel that one of the reasons why we chose the whole blood method, namely the better approximation of *in vivo* conditions, does not justify such "normalisations".

Inter-person and intra-person variabilities in cytokine measurements are well recognised.[202] In healthy persons, serum cytokine concentrations vary at different time points during the day.[203] To what degree variations in serum concentrations over the day matter to blood cultures after several days of incubation is not known. It is conceivable that serum concentrations of cytokines at the time of venous puncture influence the results after stimulation and proliferation. The fact that all the blood samples were taken between 9 and 12 am has ensured some standardisation with respect to the 24-hour differences. However, a weakness of this study with respect to inter person variability is the fact that we did not do serial measurements of the included subjects. With respect to the patient groups, such an exercise might not have validated the study any better: only a few weeks of therapy is known to have an effect on immune responses to mycobacterial antigens.

Patients were investigated at time of diagnosis before start of therapy and thus presumably had an ongoing immune response towards their illness. Consequently, the "immune state" of the patients may have been skewed towards a certain type of response at the moment of venous puncture. Furthermore compartmentalisation of cells at the site of disease may have taken place and therefore lower numbers of representative immunologically active cells may have been present in the periphery. The general condition of the patient and stage of disease are also known to influence the immune response.[204, 205] However, if one wants to study patients there are few conceivable ways to control for these possible confounders. A large, prospective, longitudinal study of healthy persons of whom a subset may present with disease at a point in time would be the best model for a study into the immune pathogenesis of TB. The practical problems of doing such a long-term study are enormous and I doubt if any research group will come up with the courage and funding to do it. The cross sectional method, as we used, is probably the method that will be favoured by most groups, but

its limitations are clear. The choice of appropriate cases and controls as well as the number of subjects included in a study of this type largely define limits of its interpretation. With regard to the choice of patients, I can say that they were consecutively recruited over the study period, whenever we got word of there being a possible candidate for the study, via the hospital or one of the participating community health clinics. Not all the information necessary for inclusion in the study was always available at time of diagnosis, but in line with good clinical practice, treatment of the disease was not postponed. The quite rigorous inclusion criteria necessitated exclusion of a substantial proportion of patients, initially regarded as suitable for study entry, later on. The controls were all living and/or working in the area where we recruited most of the patients. There are however recognised differences in TB notifications in this relatively small catchment area.[157] What obviously is not known, is if any of the controls will develop disease in the future, which is not inconceivable in such a high incidence area. None of our healthy Mantoux positives has developed disease 1 year after inclusion in this study, but of these, the children younger than five all received prophylactic treatment.

The major drawback of all studies of this kind where cytokine responses are measured from blood or its components, is that peripheral immune responses may not be representative of the actual events at the site of inflammation. The studies that have used BAL or pleural fluids as well as peripheral cells to measure cytokine production have indeed found that within the same patient different balances of cytokines may be measured when site of disease is compared to periphery.[85, 86]. It can therefore be argued that measuring peripheral responses to mycobacterial antigens in humans is primarily to be used for the evaluation of diagnostic correlates of disease rather than speculation on the pathogenesis of human TB. However, most of us cannot resist the temptation to do the latter as well.

This was a relatively small study. The number of study subjects and the non-normal distribution of the majority of findings did not permit statistical analysis with other than

non-parametric tests. Although mathematical transformations did normalise distributions for a subset of findings and, more sensitive, parametric methods could have been used for these, I chose not to do that for three reasons. Firstly, comparison of the various results would be more cumbersome. Secondly I appreciate the preference of any researcher in this field to value cytokine production in actual pg/ml and not in log transformed pg/ml. Thirdly the uniformity of the format of presentation would be lost.
In this study, TB patients, Mantoux positive and Mantoux negative healthy controls of different ages were investigated for their cytokine response to mitogens and mycobacterial antigens. Age (in years) was a significant variable in levels (in pg/ml) of all the PHA stimulated cytokine responses that we measured. Most published studies[7] on age-related PHA induced cytokine responses have been performed on PBMCs, whereas the results from this study are derived from PHA stimulation of whole blood. PHA is a mitogen for lymphocytes and the absolute amount of lymphocytes per unit of whole blood was strongly, but inversely, correlated with age. Except for the lymphocyte proliferative response to PHA, it should be noted that neither the proliferative response to PPD, nor any of the cytokine levels correlated with the absolute amount of lymphocytes initially present in the blood sample. Thus, at younger age, even with more lymphocytes per unit of blood, less amount of cytokine is produced after stimulation with PHA.

The results of the PPD stimulated cytokine responses in relation to age are less straight forward. They were, on the whole not correlated with age, with one very notable exception: PPD stimulated IFN-γ production in healthy Mantoux positive children younger that 12 years of age was strongly correlated with age. ESAT-6 stimulated IFN-γ production was not related to age in any (sub) group. Apparently the nature of the antigen determines if there is a relationship between age and the magnitude of a cytokine response. It should then be concluded that for any individual antigen-stimulated cytokine response, normal reference values have to be examined for a possible relationship with age. In a way, this is a rather daunting prospect for researchers who study paediatric populations. Even though less blood is required for

studying cellular immune responses with a whole blood based- than with a PBMC based technique, recruiting enough healthy children of different ages to obtain meaningful results for any given antigen is a cumbersome exercise.

Our findings support the notion that lymphocytes from young children under 5 yrs are deficient in their capacity to produce IFN-γ to a number of antigens when compared to older persons. That this phenomenon is solely the consequence of the young child's relative higher number of naïve T cells seems unlikely. Given the fact that the same relative deficiency to produce IFN-γ after PHA stimulation was found after stimulation with PPD and Mantoux negative subjects did not produce appreciable amounts of IFN-γ. I therefore presume that specific memory T cells were primarily involved in the PPD driven IFN-γ response. Furthermore the relatively higher PPD stimulated IL-2 production in the young children indicates that the capacity to expand specific lymphocytes is not deficient either. A role for antigen presenting cells, as proposed in some studies[51, 52], in the lesser cytokine production in young children is a possibility. Antigen-responsiveness, as measured by lymphocyte proliferation to PPD was very much age-dependent in the healthy Mantoux pos control group younger than 12 yrs. On the other hand, in the youngest age group the level of IFN-γ production after PPD stimulation was essentially not different from production after stimulation with PHA and mitogen induced lymphocyte responses are not as dependent on the qualities of antigen presenting cells. Thus, even when charged with "memory", young children may be deficient in production of IFN-γ and speculation upon which other factors might be responsible for this phenomenon is open for debate and, preferably, further studies.

Children with TB have been rarely studied for their cellular immune response. In the study by Swaminathan et al[112], their methodology was different from ours in the sense that they stimulated PBMCs and our results are based on whole blood stimulation, but otherwise this is the only study in this field, worth comparing. In that study PPD and *M.tb* (Erdman strain) induced IFN-γ production was significantly lower in patients than in healthy Mantoux positive controls of comparable age (mean~6yrs). Their patients

produced similar amounts of IL-10 and IL-12 as compared to controls. Interestingly, their study included 24 patients of which 41% were malnourished and 22 "healthy" controls of whom 59% were malnourished. The authors do not mention if this was an intentional "matching" for malnutrition (defined as <75% of body weight for age). PHA stimulated IFN-γ production was measured in 8 patients and controls (no differences between patients and controls found), while IFN-γ mRNA expression after stimulation with *M.tb.* was lower in 5 patients compared to 5 controls. Support as to why their results (lower IFN-γ after PPD stimulation) are "real" and specific to children with TB, are argued on the basis of these additional experiments, albeit from less than a third of their original study group. They do inform us that malnourished patients produced less IFN-γ after PPD stimulation than patients who were in good nutritional health and that there were no such differences between malnourished and well nourished healthy controls. They do not inform us how they chose that small subgroup to do some additional experiments on. Nevertheless, the differences between their results and ours, with respect to PPD induced IFN-γ production, should not be disregarded off hand solely on the basis of technical methodology and numbers, but underline the need for further studies in paediatric populations. The one other study known to me[206] where cytokines were measured in children of different ages with and without TB was, in my opinion, methodologically completely flawed and the publication so riddled with mistakes that I will not discuss it in any detail. Furthermore they could not measure any IFN-γ in the supernatants of the cell cultures. Dieli et al[111] studied lymphocyte proliferation after stimulation with PPD and their patients had equal responses when compared to the Mantoux positive controls (mean age ~7 yrs). The cut-off point for a positive Mantoux test in this study from Italy was a response of 5mm to 1U PPD and this may have influenced the results; it is likely that a number of their healthy controls were sensitised by mycobacteria other than *M.tb.*[207]

Previous "exposure" to the actual antigen, as described in the introduction chapter on age and the development of the immune system, came out as an important variable also

in this study. The immunological responses to PPD that we measured were negligible in the Mantoux negative subjects when compared to the Mantoux positive subjects. This is an important finding for three reasons. Firstly it validates the specificity of the PPD induced responses in the patients and healthy Mantoux positive groups. Secondly it gives a handle to the prospect of evaluating infection with *M.tb* by way of an *in vitro* test, rather than a skin test in children. The restrictions for drawing such a conclusion from this study are already mentioned: the study was not designed to investigate this aspect and we had few subjects with intermediate (0-15mm) reactions. Further studies in this field are clearly needed. Thirdly, PPD induced responses were apparently not dependent of BCG vaccination. Apparently, because we did not have proven records of BCG vaccination in any of the included subjects and, unfortunately, insufficient BCG scar notifications to make a valid statement. Recently the method of using BCG scars as a reliable indicator of BCG vaccination has been questioned anyway, at least in persons vaccinated BCG at very young age, where the scar seems to vanish with time in about 25% of persons.[165] There is a high coverage of BCG in this population (more than 90%), but the timing of vaccination is in the neonatal period. It has been argued many times that responses induced by PPD are not specific enough for *M.tb*, because PPD shares many proteins with other mycobacterial species, including BCG. I do not dare go against this argument, if only for theoretical reasons, but on the assumption that at least 70% of the Mantoux negative subjects from our study were vaccinated with BCG, BCG exposure did not result in PPD induced cytokine responses of any important magnitude. The research group in the Gambia has shown that in 2 months' old children, vaccinated with BCG at birth, PPD induced IFN-γ production (by PBMCs) in the first months of life period was not different from adults with a BCG scar who were vaccinated "in childhood" [113, 208] A potentially important difference in the vaccination technique is that children in South Africa until now have been vaccinated via the percutaneous route and children in the Gambian study via the intra-dermal route. The percutaneous route is potentially less immunogenic if the magnitude of skin test induration and the presence of a BCG scar are taken as clinical correlates for immunogenicity.[209] Studies into the route of vaccination as a potential determinant of the quality of TB related immune

responses are currently under way in the Cape Town region, while South Africa is planning intra-dermal vaccination. Other differences that make our results difficult to compare with the findings from the Gambia are related to the fact that we had only 1 child under the age of 1 yr in our Mantoux negative study group. We have not yet been informed on the longer term follow-up of the Gambian children and perhaps BCG induced PPD responsiveness wenes, as a percentage of BCG scars do, when BCG is given in the neonatal period.

The virtual absence of ESAT-6 stimulated IFN-γ production in the Mantoux positive healthy controls older than twelve years, while being vigorous in the younger controls, suggests that, with time, weaning of specific antigen responses does occur. Since this was a cross sectional study and not a longitudinal study, the finding is only suggestive: it is not known if any of the Mantoux positive healthy controls were ever capable of mounting a response to ESAT-6. It may well be that the incapacity to mount a response to ESAT-6 will prove to be prognostic for a propensity to develop TB at a later time point. Studies on healthy household contacts have shown variable ESAT-6 responses[116], ranging from negative to higher than the index cases. Longitudinal follow-up of these contacts will be very valuable for interpreting the (prognostic) value of a positive ESAT-6 response. From our study, one may conclude that a positive ESAT-6 response in children younger than twelve years does not have an added value above a PPD induced response or, for that matter, a positive skin test (>15mm). However, since our patients and Mantoux positive controls had median Mantoux skin indurations well above 15mm, it would be interesting to study responses from children with intermediate skin test results (5-15mm).

A uniform classification system for primary and postprimary TB by severity of disease does not exist and thus was not an option in comparing patients with primary and postprimary disease. Classification of patients along any severity scale heavily depends on the time of presentation to the doctor: today's patient with minor disease is probably tomorrow's patient with major disease. Based on the extent of radiological

abnormalities, a grading system for classifying severity and predicting outcome of disease is in use for adults with postprimary TB. A staging scale for TBM, based on clinical findings, is also in practice and mostly used to predict neurological outcome of disease.[210] No such official classification system exists for primary intrathoracic TB, although clinicians tend to relate the type and extent of radiological abnormalities in combination with clinical findings to a prognosis. In our study, where patients with fundamentally different types of disease are compared, any personal attempt at stratification by "degree of illness" seemed a pointless operation to me. However, the notion that primary intrathoracic TB in children is a relatively benign disease has been shown in studies from the pre-chemotherapy era. One of the most instructive of these is from the child contacts clinic of the Brompton Hospital in London[212] where 2377 children followed up for a mean of 9 years from 1930 till 1952. 50% of their mortality was in children with disseminated miliary and meningeal TB (mostly children younger than 5 yrs). The other 50% died from " adult type" postprimary TB (only 6% of their children with postprimary disease were younger than 10 yrs). The relative good production of IFN-γ compared to age-related controls in our children with primary disease might well be a reflection of the relatively benign nature of this illness.

The analysis on cytokine ratios by expression of disease is an important part of this thesis for several reasons. Firstly, the increase in numbers per newly stratified age-group did not significantly change the results on single cytokine responses when compared to those obtained by analysing along the initial stratification by age, whereby each group contained smaller numbers of subjects. Secondly, because the suggestion, made by Ellner et al[200], to use cytokine ratios to obtain a more universal indicator of disease, rather than only the variable IFN-γ production in response mycobacterial antigens, indeed did result in a significant difference between TB patients and healthy Mantoux pos subjects, regardless of age. Lastly, and most importantly to me, it puts into focus the somewhat artificial division into "good" and "bad" cytokines with respect to TB. Over the last decade, we have grown accustomed to the use of "type 1" *vs* "type 2" when talking about immune regulatory pathways. The origin of this distinction stems from

research into experimental models of disease in inbred mice, living under laboratory conditions. I have no doubt that the divide is conceptually very useful, especially when the notion of "cross-talk" between the two types of responses is introduced. Effectively this leads to a more balanced view of the immune system, whereby we are forced to acknowledge that most reactions in nature are contra-regulated by another to produce a stationary phase, whereby life is made easier. In the particular case of TB, there is enough reason to assume that type1 immune responses are beneficial for outcome of disease, given the evidence that containment of mycobacterial infections is deficient in people with INF-γ and IL-12 receptor abnormalities.[81] A definite role for a relative overproduction of type2 cytokines in the pathogenesis of human TB is less clear either peripheral or at site of disease[211], although, certainly, a number of studies have shown increased TGF-β, IL-4 and IL-10 production by PBMCs in TB patients as compared to healthy controls. But if an imbalanced type 2 immune response is cause or effect in human disease has not been convincingly shown, not even in murine models.[104] TNF-α is primarily regarded as a pro-inflammatory cytokine, but also implicated in type 1 immune pathways. INF-γ, the archetypal type1 cytokine, can be a potent amplifier of TNF-α production.[179] Most researchers in the field agree that either too much, (resulting in tissue necrosis, wasting and fever) or too little (no proper granuloma formation) of TNF-α is detrimental to the host: the principle of the balance. When our patients were compared to healthy controls, the balance (ratio) of a "good" cytokine (IFN-γ) and a potentially other "good" cytokine (TNF-α) showed a "bad" imbalance, without any type 2 cytokine in the calculation.

The findings of this study underline the necessity to include age as a variable in immunological investigations. This in itself is no surprise, as we have long known about age-dependent values for white blood cells and immunoglobulins. Specific responses to defined antigens are clearly not only dependent on age, but also on previous exposure and the subsequent development of lasting memory. Unfortunately there is much work to do in obtaining normal age-related values for all the antigen induced immune responses that we might be interested in. In light of the urgent need for

new diagnostic tool in childhood TB as well as an improved vaccine (-strategy), this is a particular cumbersome fact, demanding rationalisation in the choice of antigens to study. In view of our results, I would argue, that more culture filtrate antigens would not be my first choice for further exploring the immune response to *M.tb* in children, but rather cell wall components of *M.tb* (for example LAM). They are known to be potent macrophage activators and involved in the early encounter of *M.tb* with the immune system. The results we obtained in this study obviously need confirmation in other, possibly larger studies, of this kind and preferably in different populations. However, it seems doubtful from this study and work by others[200,113] that, as crucial as IFN-γ may be for protection from mycobacterial disease, measuring only IFN-γ production will provide us with a diagnostic tool in children.

Smith et al [213] have proposed that the ontogeny of cellular immunity could parallel the immunobiology of tuberculosis in children at different ages. Without any tuberculosis specific data from children available at the time of publication, they put forward a well-founded hypothesis that the vulnerability of young children, under the age of 2-5 yrs, to develop TB might well be related to the relative immaturity of their cellular immune system as a whole. The cross-sectional design of our study does not allow any answer with regard to susceptibility to disease, but if absolute levels of low IFN-γ production prove to be a measure of susceptibility, the results of our study might well support their hypothesis.

The perils of paediatric research are multifold. The limited amount of blood often available poses technical constraints for a variety methods. Ethical considerations are complex and obtaining informed consent from parents to let children participate in research projects difficult. However, if one wants to know more about childhood diseases, there is ultimately no other way than to set up studies that involve children. The fact that age must be a consideration, is often a confounder and sometimes a characteristic is fortunately well known to paediatricians.

Epilogue

"....Consumption, which in the commencement is easy to cure and difficult to understand, but when it has neither been discovered in due time nor treated upon proper principle, it becomes easy to understand and difficult to cure..." is a citation from the political scientist Machiavelli who lived from 1469-1527.

Since his life time the origin of TB has been discovered, the pathogenesis of disease is better defined and curative strategies have been developed. However, can we rightfully claim that we solved this problem in 2001?

Personal accountability for the work presented in this thesis

I am responsible for the design of the study, which was set up in close consultation with Prof. N. Beyers and the late Prof. A.D. Beyers.

I coordinated the recruitment process and was actively involved in it, with help from the research nurses of the TB research group at the Department of Paediatrics and Child Health at the University of Stellenbosch.

Decisions on in- or exclusion of subjects were taken by myself, without prior knowledge of outcomes of the laboratory data.

Prof. R.P. Gie was responsible for the classifications of the chest radiographs.

I did all the lab work myself, but kindly got help from D. Bowers, D. de Villiers and J. Adams at their discretion. The whole blood samples were consecutively assigned a number in the lab and the lab work was done blinded to name and/or clinical status.

Prior to statistical analysis, advice on the statistical methodology was kindly given by Dr. C.J. Lombard, Center for Epidemiological Research, MRC, Cape Town, South Africa. Analysis of the data, including the computerised statistical analysis, is my responsibility.

The labwork was supervised by Prof. A.D.Beyers.

The whole project was supervised by Prof. N. Beyers.

Acknowledgments

A very special, but sad, acknowledgement to **Albert Beyers**, who died tragically and so suddenly, shortly after the practical work presented in this thesis was completed. As my co-promoter and principal scientific guide into the world of immunology, he has been invaluable and irreplaceable. While analysing the data and writing up this thesis, I have greatly missed his ever valid and carefully considered input, but this miss is quite insignificant compared to the loss of him by others as a husband, father, relative or friend. He died while at work with his scientific files in hand and science was his greatest hobby, but he also loved fishing: that quiet pass-time of waiting for a good catch, while appreciating the natural surroundings. I'll fondly remember him as a naturally quiet and friendly man with a passion for fishing explorations, of any kind.

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Mr W Bakhuys Roozeboom Stichting

Consent Form to participate in a research project on Tuberculosis

Project: "The influence of age on the cellular immune response in patients with Tuberculosis", regnr.....approved by the Ethics Committee of the University of Stellenbosch

Patient Details:

Name: .....
First Names: .....
Date of Birth: .....
Address: .....

I, the undersigned doctor/nursing sister .....hereby declare that I have informed the patient or his/her parent and/or legal guardian on the nature, purpose, risks and possible consequences of the research project in his own language. I will take full responsibility if the study results of this person need follow-up in terms of treatment and/or counseling

....
(Signature of doctor/nursing sister) *(Date)*

I, the undersigned....., (in my capacity as.....),
(Name of person giving consent) *(Nature of relation)*
hereby give consent for the inclusion of ....in the above
(Name of person included in the study)
research project, the nature of which has been explained by .....
(Name of doctor/nursing sister)

1. The nature of the project has been explained to me

2. I understand the implication s of the research and the risks involved

3. The following procedures may be carried out as part of the project:

   i) Drawing of 12 ml of blood
   ii) Tuberculin skin testing
   iii) Chest radiograph if deemed necessary by the doctor
   iv) HIV testing

4. I understand that I can withdraw at any stage from the project without influencing my or my child's **medical treatment.**

5. I understand that there will be no financial rewards

....
(Signature of person giving consent) *(Date)*

Information for persons or parents of children participating in the research project "The influence of age on the cellular immune response in patients with Tuberculosis"

Tuberculosis (TB) is a major health problem in the world and especially in the Western Cape. It is a disease that can be cured with medication, but we need more scientific information on the nature of the disease to be able to come up with better ways of diagnosis, prevention and treatment. We also need more information on the manner in which persons of different ages react to TB. For this purpose we ask your permission to include you/ your child in a research project to investigate this.

We need to ask you some questions on the health of you/your child, do a physical examination, do a Mantoux skin test and draw blood for testing of the immune system, including an HIV test.**The proceedings and the meaning of doing a HIV test will be explained to you.**

The amount of blood drawn will be 12ml (2,5 teaspoons), devided over three tubes. This is not an amount that poses any dangers to you/your child. If, by any chance, there are signs in the blood test suggesting that you/your child need to be seen by a specialist doctor we will arrange an appointment. If the HIV test should be positive we will refer you to the Infectious Disease clinic of Tygerberg Hospital
The Mantoux skin test will be done with a small needle, whereby a little amount of fluid will be put into the skin. After 2-3 days we will read the skin test to see if it is positive. If positive, which means that you/your child has been in contact with TB, we will arrange for you or your child to have a chest radiograph taken, so that we can look if there are any signs of lung disease. If there are signs of disease we will refer you to the local health clinic for further treatment.

The procedures mentioned are not dangerous for your health and the only side-effect is a possible positive skin reaction that can be itching or ulcerating for which we can give you a steroid cream to be used for a couple of days until the reaction has stopped.
You are completely free to choose whether or not you decide to take part in this study. Refusal to participate will not involve any penalty or change in the standard of medical attention in the hospital or clinic. You will not receive any financial reward for participating in this study.
All personal information obtained from this study will be strictly confidential. The results of the study will be published in medical journals, but without mentioning the name of any person on whom blood was taken.

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