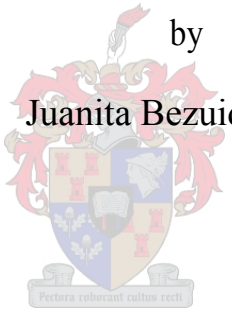
CYTOKINES AND TUBERCULOSIS

An Investigation of Tuberculous Lung Tissue
and a
Comparison with Sarcoidosis

by

Juanita Bezuidenhout



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DECLARATION

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

Signature …….. Date: December 2005

ABSTRACT
The formation of granulomas at the site of antigen presentation in both tuberculosis and sarcoidosis is an essential component of host immunity for controlling inflammation. Granuloma formation is a complex process that also requires recruitment and activation of lymphocytes and macrophages to the site of infection and arrangement into a granuloma. It is dependant on the activation of especially IFNγ secreting CD4+ T cells, resulting in a Th1 profile. However, it is suggested that a persistently high IFNγ is responsible for the damage caused by granulomatous disease and that moderating cytokines, resulting in a Th0 profile, are necessary to down-regulate the IFNγ response to more appropriate levels later in the disease process, after the antigen has been effectively contained.

I propose that: "*Cytokine profiles determine clinical and histopathological phenotypes of disease. This thesis tests the hypothesis that it will be reflected by cytokine expression profiles in granulomas in different forms of tuberculosis and in sarcoidosis*." To examine this, biopsy tissue was obtained from patients with pulmonary cavitary tuberculosis, pleural tuberculosis in HIV sero-negative and sero-positive patients, and sarcoidosis. The diagnosis of tuberculosis or sarcoidosis was confirmed, granulomas were characterised as necrotic or non-necrotic, sarcoidosis cases were graded histologically and in situ hybridisation was performed for IL-12-, IFNγ-, TNFα- and IL-4-mRNA.

In all patients with pleural tuberculosis, a Th0 profile was noted, while necrotic granulomas were more evident in HIV positive than HIV negative patients. There was a clear association between TNFα and necrosis in tuberculous granulomas that may be ascribed to the increased apoptotic activity of TNFα. An increase in IFNγ correlated with an increase in necrosis, supporting the theory that high IFNγ levels later in disease is detrimental. This effect may be enhanced by a strong presence of TNFα positive cells. An increase in both Th1 and Th2 cytokine mRNA in HIV positive patients supports the theory that an overproduction of cytokines may be a mechanism to compensate for the failure of another immune effector mechanism. Findings in pulmonary tuberculosis were similar to those in pleural tuberculosis.

In all sarcoidosis cases the presence of a very strong Th1 and TNFα, but no Th0 response was confirmed. None of the differences in either the histological grading, or the clinical outcome of patients were reflected in the cytokine profile. It is possible that this profile does not reflect the histological grade of disease or that it may reflect various stages of disease. These findings support the theory that a strong Th1 presence later in disease, in conjunction with TNFα may induce fibrosis, as most of these cases showed signs of at least focal fibrosis.

Numerous aspects, including a T helper response are involved in granulomatous inflammation. The earlier dogma of *good*, beneficial (Th1) versus *evil,* detrimental (Th2), is an oversimplification of a very complex process. It is clear that the effect of a cytokine depends at least partially on the stage of disease. The balance between the various cytokines, and the levels of these cytokines contribute to their role in resolution or disease progression. An early, pure Th1 response may be beneficial if effectively clearing the granuloma-inducing antigen. At this stage, a Th2 presence will be harmful as clearing of the antigen will not be as effective. In chronic disease where failure to remove the antigen results in progression of granulomas with subsequent necrosis and/or fibrosis, a pro-inflammatory Th1 response may be detrimental and minimising of this effect is needed. An overly strong presence of the various cytokines may also be detrimental, while lower levels will be beneficial.

ABSTRAK

Die vorming van granulome by die plek waar die antigeen presenteer word in beide tuberkulose en sarkoïedose is ‘n noodsaaklike komponent van die gasheer immuniteit in die beheer van inflammasie. Granuloomvorming is ‘n komplekse proses wat onder andere werwing en aktivering van limfosiete en makrofae na die plek van infeksie en rangskikking in ‘n granuloom insluit. Dit is afhanklik van aktivering van veral IFNγ sekreterende CD4+ T selle, wat lei tot ‘n Th1 respons. Dit is egter moontlik dat persisterende hoë IFNγ verantwoordelik is vir die skade veroorsaak deur granulomateuse siekte en dat modererende sitokiene, wat lei tot ‘n Th0 profiel, nodig is om IFNγ af te reguleer na aanvaarbare vlakke later in die siekteproses, sodra die antigeen onder beheer is.

Ek postuleer dat: “*sitokien profiele die kliniese en histopatologiese fenotipes van siekte bepaal. Hierdie tesis toets die hipotese dat dit reflekteer sal word deur die sitokien profiele in granulome in verskillende vorms van tuberkulose en sarkoïedose”.* Om dit te ondersoek is weefsel biopsies verkry van pasiënte met holtevormende long tuberkulose, pleurale tuberkulose in HIV sero-negatiewe en positiewe pasiënte en sarkoïedose. Die diagnose van tuberkulose of sarkoïedose is bevestig, granulome is geklassifiseer as nekroties of nie-nekroties, sarkoïedose gevalle is histologies gegradeer en in situ hibridisasie is gedoen vir IL-12-, IFNγ-, TNFα- en IL-4-mRNA.

‘n Th0 profiel is bespeur in alle pleurale tuberkulose pasiënte en daar is meer nekrotiese granulome in HIV positiewe as HIV negatiewe pasiënte. Daar is ‘n duidelike assosiasie tussen TNFα en nekrose in tuberkuleuse granulome, wat toegeskryf mag word aan verhoogde apoptotiese aktiwiteit van TNFα. ‘n Toename in IFNγ korreleer met ‘n toename in nekrose, wat die teorie ondersteun dat hoë vlakke van IFNγ later in die siekteproses nadelig kan wees. Hierdie effek mag versterk word deur die sterk teenwoordigheid van TNFα positiewe selle. ‘n Toename in Th1 en Th2 sitokien mRNA in HIV positiewe pasiënte ondersteun die teorie dat die oorproduksie van sitokiene ‘n meganisme is om te kompenseer vir die versaking van ‘n ander immuun effektor meganisme. Soortgelyke bevindings word in die long tuberkulose gevalle gesien.

‘n Sterk Th1 en TNFα respons, met ‘n afwesige Th0 respons is in al die sarkoïedose gevalle gesien. Geen van die verskille in histologiese gradering of kliniese uitkoms is gereflekteer in die sitokien profiel nie. Dit is moontlik dat hierdie profiel nie die histologiese graad reflekteer nie, of dat verskillende fases van die siekte gereflekteer word. Hierdie bevindinge ondersteun die teorie dat ‘n sterk Th1 profiel, later in siekte, tesame met TNFα fibrose kan induseer, aangesien meeste van die gevalle ten minste fokale fibrose getoon het.

Verkeie aspekte, insluitende ‘n T helper respons, is betrokke in granulomateuse inflammasie. Die vroeëre dogma van *goed*, voordelig (Th1) teenoor *kwaad*, nadelig (Th2) is ‘n oorvereenvoudiging van ‘n baie komplekse proses. Dit is duidelik dat die effek van ‘n sitokien ten minste deels berus op die stadium van siekte. Die balans tussen die verskillende sitokiene en die vlakke van hierdie sitokiene dra by tot hulle rol in resolusie of progressie van siekte. ‘n Suiwer Th1 respons vroeg in siekte is waarskynlik voordelig indien die granuloom-induserende antigeen verwyder word. Op hierdie stadium sal ‘n Th2 teenwoordigheid nadelig wees en antigeen verwydering sal nie so effektief wees nie. In chroniese siekte, waar die onvermoë om die antigeen te verwyder lei tot progressie van granulome met gevolglike nekrose en/of fibrose, mag ‘n pro-inflammatoriese Th1 respons nadelig wees en die verlangde effek verdun. ‘n Oormatige sterk teenwoordigheid van die spektrum van sitokiene mag ook nadelig wees, terwyl laer vlakke voordelig kan wees.

I dedicate this work to prof. L Dreyer and
prof. I Simson, my two undergraduate Anatomical
Pathology professors at the University of Pretoria,
who inspired me to become a pathologist.

Our imagination loves to be filled with an object or to grasp at anything that is too big for it's capacity. We are flung into a pleasing astonishment at such unbounded views and feel a delightful stillness and amazement in the soul at the apprehension of them.

Joseph Addison (1672 - 1719), 1712

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PUBLICATIONS AND PRESENTATIONS BASED ON THIS WORK

Publications

1. "*In Situ* Production of Gamma Interferon, Interleukin-4, and Tumor Necrosis Factor Alpha mRNA in Human Tuberculous Granulomas". Fenhalls G, Wong A, Bezuidenhout J, Van Helden P, Bardin P, Lukey PT. *Infection and Immunity,* 2000 May: 68 (5) 2827-2836.
2. Distribution of IFN-gamma, IL-4 and TNF-alpha protein and CD8 T cells producing IL-12p40 mRNA in human lung tuberculous granulomas. Fenhalls G, Stevens L, Bezuidenhout J, Amphlett GE, Duncan K, Bardin P, Lukey PT. *Immunology*. 2002 Mar;105(3):325-35.

Published abstracts

1. "Cytokine mRNA in Human Tuberculous Granulomas". J Bezuidenhout, G Fenhalls, A Wong, P Lukey, P Bardin. *Abstract in SAMJ,* 1999, 89:3.
2. "The Histological Grading of Pulmonary Sarcoidosis". J Bezuidenhout, EM van Schalkwyk, PG Bardin, JR Joubert. *Abstract in SAMJ,* 1999, 89:3.

Presentations

International level:

1. 2002, June 27-30: "Immune responses in the human lung tuberculous granuloma do not conform to the Th1/Th2 dichotomy". *Gael Fenhalls, Liesel Stevens, Gillian Amphlett, Ken Duncan, Juanita Bezuidenhout, Paul van Helden and Pauline T. Lukey.* Fifth International Conference on the Pathogenesis of Mycobacterial Infections" in Saltsjobaden, Stockholm.
2. 2002, June 27-30: "Myeloid cells produce T-cell cytokines and T-cells produce myeloid cytokines in human lung tuberculous granulomas". *Gael Fenhalls, Liesel Stevens, Ken Duncan, Juanita Bezuidenhout, Paul van Helden and Pauline T. Lukey.* Fifth International Conference on the Pathogenesis of Mycobacterial Infections" in Saltsjobaden, Stockholm.
3. 2000, 22-24 March: "IL-12 Gene Expression Correlates with IFN-gamma Protein in Human Tuberculous Granulomas". *Gael Fenhalls, Liesel Stevens, Juanita Bezuidenhout, Philip Bardin and Pauline T. Lukey.* Annual Action TB Meeting, Glaxo Wellcome Medicines Research Centre, Stevenage, UK
4. 1997, 31 August - 4 September: "Cytokine Immunoreactivity in Human Tuberculous Granulomas". *J Bezuidenhout, DG du Plessis, CJF Muller, MS Pretorius, G Walzl, PG Bardin.* 14th Southern African Pulmonology Congress, Windhoek.
5. 1997, 31 August - 4 September. "Cytokine Immunoreactivity in Human Tuberculous Granulomas". *J Bezuidenhout, DG du Plessis, CJF Muller, MS Pretorius, G Walzl, PG Bardin.* 14th Southern African Pulmonology Congress, Windhoek.
6. 1995, 5 - 6 October: "Cellular Definition of Granulomatous Tissue Employing Immunohistochemistry*". C Muller, S Ferris, M Pretorius, J Bezuidenhout, D du Plessis, B van de Wal, J Joubert, P Bardin.* Glaxo Action Tb Scientific Conference and Workshops. Cape Town.

National level:

1. 1999, 21 - 25 March. "Cytokine mRNA in Human Tuberculous Granulomas". *J Bezuidenhout, G Fenhalls, A Wong, P Lukey, P Bardin.* 15th South Africal Pulmonology Congress, Wild Coast Sun.
2. 1999, 21 - 25 March. "The Histological Grading of Pulmonary Sarcoidosis" *J Bezuidenhout, EM van Schalkwyk, PG Bardin, JR Joubert.* 15th South Africal Pulmonology Congress, Wild Coast Sun.
3. 1998, 12 August. "TNF-α, IFN-γ and IL-4 mRNA in Human Tuberculous Granulomas". *G Fenhalls, J Bezuidenhout, P Lukey, N Beyers, AD Beyers, P Bardin.* First Annual Medical Research Day, Medical School, University of Cape Town.
4. 1997, 29 June - 2 July: "Cytokine Immunoreactivity in Human Tuberculous Granulomas". *Juanita Bezuidenhout, Daniel du Plessis, Christo Muller, Marsia Pretorius, Gerhard Walzl and Philip Bardin.* 37th Annual Congress Federation of the South African Societies of Pathology. Cape Town.
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Faculty level:

1. 1999, 18 - 19 Augustus. "Cytokines In Pulmonary Human Tuberculosis: Immunocytochemistry For Tnf-α, Ifn-γ And Il-4". *Juanita Bezuidenhout, Gael Fenhalls, Liesel Stevens, Pauline Lukey and Phillip Bardin.* 43rd Academic Year Day, Faculty of Medicine and Dentistry, University of Stel44th Academic Year Day, Faculty of Medicine and Dentistry, University of Stellenbosch.
2. 1999, 18 - 19 Augustus. "Tnf-α, Ifn-γ And Il-4 Mrna Produced *In Situ* In Human Tuberculous Granulomas". *Gael Fenhalls, Anthony Wong, Juanita Bezuidenhout, Paul van Helden, Phillip Bardin and Pauline T. Lukey.* 43rd Academic Year Day, Faculty of Medicine and Dentistry, University of Stellenbosch.
3. 1999, 18 - 19 Augustus. "**STATE OF THE ART:** Immunopathology of Tuberculosis: The Role of Cytokines in

the Pathogenesis". *J Bezuidenhout*. 43rd Academic Year Day, Faculty of Medicine and Dentistry, University of Stellenbosch.

4. 1998, 19 - 20 August. "Cytokine mRNA in Human Tuberculous Granulomas: in situ hybridisation". *G Fenhalls, J Bezuidenhout, L Scholvink, N Beyers, AD Beyers, P Lukey, P Bardin.* 42nd Academic Year Day, Faculty of Medicine and Dentistry, University of Stellenbosch.
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List of abbreviations

CMI: Cell-mediated immunity
AIDS: Acquired immunodeficiency syndrome
APCs: Antigen presenting cells
BAL: bronch-alveolar lavage
BCG: bacille Calmette-Guérin
CXR: chest X-ray
DTH: type IV (delayed type) hypersensitivity reaction
HIV: human immunodeficiency virus
IFNγ: Interferon-gamma
IL-10: interleukin 10
IL-12: Interleukin-12
IL-4: interleukin 4
ISH: *in situ* hybridisation
M.tb: Mycobacterium tuberculosis
MNPS: mononuclear phagocyte system
mRNA: messenger-RNA
NK cells: natural killer cells
PBMC: peripheral blood mononuclear cells
PCR: polymerase chain reaction
TB: Tuberculosis
TGFβ: transforming growth factor-β
TH0: T helper 0
TH1: T helper 1
TH2: T helper 2
TNFα: Tumour necrosis factor-alpha

CYTOKINES AND TUBERCULOSIS:

An Investigation of Tuberculous Lung Tissue and a Comparison with Sarcoidosis

1. Literature Review

1.1 Introduction

Defense against micro-organisms is mediated by the early reactions of innate immunity in a previously unexposed/unsensitised individual, or the later response of adaptive immunity in a previously sensitised individual. Innate immunity is not specific and consists of cellular and biochemical defenses that exist even before infection and are ready to respond rapidly to infections[3,4].

In contrast, adaptive immunity is acquired by exposure to infective agents and increases with each successive exposure to the same antigen. Adaptive immunity is exquisitely specific and has the ability to remember and respond more vigorously to subsequent exposures[4]. There are two types of adaptive immunity, called humoral immunity and cell-mediated immunity[5]. Cell-mediated immunity (CMI) is mediated by T lymphocytes and aimed at intra-cellular micro-organisms, such as viruses and mycobacteria that survive within phagocytic cells, like macrophages, or infect non-phagocytic cells. The function of macrophages is to ingest and kill micro-organisms. Many organisms have developed defense mechanisms that help them survive and even replicate in macrophages and the function of CMI is to enhance the microbiocidal effect of macrophages and eradicate the organism.

Adaptive immunity has a number of fundamental properties that reflect the properties of the lymphocytes that mediate the responses. These are[6,7]:

1. Specificity – distinct antigens elicit specific responses.
2. Diversity – enables the immune system to respond to a large variety of antigens.
3. Memory – leads to enhanced responses to repeated exposures to the same antigens.
4. Specialisation – generates responses that are optimal for defense against different types of micro-organisms.
5. Self-limitation – after eliminating the antigen, the immune system returns to a resting state until the next exposure.
6. Non-reactivity to self – prevents injury to the host during these responses.

Abnormalities in this process for instance lead to the so-called auto-immune diseases, allergies infections and tumours.

Granulomatous inflammation is a distinctive pattern of chronic inflammation and adaptive immunity, and the granuloma is the hallmark of many human diseases of great significance, the most important of which must be tuberculosis[8,9]. Its formation is firmly linked to the type IV (delayed type) hypersensitivity reaction (DTH), which is a form of adaptive cell- mediated immunity that is mediated by activated T lymphocytes and their products, called cytokines. Recognition of granulomatous inflammation on histology is very important because of the fairly limited number of conditions (table 1) that give rise to granulomas and the clinical significance of these diagnoses.

Table 1: Examples of granulomatous inflammation[10]

Infectious	Mycobacteria	Tuberculosis, leprosy, atypical mycobacteria
	Other bacteria	Cat-scratch disease, brucellosis, meliodosis
	Fungi	Cryptococcosis, candidiasis, aspergillosis
	Spirochetes	Syphilis
	Parasites, larvae, eggs, worms	
Foreign bodies/particles	Endogenous	Keratin, necrotic bone, cholesterol crystals, sodium urate
	Exogenous	Tattoos, talc, silica, suture material, oils, silicone, thorns, splinters
		Hypersensitivity pneumonitis
Chemicals		Beryllium
Drugs		Allopurinol, phenylbutazone, sulphonamides
Idiopathic		Sarcoidosis
		Wegener's granulomatosis
		Crohn's disease
Neoplasia	Lymphomas	Hodgkin's lymphoma

1.1.1 Definition of a Granuloma

A granuloma is defined morphologically as follows: a microscopic aggregation of epithelioid (activated) macrophages, usually surrounded by a collar of lymphocytes (fig. 1)[11]. Despite the fact that granulomas in specific diseases occasionally have a specific histological appearance, this is usually not pathognomonic of a particular disease and additional investigations are required to determine the exact cause of the granulomatous disease[12].

1.1.2 The Cells Involved in Granuloma Formation

Two main cell types are involved in an immunogenic granuloma, namely antigen presenting cells and T lymphocytes.

1.1.2.1 Antigen presenting cells (APCs)

Antigen presenting cells are specialised to capture and process microbial and other antigens, present them to lymphocytes, and provide signals that stimulate proliferation and differentiation of lymphocytes[13].

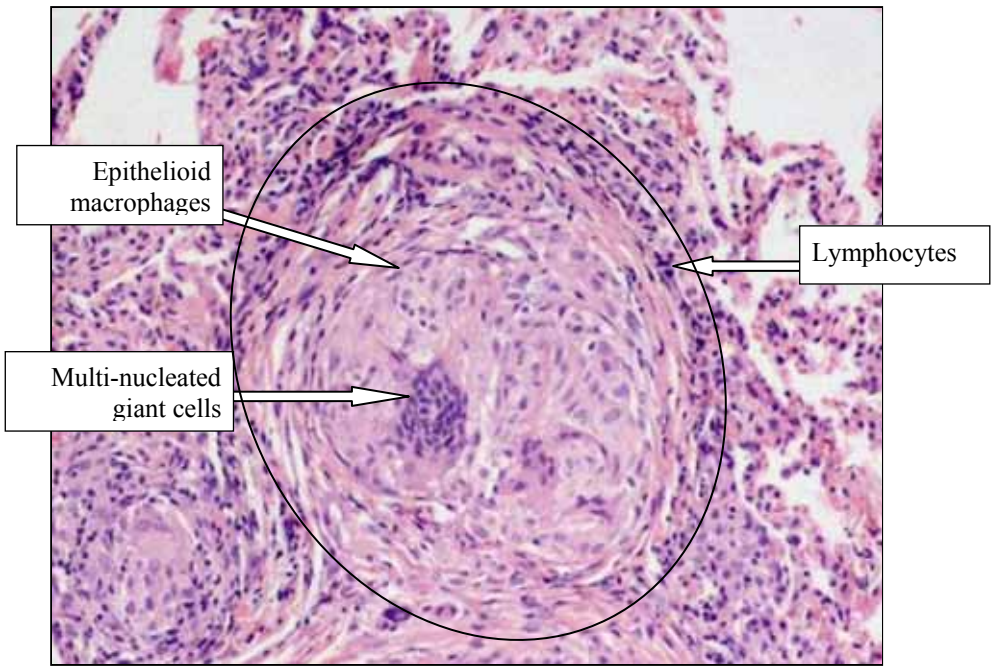


Figure 1: An example of a classical granuloma.

The granulomas contain all the cellular components required, as described in the text. Epithelioid macrophages are present in the centre and some multi-nucleated giant cells can be seen. The central area is surrounded by lymphocytes. Granuloma circled in black. Haematoxylin and eosin (H + E) stain[14]. X200 magnification

1.1.2.1.1 Macrophages

The macrophage is central in chronic inflammation because of the vast number of substances an activated macrophage can produce[13]. It forms part of the mononuclear phagocyte system (MNPS), arising from a common bone marrow precursor. The MNPS consists of blood monocytes and tissue macrophages. Tissue macrophages are scattered in connective tissue or clustered in organs such as the liver (Kupffer cells), spleen, lymph nodes (sinus histiocytes) and lungs (alveolar macrophages) and results

from blood monocytes (with a half-life of about one day) migrating into various tissues to transform into macrophages with a half-life of months. This whole process is regulated by a variety of growth and differentiation factors, cytokines, adhesion molecules and cellular interactions[13,15].

Macrophages are primarily phagocytic cells, but have the potential to be activated, resulting in increased cell size, resulting in an epithelioid appearance. Lysosomal enzyme levels and metabolism are increased and the cells can then more effectively phagocytose and kill ingested organisms. In addition, more MHC class II molecules are expressed on the surface. This facilitates antigen presentation as $CD4^+$ lymphocytes only recognise antigens in the presence of MHC class II expression. Several growth factors are also secreted, that stimulate fibroblast proliferation and collagen synthesis. Activation signals include cytokines, produced by T lymphocytes, bacterial endotoxins, other chemical mediators and certain extra-cellular matrix proteins. After activation the macrophage secretes a wide variety of biologically active products[8,15].

In an H + E stained slide epithelioid cells have abundant pale pink cytoplasm with indistinct cell borders, giving the impression of merging into one another. The cell has a vesicular nucleus, often with a distinct nucleolus. Epithelioid cells frequently fuse to form multi-nucleated giant cells of various configurations. They are usually seen on the periphery of a granuloma, but can be present anywhere in the granuloma.

1.1.2.1.2 Dendritic cells

Dendritic cells play an important role in antigen capture and the induction of T cell responses, especially in naïve T cells[16]. They are present in lymphoid organs, the skin, the gastro-intestinal and respiratory tracts and most parenchymal organs. Dendritic cells have spine-or finger like projections of their cell membranes, resulting in a vastly increased surface area of each cell. These cells capture protein antigens and transport them to the draining lymph nodes. During this journey the dendritic cells mature and become extremely efficient in presenting antigens to naïve T cells[17].

1.1.2.2 Lymphocytes

Lymphocytes are the only cells in the body capable of specifically recognising and distinguishing different antigens[18]. They are therefore responsible for both the specificity and memory that are the defining characteristics of the adaptive immune response. Lymphocytes that have not been stimulated by antigens are called naïve lymphocytes and are small cells, measuring 8-10μ in diameter, which are in the G_0 stage of the cell cycle. Once stimulated they become larger, with more cytoplasm, organelles and cytoplasmic RNA. The lymphocytes involved in the cell mediated adaptive immune response are $CD3^+$ T cells, usually of the T helper type ($CD4^+$). Once activated they differentiate into effector and memory cells. This activation follows a series of steps[7,15,17,18].

1. Early after antigen presentation (the stimulus), the lymphocytes start transcribing previously silent genes, to synthesize new proteins, including:

1.1. Cytokines, to stimulate growth and differentiation of lymphocytes and other cells

1.2. Cytokine receptors

1.3. Other proteins involved in gene transcription and cell division

2. Cellular proliferation

The antigen specific lymphocytes undergo mitotic division in response to antigen and growth factors, resulting in clonal expansion.

3. Differentiation into effector cells. These cells express surface proteins that interact with other cells, and secrete cytokines.

4. Differentiation into memory cells. The function of these cells is to mediate rapid and enhanced responses to second and subsequent exposure to antigens.

Most T cells recognise only peptides and are specific for amino acid sequences of peptides. Therefore, if an animal is immunised with native protein, the antigen specific T cells that are stimulated will respond to denatured or even digested forms of that protein. Different T cells can distinguish peptides that differ at single amino acid residues. T cells recognise and respond only to foreign peptide antigens when they are attached to the surface of APCs, and only in the presence of that individual's own MHC molecules[17,19].

1.1.3 Cytokines in Granulomatous Disease

Cytokines are small protein molecules that regulate immunological responses at a cellular level. This response helps the body to defend itself against invasion by micro-organisms and particularly those organisms that localise intra-cellularly to escape tissue

defences situated in the blood and interstitial fluid compartments. Different cytokines stimulate varied responses of the cells involved in immunity and inflammation[20]. In the activation phase they stimulate the proliferation and differentiation of lymphocytes and in the effector phase they activate effector cells to eliminate micro-organisms and other antigens. Cytokines can prime and either divert or target various effector cells to produce other mediators or toxic substances, such as oxygen radicals produced in the macrophages in response to IFNγ stimulation, which will kill intracellular organisms[20].

1.1.3.1 General properties of cytokines

Cytokine secretion is a transitory, self-limiting event. Cytokines are not stored as pre-produced products, but their synthesis is stimulated by cellular activation, via new gene transcription[21]. To identify the presence of a specific cytokine *in situ* in a specific cell, it is often necessary to perform messenger-RNA (m-RNA) *in situ* hybridisation (ISH) due to the rapid secretion and degeneration of cytokine protein[22]. The mRNA can also be identified for only a brief period, as the particular gene is switched off again and the mRNA is unstable[21].

The actions of cytokines are often pleiotropic and redundant. One cytokine has the ability to act on different cell types, which allows a cytokine to mediate diverse biological effects. Multiple cytokines also have the same functional effect[21]. Cytokines manipulate the manufacture and actions of other cytokines. This ability leads to a cascade effect and often cytokines that stimulate the production of others are affected by these cytokines. They may also have opposing, additive or synergistic effects[21].

Effects are local or systemic. Most cytokines act in an autocrine or paracrine fashion, but when produced in large amounts cytokines may enter the systemic circulation and act from a distance in an endocrine fashion[21]. Cytokines initiate their actions by binding to specific membrane receptors on target cells. They often bind their ligands with high affinities, consequently often only small quantities of cytokines are needed to elicit biological effects[21].

External signals regulate the expression of cytokine receptors and therefore the responsiveness of cells to cytokines. This mechanism maintains the specificity of immune responses as only antigen stimulated cells express increased numbers of cytokine receptors. Receptor expression is also controlled by cytokines themselves, either by positive amplification or negative feedback[21].

1.1.3.2 Specific cytokines

1.1.3.2.1 Interleukin-12 (IL-12)

IL-12 is a principal mediator of the early innate immune response to intra-cellular bacteria and is a key player in the induction of cell-mediated immunity. Its most important function is to stimulate IFNγ production by T cells and NK cells. IL-12 is produced by macrophages and dendritic cells. A recent publication by Fenhalls et al[1] also demonstrated the presence of IL-12p40 mRNA in CD8+ lymphocytes, indicating that these lymphocytes produce IL-12. IL-12 is critical for the induction of the Th1 response and the subsequent DTH reaction[23,24,25,26]. On initial encounter with an organism, resting macrophages attempt to phagocytose and kill the organism, but are often not successful. This encounter, however, leads to the production and secretion of

IL-12 by the macrophage. IL-12 is produced in response to many stimuli, including intra-cellular bacteria and viruses.

Antigen stimulated T cells also induce IL-12 production, via the CD40 ligand on T cells engaging the CD40 receptor on producing cells. T cells and NK cells also produce IFNγ that stimulates IL-12 production. IL-12 is therefore produced during the induction and effector phases of the cell-mediated immune response[23,24,25,26].

IL-12 is crucial in initiating the responses resulting in the eradication of intra-cellular bacteria[23,24,25,26]:

- IL-12 induces the production of IFNγ by T cells and NK cells. IFNγ activates the macrophages to kill phagocytosed organisms.
- IL-12 stimulates the differentiation of CD4 T helper cells into Th1 cells. These cells then activate phagocytes in cell-mediated immunity.
- IL-12 enhances the cytolytic activity of activated CD8 cytolytic T cells and NK cells.
- IL-12 induces autocrine stimulation of macrophages, leading to additional IL-12 secretion by macrophages.
- IL-12 suppresses IgG1 and IgE production.

1.1.3.2.2 IL-2

IL-2 is a growth factor for antigen-stimulated T lymphocytes and is responsible for the clonal expansion of T cells after antigen recognition. IL-2 is produced by $CD4^+$T cells and sometimes also by $CD8^+$ T cells. IL-2 production is transient, with a peak 8-12

hours after stimulation. Antigen stimulation enhances IL-2 receptor expression on T cells, thereby augmenting the preferential proliferation of antigen stimulated T cells[20,27].

IL-2 plays several important roles in the adaptive immune response:

- IL-2 is the most important T cell growth factor, and specifically for antigen stimulated T cell proliferation. This is achieved by promotion of cell cycle progression.
- IL-2 promotes survival of cells by inducing bcl-2, an anti-apoptotic protein, thereby preventing cell death.
- IL-2 increases production of IL-4 and IFNγ by T cells.
- IL-2 promotes the proliferation and differentiation of other immune cells, including NK cells. It stimulates growth and enhances cytolytic function. It also stimulates B cell growth and antibody synthesis.
- Repeated activation of $CD4^+$ T cells in the presence of IL-2 makes these cells sensitive to apoptosis via the Fas pathway. This functions as a control to switch off the immune response.

1.1.3.2.3 Interferon-gamma (IFNγ)

IFNγ is the principal activator of macrophages and plays a role in both innate and adaptive cell-mediated immunity. In innate immunity, natural killer (NK) cells secrete IFNγ, stimulated by IL-12, in response to recognition of unknown organisms. In adaptive immunity, T cells secrete IFNγ in response to antigen stimulation, as well as IL-12 and IL-18 secretion[28,29].

The functions of IFNγ are especially important in adaptive cell-mediated immunity:

- IFNγ is a macrophage-activating cytokine, enhancing the microbiocidal activities of macrophages. It stimulates the synthesis of reactive oxygen intermediates and nitric oxide. These active molecules are produced in lysosomes and destroy organisms within the phagolysosome[20,28,29,30].
- IFNγ stimulates expression of class I and II MHC molecules and costimulators on APCs. It also stimulates the expression of many antigen-processing proteins. In this way it enhances MHC-associated antigen presentation and amplifies the recognition phase of immune responses by increasing expression of the ligands that T cells recognise.
- IFNγ activates vascular endothelial cells and potentiates some of the action of TNFα. These actions promote lymphocyte adhesion and extravasation to the site of infection.
- IFNγ promotes the differentiation of naïve $CD4^+$ T cells to the Th1 subset.
- IFNγ inhibits the proliferation of Th2 cells.
- In B cells, IFNγ promotes the switching of certain IgG subclasses.
- IFNγ activates neutrophils and stimulates cytolytic activity of NK cells.
- Although IFN production is induced by IL-12, IFNγ in turn initiates or augments IL-12 secretion.
- IFNγ also enhances the number of IL-12 binding sites expressed on individual macrophages.

The net effect of these activities is to promote a macrophage rich inflammatory environment, while inhibiting IgE-dependent reactions[20,28,29,30].

1.1.3.2.4 Tumour necrosis factor-alpha (TNFα)

TNFα is the principal mediator of the acute inflammatory response. It is also, in an endocrine fashion, responsible for many of the systemic complications of severe infections. TNFα is mainly produced by macrophages, but T cells, NK cells and mast cells can also produce TNFα.

TNFα has important effects on endothelial and inflammatory cells[9,15,20,31].

- Increased secretion of prostacyclin, which leads to increased blood flow by vasodilation.
- Increased expression of amongst others, E-selectin, an adhesion molecule that promotes attachment of lymphocytes and monocytes to the endothelium in the vicinity of the inflammatory process.
- Induction and secretion of chemotactic factors such as IL-8.
- Stimulation of mesenchymal cells such as fibroblasts and smooth muscle cells.
- Suppresses IL-12 p40 transcription [32].
- Induces IL-10 production [33]

These actions all facilitate extravasation of lymphocytes and macrophages at the site of reaction.

1.1.3.2.5 IL-10

IL-10 is an inhibitor of activated macrophages and dendritic cells. It is produced by activated macrophages and therefore acts as a negative feedback regulator. It is however, not clear what stimulates the production of IL-10[9,20,34].

- IL-10 inhibits the expression of costimulators and class II MHC molecules on macrophages and dendritic cells. This leads to inhibition of T cell activation and termination of cell-mediated immune reactions.
- IL-10 inhibits the production of IL-12. IL-10 consequently down-regulates the induction of innate and cell-mediated immune reactions.
- On the other hand IL-10 promotes the differentiation of immature dendritic cells into macrophages.

1.1.3.2.6 IL-4

IL-4 is the major stimulus for the production of IgE antibodies and for the development of Th2 cells from naïve CD4 T cells. IL-4 is, in turn, also produced by Th2 cells, activated mast cells and basophils, thereby stimulating Th2 differentiation and proliferation in an autocrine fashion[20,35,36,37].

- IL-4 stimulates B-cell Ig heavy chain class switching to the IgE isotype.
- IL-4 mediates recruitment and activation of mast cells.
- IL-4 stimulates the development of Th2 cells from naïve CD4 T cells and functions as an autocrine growth factor for differentiated Th2 cells. Therefore IL-4 induces and expands this subset of T cells.
- IL-4 suppresses macrophage-derived production of Il-12, thereby inhibiting differentiation of Th1 cells.
- IL-4 antagonizes the macrophage activating effects of IFNγ and consequently inhibits cell-mediated immune reactions.

- IL-4 is able to block or suppress the monocyte derived cytokines, including IL-1, TNFα, IL-6, IL-8 and macrophage-inflammatory protein-1α[38,39,40,41].
- It has also been shown to suppress macrophage cytotoxic activity, parasite killing and macrophage-derived nitric oxide production[42].
- IL-4 can potentiate proliferation of vascular endothelium and skin fibroblasts, and decrease proliferation of human adult astrocytes and vascular smooth muscle cells[38,43].
- In addition IL-4 induces potent cytotoxic responses in tumours, possibly by stabilizing disease and modifying tumour growth rates[44].

1.2 Pathogenesis of DTH and Granuloma Formation

There are 5 distinct phases of adaptive immunity[4,7,45], namely

- Recognition of antigens
- Activation of lymphocytes
- Effector phase (elimination of antigens)
- Decline (return to resting state)
- Memory

The current model for granuloma formation is based on the delayed-type hypersensitivity (DTH) reaction, as epitomized by the tuberculin reaction[46]. With the first exposure of a person to tubercle bacilli, naïve CD4+ T cells recognise captured peptides derived from these bacilli in association with class II molecules on antigen

presenting cells, namely dendritic or Langerhans cells. This initiates the differentiation of these T cells to sensitised Th1 cells. Some of these Th1 cells enter the circulation and remain in the T cell memory pool for years[18,19].

On subsequent intracutaneous injection of tuberculin, the DTH process is initiated by these sensitised Th1 cells, following antigen presentation by macrophages[47,48]. In a previously sensitised person, a response appears on site within 8-12 hours after injection, in the form of reddening and induration. This reaches a peak within 24-72 hours and thereafter recedes. The redness and induration are due to vasodilation with perivascular cuffing of predominantly CD4 (helper) T lymphocytes around small veins and venules[18,19].

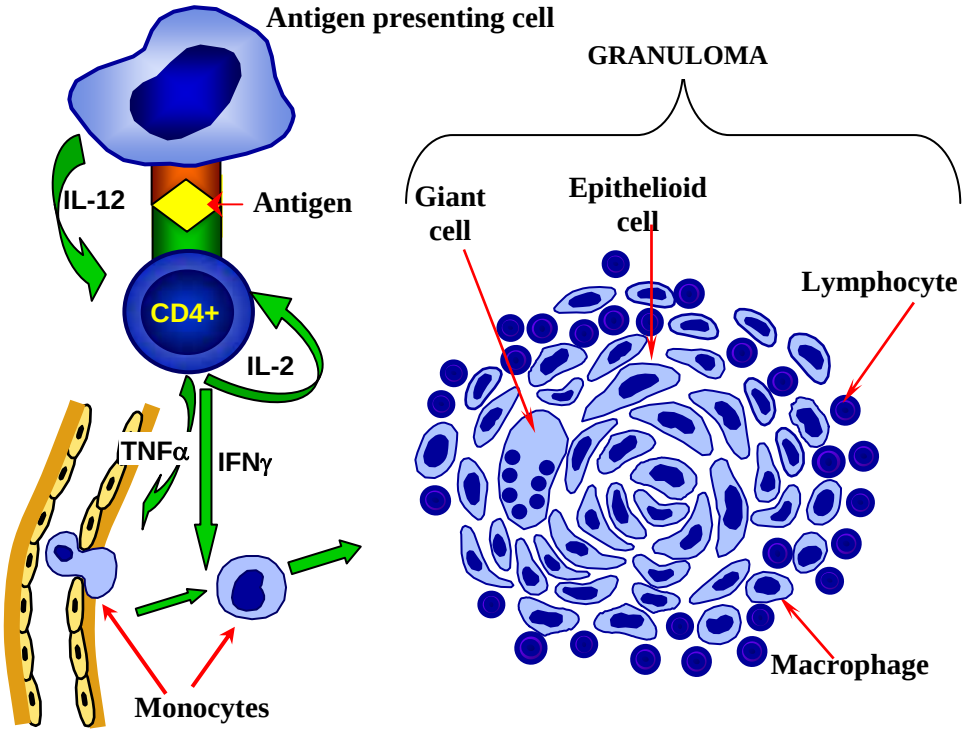


Figure 2: A schematic illustration of granuloma formation during the DTH reaction.
After capture of the antigen, antigen presenting cells present the antigen to the CD-4 lymphocytes, while producing IL-12 which stimulates Th1 differentiation. The pro-inflammatory cytokines TNFα and IFNγ drive the inflammation, resulting in granuloma formation.

An increase in vascular permeability results in the escape of plasma proteins and fluid, causing dermal oedema and fibrin deposition, leading to induration. With persistence of the antigen, the lymphocytic infiltrate is largely replaced by macrophages over the next 2-3 weeks. These macrophages undergo morphological transformation to epithelioid cells and thus a granuloma is formed[9].

1.2.1 Antigen Capture, Processing and Presentation

Micro-organisms stimulate the innate immune response and induce the secretion of inflammatory cytokines in an effort to kill the organisms. Innate immunity against intra-cellular organisms may limit bacterial growth, but usually fails to eradicate the organisms and then requires adaptive CMI for eradication. This process starts with the capturing of an antigen by an antigen presenting cell[9,16,17].

Once the protein antigen is captured and phagocytosed by the APC, it is converted to a peptide and then displayed as a peptide-MHC II complex on the surface of the APC, for recognition by T cells[17]. The initial capturing is performed by immature dendritic cells (DCs), and occurs at the site of infection/exposure to the antigen, which is usually the skin, GIT, respiratory tract or other mucosal surfaces. These cells express membrane receptors like mannose and Toll-like receptors that capture micro-organisms, endocytose them and start processing the proteins into peptides that can bind to MHC II molecules for presentation to $CD4^+$ T cells.

This conversion is called antigen processing. These actions activate the DCs. The cells lose their adhesiveness for epithelium and start migrating to the draining lymph nodes of those areas via afferent lymphatics. During the migration the DCs mature and are

able to present antigens to naïve $CD4^+$ T cells and activate these cells. Mature DCs express high levels of Class II MHC molecules with bound peptides as well as the costimulators required for T cell activation[21].

1.2.2 Activation, Proliferation and Differentiation of Lymphocytes

DCs present antigens to naïve $CD4^+$ T cells in lymphoid tissue and during this process the naïve lymphocytes are activated. This results in a clonal expansion of antigen-specific lymphocytes and the differentiation of these cells into effector cells and memory cells. Some of these cells re-enter the systemic circulation and migrate to the site of infection. Here the cells again encounter the antigen and respond to eliminate the antigen[49].

The activation of T cells requires[18,19,47,49]:

- Antigen recognition by the T cells.

This is the first signal for activation and provides the ensuing specificity of the T cells. $CD4^+$ T cells recognise MHC II specifically. Adhesion molecules stabilise the attachment of T cells to DCs. This ensures prolonged exposures to ensure that the functional responses of the T cells are triggered. T cell receptors for costimulators on DCs provide second signals for T cell activation. During this process cytokines are produced that drive the proliferation and differentiation of the T cells[18,19,47,49].

- Costimulators and
- Cytokines produced by DCs and T cells themselves.

1.2.3 Functional Responses of T Cells

1.2.3.1 Cytokine production and secretion

One of the earliest responses to antigen recognition by the T cells is cytokine production and secretion. The principal cytokine produced by naïve T cells is IL-2. IL-2 functions as a growth factor for T cells. Antigen stimulation also increases expression of receptors for various cytokines by T cells[18,19,50,51].

1.2.3.2 Proliferation

This is mediated in a primarily autocrine manner via IL-2. The T cells respond to their own secreted cytokines and also express surface receptors for these cytokines[18,19,50,51].

1.2.3.3 Differentiation

On antigen stimulation some of the T cells differentiate into effector cells and others into memory cells. This is also dependant on antigen stimulation, costimulators and cytokines[18,19,50,51]. The offspring of $CD4^+$ T cells can differentiate into two distinct subsets: called Th1 and Th2[19,47,52,53]. These cells are recognised by the principal cytokines they produce. Th1 cells produce IFNγ and Th2 cells IL-4 and IL-5.

Th1 and Th2 subsets develop from the same naïve T cells and the differentiation is determined by the stimuli, especially cytokines, present early in the immune response. IL-12 induces Th1 and IL-4 induces Th2 differentiation[19,47,52,53,54,55]. Th1 differentiation is the response to organisms that infect/activate macrophages or NK cells, while Th2 differentiation follows on helminth and allergen exposure.

1.2.3.3.1 Th1

Following the innate immune response, macrophages activated by intra-cellular organisms secrete IL-12[17]. The organisms may trigger this directly by binding Toll-like receptors on the macrophages or indirectly by stimulating NK cells to produce IFNγ, which then activates macrophages to secrete IL-12[7,15,17]. T-cells further enhance this process via CD40L on T cells engaging CD40 on APCs, stimulating IL-12 gene transcription.

IL-12 binds to receptors on activated $CD4^+$ cells and activates the transcription of STAT4, a transcription factor that promotes the differentiation of Th1 cells. IFNγ induces another TH1 transcription factor, called T-bet[7,15]. Simultaneously, IFNγ stimulates IL-12 production by macrophages and IL-12 receptor expression on T lymphocytes. In addition, IFNγ suppresses Th2 proliferation[54,55].

1.2.3.3.2 Th2

Th2 differentiation is dependant on IL-4, which activates a transcription factor, STAT6, that stimulates Th2 development[35]. Th2 cells are, however, the major source of IL-4 during the immune response. This poses the interesting question of where IL-4 comes from before the Th2 cells differentiate. A possible explanation is that, from the point of activation, $CD4^+$ T cells secrete small amounts of IL-4. If the antigen then persists and does not induce IL-12 secretion, Th2 differentiation will follow[54,55]. Other factors that influence differentiation are the amount of antigen and costimulators expressed on APCs and the genetic structure of the host. In mice some strains develop Th2 responses to stimuli that elicit Th1 responses in most strains. IL-10, also produced by Th2 cells, inhibits activation of Th1 cells[20].

1.2.3.4 Migration of effector cells to site of antigen

The migration of the effector cells is mediated by cytokines[18,56]. These cytokines induce the expression of adhesion molecules on endothelial cells and the chemotaxis of leukocytes.

Cytokines are produced by macrophages and endothelial cells, stimulated by microbial products and later by activated T cells, responding to the antigen. The most important cytokines involved are TNFα, IL-12 and chemokines. Both TNFα and IL-1 activate endothelial cells to express ligands for leukocyte adhesion molecules.

Table 2:A comparison of the Th1 and Th2 subsets

	Th1 subset	Th2 subset
Cytokines produced		
IFNγ	+++	-
IL-4, IL-5, IL-13	-	+++
IL-10	+/-	++
IL-3, GM-CSF	++	++
Cytokine receptor expression		
IL-12R β chain	++	-
IL-18R	++	-
Chemokine receptor expression	+/-	++
CCR4	++	+/-
CXCR3, CCR5		
Ligands for E- and P-selectin	++	+/-
Antibody isotypes stimulated	IgG2a (mouse)	IgE, IgG1 (mouse) IgG4 (humans)
Macrophage activation	+++	-

Cytokines are produced by macrophages and endothelial cells stimulated by microbial products and later by activated T cells, responding to the antigen. The most important

cytokines involved are TNFα, IL-12 and chemokines. Both TNFα and IL-1 activate endothelial cells to express ligands for leukocyte adhesion molecules.

Chemokines increase the affinity of leukocyte adhesion molecules for their ligands on endothelial cells and also promote trans-endothelial migration of leukocytes[54,55].

TNFα stimulates endothelial cells to produce prostacyclin, a vasodilatory substance that causes increased blood flow and increased delivery of leukocytes to the site of inflammation[19].

TNFα and IFNγ induce endothelial cells to change shape and remodel basement membrane, supporting extravasation of cells and leakage of macromolecules like fibrinogen[19,30]. This leakage of macromolecules forms the basis of the induration seen in DTH reactions[50,51]. Fibrinogen, and its product fibrin, provides the scaffolding that facilitates migration and retention of leukocytes in the extravascular tissue.

The migration of effector T cells to the site of inflammation is not antigen specific[18,50,51]. All activated T cells will migrate to this region, including effector and memory T cells that might be specific for the antigen in question. If these specific T cells recognise the antigen presented, the affinity of these cells' adhesion molecules for their ligands is increased, resulting in the preferential retaining of antigen specific effector and memory cells. T cells not specific for the antigen may return to the circulation via the lymphatic system.

1.2.3.5 Effector phase

Effector T cells can respond to antigens presented by a wider variety of APCs than naïve cells. Both effector and memory cells are less dependant on costimulators and require less antigen to be activated. Once the effector cells reach the site of antigen challenge, they are activated to perform their effector duties[57]. In cell-mediated immunity, the main effector function of the $CD4^+$ cells is to stimulate the microbiocidal activities of macrophages and other leukocytes.

Monocytes are recruited from the blood and are then exposed to Th1 effector cells that activate them into macrophages, able to kill micro-organisms[50,51]. Stimulated effector Th1 cells secrete IFNγ and express CD40L. Macrophages are activated by the IFNγ and CD40L-CD40 (CD40 expressed on macrophages) interaction. The requirement for CD40L-CD40 interaction ensures that the macrophages presenting antigen to T cells are also the macrophages activated by the T cells[7].

In chronic DTH reactions, activated macrophages also undergo changes in response to persistent cytokine signals. They then acquire the morphological appearance of epithelioid cells. These macrophages have an increase in cytoplasm and organelles and a collection of these forms a granuloma. They may also fuse to form multi-nucleated giant cells.

1.2.3.5.1 Function of activated macrophages

Killing of phagocytosed micro-organisms[58]

- Macrophage activation leads to increased synthesis of reactive oxygen intermediates and nitric oxide, which are potent microbiocidal agents, produced in the lysosomes of macrophages. These substances kill phagocytosed organisms after fusion of phagosome with lysosome. They may also be released into the adjacent tissue, to kill extra-cellular organisms and may then cause tissue damage[15.]

Stimulation of acute inflammation

- Activated macrophages stimulate acute inflammation through the secretion of cytokines, chemokines and lipid mediators. This results in local inflammation rich in neutrophils, to phagocytose and destroy organisms.

Repair

- Activated macrophages remove dead tissue and therefore facilitate repair.
- They also produce growth factors that stimulate formation of repair tissue. These include platelet derived growth factor (fibroblast stimulation), transforming growth factor-β (collagen synthesis) and fibroblast growth factor (angiogenesis) [15].

1.2.3.6 Memory phase

Memory T cells are an expanded population of T cells specific for an antigen that can respond rapidly to subsequent encounters with the same antigen, differentiate into effector cells and eliminate the antigen. The mechanisms of memory cell generation are not known.

1.3 Tuberculosis

Tuberculosis (TB) is an important cause of serious pulmonary disease and mortality. Previous research into the pathogenesis has been neglected because of the low public health priority of tuberculosis, but the advent of HIV infection and the subsequent rise in the incidence of tuberculosis refocused attention on this disease. Another important gap in current knowledge is an understanding of how host resistance to infection is mediated and how infection may be resisted or heals spontaneously.

Tuberculosis is caused by the organism *Mycobacterium tuberculosis (M.tb)*, which is an aerobic, non-spore forming, non-motile bacillus with a waxy coating that retains red dye when treated in acid-fast stains. *M.tb* is transmitted by inhalation of infective droplets. The lungs are the usual sites of infection. The ensuing granuloma of TB has a very characteristic appearance. Early in the disease typical granulomas as previously described can be seen. With progression of disease, however, central necrosis develops in these granulomas, bestowing these granulomas with the typical caseating centre seen in tuberculosis[59,60].

1.3.1 Pathogenesis

Tuberculosis is divided into a primary and a post-primary (secondary) type.

1.3.1.1 Primary tuberculosis

This phase takes place in a previously unexposed host. The organism is inhaled and in the lung, usually in the periphery, the organism is phagocytosed and innate immunity is initiated. The phagocytosed organism is transported to the hilar lymph nodes and

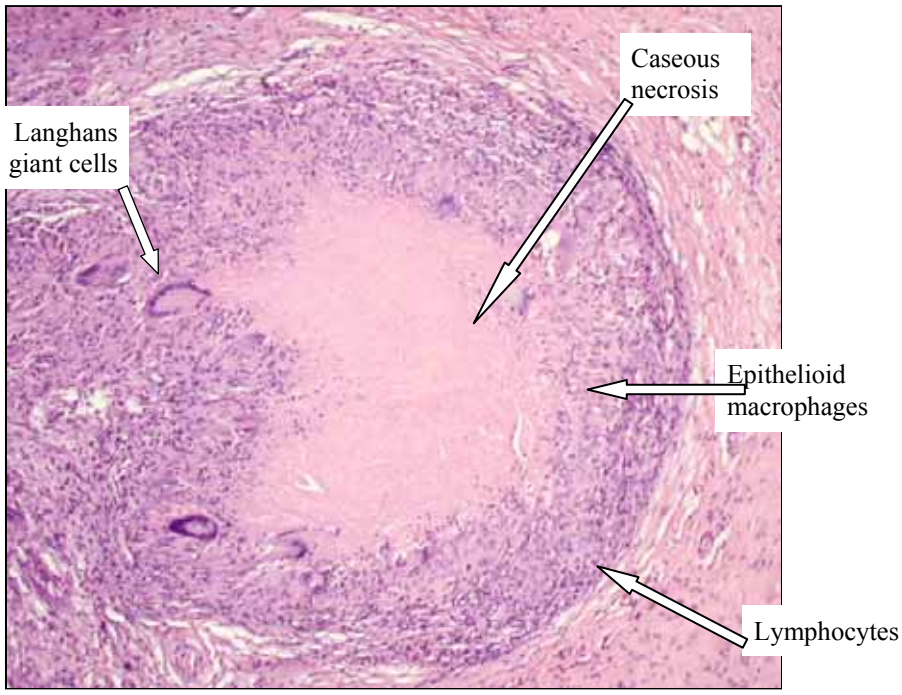


Figure 3: Microscopic appearance of caseous necrosis.
A well-formed granuloma containing epithelioid macrophages, with a rim of lymphocytes and several Langhans giant cells can be seen. Centrally, caseous necrosis is apparent as amorphous pink material.

presented to naïve T cells, stimulating the process of activation, proliferation and differentiation of these T cells. If the organism is not killed, the process of adaptive immunity takes control and a DTH response commences. This response occurs predominantly at the primary site of infection, i.e. the periphery of the lung and granuloma formation ensues, classically typified by central (caseating) necrosis. In most patients infected in this manner the infection is contained, usually by fibrosis, and a calcified scar in the periphery of the lung, also referred to as a Ghon focus, is all that remains. Sometimes the hilar lymph nodes are also involved in this process and the lesion is the referred to as a Ghon complex[59,60]. In a small number of patients, usually due to some form of immune-suppression, active disease develops immediately following primary infection. This process is called progressive primary tuberculosis and is often a disseminated form of tuberculosis.

1.3.1.2 Secondary tuberculosis

Some patients become reinfected with mycobacteria or reactivate dormant disease. *M. tuberculosis* infection is latently present in one third of the world population. Less than 10% however, develop active disease. The remainder generate an effective immune response, which allows containment of the bacilli within granulomas. The bacteria persist for long periods of time in this dormant state, but fail to transmit infection. Re-activation of the dormant infection and the onset of active disease will lead to the transmission of disease to a new host. The clinical and morphological pictures of these events are similar.

Granulomas of secondary TB occur most often in the apices of the lungs, but may be widely disseminated, depending on a large variety of factors. Again, the most prominent site affected is the lung and the granulomas, which fail to contain the bacteria, are a major cause of tissue destruction, due to the extensive caseous necrosis[59,60]. Once these localised granulomas develop, the subsequent course is variable. They may heal spontaneously or after therapy, resulting in a fibro-calcified nodule, or they may progress as follows:

Caseating granulomas may erode into a bronchiole or a bronchus. The result is cavitation and this facilitates organism growth due to the favourable oxygen tension. In most cases the cavity is walled off by a fibrotic capsule and the lesion remains localised. However, in certain circumstances the infective material may disseminate[59,60].

This happens in one or more of the following ways:

- The infective caseous material may be coughed up and spread in the following manner:
    - It can spread along the airways and affect the trachea, larynx, and the rest of the upper respiratory and digestive tract.
    - The infective material can be inhaled, causing a bronchopneumonic picture.
    - It can be swallowed, leading to intestinal TB, usually in the region of the distal ileum or ileo-caecal junction, as this is where the lymphoid tissue of the GIT is present in the form of Peyer's patches.
- The granulomas may spread into a branch of the pulmonary artery, leading to:
    - Haemorrhage into the cavity, resulting in haemoptysis
    - Localised spread of the infective material to the area of the lung supplied by that section of the pulmonary artery, causing localised miliary TB.
- The granulomas may spread through the lymphatics to lymph nodes:
    - Spreading retrogressively through other lymphatics to other areas of the lung, or other organs.
    - Spread to both lungs via the right heart back through the pulmonary arteries, causing diffuse miliary TB.
- The granulomas may spread into the pulmonary veins, again resulting in:
    - Haemoptysis
    - Spreading to the systemic circulations via the pulmonary veins to the left heart and through the aorta.

In the progression of pulmonary disease, the pleura is often inevitably involved. This can take on the form of serous effusions, tuberculous empyema or massive obliterative fibrous pleuritis[61].

Spread to the systemic circulation can lead either to individual organs being affected or to miliary TB, depending on the immune status of the patient and the virulence of the organism. Organs that are often affected include the kidneys, spleen, brain, adrenals, testes, etc.

1.3.1.3 Miliary TB

In the miliary type of TB, individual lesions vary from one to several millimetres in diameter and are distinct, yellow-white nodules. There is no visible central necrosis or cavitation present. Histologically, however, it has the characteristic necrotising granulomatous appearance. Organs often involved in miliary TB include the bone marrow, liver, spleen and eye. This is usually an aggressive form of the disease with a fulminant course[62].

1.3.1.4 Pleural tuberculosis

Primary pleural effusion, as a presenting symptom of TB, usually occurs in adolescents and adults. It occurs on the same side as the primary (Ghon complex), suggesting that a sub-pleural tuberculous focus or lymph node has extended into the pleural space[63]. Bilateral or opposite effusion should raise the possibility of miliary TB[64,65]. In primary pleural effusions granulomas are seen on both the visceral and the parietal pleural surfaces and have the typical appearance of TB granulomas.

Primary pleural TB usually resolves without treatment. In young adults especially, it does, however, carry the risk of developing into other forms of TB, usually within 5-10 years. Full therapy is therefore justified. Local complications that may ensue include focal or diffuse fibrosis, a large caseous lesion or cavity that may rupture into the pleura, creating a pulmonary-pleural fistula or spontaneous pneumothorax, para-vertebral abscess and osteitis of the ribs.

These manifestations of TB are caused by the balance between the virulence of the organism and the host immune response. Little is known about the virulence factors of M.tb, but current research, including the now completed sequencing of the organism's genome will advance our understanding of the organism.

Understanding the human host response is also important, as only a complete understanding of this process will assist in the development of new vaccines and possible immuno-modulatory adjuvants to the treatment of tuberculosis.

1.3.2 Cellular Immunity and Tuberculosis

One of the examples of an organism of low virulence forcing a host response is that of *Mycobacterium leprae* that appears to be able to elicit two very different clinical manifestations of the disease leprosy, with a spectrum of changes between these two poles and has therefore been described as a polar disease[66]. Leprosy has been investigated extensively and has a vast spectrum of clinical manifestations, from lepromatous leprosy, which is the most severe form of leprosy, to tuberculoid leprosy, which is the mildest form of leprosy. Studies of lepra have yielded some insights into the cellular and molecular mechanisms of mycobacterial granulomatous inflammation and suggest that the Th1 cytokines such as IFNγ and TNFα may have an important role in resistance to infection as they are found in tuberculoid lepra[66,67].

Conversely, a predominance of Th2 cytokines (IL-4, IL-10) may be associated with spread of disease as can be observed in lepromatous lepra[68,69]. Patients with tuberculoid leprosy exhibit paucibacillary disease with a well-developed Th1 response. The T cells in these patients proliferate vigorously in response to mycobacterial antigens and large amounts of IFNγ are present in these lesions. On the other hand, patients with lepromatous leprosy have a high bacillary load and a poor proliferative response to mycobacterial antigens. Whether this is due to a Th2 response or a lack of Th1 response is debatable. Attempts have been made to extrapolate these observations in leprosy to patients with TB.

It is generally accepted that the cellular immune system plays the most important role in host defense against *M.tb*. All the components in this immune system, from the

initial innate immunity, through to the late stages of granuloma formation may play an important role in this host defense. However, for the purposes of this study I will focus on the granuloma as such and specifically some of the cytokines involved in this process.

Clinical studies are restricted by certain common features. Most studies involve post-clinical observation of chronic infection, where the dose of the organism and the level of exposure are unknown. The patient also may have chronic infection-induced anergy, for instance when infected by helminths, in several stages of the parasite life cycle, each stage being able to stimulate different types of immune responses. In animal models, a more controlled environment for study exists. Despite the vast number of variables that have to be considered when studying human disease, it is clear that similarities exist between the immune response in humans and those in mouse models.

It has become quite apparent that T cells play a major role in the host response against *M.tb.* This is clearly illustrated in HIV/AIDS where patients develop tuberculosis at a rate of 10% per year, rather than the norm of 10% per lifetime[70]. During the last few years a substantial case has been built to prove that IFNγ, the hallmark cytokine of the Th1 response, plays a key role in the defence against tuberculosis. IFNγ is a pro-inflammatory cytokine promoting a macrophage rich inflammatory environment that is beneficial for the inhibition and killing of mycobacteria. It is clear from the literature that IFNγ is crucial in the defence against mycobacteria, as individuals with a genetic defect in IFNγ production or receptors, or who do not respond to IFNγ, develop severe systemic infection[71,72]. It has also been shown that patients with lymphocytic alveolitis

and significant local production of IFNγ develop mild disease, as opposed to patients without this response, who develop cavitary disease[73]. In patients with multi-drug-resistant (MDR) TB, who failed medical treatment, clinical and radiological improvement was noted after IFNγ aerosol treatment[73].

On the other hand it is suggested that a persistent high IFNγ is responsible for the damage caused by granulomatous disease and that moderating cytokines are necessary to down-regulate the IFNγ response to more appropriate levels later in the disease process, once the organisms have been effectively contained.

Mouse models demonstrate the existence of a non-static, adaptive immune response that can change over time given the relevant stimulus. Longitudinal studies suggest that IL-4 has a controlling effect on IFNγ[74]. Thus, in tuberculosis a dual role for INFγ in both protection and immunopathology is suggested, where a proper CMI is responsible for containment, but an over-exuberant CMI is responsible for tissue destruction. In cattle, the peak level of IFNγ production did not coincide with protection, and it is suggested that the over-production of IFNγ might lead to immune dysregulation and be damaging to the host[75].

An *in vitro* study of the production of cytokines by normal peripheral blood mononuclear cells (PBMC) in response to BCG described an evolving cytokine response, starting with the production of monokines, followed by a Th1 response and ending with a Th2 response[76]. In a cattle model, cattle were infected with virulent *M. bovis* and the cytokines monitored for 20 weeks. By this time lesions had formed in the

upper respiratory lymph nodes and lungs. A strong, sustained IFNγ response was present throughout the infection period. IL-4 was detected in a peak from 8-10 weeks. Before and after this peak IL-4 was undetectable[77]. In the same study a model with cattle infected by onchocerciasis demonstrated exactly the inverse cytokine profile. These observations suggest that a prolonged cytokine bias results in immunopathology and that a balanced immune response is necessary for host protection and the resolution of infection.

Some studies suggest however, that an increase in IL-4 and IL-10 production is responsible for the immunopathology of tuberculosis. It is suggested that an increase in IL-4 suppresses IFNγ production, followed by reduced killing of organisms[78]. A study on PBMCs in patients with cavitary TB and normal controls demonstrated an increase in IL-4 producing cells in patients with cavitary TB, whereas IFNγ production was the same. Similar findings were also reported by Seah et al [79].

Another cytokine that is of key importance is IL-12. It has been demonstrated that IL-12 production is essential to initiate the Th1-response and that persistent IL-12 production is necessary to sustain it. This is due to the fact that IL-12 is a potent inducer of IFNγ production in many different cell types[80] as well as the key inducer of Th1 differentiation.

In mice, enhanced protection was noted in the group where IL-12 was used as an adjuvant to bacille Calmette-Guérin (BCG) immunisation[81]. Other studies performed in mice confirmed this. In IL-12p40$^{-/-}$ mice there was an inability to control bacterial

growth and IFNγ and TNFα mRNA levels as well as IL-4 are reduced[82]. There were also a lower number of lymphocytes in the granulomas. After infection with BCG, IL-12p40$^{-/-}$ mice demonstrated a lack of both Th1 and Th2 cytokines in the lung and blood. They also had an impaired inflammatory response, lacking macrophages, neutrophils, CD4, CD8 and NK cells in the lung [83]. There is also however, evidence for the destructive effect of IL-12. A study on mice[84] demonstrated that granuloma disintegration and death was dependant on T cells and IL-12 production, while depletion of T cells subsets and IL-12 led to well formed compact granulomas and prolonged survival.

TNFα has a central role to play both in the host immune response to *M.tb* and the immunopathology of tuberculosis[85]. The release of TNFα in response to *M.tb* has several beneficial effects. In-vitro studies have demonstrated that TNFα increases the ability of macrophages to phagocytose and kill mycobacteria[86]. This cytokine is required for granuloma formation, which sequesters mycobacteria and prevents dissemination of the organism[87]. It is accepted that granuloma formation is protective and indicates a successful immune response.

Several studies in mice have demonstrated the importance of TNFα. Roach et al[88] demonstrated that TNFα was required for the early expression of mRNA encoding chemokines and leucocyte recruitment. Lymphocytes and macrophages also failed to form granulomas and prevent progressive infection. In a study by Ehlers et al[89], granulomas in TNFR1-deficient mice underwent progressive necrosis and resulted in death of the mice. Blocking of TNFα caused fatal reactivation of persistent

tuberculosis, as well as an increase in bacillary load and increased production of IL-10[90], while inhibition of TNFα synthesis increased macrophage degeneration and tissue destruction[91]. TNFα depletion in TNFR1-deficient mice also resulted in abnormal granuloma formation[92]. TNFα also assisted in containing mycobacterial growth by inducing apoptosis of ineffective macrophages[93,94,95].

Although enough evidence exists to support a protective role for TNFα, excessive production of TNFα and an increased sensitivity to the cytokine have been implicated in the immunopathology of tuberculosis, for example, caseous necrosis. A study on mice demonstrated the opposite effects of TNFα[96]. On the one hand TNFα is necessary for the accumulation of cells into well-organised granulomas, the activation of T-cells and the differentiation of dendritic cells to enhance IL-12 production. On the other hand, TNFα has detrimental effects. A high dose of TNFα resulted in damaging inflammation, due to the systemic effects of this cytokine. With a high dose of TNFα in this study there was an aggressive and rapid response in the lung, with extensive cellular recruitment in the lung, compromising lung function. While TNFα is an essential component of the host immune response against mycobacterial infection, high levels of the cytokine at the site of infection induce an excessive inflammatory response that overwhelms the beneficial effects of the cytokine.

It is also possible that the type two cytokines may mediate local tissue inflammation and necrosis through their effect on TNFα mediated cytotoxicity[97,98]. This is supported by findings that the IFNγ:IL-4 mRNA copy number ratios were significantly lower in

patients with tuberculosis than in healthy controls. This ratio was even lower in patients with more severe radiological disease[79].

The role of cytokines such as TNFα may be to modulate and fine-tune this process, depending on the spectrum of cytokines already present. It may be of particular importance in TB where TNFα superimposed on a Th1 cytokine profile may lead to a protective granulomatous response, whereas a Th2 background may result in tissue necrosis and breakdown with cavitation, thus favouring spread of organisms.

1.4 Pleural Tuberculosis

Primary pleural effusion, as a presenting symptom of TB, usually occurs in adolescents and adults, in up to 10% of recently infected persons. It occurs on the same side as the primary (Ghon complex), suggesting that disease in a sub-pleural tuberculous focus or lymph node spread into the pleural space[63]. Contralateral or bilateral effusions should raise the possibility of miliary TB[64,65]. A tuberculous pleural effusion may also be the result of post primary/secondary tuberculosis, years after primary infection.

Primary pleural TB usually resolves without treatment. In young adults especially, it does however carry the risk of developing into other forms of TB, usually within 5-10 years[99]. Full therapy is therefore justified. Local complications that may ensue include focal or diffuse fibrosis, occasionally a large caseous lesion or cavity may rupture into the pleura, creating pulmonary-pleural fistula or leading to spontaneous pneumothorax, para-vertebral abscess and osteitis of the ribs.

The clinical importance of tuberculosis in general and of tuberculous pleural effusion in particular is not the same worldwide. Eight million people developed tuberculosis in 1990 and 95% of these people lived in developing countries[100]. The frequency of pleural effusion in these tuberculous patients is currently approximately 31% [101,102]. Fifty percent of all individuals infected with HIV worldwide are also infected with tuberculosis. Of all HIV-related deaths, approximately 33% are ascribed to tuberculosis. Furthermore, the lifetime risk of an HIV positive person of developing tuberculosis is 50%, compared to 5-10% in an uninfected person[103]. There are contradictions regarding the frequency of pleurisy in HIV-positive and HIV-negative

patients with tuberculosis. A South African study reported a higher frequency in HIV-positive patients [104], but some Central African studies could find no such differences, [103, 105]. Initial data suggest that HIV patients tend to develop tuberculous pleurisy in the early stages of immunosuppression, when the CD4+ T-lymphocyte (helper-inducer) count >200 cells·mL-1 (27%) as opposed to CD4+ T-lymphocyte count <200 cells·mL-1 (10%)[106].

It is clear from these figures that co-infection by tuberculosis and HIV is a very dangerous combination. This problem is compounded in South Africa, with its high incidence of both HIV and tuberculosis.

1.4.1 Pathogenesis

The current hypothesis for the pathogenesis of primary tuberculous pleural effusion is that a subpleural caseous focus in the lung ruptures into the pleural space 6–12 weeks after a primary infection[63,64]. Mycobacterial antigens enter the pleural space and elicit a delayed hypersensitivity reaction and the accumulation of fluid. It appears that the DTH reaction results in an increased permeability of pleural capillaries to serum proteins[65], leading to increased oncotic pressure in the pleural fluid and the development a pleural effusion.

In primary pleural effusions, granulomas are seen on both the visceral and the parietal pleural surfaces and have the typical appearance of TB granulomas. These granulomas tend to follow the lymphatics on the visceral surface[107] and focal or diffuse fibrosis may follow. The lymphatic system is probably also involved and may contribute to the

development of a pleural effusion. Impaired clearance of proteins from the pleural space has been reported in human tuberculous effusions[65].

Clearance of proteins and fluid from the pleural space is performed by lymphatics in the parietal pleura. Access to the lymphatics is through openings in the parietal pleura called stomata[108]. In tuberculous pleuritis, with diffuse involvement of the parietal pleura, damage to or obstruction of the stomata could be an important mechanism leading to accumulation of pleural fluid.

It appears that tuberculous pleurisy is due to release of isolated mycobacterial antigens into the pleural space, eliciting a delayed hypersensitivity reaction rather than to a tuberculous infection by viable organisms[109]. This hypothesis is supported by several facts. Cultures of pleural specimens from patients with tuberculous pleurisy are positive in one-fifth to two thirds of patients[110] who are HIV negative[111]. On histological examination, acid-fast bacilli are demonstrated in 5- 18% of cases[112]. The yield is improved when a combination of fluid culture as well as culture and histology of tissue are performed. The diagnosis is then confirmed in more than 95% of patients[113]. A lymphocytic pleurisy can be produced in sensitised guinea pigs by the intrapleural instillation of heat-killed bacilli Calmette- Guérin (BCG)[114]. Moreover, pleural effusion also develops in nonsensitised animals that have received cells from immunized animals[115]; and effusion does not develop in sensitised animals if they are given antilymphocyte serum[116].

The same process of granuloma formation previously described takes place in the pleura. Some studies suggest that the mesothelial cell plays a role in the recruitment of the cells participating in tuberculous pleuritis[117]. As expected, most of the lymphocytes in a tuberculous pleural effusion are CD4+ T-lymphocytes[118], with a mean CD4:CD8 (helper:suppressor) ratio of about 4.3 in pleural fluid, and 1.6 in peripheral blood[119]. Pleural tuberculosis in HIV-negative subjects has been taken to represent a protective immune response to *Mycobacterium tuberculosis* because a high proportion of individuals can recover without antibiotic therapy[120]. A tuberculous pleural effusion in HIV-negative subjects is usually associated with a prominent cell-mediated immune response, represented by an infiltration of CD4+ T cells [121] and high levels of pro-inflammatory cytokines, including IFNγ and TNFα[122]. This appears to indicate a key role for these responses in protective immunity against tuberculosis in humans, in keeping with evidence that IFNγ and TNFα are necessary for protective immunity to tuberculosis in animals[123,124,125], and that abnormalities of the IFNγ receptor are associated with susceptibility to mycobacterial disease in humans[126,127].

1.4.2 The Effect of HIV on TB

Increased nosocomial[128,129] and community[130] exposure to *M.tb* may play a role in the increased risk of contracting tuberculosis in HIV infected patients, but HIV-associated impairment of one or more immunological mechanisms plays an important role[131]. These mechanisms include impairment of pulmonary innate immune defences, impairment of cellular recruitment and establishment of the cell-mediated granulomatous response to recent *M.tb* infection, and functional impairment of established granulomas containing latent *M.tb* infection.

There are no epidemiological data that clearly demonstrate a higher rate of initial *M.tb* infection among HIV-1- infected individuals than among non-infected individuals following a similar exposure[132]. It therefore appears that the risk of infection by *M.tb* is similar for both HIV positive and HIV negative persons.

The risk of developing progressive primary TB following *M.tb* infection is however higher among HIV- 1-infected persons than among immunocompetent persons[128,133]. In addition, the estimated lifetime risk of developing active TB among immunocompetent individuals with latent *M.tb* infection is only 2–23%[134]. However, in HIV-infected persons this number is much higher and is estimated to be approximately 10% per year[132,135]. Therefore, the risk of developing active TB among HIV-1-infected persons with latent *M.tb* infection appears to be very high.

These data suggest that HIV-1 infection affects the ability of established granulomas to contain *M.tb*, resulting in increased reactivation of latent mycobacteria. The relative contribution of endogenous reactivation and recently acquired exogenous TB to the overall increased risk of TB among HIV-1-infected persons is not clear and is likely to vary depending on the prevalence of TB and *M.tb* infection in a given community.

HIV-1 replication is closely regulated by the host cell transcriptional machinery, and is therefore under the influence of a complex network of pro-inflammatory and immunoregulatory cytokines[136,137,138]. TNFα plays a pivotal role in HIV-1 pathogenesis[139] by inducing HIV-1 transcription in both macrophages and T lymphocytes via the NF-κB pathway[138,139, 140,141]. Other proinflammatory cytokines (IL-1, IL-2, and IL-6) also induce HIV-1 replication[142]. IL-6 synergizes with TNFα to

enhance HIV-1 replication at transcriptional and posttranscriptional levels in monocytic cells but not lymphocytes[143]. IL-1 increases HIV-1 replication in promonocytic cell lines by enhancing TNFα mediated induction of NF-κB[144]. In addition to these proinflammatory cytokines, it is possible that other host-encoded immune mediators may play a role in the induction of HIV-1 transcription during the inflammatory response[145,146].

It has been hypothesized that qualitative T-helper-type responses may also impact AIDS pathogenesis. A switch in the predominant response from a Th1 to a Th2 response and the production of associated cytokines may facilitate disease progression[147,148,149]. Some studies indicate that HIV-1 disease progression is associated with increasing secretion of IL-10[150,151]. However, these findings and the supposed role of modulation of T helper responses in HIV pathogenesis remain controversial[152,153].

This controversy concerning the role of T helper-type responses in HIV-1 pathogenesis raised the question whether coinfections that modulate these Th responses might also impact on the natural history of HIV-1 *in vivo*[154,155]. Parasitic diseases typically induce a dominant Th2 response and also modulate subsequent responses. For example, mice with schistosomiasis mount Th2 responses to nonparasite antigens that normally induce Th1 responses[156,157,158]. An example in humans is that of schistosomiasis that impairs Th1 responses to tetanus toxoid immunization[159]. It is therefore possible that Th2 modulation of the immune system resulting from coinfections with parasitic diseases may facilitate HIV-1 replication in coinfected persons[154,160], although data to support this are lacking.

In 1994 Lucas et al described 3 histological stages of cellular immune response that correlate with depletion of the peripheral blood CD4+ lymphocyte count[161], namely:

- in immunocompetent individuals with HIV-1 infection, TB granulomas are characterised by abundant epithelioid macrophages, Langhans giant cells, peripherally located CD4+ lymphocytes, and a paucity of bacteria
- in individuals with moderate HIV-associated immunodeficiency, Langhans giant cells are not seen, epithelioid differentiation and activation of macrophages are absent, there is CD4+ lymphocytopaenia, and acid-fast bacilli (AFB) are more numerous
- in individuals with advanced HIV associated immunosuppression and AIDS, there is a striking paucity of granuloma formation with little cellular recruitment, very few CD4+ lymphocytes, and even larger numbers of AFB.

It is therefore apparent that HIV-1 coinfection weakens the granulomatous host response to *M.tb*.

HIV-1 infects macrophages and CD4+ T cells that are essential in granuloma formation in tuberculosis. Co-infection will therefore impair cell-mediated immune responses to *M.tb* infection. Several possible levels of impairment exist.

Macrophages are central to the pathogenesis of both TB and HIV-1 infection. They are the initial effector cells against *M.tb* and serve as reservoirs for the intracellular growth of both organisms. Several studies indicate that HIV-1 impairs intrinsic macrophage-mediated defences against a variety of intracellular pathogens,[162,163,164,165,166,167] including increased intra-cellular growth of *M.tb* [166] and suppression of IL-12

production[32]. TB and HIV-1 coinfection are also likely to be associated with extensive virus-induced and activation-induced cell loss and with the suppression of lymphocyte regeneration and maturation[168,169]. HIV may also block the ability of the host to mount an effective proliferative T- lymphocyte response to *M.tb*[170]. It is well recognized that CD4+ lymphocytes in HIV-infected persons are functionally impaired[171,172,173,174,175,176].

According to the dogma, effective cell-mediated immunity to *M.tb* is characterised by a strong Th1 response. This is supported by the susceptibility to tuberculosis among individuals with IL-12- receptor or IFNγ- receptor deficiencies[177]. Numerous studies have shown that during the progression of HIV-1 disease, mononuclear cells lose their ability to secrete Th1 cytokines and instead produce increased levels of the Th2 cytokines[178].

1.4.2.1 The effect of HIV on the granuloma

The several changes already described above, as well as the progressive immunosuppression associated with the development of AIDS result in failure of epithelioid differentiation of macrophages, no formation of Langhans giant cells, and no caseous necrosis[161]. Tuberculous hypersensitivity-type granulomas are thought not to be immunologically quiescent structures but rather to have a continual level of mononuclear cell-death and cell replacement by active recruitment[179]. Very little is known, however, about the mechanisms whereby HIV-1 coinfection actually causes dysfunction of established granulomas and leads to the reactivation of latent TB.

It is possible that HIV-1 infection might affect granuloma function via two processes:

- Systemic depletion of the mononuclear cells required for the ongoing maintenance and functioning of granulomas and
- The effects of HIV-1-infected cells (either lymphocytes or macrophages) trafficking into the granuloma itself.

It is clearly documented that tuberculosis is an early occurrence in HIV, before any reduction in CD4+ count[180]. The effect of HIV infected cells trafficking into the granuloma is therefore probably the more important pathway for granuloma disruption.

There are many areas in which concomitant HIV infection may tip the balance in tuberculous infection towards progressive disease. Individuals with both infections have reduced T cell lymphoproliferative responses and less production of IFNγ and IL-2 *in vitro* when stimulated with tuberculous antigen in comparison to patients infected only with TB. Thus, Th1 type cytokines are reduced. However, there is little evidence for an enhanced Th2 response as IL-4 production appears to be the same in co-infected and tuberculous-only individuals[103].

CD4+ T lymphocyte depletion is crucial for the pathogenesis of HIV infection. It has been demonstrated that *M.tb* infection enhances HIV replication and CD4+ T lymphocyte depletion. High CD4+ T lymphocyte decline rate was observed in patients with active tuberculosis disease, whereas *M.tb* -infected asymptomatic patients showed similar rates of decline and similar levels of HIV load as patients without latent tuberculosis infection[181,182].

M.tb can also modulate HIV replication by generating a cytokine microenvironment favouring viral infection and spread in local mononuclear cells. In fact, in the context of the specific anti- MTB immune response, a number of cytokines are produced in large amounts in the infected organs[183,184,185,186,187,188]. Several of these cytokines have *in vitro* upregulating (TNFα, IL-6, GM-CSF), or bifunctional activities on HIV replication (IL-10, IFNγ, TGFβ)[189,190]. In support of these data, Garrait *et al*. found that pleural fluid obtained from tuberculosis patients, but not from subjects with congestive heart failure, induced *in vitro* HIV replication and this effect was mediated by TNFα and IL-6[191].

1.5 Sarcoidosis

Sarcoidosis is a multi-system granulomatous disease of unknown aetiology, although several organisms have been investigated as possible causes, amongst these *M. tb*[192,193,194,195]. It is characterised by enhanced cellular hypersensitivity at sites of involvement. Elsewhere in the body, cellular hypersensitivity is depressed so that reactions to common allergens such as tuberculin are negative. The disease is characterised by non-caseating granulomas, which either resolve or progress to fibrosis. The severity of disease varies and there are individuals whose disease is asymptomatic or sub-clinical[196].

Sarcoidosis occurs worldwide, but appears to be more prevalent in higher altitudes, especially Scandinavia, other northern European countries and North America. However, Black Americans, and West Indian and Asian immigrants in the United Kingdom have a much higher incidence than any other racial group[197]. Amongst some races the disease is extremely rare. Most cases occur between the ages of 20 and 40 years and are often discovered during evaluation for other conditions[198]. Symptoms are worst in older women. Approximately 90% of patients have signs of involvement on chest X-ray, with/without lymphadenopathy[199].

The radiological picture of pulmonary sarcoidosis may vary considerably, but the histological picture is always that of granulomatous disease, with or without accompanying fibrosis. Ninety percent of patients present with hilar lymphadenopathy or pulmonary involvement on chest X-ray (CXR). Skin and eye lesions are also fairly common. Patients present with a variety of symptoms, often respiratory, but in 10% of

cases this disease is discovered during evaluation for other conditions. Respiratory symptoms include shortness of breath, cough, chest pain and haemoptysis, which can be massive. Although the CXR findings may vary enormously, the classical finding is that of a reticulonodular infiltrate, implying delicate alveolar septum involvement (reticular) as well as aggregates of granulomas (nodular)[200]. Radiological staging systems for sarcoidosis have been in use for many years and the system described by Berkmen is as follows:

Stage 0: Normal CXR (5-10%)

Stage I: Lymphadenopathy only (50%)

Stage II: Lymphadenopathy associated with pulmonary infiltrates (25-30%)

Stage III: Pulmonary infiltrates without lymphadenopathy (15%)

Stage IV: Fibrosis and end stage lung disease - ± 20% progress to this stage

The macroscopic size and distribution of the granulomas differ significantly and explains the variation in radiological findings.

In a study by Möllers et al[201], patients were divided into two groups according to the duration of symptoms. Patients with symptoms lasting < 2 years at presentation were regarded as having acute disease and patients with symptoms > 2 years were regarded as having chronic disease. All patients with acute disease had a stage I CXR and all patients with chronic disease had either a stage II or III CXR.

Since other diseases, including mycobacterial and fungal infections, can also produce noncaseating granulomas, the diagnosis of sarcoidosis is one of clinicopathological correlation. However, the granulomas in sarcoidosis do have a characteristic appearance, suggestive of the diagnosis on histology[202]. The granulomas are well formed, consisting of tightly clustered epithelioid macrophages, usually with Langhans or foreign body-type giant cells, and often lacking significant surrounding inflammation. Concentric fibrosis, surrounding the granuloma, is often a feature (fig. 4). A variety of changes may be seen in the granulomas, but none are specific to this disease. These include small foci of fibrinoid necrosis and giant cells with inclusions like Schaumann bodies, asteroid bodies and calcium oxalate crystals.

The granulomas are distributed along the pulmonary lymphatics in the pleura and septa and along pulmonary arteries, veins and bronchi, often involving these structures. An interstitial infiltrate of lymphocytes and plasma cells often occurs in the alveoli adjacent to granulomas. The lymphatic distribution is classical of sarcoidosis and assists in distinguishing sarcoidosis from other granulomatous diseases. More than 50% of cases show histological involvement of pulmonary arteries and/or veins (fig. 4). This can probably be explained by the lymphatic distribution of this disease. Both small and large airways are frequently involved by granulomas and are often visible as small submucosal nodules on bronchoscopy. An interstitial infiltrate of lymphocytes and plasma cells are frequently present in sarcoidosis, especially around the granulomas.

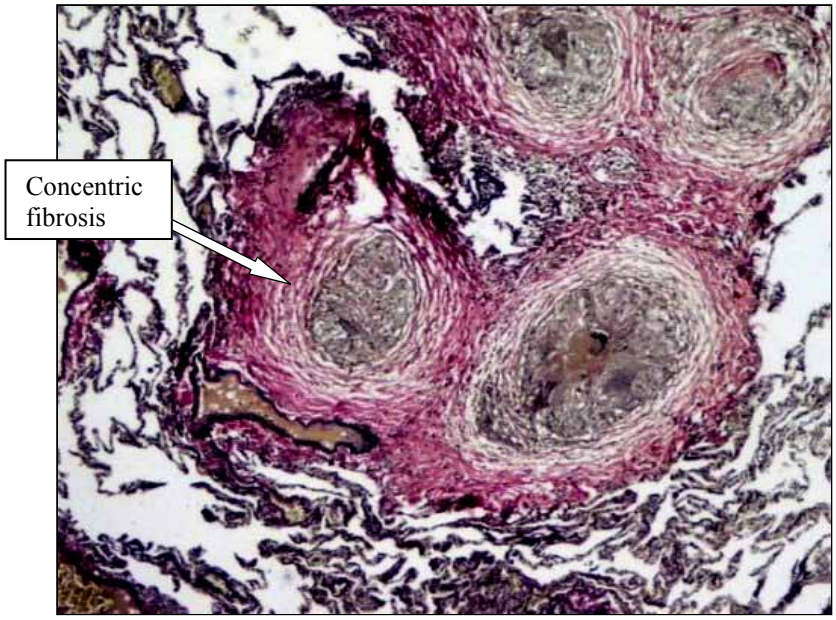


Figure 4: Concentric fibrosis around granulomata evident (x100 magnification, Verhoef van Giesson stain). The concentic fibrosis surrounding the granulomas can be seen staining red in this section.

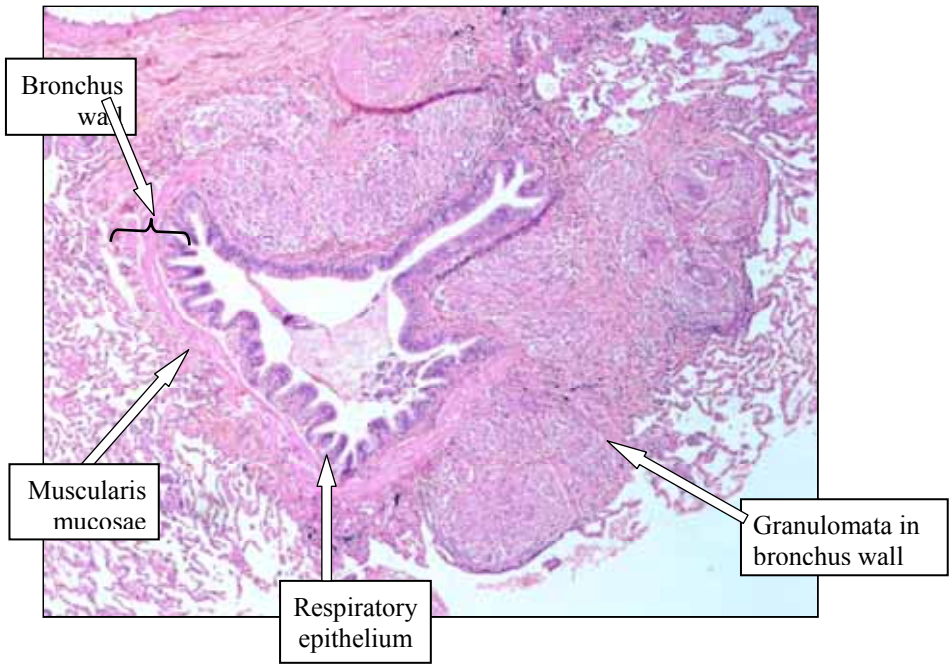


Figure 5: Brochocentric distribution of the granulomata (x100 magnification). Numerous granulomata can be seen, invading the bronchial wall.

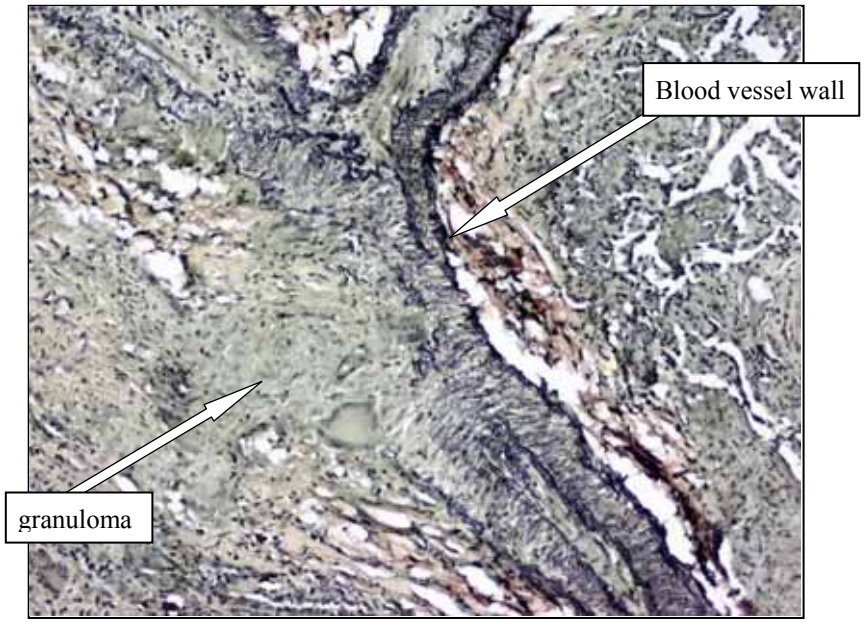


Figure 6: Granulomatous angiitis (x200 Verhoef van Giesson stain). A granuloma can be seen clearly invading the blood vessel wall, with destruction of the wall.

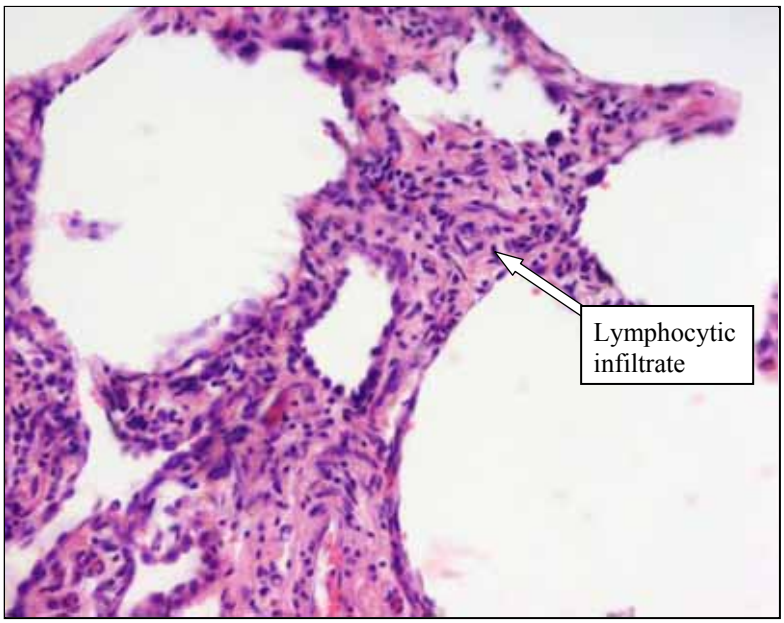


Figure 7: Lymphocytic pneumonitis in sarcoidosis (x200 magnification). An interstitial lymphocytic infiltrate is present in the alveolar interstitium, removed from the granulomata.

This infiltrate varies from very mild, to sometimes quite severe. Apart from interstitial inflammation, interstitial fibrosis can also be seen and is a precursor of honeycombing. Although the clinical and radiological features are often so typical that a presumptive diagnosis of sarcoidosis can be made without histological confirmation, histological confirmation should be obtained before treatment.

A histological grading system exists to determine the severity of pulmonary involvement, based on the extent of specific pathological changes, including granulomas, interstitial pneumonitis, fibrosis and overall change. The extent of airway involvement can also be determined histologically[203].

PULMONARY INVOLVEMENT[203]

- **a. Extent of specific pathological change**
  - *i. Granulomas*
    1. Minimal: a few scattered granulomas
    2. Moderate: more than a few, but occupying less than two thirds of the area
    3. Extensive: numerous granulomas, often conglomerated and occupying more than two thirds of the area
  - *ii. Interstitial pneumonitis (nongranulomatous inflammation of the alveolar walls, predominately lymphocytic)*
    1. Minimal: lesions occupying less than one third of the area
    2. Moderate: lesions occupying one to two thirds of the areas
    3. Extensive: lesions occupying more than two thirds of the area
  - *iii. Granulomatous angiitis (granulomas within the walls of veins and/or arteries)*
    1. Absent: no granulomatous angiitis
    2. Minimal: one to two foci
    3. Moderate: more than two foci
  - *iv. Fibrosis (fibrosis of alveolar wall or interstitium)*
    1. Absent: no fibrosis
    2. Focal: focal and scattered fibrosis
    3. Diffuse: diffuse and extensive fibrosis
- **b. Evaluation of overall pathologic change**
  - i. Mild: no fibrosis, granulomas and interstitial pneumonitis minimal and granulomatous angiitis absent or minimal
  - ii. Moderate: by exclusion from mild and severe categories
  - iii. Severe: diffuse fibrosis, granulomatous and interstitial pneumonitis both extensive, granulomatous angiitis moderate

1.5.1 Pathogenesis

In patients with sarcoidosis, the hypersensitivity reaction is viewed as the consequence of a chronic, exaggerated Th1 immunological response against an unidentified antigen that persists at the sites of disease involvement[204]. Initial granuloma formation has the same pathogenesis as previously described. The accumulation of CD4+ Th1 cells and macrophages at the site of inflammation is the earliest step in the events that lead to granuloma formation. This accumulation is followed by granuloma formation.

Many of the immunological studies performed on the cells involved in sarcoidosis are based on cells present in broncho-alveolar lavage or cell suspensions, while very little is known about the *in situ* profile of these cells.

In general, there is a depression of DTH, an imbalance of CD4/CD8 T cell subsets, an influx of Th1 cells to the site of activity, hyper-reactivity of B cells and circulation on immune complexes. In the early phases of the disease, T cells in the granulomatous areas show a predominantly Th1 profile, expressing elevated mRNA and protein levels of IFNγ, IL-2 and IL-12[205,206,207]. According to these studies the net effect of the Th1 response is the organization of the local inflammatory process into the granuloma and the inhibition of the fibrogenetic process[208,209,210].

TNFα is thought to play a key role as mediator of inflammation and cellular response, due to its pivotal role in granuloma formation[211,212,213]. As TNFα also stimulates fibroblast proliferation and collagen synthesis it may be regarded as a key mediator for the induction of pulmonary fibrosis in sarcoidosis[214,215,216].

Some studies suggest that a switch to Th2 cells occurs in patients with progressive disease who evolve towards lung fibrosis[207]. According to these studies the T cells in the lungs release Th2 cytokines, including IL-4, which stimulate the production of extra-cellular matrix proteins and is chemo-attractant for fibroblasts, as has been demonstrated in cryptogenic fibrosing alveolitis[217,218]. This results in hyperplasia of fibroblasts in granulomas, as well as the expansion of the mesenchymal cell population and subsequent increased deposition of extra-cellular matrix components in the environment surrounding the granulomas.

On the other hand, it has been demonstrated that in newly diagnosed sarcoidosis patients with progressive disease, cells in their broncho-alveolar lavage specimens are characterised by significantly higher release of the pro-inflammatory cytokines TNFα, IL-8 and macrophage inflammatory protein 1 (MIP-1), compared to patients with stable disease[204,219]. Furthermore patients with high levels of spontaneous TNFα release by alveolar macrophages at time of diagnosis had a significantly higher risk of disease progression (in patients not yet on systemic steroid therapy), or a higher rate of relapse (in patients on systemic steroid therapy)[220,221]. In support of these findings, two studies demonstrated a significant fall in alveolar macrophage TNFα release in patients who responded to corticosteroid or immuno-suppressive treatment[222,223]. Another study also demonstrated that in patients with long-standing sarcoidosis and progressive disease (not on treatment) and patients with corticosteroid resistant disease, TNFα was significantly elevated.

A number of studies have indicated the relationship between raised TNFα levels and steroid therapy resistance. As soon as anti-TNFα treatment is applied, a positive response to steroid treatment is noticed[224,225,226,227,228,229]. Increased levels of IL-12 have also been demonstrated in untreated sarcoid patients with disease progression, indicating a potential prognostic role for this parameter[230]. Möllers et al demonstrated that patients with acute onset of disease expressed more Th1 cytokines than patients with chronic disease. However, Th2 expression was similar in both groups and the expected Th2 shift was not observed.

1.5.2 Causal Agent of Sarcoidosis

Numerous studies have been performed in patients with sarcoidosis to determine the cause of this disease. Varying results point towards several organisms, including *M.tb*, *Propionibacterium acnes, Rickettsia helvetica* and *Human herpesvirus 8* [192,193,194,1957]. These results were never conclusive, but there are many indications that an infective agent may be responsible for the development of sarcoidosis. Unpublished data by Fenhalls demonstrated the presence of *M.tb* DNA, RNA and protein in the granulomas of patients diagnosed with sarcoidosis (personal communication).

1.6 Apoptosis

Cell death in living organisms presents either as necrosis or apoptosis, also known as programmed cell death (PCD). Each has distinctive morphological and biochemical characteristics. Necrosis is unregulated, resulting in disintegration of the cell membrane and its organelles[231]. Apoptosis on the other hand, results from the activation of an internally encoded suicide program induced by a variety of extrinsic and intrinsic signals. It is a form of cell death that serves to eliminate cells that are no longer needed[232]. Differences between apoptosis and necrosis are listed in table 3.

Table 3: Differences between apoptosis and necrosis

	Apoptosis	Necrosis
Cellular role	Active	Passive
Cellular membrane	Preserved	Loss of integrity
Cellular architecture	Preserved	Interrupted
Induction	Slow (hours)	Rapid (seconds)
Chromatin condensation	Present	Absent
Cell removal	Rapid	Slow
Inflammatory response	Absent	Present
Regulation	Gene regulated	Not regulated

It was first identified as a distinct form of cell death on the basis of its morphology, where tissue architecture is preserved and inflammation is minimal[231]. Subsequent studies of its incidence, biochemistry and genetics have upheld predictions of its general role in controlled cell deletion and its genetically determined nature. The classic example of the role of apoptosis in embryogenesis is the programmed cell death of various human embryonic tissues, such as deletion of the finger webs. Apoptosis also plays a very important role in the development of T cells, by selecting self-tolerant T cells.

The earliest morphological changes in apoptotic cells include cell shrinkage and condensation of the cytoplasm. The nuclei become pyknotic and basophilic chromatin marginates to the periphery. Endonucleases cleave the nuclear DNA at 180-200-base pair intervals. The DNA and cell content do not spill into the extra-cellular space and therefore do not provoke an inflammatory response. Eventually cells break into fragments, forming apoptotic bodies that are phagocytosed and removed[232].

Apoptosis is the net result of the balance between pro-apoptotic and anti-apoptotic stimuli, including cytokines, death receptors, caspases, mitochondria bcl-2 proto-oncogenes and certain tumour-suppressor genes.

Understanding apoptosis is important because it can potentially be controlled or manipulated to gain therapeutic advantage in certain disease states. A delicate balance between apoptosis and survival of activated inflammatory cells is a fundamental mechanism for the homeostasis of the immune system.

1.6.1 Pro-Apoptotic Factors

Apoptosis is the endpoint of an energy-dependent cascade of molecular events, initiated by certain stimuli and consisting of 4 separable but overlapping events, namely:

- Initiation of apoptosis via signalling pathways
- Control and integration
- Execution
- Removal of dead cells

Apoptotic stimuli generate signals that are either transmitted across the cell membrane to intra-cellular regulatory molecules or directly affect targets within the cell. Death receptors are situated on the surface of certain cells and act as sensors to extra-cellular apoptotic signals. These include Fas (CD95) and TNFreceptorI and TNF-RII, and their corresponding ligands Fas ligand (FasL) and TNFα.

TNFα is an established pro-apoptotic cytokine, released during the inflammatory process. TNFα stimulation does not necessarily lead to apoptosis, but also to activation and proliferation, depending on the cell type, the receptor and other regulators. Fas ligand is released by activated T cells, neutrophils and monocytes. Following activation, the signal for apoptosis is mediated by cleavage of caspases.

In tuberculosis the first step in host defence is the ingestion and uptake of *M. tb* organisms by alveolar macrophages and other cells. Apoptosis is an important host defence mechanism to contain mycobacterial survival and growth[233]. By inducing apoptosis of macrophages containing organisms, the organism is deprived of its intracellular sanctuary and the process of apoptosis therefore favours the host[233,234]. It is possible that dissemination in tuberculosis depends at least in part on a paucity of apoptosis. Insufficient T cell apoptosis may also interfere with clonal deletion and maintenance of tolerance, resulting in inappropriate T cell accumulation, contributing to chronic inflammation. This may lead to maintenance of the granulomas and to consequent fibrosis[235].

Several factors control apoptosis. Dysregulation of the TNF-receptor and TNF-ligand superfamilies are involved in T-cell apoptosis, which occur in a number of fibrotic lung diseases[235]. The ratio of Th1/Th2 cells is also an important factor in regulating apoptosis through cytokine release. Th1 cytokines inhibit apoptosis, while Th2 cytokines enhance apoptosis.

1.7 Hypothesis

Cytokine profiles determine clinical and histopathological phenotypes of disease. This thesis tests the hypothesis that this will be reflected by cytokine expression profiles in granulomas in different forms of tuberculous and in sarcoidosis.

Sarcoidosis has been described as the prototype of a Th1 disease and one of the organisms considered as the aetiology in sarcoidosis is *M.tb* and is, apart from the overwhelming incidence of tuberculosis in the Western Cape, one of the relatively common granulomatous lung diseases diagnosed in the Western Cape. On the other hand, in pulmonary tuberculosis, it is suggested that defective Th1 reactions result in immunopathology and caseous necrosis. Furthermore, pleural tuberculosis is often self-healing, and may offer insights into more appropriate antimycobacterial immune responses.

An *in situ* assessment of the cytokine profile of each of these diseases as well as a comparison between the various profiles will add to the understanding of the pathogenesis of these diseases.

2. Aims

- To describe the Th1 and Th2 cytokine profile in patients with:
    - pleural tuberculosis,
    - sarcoidosis and
    - pulmonary tuberculosis

  by *in situ* hybridisation (ISH) and/or immunohistochemical techniques.

  The roles of the following cytokines will be investigated:

  IL-12

  IFNγ

  IL-4

  TNFα

- To compare the cytokine profile of necrotic and non-necrotic granulomas in each of the diseases

- To compare the cytokine profile in patients with tuberculosis and patients with sarcoidosis

3. Materials and Methods

In situ hybridisation is a valuable tool in investigating tissue sections as the preservation of tissue morphology permits patterns of specific nucleic acid sequences to be localised in their cellular environment. Immunohistochemistry, on the other hand, is a technique that applies immunological principles to tissue to identify cellular or tissue antigens through antigen-antibody interactions. The site of antibody binding is identified either by direct labelling of the antibody, or by use of a secondary labelling method. Excellent descriptions of the principles of both these techniques, as well as the advantages, disadvantages, pitfalls and false positive or negative results exist in the literature[236,237,238].

3.1 Pleural Tuberculosis

To investigate the cytokine profile of patients with pleural tuberculosis, two groups of patients were examined. The one group consisted of 6 patients presenting with tuberculous pleural effusion and sero-negative for human immunodeficiency virus. The second group consisted of 6 patients presenting with tuberculous pleural effusions and sero-positive for human immunodeficiency virus. The clinical information available on these patients are summarised in Table 4.

3.1.1 Tissue Specimens

Pleural biopsy tissue was obtained from 12 patients presenting with pleural effusions at Tygerberg Hospital. The biopsies were performed for diagnostic purposes. After the diagnosis of tuberculosis was confirmed, the patients were put on standard, fixed-dose

anti-tuberculous treatment and referred back to their respective clinics. No follow-up data on these patients were available.

Table 4: Clinical information on patients with pleural tuberculosis

Patient	Age /sex	previous TB	Current R_X	Clinical presentation	HIV serology
1	39 / F	No	None	(L) pleural effusion	positive
2	21 / F	No	None	(R) pleural effusion	positive
3	31 / M	No	None	(R) pleural effusion	positive
4	39 / F	No	None	(R) pleural effusion	positive
5	43 / F	No	None	(L) pleural effusion	positive
6	34 / M	No	None	(L) pleural effusion	positive
7	71 / M	No	None	(L) pleural effusion	negative
8	38 / M	No	None	Bilateral pleural effusions	negative
9	41 / F	No	None	(L) pleural effusion	negative
10	40 / M	No	None	(L) pleural effusion	negative
11	42 / M	No	None	(R) pleural effusion	negative
12	23 / M	No	None	Bilateral pleural effusions	negative

Age in years F = female, M = Male R_X = treatment (L) = left, (R) = right

There were seven male and five female patients. Tissue biopsies were routinely processed in the department of anatomical pathology and the diagnosis of tuberculosis was made, based on the presence of granulomatous inflammation and Ziehl-Neelsen positive bacilli[239]. All patients were subsequently culture positive for drug-sensitive *M. tuberculosis*. HIV serology was performed on all 12 patients by ELISA.

3.1.2 Preparation of Riboprobes

Peripheral mononuclear blood cells (PBMCs) were isolated from 10 ml of blood collected from a healthy volunteer. These cells were stimulated with phytohemagglutinin for 18 h prior to RNA extraction. The Tri-Reagent (Sigma Aldrich) was used to extract total RNA from the cells and quantified by measurement of absorbance at 260 nm. It was shown that the RNA was undegraded following electrophoresis of an aliquot in a 1% agarose gel containing 8% formaldehyde and

visualization of the RNA by ethidium bromide staining. The Titan (one-tube RT-PCR) system (Boehringer Mannheim, Mannheim, Germany) was used to prepare cDNA and PCR products for IL-4, TNFα, IFNγ, and β-actin from 2 mg of RNA, using PCR conditions and primer sequences as published elsewhere[240,241].

The vector pGEM7Zf (Promega U.K.), was digested with *Sma*I (Boehringer Mannheim) and the PCR products were blunted and cloned into this vector. Sequencing the clones confirmed the DNA sequence (Table 5) and ascertained the orientation of the PCR product in order to synthesize sense and antisense riboprobes.

Table 5: Sequences of cytokine riboprobes used in ISH

Riboprobe	Length (bp)	Sequence (5′–3′)
TNFα	124	TCTCGAACCCCGAGTGACAAGCCTGTAGCCCATGTTGTAGCAAACCCTCAAGCT GAGGGGCAGCTCCAGTGGCTGAACCGCCGGGCCAATGCCCTCCTGGCCAAT GGTGTGGAGCTGAGAGATA
IFNγ	356	AGTTATATCTTGGCTTTTGAGCTCTGCATCGTTTTGGGTTCTCTTGGCTGTTACT GCCAGGACCCATATGTACAAGAAGCAGAAAACCTTAAGAAATATTTTAATG CAGGTCATTCAGATGTAGCGGATAATGGAACTCTTTTCTTAGGCATTTTGAA GAATTGGAAAGAGGAGAGTGACAGAAAAATAATGCAGAGCCAAATTGTCT CCTTTTACTTCAAACTTTTTAAAAACTTTAAAGATGACCAGAGCATCCAAAA GAGTGTGGAGACCATCAAGGAAGACATGAATGTCAAGTTTTTCAATAGCAA CAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAATTATTCGGT
IL-4	317	CTTCCCCCTCTGTTCTTCCTGCTAGCATGTGCCGGCAACTTTGTCCACGGACACA AGTGCGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAG AGCAGAAGACTTGTGCACCGAGTTGACCGTAACAGACATCTTTGCTGCCTC CAAGAACACAACTGAGAAGGAAACCTTCTGCAGGGCTGCGACTGTGCTCCG GCAGTTCTACAGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGC ACAGCAGTTCCACAGGCACAAGCAGCTGATCCGATTCCTGAAACGGCTCGA CAGGAA
IL-12	301	CCAACAACTTGCAGCTGAAGCCATTAAAGAATTCTCGGCAGGTGGGAGTACCC TGACACCTGGAGTACTCCACATTCCTACTTCTCCCTGACAGGTCCAGGGCAA GAGCAAGAGAGAAAAGAAAGATAGAGTCTTCACCGACCACGGTCATCTGC CGCAAAAATGCCAGCATTAGCGTGCGGGCCCAGGACCTCATCTTGGAGCGA ATGGGCATCTGTGCCCTGCAGTTAGGTTCTGATCTTTGGAGGAAAAGTGGA AGATATTAAGCAAAATGTTTAAAAGACACAACGGAATAGACCCA

The fragments were separated by electrophoresis on 1% low-melting-point temperature agarose gels and isolated from the gel by using the Boehringer Mannheim DNA extraction protocol, after digestion with *Pvu*I. T7 and SP6 RNA polymerases were used to transcribe antisense and sense digoxigenin-labeled RNA as instructed by the

manufacturer (Boehringer Mannheim). In addition to preparing cDNA and PCR product for TNFα, IFNγ and IL-4, it was also prepared for IL-12p40. The IL-12 PCR product was then cloned into the vector pGEMTeasy (Promega, Southampton, UK) and sequenced. A 600-base pair fragment containing the IL-12 cDNA was obtained. Labelling of the probes was subsequently confirmed by Northern blot analysis, by first incubating filters with antidigoxigenin alkaline phosphatase-conjugated F(ab9)2 fragments (Boehringer Mannheim), washing, and then incubating with BCIP-NBT-INT (5-bromo-4-chloro-3-indolylphosphate –nitroblue tetrazolium–iodonitrotetrazolium violet; Dako, Glostrup, Denmark)[242] .

3.1.3 RNA-RNA *In Situ* Hybridisation

Paraffin-embedded lung tissue was cut into 5-mm sections using a microtome. As previously described[1,243], consecutive sections were applied to RNase-free slides that were previously coated with aminopropyltriethoxysilane (5 mg/ml; Sigma Aldrich). Deparaffinisation in xylene, rehydration through graded ethanols and diethyl pyrocarbonate-treated water, and finally incubation in phosphate-buffered saline (PBS) of the sections followed. To facilitate antigen retrieval, the sections were treated with 1 mg of proteinase K per ml in 10 mM Tris-HCl (pH 7.5)–5 mM EDTA for 45 min at 37°C. Sections were washed with PBS, refixed in 0.4% paraformaldehyde and acetylated in a 400:1 (vol/vol) solution of triethanolamine-acetic anhydride for 10 min. Subsequently the slides were then rinsed in PBS, dehydrated in graded ethanols, and air dried.

Hybridisation followed after drying of the slides. The first step was to incubate sections for 30 min at 50°C in a prehybridisation mixture containing 25% dextran sulfate, 25 mM Tris-HCl (pH 8.0), 2.53 Denhardt's solution, 2.5 mM EDTA, 25 mM dithiothreitol, 1.25 mg of herring sperm DNA per ml, 0.06 mg of tRNA per ml, and 50% deionized formamide. Digoxigenin-labeled riboprobes (5 ng/ml) were added to a hybridisation mixture made up of 20% dextran sulfate, 12.5 mM Tris-HCl (pH 8.0), 2.53 Denhardt's solution, 2.5 mM EDTA, 12.5 mM dithiothreitol, 0.01 mg of herring sperm DNA per ml, 0.002 mg of tRNA per ml, and 50% deionized formamide. This mixture was added to the sections and the sections were hybridised for 18 hours at 50°C in a humidified chamber. Afterwards the slides were twice washed at room temperature in 23 SSC (saline sodium citrate; 13 SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min at a time and then twice at 43°C with 0.13 SSC for 15 min at a time. The slides were then incubated for 5 min in 100 mM Tris-HCl (pH 7.5)–150 mM NaCl (buffer A). After incubation it was placed in buffer A containing 2% normal sheep serum, and washed in buffer A plus 0.05% Tween 20.

The final steps were incubation of the sections with antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) for 30 min at room temperature, and detection of the signal by BCIP-NBT-INT (Dako). A psotive reaction was demonstrated by the appearance of a brown colour (up to 60 min), and the slides were then counterstained with Mayer's hematoxylin (Sigma Aldrich) for 15 s, rinsed in distilled water, and mounted with Dako Faramount. After proper drying of the coverslips, the slides were viewed under a light microscope.

3.1.4 Assessment of Slides

A map of each section was created by photographing the slides at x25 magnification and printing out a copy of the photograph (fig. 8). Each granuloma was numbered.

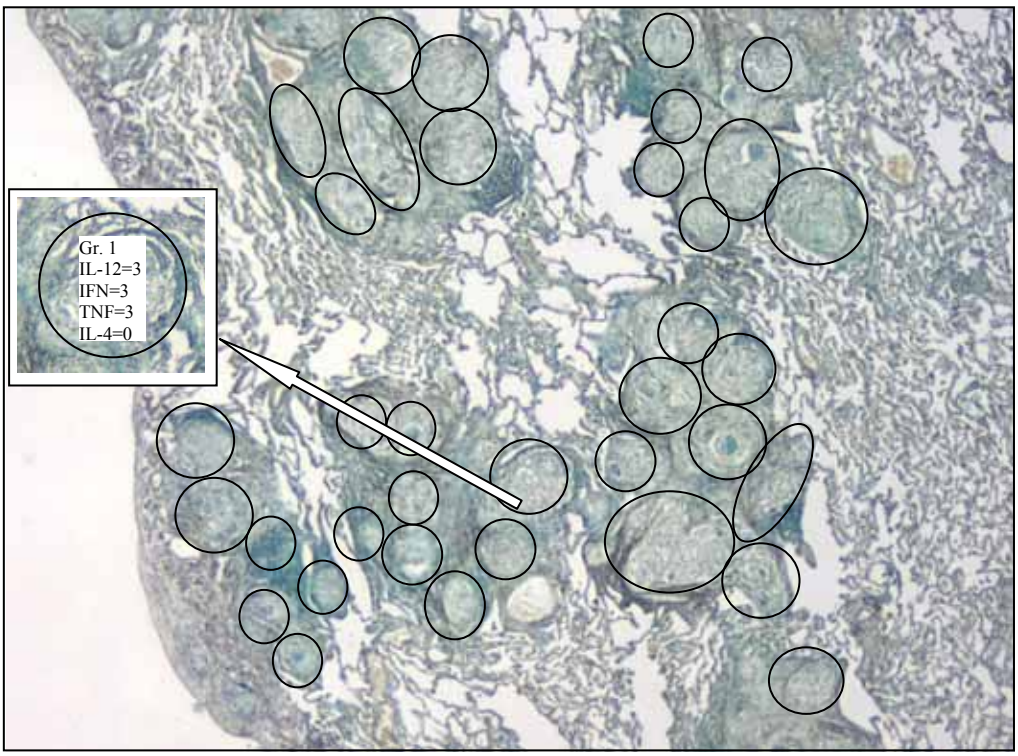


Figure 8: An example of the granuloma map for one of the cases. Each granuloma is circled, numbered and scored according to the number of positive cells present.

The slides were then examined under the light microscope and the cytokine profile of each numbered granuloma was recorded (at x400 magnification) on the photographic map as follows:

- >75% of cells in a granuloma positive: documented as a score of 3
- <75% but >25% of cells in a granuloma positive: documented as a score of 2
- <25% of cells in a granuloma positive: documented as a score of 1
- no cells positive: documented as a score of 0

3.1.5 Statistical Analysis

All granulomas were regarded as independent parameters.

Statistical analysis was performed to investigate the following, applying Pearson Chi square and Spearman rank R tests:

- The association between HIV serology and presence of necrosis.
- The association between HIV and individual cytokines
- Comparisons in association patterns between various cytokines were compared between HIV negative and HIV positive granulomas.
- The association between necrosis and individual cytokines.
- Comparisons in association patterns between various cytokines were compared between necrotic and non-necrotic granulomas.
- Comparisons in association patterns between various cytokines, HIV serology and necrosis were compared between the various cytokines at a given percentage.

3.2 Sarcoidosis

To investigate the cytokine profile of patients with sarcoidosis, open lung biopsies from a group of 15 patients with sarcoidosis were examined. The clinical information available on these patients are summarised in Table 6. The diagnosis of sarcoidosis was made in consultation with the pulmonologists and radiologists, based on the clinical, radiological and histological appearance of the disease and other possible causes of granulomatous lung disease had been excluded.

Table 6: Clinical information and radiological staging of patients

Patient	Age/Sex	Previous TB	Rx at diagnosis	Radiological staging	Follow up
S1	35/F	No	None	2	2 years, healthy
S2	21/F	No	None	3	2 years, healthy
S3	27/F	No	None	2	6 years, healthy
S4	36/F	No	None	2	3 years, respiratory failure
S5	35/F	No	None	2	12 years, healthy
S6	27/F	No	None	3	1,5yrs
S7	26/F	No	None	2	6 years, Grade I dyspnoea
S8	54/M	No	None	2	2 years, died
S9	36/F	No	None	3	5 years, grade II dyspnoea
S10	29/F	No	None	2	1 year, healthy
S11	37/F	No	None	2	1,5 years, died
S12	25/M	No	None	2	5 years
S13	36/F	No	None	2	1 year, healthy
S14	23/F	No	None	2	2yrs
S15	45/F	No	None	3	2 years, healthy

Age in years F = female, M = Male R_X = treatment

3.2.1 Tissue Specimens

Lung tissue for histological examination was obtained from 15 patients at Tygerberg Hospital, with the clinico-radiological appearance of pulmonary sarcoidosis. The

biopsies were performed for diagnostic purposes. Tissue biopsies were routinely processed in the department of anatomical pathology and after clinico-pathological consultation and exclusion of other possible causes of pulmonary granulomatous disease, the diagnosis of sarcoidosis was confirmed and the patients were put on treatment. Follow-up on these patients was available (table 6). There were two male and thirteen female patients.

3.2.2 Histological Grading

The histological grading as discussed in the introduction was applied to these 15 cases.

3.2.3 Preparation of the Riboprobes, RNA-RNA *In Situ* Hybridisation and Assessment of Slides

The methodology used for the preparation of the riboprobes, performance of *in situ* hybridisation and assessment of slides was identical to that described for the pleural tuberculosis case above, except for the following additions and changes[1,2,238].

TNFα, IFNγ, IL-4 and IL-12 PCR product was cloned into the vector pGEMTeasy (Promega, Southampton, UK) and sequenced. To synthesize sense and antisense biotinylated, labelled riboprobes, T7 or SP6 RNA polymerases were then added according to the manufacturers' instructions (Gibco BRL, Basel, Switzerland).

Northern blot analysis using streptavidin-conjugated alkaline-phosphatase (Gibco BRL) and the substrate nitroblue tetrazolium chloride/5-bromo-4-chloro-3 indolyl- phosphate p-toluidine salt (NBT/BCIP) (Gibco BRL) confirmed labelling of the probes.

3.2.4 RNA: RNA *in situ* hybridisation

Proteinase K treatment, prehybridisation and hybridisation protocols were as previously described. After hybridisation, the slides were washed in 2x saline sodium citrate for 15 min at room temperature and repeated once, after which the sections were blocked with 100 µl blocking reagent (Gibco BRL) for 15 min at room temperature, followed by 100 µl streptavidin-conjugated alkaline phosphatase diluted 1:10 in conjugate buffer for 30 min also at room temperature. Slides were then washed twice in 100 mM Tris-HCl (pH 7-5), 150 mM NaCI for 15 min at room temperature and the signal was then detected using NBT/BCIP. A blue/purple colour appeared when positive and the sections were counterstained with methyl green (Dako) for 10 seconds, rinsed in distilled water and mounted with Dako faramount. After proper drying of the coverslips, the slides were viewed under a light microscope.

β-Actin was used as a positive control for the presence of mRNA within the tissue sections. β-Actin mRNA is ubiquitous in cells and therefore both a perfect positive control for the technique (but not for the specific probe) and also an indication of the immunoreactivity of the tissue. Pretreatment of the sections by ribonuclease (RNase) before hybridisations further confirmed the specificity of the hybridisation signal.

3.2.5 Assessment of the slides

The same principal described in the section on pleural tuberculosis was applied in sarcoidosis.

3.2.6 Statistical Analysis

Due to the uniform nature of the sarcoidosis results as can be seen in Chapter 5, there was no requirement to perform any statistical analysis on the results.

3.3 Pulmonary Tuberculosis

To investigate the cytokine profile of patients with pulmonary tuberculosis, two groups of patients were examined. Two different patient groups had to be tested, as there was an insufficient amount of tissue available to perform the complete set of investigations on one group alone. Additional ISH, as well as immunohistochemistry had to be performed on sections from the new group of patients with a similar clinical presentation to the first group. The 2 groups will be discussed separately and are labelled "first group of patients" and "second group of patients".

3.3.1 First Group of Patients

3.3.1.1 Tissue Specimens

Adult lung tissue obtained from five patients undergoing surgery for haemoptysis at Tygerberg Hospital was examined. There were four males and one female with TB and all had upper lobe cavitation. All five had received treatment for between 2 and 3 months prior to surgery (Table 7). Diagnosis was confirmed by Ziehl-Neelsen staining, all patients had been culture positive for drug-sensitive *M. tuberculosis* and sero-negative for HIV by ELISA.

Table 7:Clinical details of first group of patients with pulmonary tuberculosis

Patient	Age / sex	Time since previous TB (years)	Preoperative treatment duration (months)	Chest radiograph
A1	39/M	3	2	RUL cavity
A2	35/M	15	3	RUL cavity
A3	26/F	3.4	2	LUL cavity
A4	23/M	3	2	LUL cavity
A5	31/M	5	3	LUL cavity

Age in years M = male, F = female RUL = Right upper lobe; LUL = Left upper lobe

The patients had recurrent disease, as they had all been successfully treated for tuberculosis between 3 and 15 years previously (Table 7). Directly after surgery, tissue was selectively dissected for formaldehyde fixation. All patients successfully completed their anti-TB therapy, but were lost for follow-up thereafter.

3.3.1.2 Preparation of the Riboprobes, RNA-RNA *In Situ* Hybridisation and Assessment of Slides

Preparation of the probes and ISH were performed according to the methods described earlier. BCIP-NBTINT (Dako) was used for detection. The slides were examined under the light microscope and the results were recorded as positive (1) or negative (0) at x400 magnification. The number of granulomas present in each biopsy was counted and the presence or absence of cytokines was noted in each of the granulomas.

3.3.1.3 Statistical Analysis

Statistical analysis was not originally performed on these cases, but as part of this thesis Pearson Chi square and Spearman rank R tests were performed.

3.3.2 Second Group of Patients

Adult lung tissue was obtained from seven patients undergoing surgery for haemoptysis at Tygerberg Hospital (Table 8). Diagnosis was confirmed by Ziehl-Neelsen staining, all patients were culture positive for drug-sensitive *M. tuberculosis*, and sero-negative for HIV by ELISA. All patients completed their anti-tuberculosis therapy and follow-up varied from none to 8 years.

Table 8: Clinical details of patients of 2nd group of patients with pulmonary tuberculosis

Patient	Age/Sex	Extent of disease	Time since previous TB	Pre-operative treatment	Follow-up
B1	23/M	RUL collapse	2 years	1 month	8 years, healthy
B2	43/M	LUL cavitation with active TB	3 years	1 month	1 years, healthy
B3	33/F	LUL large cavity, RUL and LLL infiltration	11 years	3 months	9 years, chronic asthma
B4	31/F	Mediastinal shift left, pleural effusion, scarring RUL	3 years	3 months	4 years, healthy
B5	22/M	Cavitations RUL and RML, coughing up ascaris	2.5 years	3 months	None
B6	21/F	Total destruction RUL, cystic lesions in LUL	17 years	2 months	1 month, healthy
B7	41/M	R lung collapse, fibrosis. L lung unaffected	6 years	3 months	5 years, chronic empyema

Age in years M = male, F = female RUL = Right upper lobe; LUL = Left upper lobe; LLL = Left lower lobe; RML = Right middle lobe

3.3.2.1 Immunohistochemistry

Sections were dewaxed, rehydrated through graded alcohols and washed in distilled water. Endogenous peroxidases were blocked and antigen retrieval performed by

microwaving of the sections in 0.01M citrate buffer pH 6-0 for 20 min. Care was taken not to allow the citrate buffer to evaporate and after 5 min the citrate buffer was topped up. Sections were left in the microwave for 15 min and then placed into phosphate-buffered saline (PBS) for 5 min. Five percent rabbit serum for 10 minutes was used to block non-specific proteins. Goat anti-human IFNγ, IL-4 and TNFα antibodies (R & D Systems, Oxford, UK) were diluted 1:400 in rabbit serum (final concentration 250 ng/ml) and incubated on the sections overnight at 4°C.

The following day the sections were then washed for 15 min in PBS. This was followed by incubation of the section with a secondary antibody, biotinylated rabbit anti-goat immunoglobulin G (IgG; R & D Systems), diluted 1:100 for 1 hr at 4°. The sections were again washed for 15 min in PBS and incubated with streptavidin-horse radish peroxidase (Dako, Cambridge, UK) for 30 min. The 3'3-diaminobenzedine substrate (Dako) was then added to the sections (according to the manufacturer's instructions), counterstained with haematoxylin (Dako), washed in running tap water and mounted with Paramount (Dako).

A control antibody (protein G-purified goat IgG, Santa Cruz, CA) was included, which was diluted to a final concentration of 250 ng/ml. As an additional control the primary antibody was also excluded from the overnight incubation step and these sections were only incubated with the secondary antibody. The specificity of the polyclonal antibodies was also tested. Preincubation of the primary antibody with the relevant recombinant cytokine established specificity. The antibodies (250 ng/ml) and the respective antigens (IFNγ, TNFα and IL-4 all at 50 μg/ml, all from R & D Systems)

were incubated together in for 30 min at room temperature. Antibodies were also incubated without their respective recombinant cytokine. Staining was performed as described above on sections from one IL-4-positive patient.

3.3.2.2 Preparation of the Riboprobes, RNA-RNA *In Situ* Hybridisation and Assessment of Slides

Preparation of the riboprobes, RNA-RNA *in situ* hybridisation and assessment of slides were performed as described earlier. Signal was detected using NBT/BCIP.

3.3.2.3 Assessment of slides

The sections were examined to determine to the presence or absence of the relevant colour reaction. The individual granulomas from all of the patients were scored as either positive or negative for each cytokine and caseous necrosis.

3.3.2.4 Statistical analysis

To test for association between pairs of cytokines and necrosis a two-tailed Fisher's exact test was used. Comparisons of association patterns between IL-4-positive and IL-4-negative patients were made using a Mantel Haenszel x^2 test. Comparisons between those granulomas staining alike with those staining differently for two cytokines were compared between IL-4-positive and IL-4-negative patients using Fisher's exact test.

3.4 Controls

Lung tissue specimens were obtained from 2 patients undergoing surgery for pulmonary malignancies. Tissue samples were routinely processed and ISH for mRNA of all 4 cytokines was performed on the sections. Consecutive tissue sections of these cases were used as negative controls and the antisense probe was omitted.

As negative controls, consecutive tissue sections of the cases studied were used and the antisense probe was omitted.

4. Results: Pleural Tuberculosis

Pleural tuberculosis in HIV-negative subjects has been taken to represent a protective immune response to Mycobacterium tuberculosis because a high proportion of individuals recover without antibiotic therapy [120]. A tuberculous pleural effusion in HIV-negative subjects is usually associated with a prominent cell-mediated immune response, represented by an infiltration of CD4+ T cells[121] and high levels of pro-inflammatory cytokines, including IFNγ and TNFα [122], suggesting a predominantly Th1 response.

This appears to indicate a key role for these responses in protective immunity against tuberculosis in humans, in keeping with evidence that IFNγ and TNFα are necessary for protective immunity to tuberculosis in animals[123,124,125], and that abnormalities of the IFNγ receptor are associated with susceptibility to mycobacterial disease in humans[126,127].

In HIV-positive patients, it has been hypothesized that qualitative T-helper-type responses may also impact on AIDS pathogenesis. A switch in the predominant response from a Th1 to a Th2 response and the production of associated cytokines may facilitate disease progression[147,148,149]. Numerous studies have shown that during the progression of HIV-1 disease, mononuclear cells lose their ability to secrete Th1 cytokines and instead produce increased levels of the Th2 cytokines[178].

A Th phenotype switch in HIV positive patients should also be reflected in the granulomatous response to *M.tb* and differences in cytokine gene expression at the site

of disease in HIV positive and negative patients might offer important insights into appropriate antituberculous responses.

4.1 Experimental Design

To investigate the cytokine profile of patients with pleural tuberculosis, two groups of patients presenting with pleural effusions were examined, one group of 6 patients sero-negative for HIV and the second group sero-positive for HIV. Pleural biopsy tissue was obtained, the diagnosis of tuberculosis was confirmed, and ISH was performed for IL-12-, IFNγ-, TNFα- and IL-4-mRNA on all 12 cases.

The cytokine profile of each granuloma was recorded on the photographic map as follows:

- >75% of cells in a granuloma positive: documented as a score of 3
- <75% but >25% of cells in a granuloma positive: documented as a score of 2
- <25% of cells in a granuloma positive: documented as a score of 1
- no cells positive: documented as a score of 0

Statistical analysis was performed to test for the association between:

- HIV serology and presence of necrosis.
- Necrosis and individual cytokines.
- HIV and individual cytokines.
- Comparisons in association patterns between various cytokines were compared between necrotic and non-necrotic granulomas.

- Comparisons of association patterns between various cytokines were compared between HIV negative and HIV positive granulomas.
- Comparisons of association patterns between various cytokines, HIV serology and necrosis were compared between the various cytokines at a given percentage.

4.2 Results

Throughout the results and the subsequent discussion the following are used as synonyms:
For > 75% positive cells: majority of cells, high number of cells, Grade3.
For 25-75% positive cells: a moderate number, intermediate, Grade 2
For < 25% positive cells: few cells, Grade 1
Negative: a score of 0,no positive cells
Equal distribution: no significant difference between the three groups i.e.: 75% positive cells, 25-75% positive cells, < 25% positive cells.

Pleural biopsy tissue from two groups of patients presenting with pleural effusions was examined, one group of 6 patients sero-negative for HIV and the second group sero-positive for HIV. The number of necrotic and non-necrotic granulomas was determined on H + E stain and the cytokine mRNA profile was determined by ISH for IL-12-, IFNγ-, TNFα- and IL-4-mRNA on all 12 cases.

All patients with pleural tuberculosis presented with symptoms of pleuritis and had a CXR to confirm the effusion before a pleural biopsy was performed as a diagnostic procedure (table 9). Histological and microbiological confirmation of the diagnosis of tuberculosis resulted in initiation of treatment and discharge with referral to primary health clinics where the patients were further treated and followed up. No follow-up of

the patients was available in our records. An HIV ELISA test was performed on all these patients, as part of the routine work-up of possible tuberculosis cases.

The patients' age ranged from 21 years to 71 years with 9 of the patients in their 4th or 5th decade. Five patients were female and seven were male. None had any additional medical history of any significance.

Table 9: Demographic and clinical information on patients with pleural tuberculosis.
The table shows the basic demographic and clinical characteristics of 12 patients with TB pleuritis who were recruited for the histological study of closed Abrams needle pleural biopsy samples.

Patient	Age /sex	previous TB	Current R_X	Clinical presentation	HIV serology
1	39 / F	No	None	(L) pleural effusion	positive
2	21 / F	No	None	(R) pleural effusion	positive
3	31 / M	No	None	(R) pleural effusion	positive
4	39 / F	No	None	(R) pleural effusion	positive
5	43 / F	No	None	(L) pleural effusion	positive
6	34 / M	No	None	(L) pleural effusion	positive
7	71 / M	No	None	(L) pleural effusion	negative
8	38 / M	No	None	Bilateral pleural effusions	negative
9	41 / F	No	None	(L) pleural effusion	negative
10	40 / M	No	None	(L) pleural effusion	negative
11	42 / M	No	None	(R) pleural effusion	negative
12	23 / M	No	None	Bilateral pleural effusions	negative

Age in years F = female, M = Male R_X = treatment (L) = left, (R) = right

4.2.1 Interpretation of the slides

Representative examples of the light microscopic appearance of the ISH can be seen in figs. 9-16. For complete scoring please see Appendix A. Positivity is represented by brown staining of the cytoplasm as described in the methods and indicates the presence of mRNA. Cells staining brown therefore contain mRNA for the specific cytokine tested. Counterstaining is blue, as described in the methods. Cells that stain negative do not contain the specific RNA and will therefore stain blue. These photographs were selected to illustrate the quality of staining for the various cytokine mRNAs. No

difference was seen between the staining quality of sections in HIV negative versus HIV positive patients or necrotic versus non-necrotic granulomas.

The focus of the study was on the number of cells that are positive for a specific cytokine within the granuloma and the sources of the cytokines were not determined. This does not however, detract from the fact that the cytokine mRNA was present. Documented sources of these cytokines were discussed in chapter 1.

Thick irregular dark lines on the photographs represent folds in tissue sections, which often occur during sectioning.

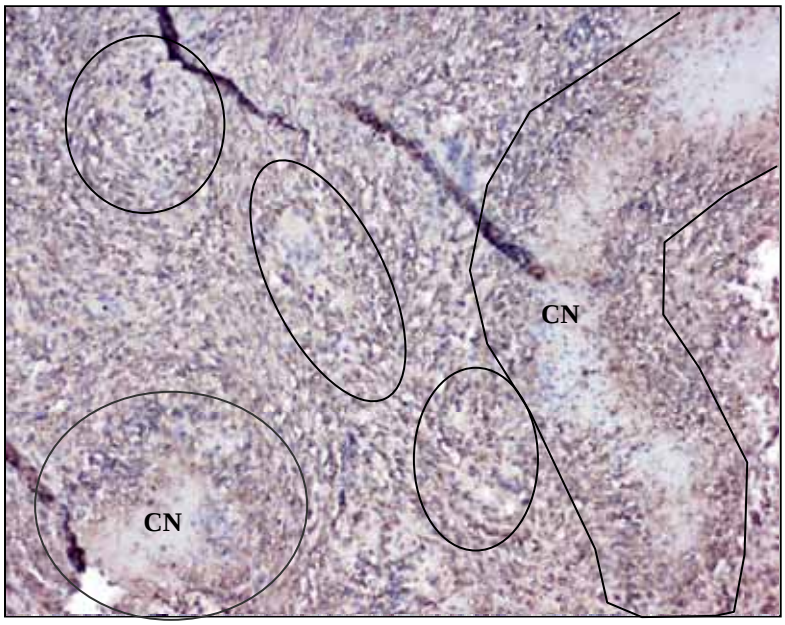


Figure 9: Pleural tuberculosis: anti-sense for β-actin diffusely positive (x100 magnification).

A representative section of a pleural needle biopsy from an HIV- patient is shown. ISH with the anti-sense β-actin probe was performed after appropriate preparation of paraffin-embedded tissue. The β-actin probes were used to determine the presence of non-degraded mRNA in sections. The brown staining represents positive signal and intact mRNA. Granulomas are outlined in black. 100 times magnification was used for this photograph. Caseous necrosis = CN.

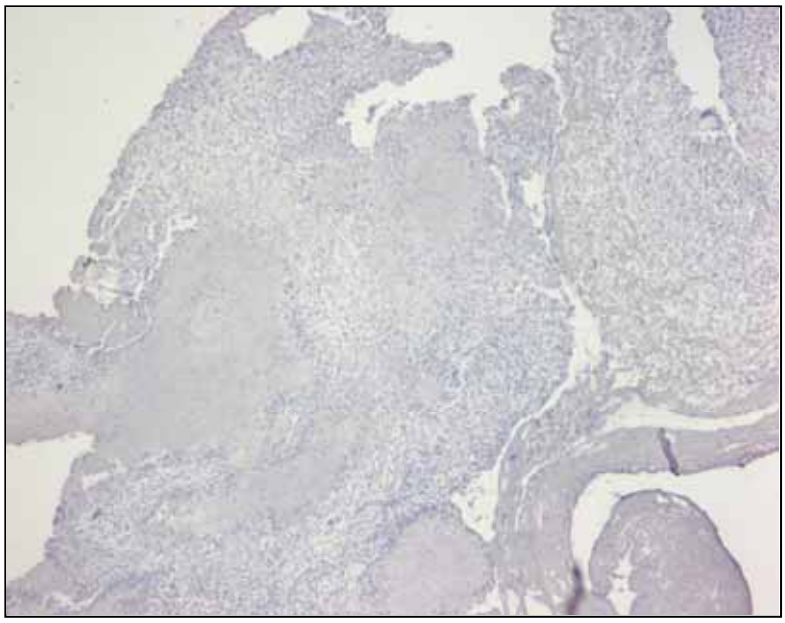


Figure 10: Pleural tuberculosis: sense probes and negative controls show no staining (x40 magnification).

A representative section of a pleural needle biopsy from an HIV- patient is shown. ISH with the sense probes for β-actin, IL-12, IFN and IL-4 was performed after appropriate preparation of paraffin-embedded tissue. No brown staining represents positive signal was observed.

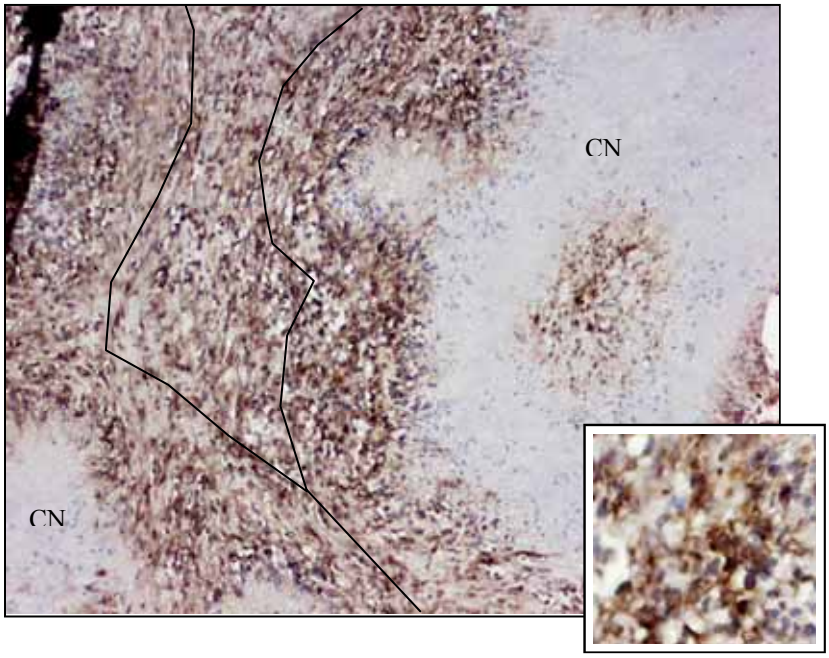


Figure 11: Pleural tuberculosis: anti-sense for IL-12, > 75% cells positive (x100 magnification).

A representative section of a pleural needle biopsy from an HIV- patient is shown. ISH with the anti-sense IL-12 probe was performed after appropriate preparation of paraffin-embedded tissue. The brown staining represents positive signal and intact mRNA. Granulomas are outlined in black. A 40 times magnification was used and a 400 times magnification for the insert, which shows that the signal is concentrated in lymphocytes. Caseous necrosis = CN.

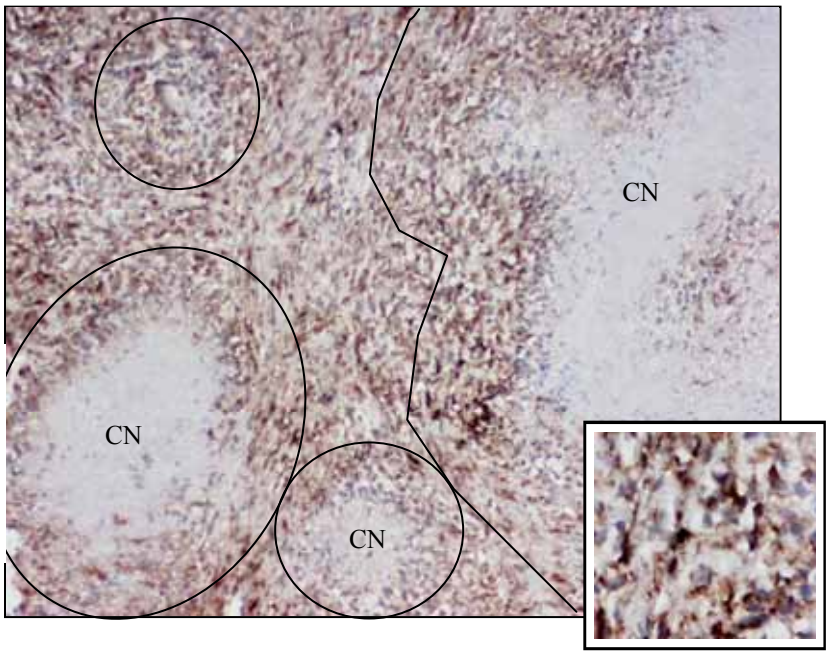


Figure 12: Pleural tuberculosis: anti-sense probe for IFNγ, > 75% cells positive positive

A representative section of a pleural needle biopsy from an HIV- patient is shown. ISH with the anti-sense IFNγ probe was performed after appropriate preparation of paraffin-embedded tissue. The brown staining represents positive signal. Granulomas are outlined in black. A 40 times magnification was used and a 400 times magnification for the insert, which shows that the signal is concentrated in lymphocytes. Caseous necrosis = CN.

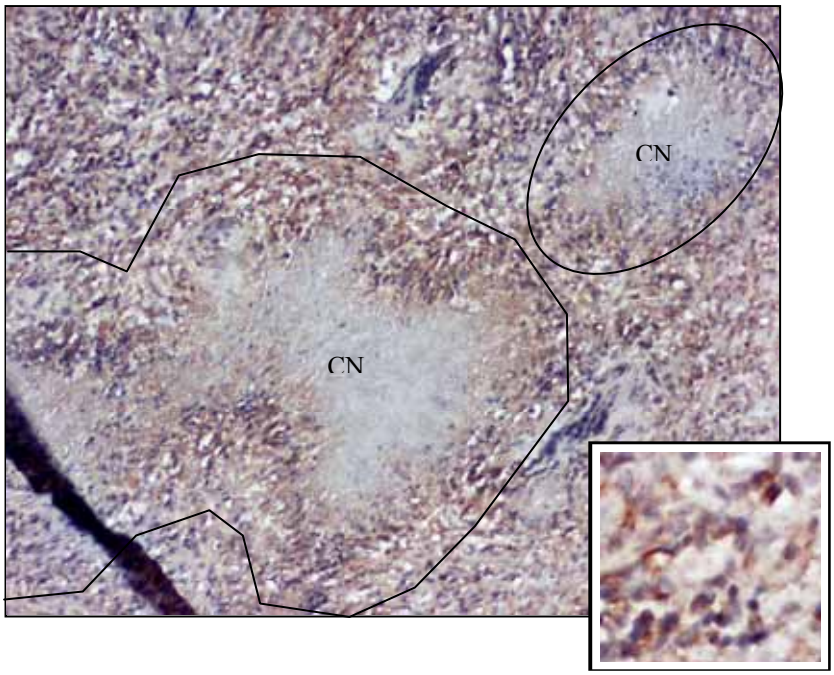


Figure 13: Pleural tuberculosis: anti-sense probe for TNFα, > 75% cells positive positive

A representative section of a pleural needle biopsy from an HIV- patient is shown. ISH with the anti-sense TNFα probe was performed after appropriate preparation of paraffin-embedded tissue. The brown staining represents positive signal. Granulomas are outlined in black. A 40 times magnification was used and a 400 times magnification for the insert, which shows that the signal is concentrated in lymphocytes. Caseous necrosis = CN.

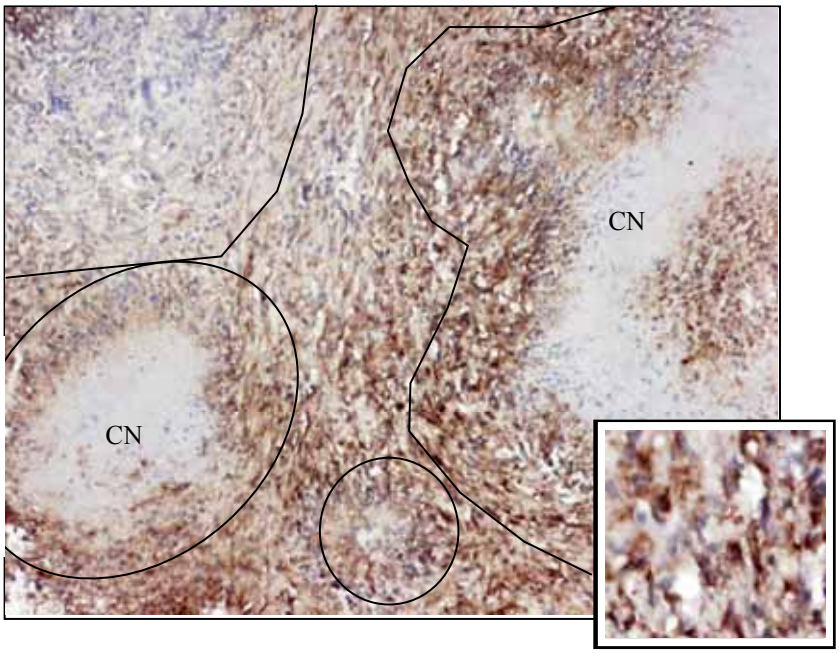


Figure 14: Pleural tuberculosis: anti-sense probe for IL-4, > 75% cells positive positive

A representative section of a pleural needle biopsy from an HIV- patient is shown. ISH with the anti-sense IL-4 probe was performed after appropriate preparation of paraffin-embedded tissue. The brown staining represents positive signal. Granulomas are outlined in black. A 40 times magnification was used and a 400 times magnification for the insert, which shows that the signal is concentrated in lymphocytes. Caseous necrosis = CN.

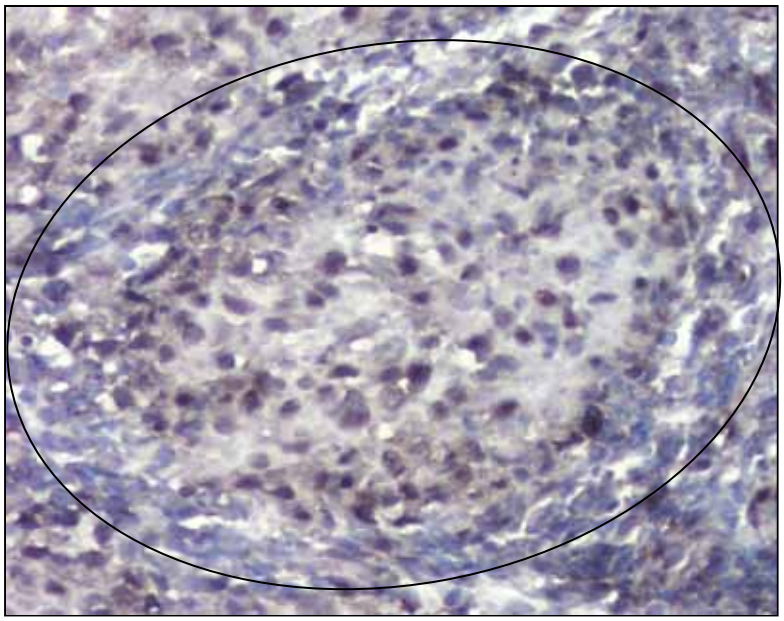


Figure 15: Pleural tuberculosis: anti-sense probe for TNFα, 25-75% cells positive

A representative section of a pleural needle biopsy from an HIV+ patient is shown. ISH with the anti-sense TNFα probe was performed after appropriate preparation of paraffin-embedded tissue. Granuloma outlined in black. No necrosis present. Although there is prominent positivity, it is clear that there is between 25-75% of the cells that are positive. The brown staining represents positive signal (200x magnification). Most negativity is seen in the lymphocyte cuff.

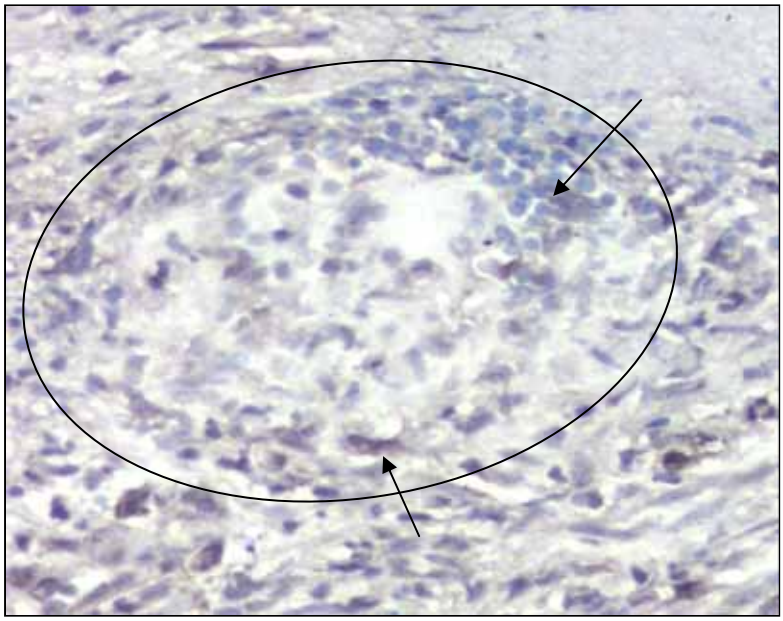


Figure 16: Pleural tuberculosis: anti-sense probe for IL-12, <25% cells positive

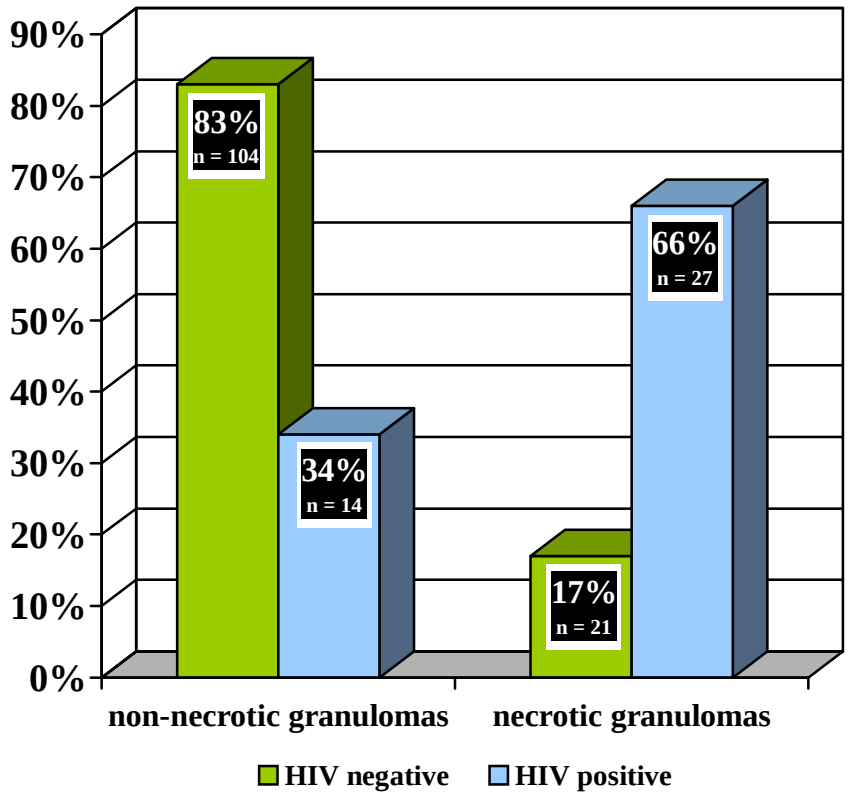
A representative section of a pleural needle biopsy from an HIV+ patient is shown. ISH with the anti-sense IL-12 probe was performed after appropriate preparation of paraffin-embedded tissue. Granuloma outlined in black. No necrosis present. The brown staining represents positive signal (200x magnification) identified by arrows. Very few cells are positive, clearly indicating <25% positivity.

4.2.2 HIV and Necrosis

Pleural biopsy tissue from two groups of patients presenting with pleural effusions was examined, one group of 6 patients sero-negative for HIV and the second group sero-positive for HIV. The number of necrotic and non-necrotic granulomas was determined on H and E stain and the cytokine mRNA profile was determined by ISH for IL-12-, IFNγ-, TNFα- and IL-4-mRNA on all 12 cases. A total of 166 granulomas was counted in these patients. In the HIV negative patients a total of 125 granulomas was counted, of which 104 were not necrotic and 21 had signs of caseous necrosis. In the HIV positive group 41 granulomas were counted, of which 34% (n = 14) were not necrotic and 66% (n = 27) showed signs of caseous necrosis (fig. 17).

Figure 17:A comparison between HIV status and necrosis in 166 granulomas.

Pleural biopsies from 6 HIV negative and 6 HIV positive patients were examined. There were 166 granulomas in total and the HIV negative patients had a total number of 125 granulomas (represented in green) of which 104 were non-necrotic and 21 necrotic. The HIV positive patients (represented in blue) had 41 granulomas of which 14 were non-necrotic and 27 necrotic (Chi-square test p = <0.01).



On the other hand in the HIV negative group 83% of granulomas were non-necrotic and 17% showed signs of necrosis. There was a clear association between HIV positivity and necrotic granulomas as well as HIV negativity and non-necrotic granulomas (Chi-square test: $p < 0.01$).

4.2.3 Necrosis and Individual Cytokines

In an analysis of the relation between the individual cytokines and necrosis in the granulomas, the tendency was for the necrotic granulomas to have more positive cells in comparison to the non-necrotic granulomas (figs. 18-21). This was applicable for cytokines, except IL-12, where there was a more equal distribution of the percentage of positive cells between necrotic and non-necrotic granulomas (fig. 18 and table 10).

Forty percent of necrotic granulomas have >75% IFNγ-positive cells (fig. 19), which is more than IL-4, but less than TNFα. With TNFα especially (fig. 20), a dramatic increase in the number of granulomas with a Grade 3 (>75%) distribution could be seen in necrotic granulomas. The percentage of IL-4 positive cells are illustrated in fig. 21. Fifty three percent of non-necrotic granulomas had either no IL-4 positive cells (3%) or less than 25% positive cells (50%). On the other hand in necrotic granulomas 77% were either Grade 2 (42%) or Grade 3 (35%).

Table 10:The % of granulomas positive for specific cytokines in the necrotic and non-necrotic groups of granulomas.
This table represents a summary of the data presented in figs. 16-19. An increase in the percentage of positive cells is noted in necrotic granulomas, especially TNFα, where 64% of granulomas have > 75% positive cells.

	Non-necrotic granulomas				Necrotic granulomas				Chi-square
	0	1	2	3	0	1	2	3	test
IL-12	0	31%	44%	25%	0	23%	54%	23%	p = 0.50
IFNγ	0	48%	35%	17%	0	35%	25%	40%	p = 0.01
TNFα	0	35%	42%	24%	0	17%	19%	64%	p < 0.01
IL-4	3%	50%	26%	21%	6%	17%	42%	35%	P < 0.01

Figure 18:Distribution of IL-12 positive cells in necrotic and non-necrotic granulomas
The presence of IL-12 positive cells was assessed by ISH in the same 166 granulomas as presented in figure 17. Positivity was graded according to the total number of cells staining positive in a specific granuloma (<25%, 25-75% and >75%). The percentage of necrotic and non-necrotic granulomas in each of these categories was calculated and is represented in this graph. The presence of necrosis did not significantly influence the percentage of IL-12 positive cells (Chi-square test p = 0.47).

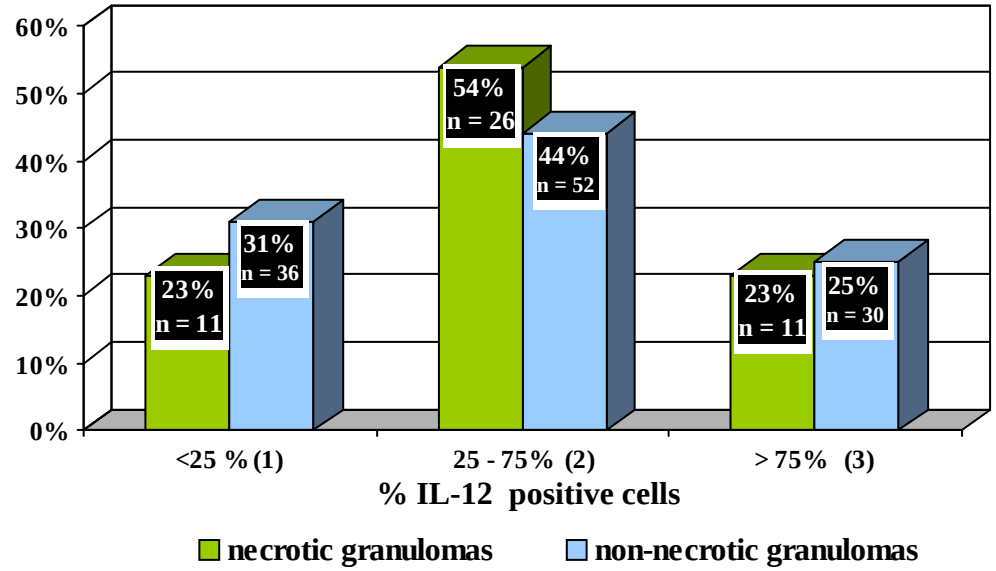


Figure 19: Distribution of IFNγ positive cells in necrotic and non-necrotic granulomas
The presence of IFNγ positive cells was assessed by ISH in the same 166 granulomas as presented in figure 17. Positivity was graded according to the total number of cells staining positive in a specific granuloma (<25%, 25-75% and >75%). The percentage of necrotic and non-necrotic granulomas in each of these categories was calculated and is represented in this graph. More necrotic granulomas contain > 75% positive of positive cells than non-necrotic granulomas (Chi-square test p = 0.01).

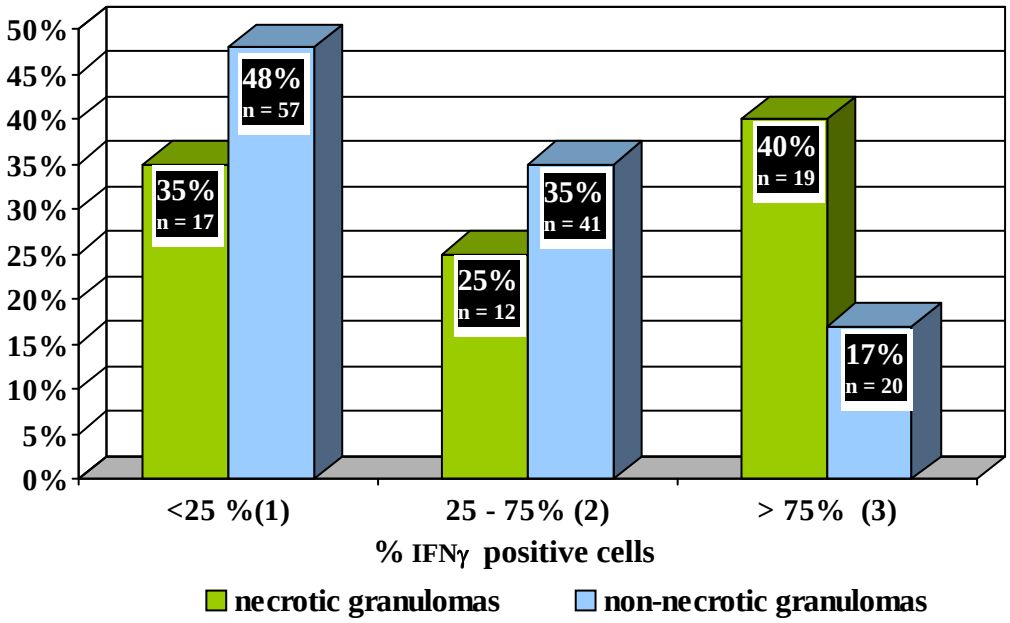


Figure 20: Necrotic granulomas are associated with increased TNFα positivity.
The presence of TNFα positive cells was assessed by ISH in the same 166 granulomas as presented in figure 17.Positivity was graded according to the total number of cells staining positive in a specific granuloma (<25%, 25-75% and >75%). The percentage of necrotic and non-necrotic granulomas in each of these categories was calculated and is represented in this graph. Necrotic granulomas had a significantly higher percentage of granulomas where > 75% of cells expressed TNFα (Chi-square test $p < 0.01$).

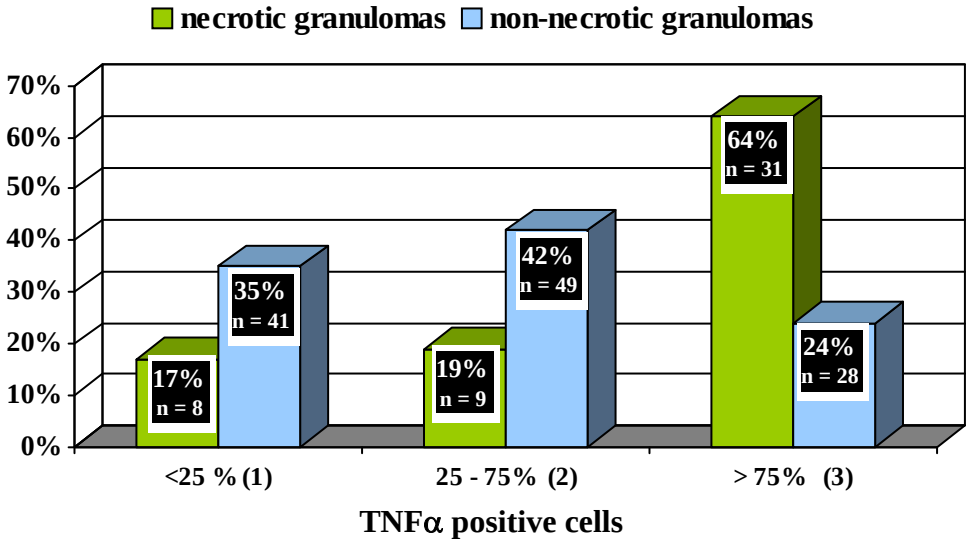
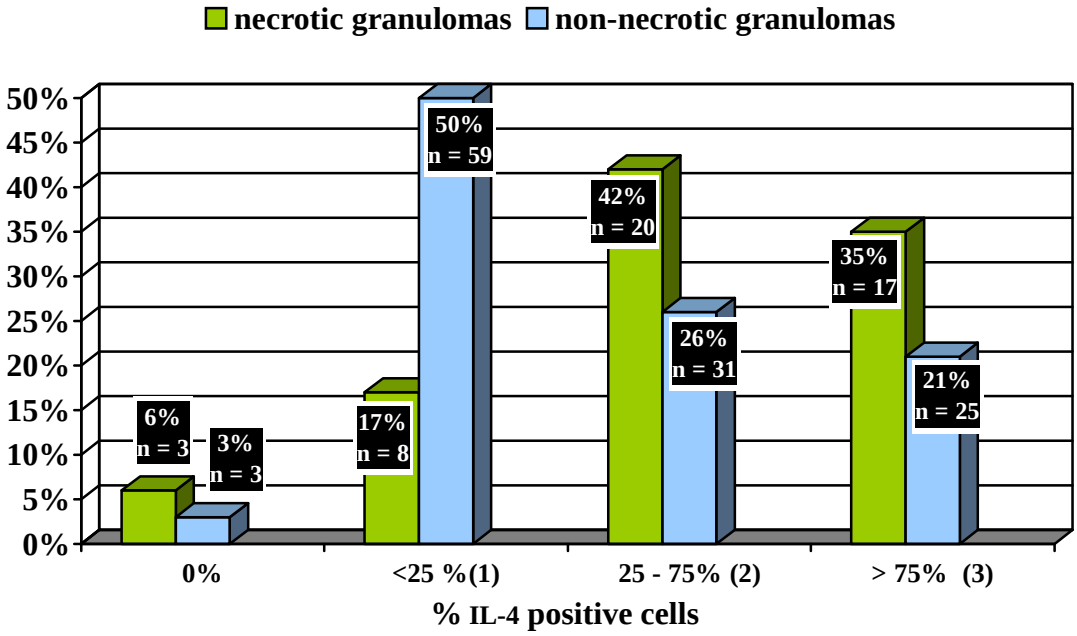


Figure 21: Distribution of IL-4 positive cells in necrotic and non-necrotic granulomas.
The presence of IL-4 positive cells was assessed by ISH in the same 166 granulomas as presented in figure 17.Positivity was graded according to the total number of cells staining positive in a specific granuloma (<25%, 25-75% and >75%). The percentage of necrotic and non-necrotic granulomas in each of these categories was calculated and is represented in this graph. Necrotic granulomas had a significantly higher percentage of granulomas where > 75% of cells were positive for IL-4 mRNA (Chi-square test $p < 0.01$).



4.2.4 HIV and Individual Cytokines

It is clear from these results presented in fig. 22 that there was also a strong association between > 75% TNFα positive cells and HIV positivity, while there was a more equal distribution of the percentage of TNFα positive cells in HIV negative granulomas (Fig. 22). In HIV negative granulomas IL-4 (Fig. 23) followed a similar pattern to the pattern in necrosis, with an almost equal distribution between Grade 1 (46%) on the one hand and Grade 2 (26%) plus Grade 3 (26%) on the other.

Figure 22: Distribution of TNFα positive cells in HIV positive and negative granulomas.
TNFα expression was examined by ISH of 166 granulomas as presented in fig 17. Positivity was graded according to the total number of cells staining positive in a specific granuloma (<25%, 25-75% and >75%). The percentage of HIV positive and negative granulomas in each of these categories was calculated and is represented in this graph. HIV positive granulomas had a significantly higher percentage of granulomas where the majority of cells expressed TNFα (Chi-square test $p < 0.01$)

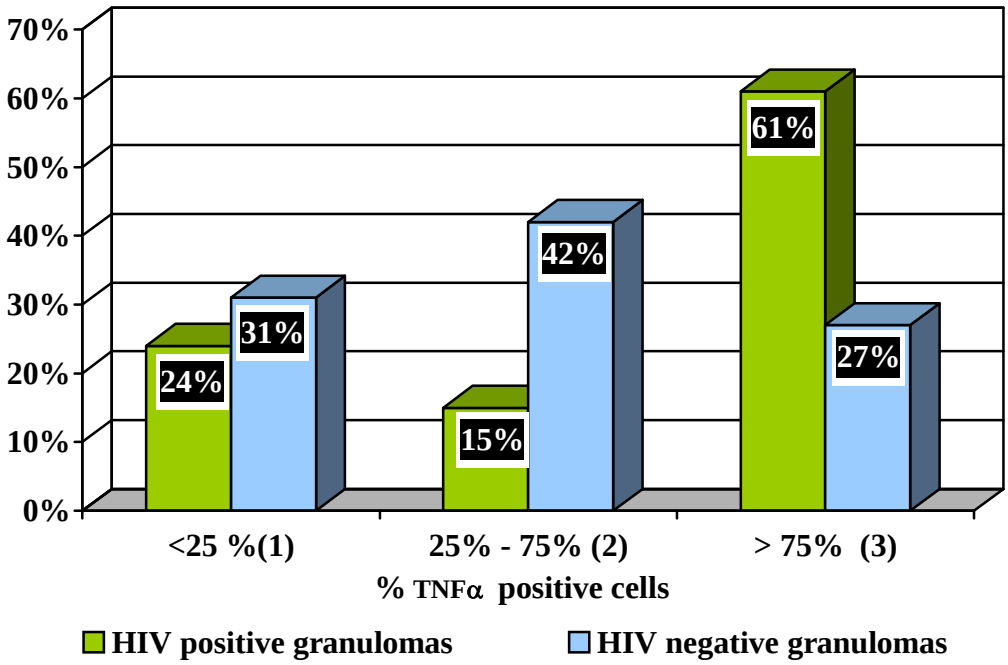
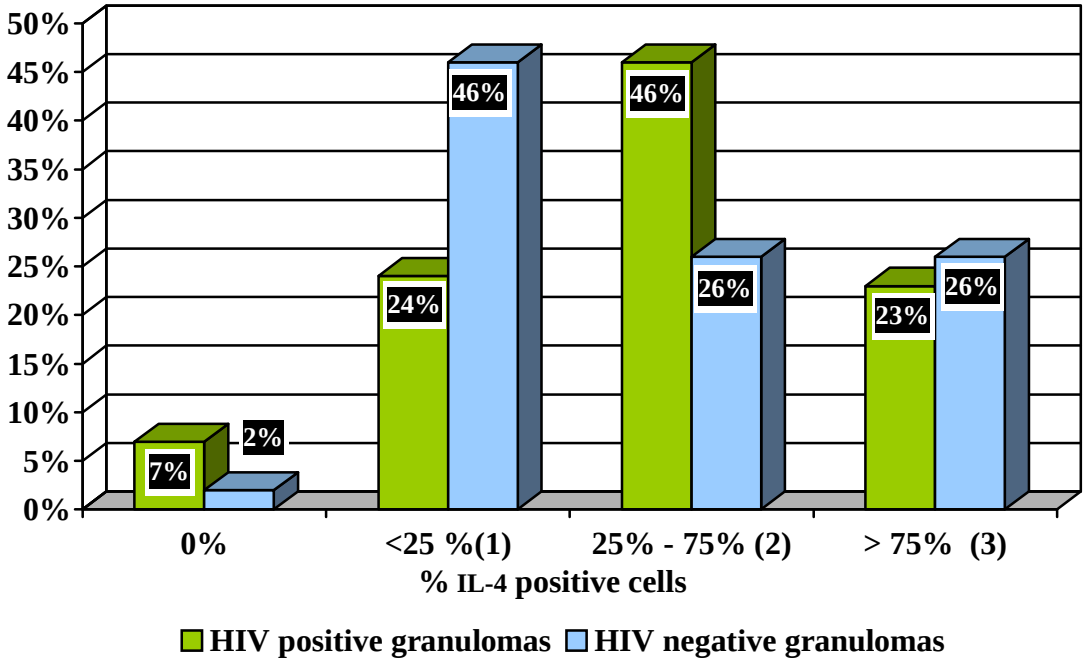


Figure 23:Distribution of IL-4 positive cells in HIV positive and negative granulomas.
IL-4 expression was examined by ISH on 166 granulomas as presented in fig 17. Positivity was graded according to the total number of cells staining positive in a specific granuloma (<25%, 25-75% and >75%). The percentage of HIV positive and negative granulomas in each of these categories was calculated and is represented in this graph. HIV negative granulomas had a significantly higher percentage of granulomas where the minority of cells expressed IL-4 (Chi-square test p = 0.02)



In HIV positive granulomas the picture changed with most granulomas either Grade 2 (46%) or 3 (22%). A similar picture could be seen with IFNγ (Fig. 24), while the IL-12 (fig. 25) picture was not as absolute, but favoured Grades 1 (31%) and 2 (46%) in HIV negative granulomas and Grades 2(49%) and 3 (31%) in HIV positive granulomas. A summary of this data can be seen in Table 11.

Table 11: The % of granulomas positive for specific cytokines in the HIV negative and HIV positive groups of granulomas.
This table represents as summary of the data presented in figs. 20-23. An increase in the number of positive cells is noted in HIV positive granulomas, and especially TNFα.

	HIV negative granulomas				HIV positive granulomas				Chi-square
	0	1	2	3	0	1	2	3	test
IL-12	0	31%	46%	23%	0	20%	49%	31%	p = 0.03
IFNγ	0	50%	30%	20%	0	26%	37%	37%	p = 0.02
TNFα	0	31%	42%	27%	0	24%	15%	61%	p < 0.01
IL-4	2%	46%	26%	26%	7%	24%	46%	23%	p = 0.02

Figure 24: Distribution of IFNγ positive cells in HIV positive and negative granulomas.

IFNγ expression was examined by ISH on 166 granulomas as presented in fig 17. Positivity was graded according to the total number of cells staining positive in a specific granuloma (<25%, 25-75% and >75%). The percentage of HIV positive and negative granulomas in each of these categories was calculated and is represented in this graph. HIV positive granulomas had a significantly higher percentage of granulomas where the majority of cells expressed IFNγ (Chi-square test p = 0.016).

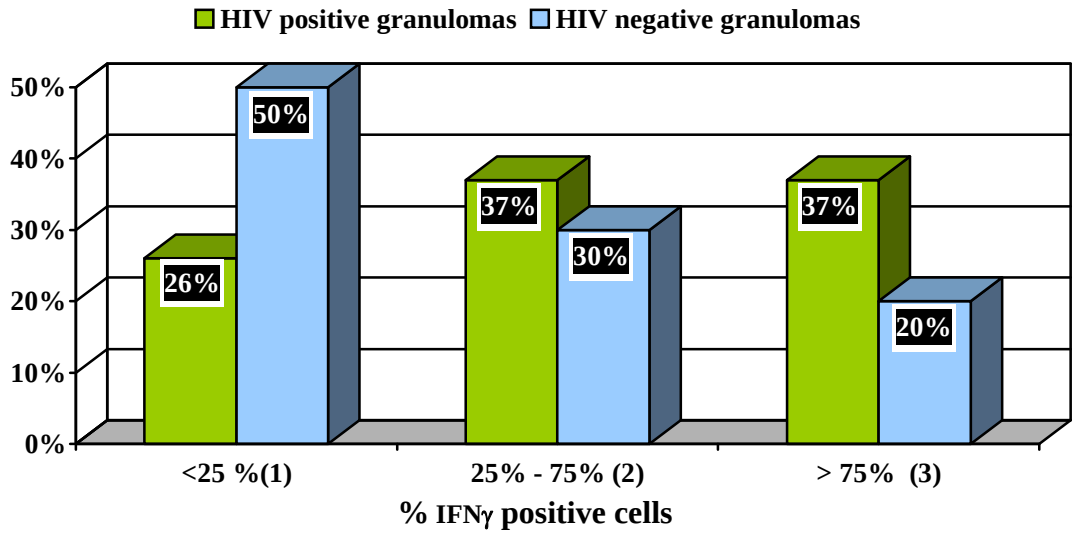
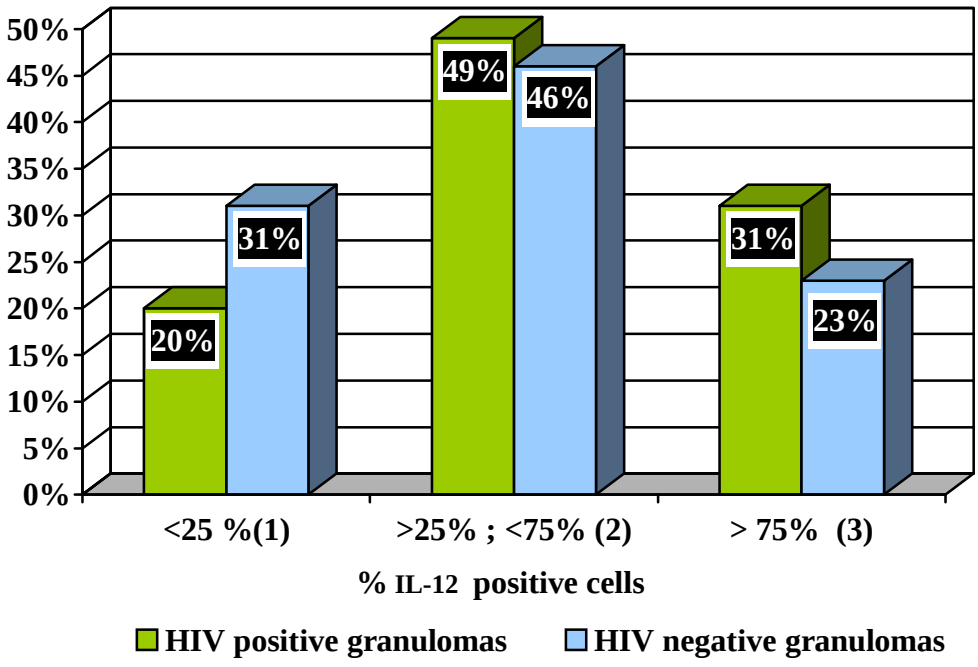


Figure 25: Distribution of IL-12 positive cells in HIV positive and negative granulomas.

IL-12 expression was examined by ISH on 166 granulomas as presented in fig 17. Positivity was graded according to the total number of cells staining positive in a specific granuloma (<25%, 25-75% and >75%). The percentage of HIV positive and negative granulomas in each of these categories was calculated and is represented in this graph. A fairly even distribution of IL-12 positive cells was seen, with almost 50% of granulomas containing 25 – 75% positive cells. (Chi-square test p = 0.027).



4.2.5 Necrosis in Granulomas

For the purpose of this section the HIV status of the patients was excluded and granulomas were only assessed on the presence of necrosis, to establish whether there was any correlation between necrosis and the cytokines, independent of HIV status. Further discussion on the relationship between HIV status, necrosis and cytokines will follow in subsequent sections.

4.2.5.1 Non-necrotic granulomas

There were 118 non-necrotic granulomas in total, 115 of which were IL-4 positive, while all 118 were TNFα, IFNγ and IL-12 positive. There were however differences in the number of positive cells in the various circumstances.

It was clear that a significant number of non-necrotic granulomas had low quantities of IL-4 and TNFα positive cells (Tables 12 and 13). Less than 25% of all the non-necrotic granulomas had a high number of either TNF, IL-4 and of these only 12% had high numbers of both. On the other hand 50% of all non-necrotic granulomas had <25% IL-4 positive cells (27% of these also <25% TNF positive cells). However, there was a clear association between the number of IL-4 positive and TNFα cells in non-necrotic granulomas. Granulomas with high numbers of IL-4 positive cells, tended also to have high numbers of TNFα positive cells (Spearman rank R - positive correlation, $r = 0.46$).

The relationship between IL-4 and IFNγ was a little less strong ($r = 0.33$), but 34% of the granulomas had low numbers of positive cells for both cytokines (Table 12 and 13).

In fact, this was a higher number of granulomas than those with a low number of IL-4 and TNF positive cells. Only 8 non-necrotic granulomas (7%) had high numbers of IL-4 and IFNγ positive cells.

A positive correlation also exists between IL-4 and IL-12 (r = 0.39), but there was a more even distribution of cells than in the previous two groups. There were less granulomas with low numbers of cells for both cytokines (20%), as opposed to the two previous groups with 27% and 34% respectively.

Table 12: Correlation of the various cytokines in necrotic and non-necrotic granulomas.
Strong positive correlations exist between most of the cytokines. See text for detailed discussion of table.

IL-4 vs TNFα

Non-necrotic granulomas p < 0.01

IL-4	TNF			
	1	2	3	Total
0	0	1.69	0.85	3
1	*27.12*	*20.34*	2.54	59
2	5.08	*12.71*	8.47	31
3	2.54	6.78	*11.86*	25
Total	41	49	28	118
Necrotic granulomas p = 0.1				
0	2.08	0	4.17	3
1	4.17	6.25	6.25	8
2	8.33	10.42	*22.92*	20
3	2.08	2.08	*31.25*	17
Total	8	9	31	48

IL-4 vs IFNγ

Non-necrotic granulomas p < 0.01

IL-4	IFN			
	1	2	3	Total
0	0	0.85	1.69	3
1	*33.9*	*11.86*	4.24	59
2	*11.02*	*11.02*	4.24	31
3	3.39	*11.02*	6.78	25
Total	57	41	20	118
Necrotic granulomas p = 0.1				
0	6.25	0	0	3
1	14.58	2.08	0	8
2	14.58	10.42	16.67	20
3	0	12.5	*22.92*	17
Total	17	12	19	48

IL-4 vs IL-12

Non-necrotic granulomas p < 0.01

IL-4	IL-12			
	1	2	3	Total
0	0.85	0.85	0.85	3
1	*20.34*	*26.27*	3.39	59
2	4.24	*15.25*	6.78	31
3	5.08	1.69	*14.41*	25
Total	36	52	30	118
Necrotic granulomas p < 0.01				
0	20.8	4.17	0	3
1	12.5	2.08	2.08	8
2	2.08	*25*	14.58	20
3	6.25	*22.92*	6.25	17
Total	11	26	11	48

IL-12 vs IFNγ

Non-necrotic granulomas p < 0.01

IL -12	IFN			
	1	2	3	Total
1	*21.19*	5.9	3.39	36
2	*27.12*	*13.5*	3.39	52
3	0	*15.25*	*10.17*	30
Total	57	41	20	118
Necrotic granulomas p = 0.1				
1	12.5	4.17	6.25	11
2	14.58	10.42	*29.17*	26
3	8.33	10.42	4.17	11
Total	17	12	19	48

IL-12 vs TNFα

Non-necrotic granulomas p < 0.01

IL -12	TNF			
	1	2	3	Tota
1	*14.41*	*14.41*	1.69	36
2	*17.8*	*20.34*	5.93	52
3	2.54	6.78	*16.1*	30
Total	41	49	28	118
Necrotic granulomas p = 0.05				
1	2.08	8.33	12.5	11
2	6.25	4.17	*43.75*	26
3	8.33	6.25	8.33	11
Total	8	9	31	48

TNFα vs IFNγ

Non-necrotic granulomas p < 0.01

IFN	TNF			
	1	2	3	Total
1	*27.12*	*19.49*	1.69	57
2	6.78	*17.8*	*10.17*	41
3	0.85	4.24	*11.86*	20
Total	41	49	28	118
Necrotic granulomas p = 0.003				
1	12.5	14.58	8.33	17
2	4.17	2.08	18.75	12
3	0	2.08	*37.5*	19
Total	8	9	31	48

Correlations are expressed as % of the total number of granulomas counted, while the figures in the total columns represent the actual number of granulomas. Percentages in italics indicate statistically significant correlations.
0 = no positive cells 1 = <25% positive cells 2 = 25 - 75% positive cells 3 = >75%positive cells

Table 13: Spearman rank R- correlation for cytokines in non-necrotic granulomas.
This correlation confirms the positive correlations noted for non-necrotic granulomas. All correlations are statistically significant.

	IL-12	IFN	TNF	IL-4
IL-12	1	*0.49*	*0.44*	*0.39*
IFN	*0.49*	1	*0.58*	*0.33*
TNF	*0.44*	*0.58*	1	*0.46*
IL-4	*0.39*	*0.33*	*0.46*	1

A similar positive correlation to the previous ones exists between IL-12 and IFNγ (r = 0.49). There were still many more granulomas where low numbers of IL-12 and IFNγ positive cells (table 12) were present (Grade 3=10%). The previous pattern of cytokine correlation was also followed by IL-12 and TNFα (r = 0.44) and IFNγ and TNFα (r = 0.58) as seen in table 13. This strong positive correlation between IFNγ and TNFα was significantly more than the association between IL-4 and IFNγ (p = 0.016). None of the other correlations were significantly less than IFNγ vs TNFα.

4.2.5.2 Necrotic granulomas

The most noticeable difference between the 48 necrotic and 118 non-necrotic granulomas was that many more cells per granuloma were positive for a specific cytokine in the necrotic granulomas. Due to the smaller number of necrotic granulomas, the differences in the results were often not statistically significant. There was however, a clearly positive correlation between IL-4 and TNFα (r = 0.34), with 31% of necrotic granulomas containing high numbers of cells of these 2 cytokines (table 14). IL-4 vs IFNγ showed a Spearman rank R - positive correlation of 0.65 (table 14), with 23% of granulomas having high numbers of positive cells for both cytokines. The correlation between IL-4 and IL-12 was not so strong (r = 0.21) and indeed most granulomas were in the grade 2 or grade 2/3 group.

Table 14: Spearman rank R- correlation of cytokines in necrotic granulomas.
This correlation confirms the positive correlations noted in table 11. All correlations in italics are statistically significant. A negative correlation is observed for TNFα versus IL-12, but it not of significance.

	Necrotic granulomas			
	IL-12	**IFN**	**TNF**	**IL-4**
IL-12	1	0.32	-0.17	0.21
IFN	0.03	1	*0.63*	*0.65*
TNF	-0.17	*0.63*	1	*0.34*
IL-4	0.21	*0.65*	*0.34*	1

A similar picture could be seen for IL-12 vs IFNγ with virtually no relationship (r = 0.03)between these two cytokines (table 14). There was a slight negative correlation between IL-12 and TNFα (r = 0.17), with the only noticeable association that of Grade 3 TNFα with Grade 2 IL-12 (44%) seen in table 12. A very strong correlation (r = 0.63)exists between IFNγ and TNFα (table 14). Thirty eight percent of cells in necrotic granulomas had more than 75% of cells positive for IFNγ and for TNFα (table 12). A positive correlation also exists between HIV positivity and IFNγ in non-necrotic granulomas, but no other correlations between HIV and cytokines were of significance, either on necrotic, or non-necrotic granulomas.

4.2.6 HIV Status and Granulomas

4.2.6.1 HIV negative

In HIV negative granulomas (table 15) a positive correlation exists between IL-12 and IFNγ (r = 0.49). This was reflected by low numbers of positive cells for both these cytokines in table 16. A positive correlation (r = 0.49) also exists between IL-12 and TNFα, but a more even distribution of positive cells was seen.

Table 15: Spearman rank R- correlation for cytokines in HIV negative granulomas.
A strong positive correlation is observed between all the cytokines. Significant correlations are highlighted in italics

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	HIV negative granulomas			
	IL-12	**IFN**	**TNF**	**IL-4**
IL-12	1	*0.49*	*0.49*	*0.47*
IFN	*0.49*	1	*0.66*	*0.52*
TNF	*0.49*	*0.66*	1	0.59
IL-4	*0.47*	*0.52*	*0.59*	1

Table 16: Relationship between cytokines in HIV positive and negative granulomas.
Strong positive correlations exist between most of the cytokines. See text for detailed discussion of table.

IL-12 vs IFNγ

	HIV negative p < 0.01			
IL -12	**IFN**			
	1	2	3	Total
1	*23.2*	5.6	2.4	39
2	*27.2*	*11.2*	*8*	58
3	0	*12.7*	*9.02*	28
Total	63	38	24	125
	HIV positive p = 073			
1	4.8	4.8	9.6	8
2	12.2	17	19.5	20
3	9.7	14.6	7.3	13
Total	11	15	15	41

IL-12 vs TNFα

	HIV negative p < 0.01			
IL -12	**TNF**			
	1	2	3	Total
1	*14.4*	*16*	0.8	39
2	*16*	*19.2*	*11.2*	58
3	0.8	6.4	*15.2*	28
Total	39	52	34	125
	HIV positive p = 0.06			
1	0	2.4	17	8
2	9.7	4.8	*34.1*	20
3	14.6	7.3	9.7	13
Total	10	6	25	41

TNF vs IFNγ

	HIV negative p < 0.01			
IFN	**TNF**			
	1	2	3	Total
1	*26.4*	*22.4*	1.6	63
2	4.8	*16*	*9.6*	38
3	0	3.2	*16*	24
Total	39	52	34	125
	HIV positive p = 0.2			
1	12.2	4.8	9.7	11
2	9.7	4.8	21.9	15
3	2.4	4.8	29.7	15
Total	10	6	25	41

IL-4 vs IL-12

	HIV negative p < 0.01			
IL -4	**IL-12**			
	1	2	3	Total
0	1.6	0.8	0	3
1	*20.8*	*23.2*	1.6	57
2	4	*15.2*	6.4	32
3	4.8	7.2	*14.4*	33
Total	39	58	28	125
	HIV positive p = 0.3			
0	0	4.8	2.4	3
1	9.7	7.3	7.3	10
2	2.4	*26.8*	17	19
3	7.3	9.7	4.4	9
Total	8	20	13	41

IL-4 vs IFNγ

	HIV negative p < 0.01			
IL -4	**IFN**			
	1	2	3	Total
0	0.8	0.8	0.8	3
1	*34.4*	*9.6*	1.6	57
2	*12*	*9.6*	4	32
3	3.2	*10.4*	*12.8*	33
Total	63	38	24	125
	HIV positive p = 0.2			
0	4.8	0	2.4	3
1	9.7	7.3	7.3	10
2	12.2	14.6	19.5	19
3	0	14.6	7.3	9
Total	11	15	15	41

IL-4 vs TNFα

	HIV negative p < 0.01			
IL -4	**TNFα**			
	1	2	3	Total
0	0.8	1.6	0	3
1	*24*	*20.8*	0.8	57
2	15.6	*12.8*	*8.8*	32
3	2.4	6.4	*17.6*	33
Total	39	52	34	125
	HIV positive p = 0.15			
0	0	0	7.3	3
1	9.7	2.4	12.2	10
2	12.2	9.7	24.3	19
3	2.4	2.4	18.18	9
Total	10	6	25	41

Correlations are expressed as % of the total number of granulomas counted, while the figures in the total columns represent the actual number of granulomas. Percentages in italics indicate statistically significant correlations.
0 = no positive cells 1 = <25% positive cells 2 = 25 - 75% positive cells 3 = >75% positive cells

The positive correlation (table 15) between IL-12 and IL-4 was just as strong (r = 0.47), with most granulomas having lower numbers of positive cells for both cytokines. The strongest positive correlations in HIV negative granulomas were between IFNγ and TNFα (r = 0.66) and IL-4 and TNFα (r = 0.59), while the positive correlation between

IL-4 and IFNγ was r = 0.52 (table 15). In all of these there were more granulomas with less positive cells for both cytokines. In the HIV negative group a positive correlation also exists between necrosis and IFNγ (r = 0.23) (data not shown), as well as necrosis and TNF (r = 0.39) (data not shown), but not as much between necrosis and IL-4 (r = 0.2) (data not shown). This difference was however not statistically significant (p=0.09).

4.2.6.2 HIV positive

Due to the smaller number of granulomas in HIV positive patients, correlations between the various cytokines were not of statistical significance, except for the negative correlation between IL-12 and TNFα of –0.45 (table 17), where high numbers of TNFα positive cells were associated with an intermediate number of IL-12 positive cells.

Table 17: Spearman rank R- correlation for cytokines in HIV positive granulomas.
A negative correlation is noted between IL-12 and TNFα.

	HIV positive granulomas			
	IL-12	**IFN**	**TNF**	**IL-4**
IL-12	1	-0.16	*-0.45*	-0.04
IFN	-0.16	1	0.38	0.22
TNF	-0.45	0.38	1	0.08
IL-4	-0.04	0.22	0.08	1

There were however, certain trends that were identifiable: namely, a higher number of cells positive in these granulomas as illustrated earlier (section 4.2.4), as well as a positive correlation between the various cytokines (not statistically significant).

4.2.7 Correlation Between HIV Status, Necrosis and the Various Cytokines when one of the Cytokines is a Constant

In the following section the relationship between the variables are discussed relative to a constant. The relationship between a specific cytokine and the other variables was examined when granulomas contained either < 25% positive cells, 25-75% positive cells or > 75% positive cells of a specific cytokine.

4.2.7.1 IL-12 as a constant

There was a significant positive correlation between all the variables when IL-12 = 1 (Table 18). The results in italics indicate statistically significant correlations (Spearman rank R-correlation) and this is further illustrated by the data in Table 19 (crosstabulation), where IL-12 positivity =1. Most of the granulomas have <25% positive cells per granuloma when the HIV-status is negative and there is no necrosis.

Table 18: Spearman rank R- correlation between variables when IL-12 is a constant.
A negative correlation is observed between HIV and cytokines when IL-12 positivity = 3, with more IFNγ, TNFα and IL-4 positive cells in HIV negative granulomas. All other significant correlations (in italics) are positive.

			IL-12 = 1		
	HIV	**necrosis**	**IFN**	**TNF**	**IL-4**
HIV	1	*0.55*	*0.43*	*0.64*	*0.2*
necrosis	*0.55*	1	0.15	*0.49*	0.03
IFN	*0.43*	0.15	1	*0.61*	0.24
TNF	*0.64*	*0.49*	*0.61*	1	0.2
IL-4	*0.2*	0.03	0.24	0.3	1
			IL-12 = 2		
HIV	1	*0.39*	*0.3*	*0.32*	*0.16*
necrosis	*0.39*	1	*0.44*	*0.56*	*0.51*
IFN	*0.3*	*0.44*	1	*0.57*	*0.51*
TNF	*0.32*	*0.56*	*0.57*	1	*0.59*
IL-4	*0.16*	*0.51*	*0.51*	*0.59*	1
			IL-12 = 3		
HIV	1	*0.53*	*-0.31*	*-0.44*	*-0.48*
necrosis	*0.53*	1	*-0.37*	-0.29	-0.17
IFN	*-0.31*	*-0.37*	1	*0.52*	0.18
TNF	*-0.44*	-0.29	*0.52*	1	0.25
IL-4	*-0.48*	-0.17	0.18	0.25	1

Table 19: Crosstabulation between HIV and necrosis when IL-12=1.
When IL-12 positivity = 1, most granulomas are non-necrotic and HIV negative (p=0.01).

HIV	Necrosis 0	Necrosis 1	Total
0	*34*	5	39
	72.3%	10.6%	82.9%
1	2	6	8
	4.2%	12.7%	17%
Total	36	11	47

Correlation expressed as actual number and as percentage of the total number of granulomas counted
0 = negative 1 = positive

This trend illustrated in Tables 18 and 19 is also applicable to the other positive correlations highlighted in italics in tables 21 to 27, which directly follows in discussions on the specific cytokines.

A typical example of the statistically significant negative correlation is that of IL-4 versus HIV when IL-12 positivity = 3 (table 20). The largest number of granulomas have >75% IL-4 positive cells when IL-12 is also high and HIV negative. The presence of numerous IL-12 positive cells appears to have an impact on the presence of other cytokine positive cells in the granuloma. More IFNγ, TNFα and IL-4 positive cells can be seen in the presence of high numbers of IL-12 positive cells, when the patients are HIV negative (table 18). There is also a clear negative correlation between IFNγ and necrosis when IL-12 =3 (table 18).

Table 20: Crosstabulation between HIV and IL-4 when IL-12 = 3.
The largest number of granulomas have >75% IL-4 positive cells in an environment with the majority of cells IL-12 positive, in HIV negative patients ($p < 0.01$).

HIV	IL-4 0	IL-4 1	IL-4 2	IL-4 3	Total
negative	0	2	8	*18*	28
	0.00%	4.8%	19.5%	*43.9%*	68.2%
positive	1	3	7	2	13
	2.44%	7.32%	17.07%	4.8%	31.7%
Total	1	5	15	20	41

Correlation expressed as actual number and as percentage of the total number of granulomas counted
0 = no positive cells 1 = <25% positive cells 2 = 25 - 75% positive cells 3 = >75%positive cells

4.2.7.2 IFNγ as a constant

When IFNγ = 1, 90% of granulomas in HIV negative patients were non-necrotic, while 100% of granulomas in HIV positive patients were necrotic($p < 0.01$). The rest of these results follow a similar pattern, with the only negative correlation being between IL-12 and necrosis as well as IL-4 and HIV (table 21).

Table 21: Correlation between variables when IFNγ is a constant.
When IFNγ positivity = 3, a negative correlation was observed between necrosis and IL-12 and IL-4 and HIV status. All other significant correlations (in italics) are positive.

			IFNγ = 1		
	HIV	necrosis	IL-12	TNF	IL-4
HIV	1	*0.76*	*0.33*	0.15	0.00
necrosis	*0.76*	1	0.17	*0.23*	-0.03
IL-12	*0.33*	0.17	1	0.1	0.14
TNF	0.15	*0.23*	0.1	1	0.08
IL-4	0.00	-0.03	0.14	0.08	1
			IFNγ = 2		
HIV	1	*0.46*	0.00	0.13	0.11
necrosis	*0.46*	1	-0.01	*0.3*	0.23
IL-12	0.00	-0.01	1	0.07	*0.36*
TNF	0.13	*0.30*	0.07	1	*0.49*
IL-4	0.11	0.23	*0.36*	0.49	1
			IFNγ=3		
HIV	1	0.07	-0.27	-0.05	*-0.4*
necrosis	0.07	1	*-0.35*	*0.32*	*0.30*
IL-12	-0.27	*-0.35*	1	0.19	0.15
TNF	-0.05	*0.32*	0.19	1	0.17
IL-4	*-0.4*	*0.30*	0.15	0.17	1

Therefore, when IFNγ was high, fewer granulomas with >75% IL-12 positive cells tended to have necrosis. When IFNγ positivity was 3, the trend was for HIV positive granulomas to have a moderate amount of IL-4 positive cells. These granulomas tended to be necrotic, but the numbers were too low to be of statistical significance.

On the other hand in HIV negative granulomas, high numbers of IL-4 cells were present in granulomas that had high numbers of IFNγ positive cells and there was an equal distribution between necrotic and non-necrotic granulomas.

4.2.7.3 TNFα as a constant

Seventy seven percent of granulomas with <25% TNFα positive cells also did not show signs of necrosis and are found in HIV negative patients (table 22), as also demonstrated by the strong positive correlation between HIV and necrosis in table 23. A negative correlation can be seen between HIV and IL-12 and HIV and IL-4 when there is >75% TNFα positive cells, with less granulomas containing >75% positive cells of either IL-12 or IL-4 in HIV positive granulomas (table 23).

Table 22; Crosstabulation between HIV and necrosis when TNFα positivity =1.

Seventy seven percent of granulomas with <25% TNF positive cells also did not show signs of necrosis and were found in HIV negative patients ($p < 0.01$).

necrosis	HIV 0	HIV 1	Row
0	*38*	3	41
	77.5%	6.1%	83.67%
1	1	7	8
	2%	14.2%	16.33%
Total	39	10	49

Correlation expressed as actual number and as percentage of the total number of granulomas counted
0 = negative 1 = positive

Table 23: Correlation between variables when TNFα is a constant.

Most granulomas with a < 25% TNFα positive cells (TNFα = 1) were not necrotic and HIV negative, with a very strong correlation. In granulomas with >75% TNFα positive cells there was a negative correlation between HIV and IL-4 and HIV and IL-12.

	HIV	necrosis	IL-12	IFN	IL-4
			TNFα = 1		
HIV	1	*0.73*	*0.58*	*0.34*	*0.32*
necrosis	*0.73*	1	*0.36*	0.02	0.22
IL-12	*0.58*	*0.36*	1	*0.51*	0.22
IFN	*0.34*	0.02	*0.51*	1	0.18
IL-4	*0.32*	0.22	0.22	0.18	1
			TNFα = 2		
HIV	1	*0.32*	0.22	0.18	0.18
necrosis	*0.32*	1	0.01	-0.19	0.10
IL-12	0.22	0.01	1	0.13	*0.35*
IFN	0.18	-0.19	0.13	1	0.20
IL-4	0.18	0.10	*0.35*	0.20	1
			TNFα = 3		
HIV	1	0.26	*-0.46*	-0.13	*-0.43*
necrosis	0.26	1	*-0.52*	0.04	-0.02
IL-12	*-0.46*	*-0.52*	1	0.06	0.18
IFN	-0.13	0.04	0.06	1	0.16
IL-4	*-0.43*	-0.02	0.18	0.16	1

In non-necrotic granulomas where TNFα positivity = 1, IL-12 numbers were also lower than in the necrotic granulomas (table 24), while non-necrotic granulomas with > 75% TNFα positive cells, also had >75% IL-12 positive cells (table 25). As in the case with IFNγ, there were also statistically significant less IL-4 positive cells in HIV positive granulomas when TNFα is 3.

Table 24: Crosstabulation between IL-12 and necrosis when TNFα positivity = 1
In non-necrotic granulomas where TNFα positivity = 1, IL-12 numbers were also lower than in the necrotic granulomas ($p < 0.01$).

Necrosis	**IL-12=1**	**IL-12=2**	**IL-12=3**	**Total**
present	*17*	*21*	3	41
	34.69%	*42.86%*	6.12%	83.67%
absent	1	3	4	8
	2.04%	6.12%	8.16%	16.33%
Total	18	24	7	49

Correlation expressed as actual number and as percentage of the total number of granulomas counted
1 = <25% positive cells 2 = 25 - 75% positive cells 3 = >75% positive cells

Table 25: Crosstabulation between IL-12 and necrosis when TNFα positivity = 3
Non-necrotic granulomas with a high number of TNFα positive cells, also had a high number of IL-12 positive cells ($p < 0.01$).

Necrosis	**IL-12=1**	**IL-12=2**	**IL-12=3**	**Total**
absent	2	7	*19*	28
	3.39%	11.86%	*32.20%*	47.46%
present	6	*21*	4	31
	10.17%	*35.59%*	6.78%	52.54%
Total	8	28	23	59

Correlation expressed as actual number and as percentage of the total number of granulomas counted
1 = <25% positive cells 2 = 25 - 75% positive cells 3 = >75% positive cells

4.2.7.4 IL-4 as a constant

Although there was a strong correlation between TNFα and HIV as well as IFNγ and necrosis when IL-4 was negative, only 6 granulomas in total were negative for IL-4 and therefore no conclusions could be drawn from these results. There was a strong correlation between HIV and necrosis when IL-4 positivity was 2 and more granulomas were HIV positive and necrotic than when IL-4 was negative (table 26).

Table 26: Spearman rank R- correlation between variables when IL-4 is a constant.

A negative correlation existed between IFNγ and necrosis when low numbers of IL-4 were observed and therefore fewer granulomas were necrotic. The opposite is true for TNFα. All other correlations were positive, except for IL-12 in the presence of high numbers of IL-4, where negative correlation with HIV status and necrosis was noted.

			IL-4 = 0		
	HIV	**necrosis**	**IL-12**	**IFN**	**TNF**
HIV	1	0.33	0.73	-0.21	*0.94*
necrosis	0.33	1	-0.21	*-0.94*	0.10
IL-12	0.73	-0.21	1	0.18	0.78
IFN	-0.21	*-0.94*	0.18	1	-0.01
TNF	*0.94*	0.10	0.78	-0.01	1
			IL-4 = 1		
HIV	1	*0.49*	0.13	*0.31*	0.24
necrosis	*0.49*	1	-0.17	-0.14	*0.26*
IL-12	0.13	-0.17	1	0.19	-0.11
IFN	*0.31*	-0.14	0.19	1	*0.32*
TNF	0.24	*0.26*	-0.11	*0.32*	1
			IL-4 = 2		
HIV	1	*0.46*	0.16	*0.28*	0.07
necrosis	*0.46*	1	0.15	0.17	0.16
IL-12	0.16	0.15	1	0.18	0.10
IFN	*0.28*	0.17	0.18	1	*0.59*
TNF	0.07	0.16	0.10	*0.59*	1
			IL-4 = 3		
	HIV	**necrosis**	**IL-12**	**IFN**	**TNF**
HIV	1	*0.39*	*-0.25*	-0.05	0.08
necrosis	*0.39*	1	*-0.33*	*0.36*	*0.32*
IL-12	*-0.25*	*-0.33*	1	0.12	0.14
IFN	-0.05	*0.36*	0.12	1	*0.45*
TNF	0.08	*0.32*	0.14	*0.45*	1

When IL-4 positivity = 1 there was also a strong positive correlation between HIV and necrosis with 80% of all granulomas being negative for both (table 27).

Table 27: Crosstabulation between HIV and necrosis when IL-4 is a constant.
In the presence of low numbers of IL-4 positive cells, few granulomas were necrotic, while in the presence of higher numbers, more necrosis was observed.

	IL-4=1		P=0.00		**IL-4=2**		p < 0.01		**IL-4=3**		p=0.01
HIV	**0**	**necrosis 1**	Total	**HIV**	**0**	**necrosis 1**	Total	**HIV**	**0**	**necrosis 1**	Total
0	*54*	3	57	0	*25*	7	32	0	*23*	10	33
	80.6%	4.5%			*49%*	13.7%			*69.7%*	30.3%	
1	5	5	10	1	6	*13*	19	1	2	7	9
	7.5%	7.5%			11.7%	*25.5%*			4.7%	16.7%	
Total	59	8	67	Total	31	20	51	Total	25	17	42

Correlation expressed as actual number and as percentage of the total number of granulomas counted
0 = negative 1 = positive

Other significant correlations included a positive correlation of 0.31 between HIV and IFNγ when IL-4 positivity was 1 (table 26), with 64% percent of HIV negative

granulomas containing low numbers of INFγ positive cells when IL-4 was 1 (data not shown). Forty percent of granulomas contained high numbers of IL-12 in non-necrotic granulomas when IL-4 positivity was 3 (data nor shown), with a negative Spearman rank R-correlation of 0.33.

4.2.8 Correlation Between the Various Cytokines when HIV Status and Necrosis are Constants

In the group of HIV negative granulomas with no necrosis, there was always a strong positive correlation between the various cytokines (tables 28, 29 and 30). In the HIV negative group without necrosis granulomas tended to have a lower number of positive cells for all the cytokines than in any of the other groups, but there still was a fairly even distribution between the various groups.

Table 28: Correlation between the various cytokines when HIV and necrosis are constants.
In the group of HIV negative granulomas with no necrosis, there was always a strong positive correlation between the various cytokines. In both groups with necrosis there was a very strong positive correlation between TNFα and IFNγ, as well as IFNγ and IL-4, but a strong negative correlation between IL-12 and TNFα.

HIV negative no necrosis				
	IL-12	IFN	TNF	IL-4
IL-12	1	*0.53*	*0.52*	*0.47*
IFN	*0.53*	1	0.57	*0.39*
TNF	*0.52*	*0.57*	1	*0.49*
IL-4	*0.47*	*0.39*	*0.49*	1
HIV positive no necrosis				
IL-12	1	-0.52	-0.38	-0.35
IFN	-0.52	1	0.27	-0.26
TNF	-0.38	0.27	1	0.2
IL-4	-0.35	-0.26	0.2	1
HIV negative with necrosis				
IL-12	1	0.27	0.43	0.41
IFN	0.27	1	*0.86*	*0.81*
TNF	0.43	*0.86*	1	*0.79*
IL-4	0.41	*0.81*	*0.79*	1
HIV positive with necrosis				
IL-12	1	-0.11	*-0.47*	0.12
IFN	-0.11	1	*0.47*	*0.51*
TNF	*-0.47*	*0.47*	1	0.01
IL-4	0.12	*0.51*	0.01	1

Table 29: Correlation between HIV negative granulomas, necrosis and specific cytokines.
In this group IL-12 positivity was evenly spread, while IFNγ and TNFα were low in non-necrotic and high in necrotic granulomas. IL-4 was also less positive in non-necrotic granulomas, but more evenly spread in necrotic granulomas, with no statistically significant differences.

necrosis	IL-12			p = 0.2
	1	2	3	Total
absent	*33%*	*43%*	*24%*	104
present	24%	*62%*	14%	21
Total	39	*58*	28	125

necrosis	IFNγ			p < 0.01
	1	2	3	Total
absent	*55%*	*37%*	*13%*	104
present	29%	19%	*52%*	21
Total	63	38	24	125

necrosis	TNFα			p < 0.01
	1	2	3	Total
absent	*36%*	*44%*	*19%*	104
present	5%	29%	*66%*	21
Total	39	52	34	125

necrosis	IL-4				p=0.01
	0	1	2	3	Total
absent	2%	*52%*	*24%*	*22%*	104
present	5%	14%	33%	48%	21
Total	3	57	32	33	125

Correlations are expressed as % of the total number of granulomas counted, while the figures in the total columns represent the actual number of granulomas. Percentages in italics indicate statistically significant correlations.
0 = no positive cells 1 = <25% positive cells 2 = 25 - 75% positive cells 3 = >75% positive cells

In the HIV negative group with necrosis, there was a very strong positive correlation between TNFα and IFNγ, with most granulomas containing high numbers of cells for both cytokines (tables 28 and 29). IL-4 and IFNγ, as well as IL-4 and TNFα showed a similar trend. In the group of HIV positive granulomas with necrosis (tables 28 and 30), there was also a strong positive correlation between IFNγ and TNFα, as well as IFNγ and IL-4, but a strong negative correlation between IL-12 and TNFα. On examining individual cytokines a similar picture could be seen, but in the case of HIV positive granulomas, only the association with IFNγ was statistically significant.

Table 30: Correlation between HIV positive granulomas, necrosis and specific cytokines.
IFNγ was the only cytokine with a statistically significant relationship with HIV and necrosis, with less positivity in the necrotic group. The other cytokines showed a more even distribution.

necrosis	IL-12			
	1	2	3	Total
absent	14.29%	50.00%	35.71	14
present	22.22%	48.15%	29.63%	27
Total	8	20	13	41

necrosis	IFNγ			p = 0.02
	1	2	3	Total
absent	0.00%	17.07%	17.07%	14
present	*26.83%*	19.51%	19.51%	27
Total	11	15	15	41

necrosis	TNFα			
	1	2	3	Total
absent	7.32%	7.32%	19.51%	14
present	17.07%	7.32%	41.46%	27
Total	10	6	25	41

necrosis	IL-4				
	0	1	2	3	Total
absent	2.44%	12.20%	14.63%	4.88%	14
present	4.88%	12.20%	31.71%	17.07%	27
Total	3	10	19	9	41

Correlations are expressed as % of the total number of granulomas counted, while the figures in the total columns represent the actual number of granulomas. Percentages in italics indicate statistically significant correlations.
0 = no positive cells 1 = <25% positive cells 2 = 25 - 75% positive cells 3 = >75% positive cells

4.2.9 Correlation Between IL-4 and Necrosis when one Cytokine is a Constant

The only significant correlations between necrosis and IL-4 when one cytokine was a constant can be seen in table 31. It is apparent that necrotic granulomas that contain high numbers of IL-4 positive cells were seen when high numbers of IFNγ positive cells were also present. A similar picture can be seen for TNFα (table 31). In the IL-12 group, high numbers of IL-4 positive cells in necrotic granulomas were seen when a moderate number of IL-12 cells were positive (table 31). Although not significant, 41% of granulomas were non-necrotic and had high numbers of IL-4 positive cells when IL-12 positivity = 3 (data not shown).

Table 31: Crosstabulation between necrosis and IL-4 when one cytokine is a constant.
The correlation between IL-4 and necrosis under specific conditions is only statistically significant when IFNγ positivity = 1 or 3, TNFα positivity = 1 or IL-12 positivity = 2.

	IFNγ=1 $p < 0.01$				
necrosis	IL-4 0	IL-4 1	IL-4 2	IL-4 3	Total
absent	0	*40*	*13*	4	57
	0.00%	*54%*	*17.5%*	5.4%	
present	3	7	7	0	17
	4%	9.4%	9.4%	0.00%	
Total	3	47	20	4	74

	IFNγ=3 p=0.04				
necrosis	IL-4 0	IL-4 1	IL-4 2	IL-4 3	Total
absent	2	5	5	8	20
	5.13%	12.82%	12.82%	20.51%	
present	0	0	8	*11*	19
	0.00%	0.00%	20.51%	*28.21%*	
Total	2	5	13	19	

	TNFα=1 $p < 0.01$				
necrosis	IL-4 0	IL-4 1	IL-4 2	IL-4 3	Total
absent	0	*32*	6	3	41
	0.00%	*65.31%*	12.24%	6.12%	
present	1	2	4	1	8
	2.04%	4.08%	8.16%	2.04%	
Total	1	34	10	4	49

	IL-12 =2 $p < 0.01$				
necrosis	IL-4 0	IL-4 1	IL-4 2	IL-4 3	Total
absent	1	31	18	2	52
	1.28%	39.74%	23.08%	2.56%	
present	2	1	*12*	*11*	26
	2.56%	1.28%	*15.38%*	*14.10%*	
Total	3	32	30	13	78

Correlations are expressed as actual numbers and % of the total number of granulomas counted, while the figures in the total columns represent the actual number of granulomas. Percentages in italics indicate statistically significant correlations.
0 = no positive cells 1 = <25% positive cells 2 = 25 - 75% positive cells 3 = >75% positive cells

5. Results: Sarcoidosis

Sarcoidosis is a multi-system granulomatous disease of unknown cause, although several organisms have been investigated as possible causes, amongst these *M. tuberculosis*[192,193,194,195]. The disease is characterised by non-caseating granulomas, which either resolve or progress to fibrosis. The histological grading is as follows[203]:

Pulmonary involvement

Extent of specific pathological change

Granulomas

Minimal: a few scattered granulomas
Moderate: more than a few, but occupying less than two thirds of the area
Extensive: numerous granulomas, often conglomerated and occupying more than two thirds of the area

Interstitial pneumonitis (nongranulomatous inflammation of the alveolar walls, predominantely lymphocytic)

Minimal: lesions occupying less than one third of the area
Moderate: lesions occupying one to two thirds of the area
Extensive: lesions occupying more than two thirds of the area

Granulomatous angiitis (granulomas within the walls of veins and/or *arteries)*

Absent: no granulomatous angiitis
Minimal: one to two foci
Moderate: more than two foci

Fibrosis (fibrosis of alveolar wall or interstitium)

Absent: no fibrosis
Focal: focal and scattered fibrosis
Diffuse: diffuse and extensive fibrosis

Evaluation of overall pathologic change

Mild: no fibrosis; granulomas and interstitial pneumonitis minimal and granulomatous angiitis absent or minimal
Moderate: by exclusion from mild and severe categories
Severe: diffuse fibrosis; granulomatous and interstitial pneumonitis both extensive; granulomatous angiitis moderate

In patients with sarcoidosis, the hypersensitivity reaction is viewed as the consequence of a chronic, exaggerated immunological response against an unidentified antigen that persists at the sites of disease involvement[204]. Initial granuloma formation has the same pathogenesis as previously described. The accumulation of CD4+ Th1 cells and macrophages at the site of inflammation is the earliest step in the events that lead to granuloma formation. According to several studies, the net effect of the Th1 response is

the organization of the local inflammatory process into the granuloma and the inhibition of the fibrogenetic process[208,209,210]. Some studies suggest that a switch to Th2 cells occurs in patients with progressive disease, evolving towards lung fibrosis[207].

5.1 Experimental Design

To investigate the cytokine profile of patients with sarcoidosis, open lung biopsies from a group of 15 patients with sarcoidosis were examined. Open lung biopsy tissue was obtained for routine diagnostic purpose: the diagnosis of sarcoidosis was confirmed; and biopsies were graded according to the histological grading system. ISH was performed for IL-12-, IFNγ-, TNFα- and IL-4-mRNA on all 15 cases. The cytokine profile of each granuloma was recorded on the photographic map as described previously.

5.2 Results

The clinical information on the patients can be seen in table 32. None of the patients had a previous history of tuberculosis. The ages ranged between 21 and 54 years and 13 of the 15 patients were female. Follow-up varied from 1 year to 12 years, and although one of the patients died after 1,5 years, none developed tuberculosis.

A spectrum of changes can be seen in the histological grading (table 33). Most of the cases (10) showed changes in keeping with moderate overall severity. Only one case showed minimal disease and 4 severe disease. Diffuse fibrosis was present in 8 cases and focal fibrosis in 3. Four cases had no fibrotic change.

Table 32: Demographic, clinical and radiological information on patients with pleural tuberculosis.
The table shows the basic demographic and clinical characteristics of 15 patients with sarcoidosis who were recruited for the histological study of open lung biopsy samples.

Patient	Age/Sex	Previous TB	Rx at diagnosis	Radiological staging	Follow up
S1	35/F	No	None	2	2 years, healthy
S2	21/F	No	None	3	2 years, healthy
S3	27/F	No	None	2	6 years, healthy
S4	36/F	No	None	2	3 years, respiratory failure
S5	35/F	No	None	2	12 years, healthy
S6	27/F	No	None	3	1,5yrs
S7	26/F	No	None	2	6 years, Grade I dyspnoea
S8	54/M	No	None	2	2 years, died
S9	36/F	No	None	3	5 years, grade II dyspnoea
S10	29/F	No	None	2	1 year, healthy
S11	37/F	No	None	2	1,5 years, died
S12	25/M	No	None	2	4 years
S13	36/F	No	None	2	1 year, healthy
S14	23/F	No	None	2	2yrs
S15	45/F	No	None	3	2 years, healthy

Age in years F = female; M =male R_x = treatment at time of diagnosis

Table 33: Histological grading of severity of disease.
A spectrum of changes can be seen, with most cases either in the overall moderate or severe categories. Fibrosis was absent in four cases.

Case	Granuloma			Pneumonitis			Angiitis		Fibrosis		Overall		
	+	++	+++	+	++	+++	Min	Mod	Foc	Dif	Min	Mod	Sev
S1		x			x		x		no	no		x	
S2			x		x			x	x			x	
S3		x			x		no	no	no	no		x	
S4			x			x		x		x			x
S5			x			x		x		x			x
S6			x			x		x		x			x
S7			x			x		x		x			x
S8		x			x		x		x			x	
S9		x		x			x		no	no		x	
S10		x			x			x		x		x	
S11	x			x			x		no	no	x		
S12		x				x		x		x		x	
S13		x				x		x		x		x	
S14		x			x		x		x			x	
S15		x				x	no	no		x		x	

+ = minimal numbers; ++ = moderate numbers; +++ = extensive; min = minimal; mod = moderate; sev = severe

5.2.1 Interpretation of the slides

Representative examples of the light microscopic appearance of the ISH can be seen in figs. 26-31. For complete scoring please see Appendix A. Positivity is represented by blue staining (see methods) of the cytoplasm and indicates the presence of mRNA. Counterstaining is green as described in methods. Cells that stain negative do not contain the specific RNA and will therefore stain green. A high concentration of positive cells in the lymphocyte cuff of the granulomas resulted in intense staining of this zone. These photographs were selected to illustrate the quality of staining for the various cytokine mRNAs.

The focus of the study was on the number of cells that are positive for a specific cytokine within the granuloma and the sources of the cytokines were not determined. This does not however, detract from the fact that the cytokine mRNA was present. Documented sources of these cytokines were discussed in chapter 1.

5.2.2 Cytokine profile

The cytokine profile of the 15 cases was identical (table 34). Numerous granulomas were present in the biopsies, ranging from 103 to 1677 granulomas per section. In all the cases diffuse positivity in the granulomas could be seen for IL-12, IFNγ and TNFα. The mRNA expression was predominantly present in the lymphocytes. On the other hand no IL-4 mRNA expression could be found in any of the cases. These results indicate the presence of a pure Th1 response in sarcoidosis.

Table 34: Cytokine profile of the granulomas in sarcoidosis. All granulomas had the same profile. All fifteen had had the same cytokine profile, showing strong positivity for IL-12, IFNγ and TNFα, but no IL-4 positivity.

Case	Number of granulomas	IL-12	IFNγ	TNFα	IL-4
S1	103	100%	100%	100%	0%
S2	1212	100%	100%	100%	0%
S3	145	100%	100%	100%	0%
S4	209	100%	100%	100%	0%
S5	426	100%	100%	100%	0%
S6	537	100%	100%	100%	0%
S7	1677	100%	100%	100%	0%
S8	452	100%	100%	100%	0%
S9	379	100%	100%	100%	0%
S10	488	100%	100%	100%	0%
S11	261	100%	100%	100%	0%
S12	604	100%	100%	100%	0%
S13	535	100%	100%	100%	0%
S14	234	100%	100%	100%	0%
S15	608	100%	100%	100%	0%

+ = positive - = negative

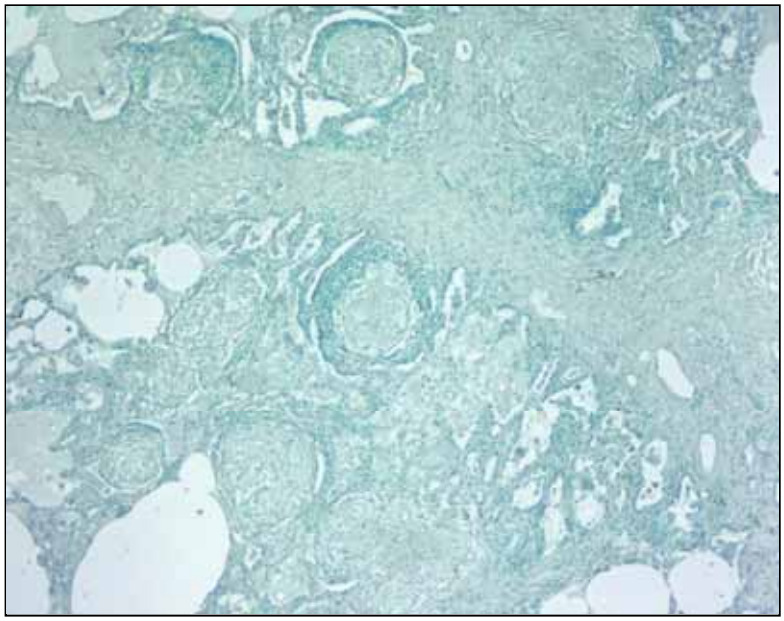


Figure 26: Both negative control and sense probes consistently negative (x40)

A representative section of an open lung biopsy from sarcoidosis patient is shown. ISH with the sense probe for all cytokines was performed after appropriate preparation of paraffin-embedded tissue. No blue staining which will indicate positive signal was noted. A 40 times magnification was used for this photograph.

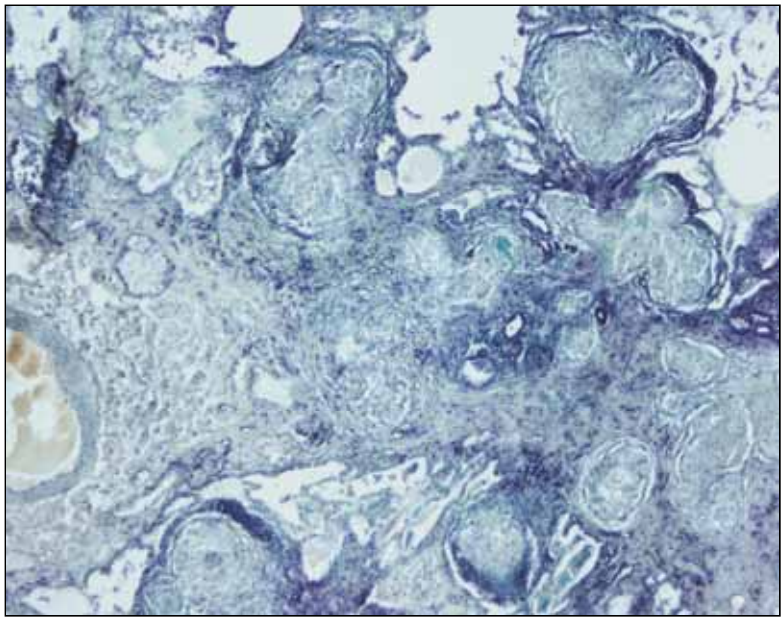


Figure 27: anti-sense probe for β-actin strongly positive (x200)

A representative section of an open lung biopsy from sarcoidosis patient is shown. ISH with the anti-sense β-actin probe was performed after appropriate preparation of paraffin-embedded tissue. The β-actin probes were used to determine the presence of non-degraded mRNA in sections. The blue staining represents positive signal and intact mRNA. A 200 times magnification was used for this photograph.

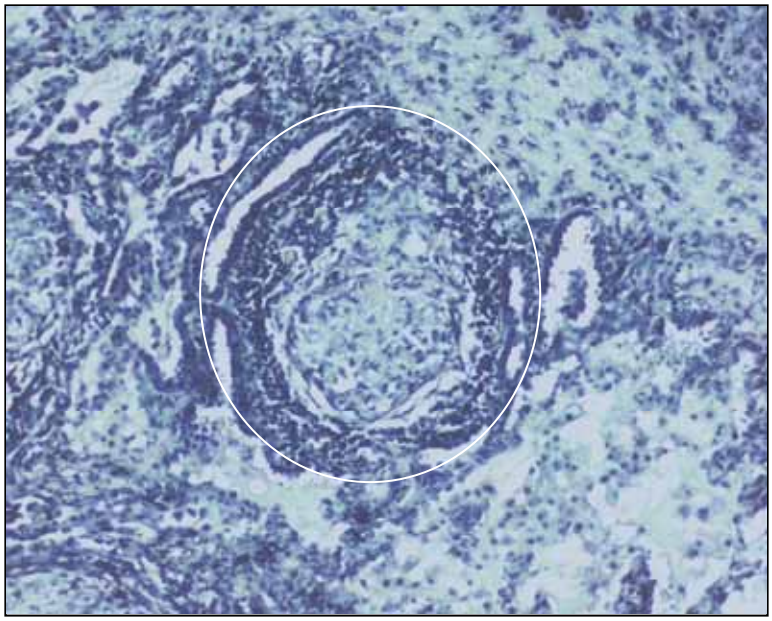


Figure 28: anti-sense probe for IL-12 strongly positive (x200)

A representative section of an open lung biopsy from a sarcoidosis patient is shown. ISH with the anti-sense IL-12 probe was performed after appropriate preparation of paraffin-embedded tissue. The blue staining represents positive signal. Granuloma outlined in white. A 200 times magnification was used.

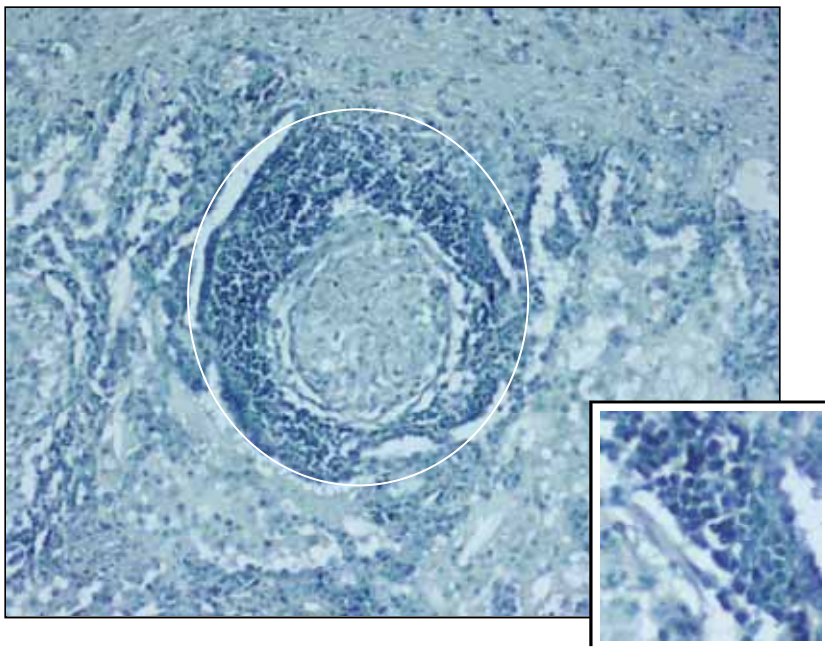


Figure 29: anti-sense probe for IFNγ strongly positive (x200, insert x400)

A representative section of an open lung biopsy from a sarcoidosis patient is shown. ISH with the anti-sense IFNγ probe was performed after appropriate preparation of paraffin-embedded tissue. The blue staining represents positive signal. Granuloma outlined in white. A 200 times magnification was used and a 400 times magnification for the insert, which shown that the signal is concentrated in lymphocytes.

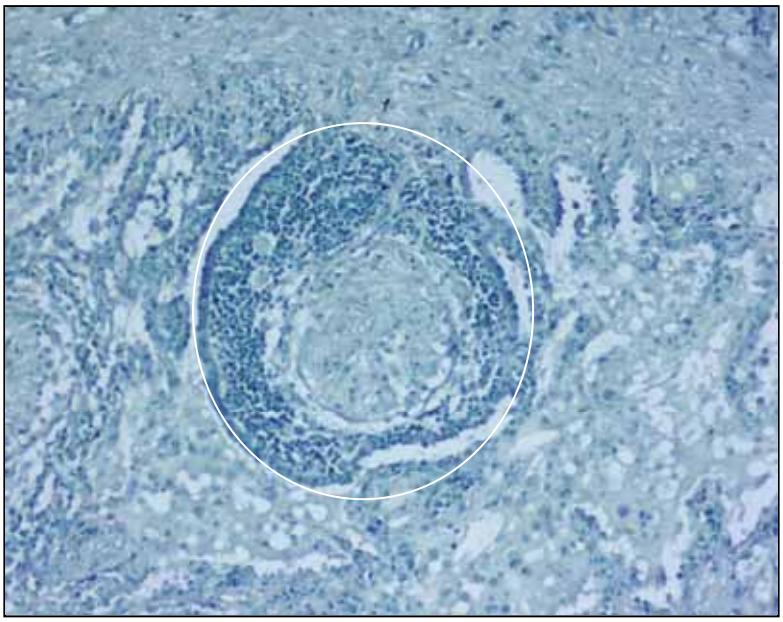


Figure 30: anti-sense probe for TNF strongly positive (x200)

A representative section of an open lung biopsy from a sarcoidosis patient is shown. ISH with the anti-sense TNFα probe was performed after appropriate preparation of paraffin-embedded tissue. The blue staining represents positive signal. Granuloma outlined in white. A 200 times magnification was used.

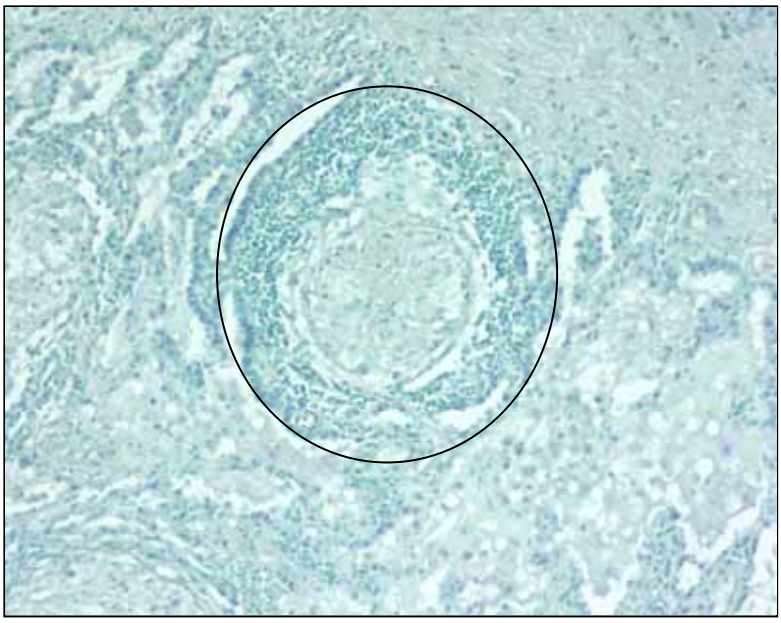


Figure 31: anti-sense probe for IL-4 negative (x200)

A representative section of an open lung biopsy from a sarcoidosis patient is shown. ISH with the anti-sense IL-4 probe was performed after appropriate preparation of paraffin-embedded tissue. A complete absence of blue staining representing positive signal is noted. Granuloma outlined in black. A 200 times magnification was used.

All the patients had radiological evidence of pulmonary involvement and all but one had at least a moderate degree of overall disease severity. Although four of the patients had no signs of fibrosis, there was no difference in the cytokine profile between these four and the remainder of the patients, as prominent IL-12, IFNγ and TNFα signals were observed, but a complete absence of IL-4 was noted in all 15 patients. Furthermore no difference in cytokine profile was found between those patients with a favourable and those with an unfavourable clinical course. Despite the spectrum of changes observed in the histological grading, none of these differences in the histological grading were reflected in the cytokine mRNA profile of these cases.

The investigations performed in the current study confirm the presence of a very strong Th1 response in all the cases of sarcoidosis as has been described in other studies[205,206,207]. Due to the uniformity of the results, no statistical analysis was could be performed. The presence of a pure Th response is in itself statistically significant. A comparison with the Th responses in pleural and pulmonary tuberculosis was performed and will be discussed in chapter 7.

6. Results: Pulmonary Tuberculosis

This section is based on original work initiated by Philip Bardin and myself and carried on by Gael Fenhalls et al in collaboration with the abovementioned (Appendix B: copies of the published articles). This work formed the basis of the subsequent work on sarcoidosis and pleural tuberculosis. Individual granuloma results are not included and only the relevant results for comparison with pleural TB and sarcoidosis are presented.

It has become quite apparent that T cells and cytokines play a major role in the host response against *M.tuberculosis* [70]. During the last few years a substantial body of evidence has emerged that IFNγ, the hallmark cytokine of the Th1 response, plays a key role in the defence against tuberculosis [71,72]. On the other hand, it is suggested that a persistently high IFNγ is responsible for the damage caused by granulomatous disease and that moderating cytokines are necessary to down-regulate the IFNγ response to more appropriate levels later in the disease process, after the organisms have been effectively contained. Thus, in tuberculosis a dual role for INFγ in both protection and pathology is suggested, where an appropriate CMI is responsible for containment, but a hyper-reactive CMI is responsible for tissue destruction.

Some studies suggest however, that an increase in IL-4 and IL-10 production is responsible for the pathology of tuberculosis [68,69]. It is suggested that an increase in IL-4 suppresses IFNγ production, followed by reduced killing of organisms[78].

Another cytokine that is of key importance is IL-12. It has been demonstrated that IL-12 production is essential for the initiation of the Th1-response and that persistent IL-

12 production is necessary to sustain it. In mice, enhanced protection was noted in the group where IL-12 was used as an adjuvant to bacille Calmette-Guérin (BCG) immunisation[81]. There is also however, evidence for the destructive effect of IL-12. In a murine study [84] demonstrated that granuloma disintegration and death was dependant on T cells and IL-12 production, while depletion of T cells subsets and IL-12 led to well-formed compact granulomas and prolonged survival.

Several studies in mice have demonstrated the importance of TNFα for the early expression of mRNA encoding chemokines and leukocyte recruitment[88], the ability of macrophages to phagocytose and kill mycobacteria [86,87], granuloma formation, prevention of necrosis [90,91] and induction of apoptosis[93,94].

Although enough evidence exist to support a protective role for TNFα, excessive production of TNFα and an increased sensitivity to the cytokine have been implicated in the immunopathology of tuberculosis, for example caseous necrosis[96]. While TNFα is an essential component of the host immune response against mycobacterial infection, high levels of the cytokine at the site of infection induce an excessive inflammatory response that overrides the beneficial effects of the cytokine.

It is also possible that the Th2 cytokines may mediate local tissue inflammation and necrosis through their effect on TNFα mediated cytotoxicity[97,98]. The role of cytokines such as TNFα may be to modulate and fine-tune this process, depending on the spectrum of cytokines already present. It may be of particular importance in TB where TNFα superimposed on a Th1 cytokine profile may lead to a protective granulomatous

response, whereas a Th2 background may result in tissue necrosis and breakdown with cavitation, thus favouring spread of organisms.

6.1 Experimental Design

To investigate the cytokine profile of patients with pulmonary tuberculosis, *two groups of patients* were examined, due to limited availability of appropriate tissue samples as previously discussed. The 2 groups will be discussed separately, and are labelled "first group of patients" and "second group of patients". Adult lung tissue was obtained, the diagnosis of tuberculosis was confirmed, and ISH was performed for IFNγ-, TNFα- and IL-4-mRNA on the first group of 5 patients, while ISH for IL-12 mRNA and immunohistochemistry for TNFα, IFNγ and IL-4 were performed on the second group of patients.

6.2 Results

6.2.1 The First Group of Patients

The first five patients presented with massive haemoptysis and on CXR all had cavity formation (Table 35). On histology, granuloma formation with caseating necrosis could be demonstrated. Ziehl-Neelsen staining revealed low levels of acid-fast bacilli in the lungs of all patients, even though all had received at least two months of multidrug therapy, which included rifampicin, isoniazid and pyrazinamide, prior to surgery.

Table 35: Clinical details of patients.
The table shows the basic demographic and clinical characteristics of 5 patients with pulmonary tuberculosis who were recruited for the histological study of lobectomy (surgical removal of one or more lobes of a lung) or pneumonectomy (surgical removal of a whole lung) specimens.

Patient	Age/sex	Time since previous TB (years)	Preoperative treatment duration (months)	Chest radiograph
A1	39/M	3	2	RUL cavity
A2	35/M	15	3	RUL cavity
A3	26/F	3.4	2	LUL cavity
A4	23/M	3	2	LUL cavity
A5	31/M		3	LUL cavity

Age in years; M=male; F=female; RUL = Right upper lobe of the lung; LUL = Left upper lobe of the lung

6.2.1.1 In Situ Hybridisation

6.2.1.1.1 General assessment of granulomas

A summary of the results can be seen in table 36. Granulomas from three patients (A1, A2 and A5) stained positive for IL-4 and IFNγ. Granulomas from two patients (A3 and A4) stained positive for IFNγ and negative for IL-4 mRNA. Granulomas from all patients were positive for TNFα mRNA.

Table 36: Summary of ISH of granulomas.
Two of the five patients have a pure Th1 profile, with no IL-4 positivity, while the remaining 3 have Th0 profile.

Patient	TNFα	IFNγ	IL-4	βactin
A1	++	+++	+	+++
A2	+++	+++	++	+++
A3	+++	+++	-	+++
A4	++	++	-	+++
A5	+++	++	++	+++

+++ strong positivity; ++ intermediate positivity; + weak positivity; - no positivity

6.2.1.1.2 Analysis of granulomas

The presence of cytokine mRNA and caseous necrosis was determined for the individual granulomas in each patient. (table 37). The number of granulomas ranged from 6 to 12 and most stained positive for IFNγ and TNFα mRNA. The IL-4 staining was negative in two patients (A3 and A4).

Table 37: Cytokine patterns of individual granulomas from five patients.
The majority of granulomas stained positive for IFNγ and TNFα mRNA. The number of TNFα mRNA positive granulomas was highest in those patients with IL-4 positive granulomas.

Patient	Total no. of granulomas	Granulomas/section (% of total)						
		IFNγ	TNFα	IL-4	+ necrosis	Th2	Th1	Th0
A1	6	100%	83%	33%	33%	0	67%	33%
A2	7	86%	71%	43%	29%	0	43%	43%
A3	12	75%	58%	0	50%	0	75%	0
A4	11	82%	64%	0	45%	0	82%	0
A5	9	67%	78%	44%	33%	0	22%	44%

The number of TNFα mRNA positive granulomas was highest in those patients with some IL-4 positive granulomas, but did not necessarily overlap with the IL-4 granulomas. No granulomas which stained positive for IL-4 and negative for IFNγ were observed; in fact, IL-4 mRNA was always detected in granulomas with low levels of IFNγ staining. Granulomas with caseous necrosis were generally larger than those without central necrosis and were either negative or only weakly positive for IFNγ and IL-4 mRNA.

Due to the relatively small number of granulomas, statistical analysis was not always viable, but significant differences were still present. Necrotic granulomas were more abundant in those patients with no evidence of IL-4 staining (fig. 32) and non-necrotic granulomas were positive for IL-4. One hundred percent of non-necrotic granulomas were positive for IFNγ (fig. 33), and 61% of necrotic granulomas were positive for IFNγ. However, these necrotic granulomas were all IL-4 negative. In general, necrotic granulomas were also negative to intermediately positive for TNFα mRNA. Cytokine mRNA staining, where present, was generally in the periphery of the granulomas and not in or around the necrotic centre, despite the periphery being positive for β-actin mRNA.

In summary, the two patients with IFNγ positive, IL-4 negative patterns of cytokine production expressed lower levels of TNFα mRNA and had more necrotic granulomas than the three patients whose granulomas were positive for both IFNγ and IL-4. In the IL-4 positive patients none of the IL-4 positive/IFNγ positive granulomas were necrotic.

Figure 32:Correlation between necrosis in granulomas and IL-4.

No necrotic granulomas showed any IL-4 mRNA positivity, while 67% of non-necrotic granulomas had some IL-4 mRNA positivity (Chi-square test p = 0.0006).

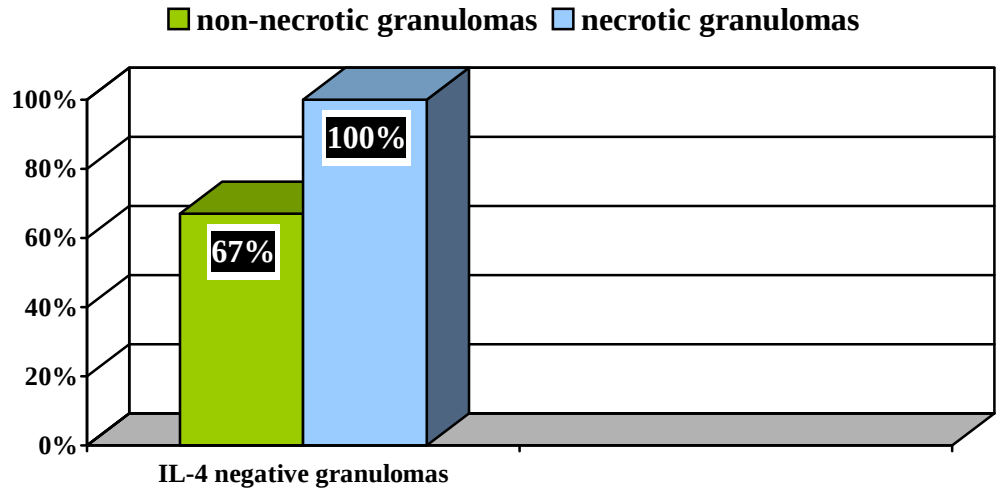
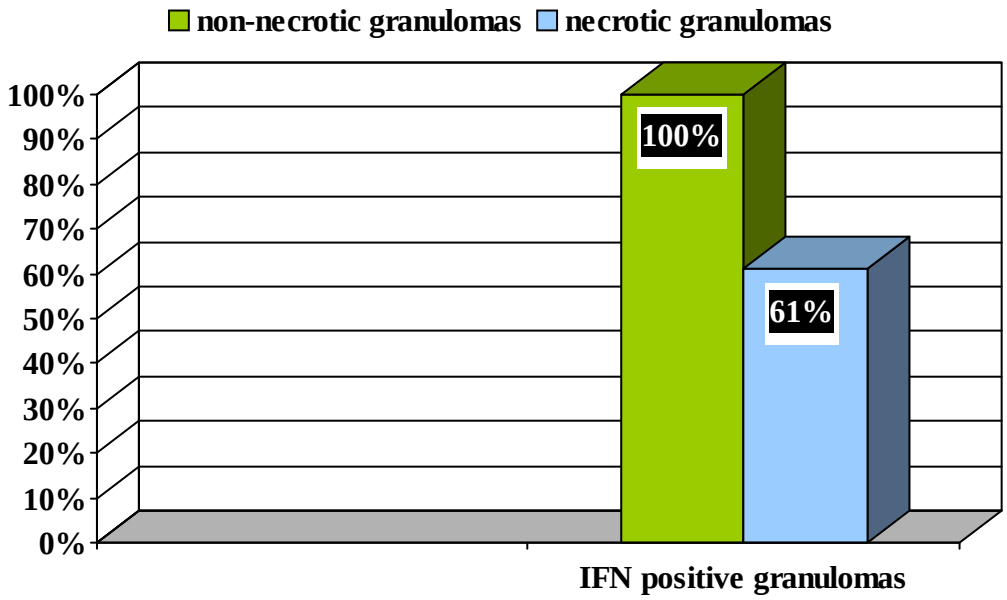


Figure 33:Correlation between necrosis in granulomas and IFNγ.
All non-necrotic granulomas had some IFNγ presence, but only 61% of necrotic granulomas showed any IFNγ positivity (Chi-square test p = 0.0004).



6.2.2 The Second Group of Patients

The seven patients in the second group of tuberculosis patients again presented with massive haemoptysis and on chest X-ray (CXR) all had cavity formation (Table 38). On histology, granuloma formation with caseating necrosis could be demonstrated. Ziehl-Neelsen staining revealed low numbers of acid-fast bacilli in the lungs of all patients, even though all had received at least one month of multidrug therapy, which included rifampicin, isoniazid and pyrazinamide, prior to surgery.

Table 38: Clinical details of patients.
The table shows the basic demographic and clinical characteristics of 5 patients with pulmonary tuberculosis who were recruited for the histological study of lobectomy or pneumonectomy specimens.

	Age/Sex	Extent of disease	Time since previous TB	Pre-operative treatment	Follow-up
B1	23/M	RUL collapse	2 years	1 month	8 years
B2	43/M	LUL cavitation with active TB	3 years	1 month	1 years
B3	33/F	LUL large cavity, RUL and LLL infiltration	11 years	3 months	9 years
B4	31/F	Mediastinal shift left, pleural effusion, scarring RUL	3 years	3 months	4 years
B5	22/M	Cavitations RUL and RML, coughing up ascaris	2.5 years	3 months	None
B6	21/F	Total destruction RUL, cystic lesions in LUL	17 years	2 months	1 month
B7	41/M	R lung collapse, fibrosis. L lung unaffected	6 years	3 months	5 years

Age = years RUL = Right upper lobe; LUL = Left upper lobe; LLL = Left lower lobe; RML = Right middle lobe

6.2.2.1 In Situ Hybridisation and Immunohistochemistry

6.2.2.1.1 Scoring of individual granulomas for cytokine patterns and immunophenotype

Individual granulomas from the seven patients were scored for the presence of

cytokines, phenotypic markers, and caseous necrosis and the data are summarised in Table 39. Each case contained 10 and 40 granulomas. All seven patients had some granulomas positive for IFNγ, TNFα, IL-12p40 and central necrosis. However, only four of the seven patients had any granulomas staining positive for IL-4 (Table 39: patients B3, B4, B5 and B7). These patients tended to have the highest percentage of necrotic granulomas and the lowest percentage of TNFα positive granulomas. Staining intensity did not necessarily correlate with protein levels, being dependent also on the affinity of the primary antibody for the antigen.

Table 39: Cytokine patterns and immunophenotyping of the individual granulomas for seven patients.
All 7 patients had some granulomas positive for IFNγ, TNFα, IL-12 and central necrosis. Only 4 of the 7 patients had any granulomas staining positive for IL-4 and these patients tended to have the highest percentage of necrotic granulomas and the lowest percentage of TNFα positive granulomas.

	Total no. granulomas	Caseous necrosis	IFNγ	TNFα	IL-4	IL-12p40 mRNA
B1	10	2 (20%)	9 (90%)	10 (100%)	0 (0%)	9 (90%)
B2	40	9 (23%)	37 (93%)	30 (75%)	0 (0%)	29 (73%)
B3	12	4 (33%)	11(92%)	4 (33%)	4 (33%)	9 (75%)
B4	15	8 (53%)	10 (67%)	8 (53%)	6 (40%)	11 (73%)
B5	23	9 (39%)	17 (74%)	15 (65%)	5 (22%)	17 (74%)
B6	29	7 (24%)	26 (90%)	21 (72%)	0 (0%)	25(86%)
B7	12	4 (33%)	9 (75%)	4 (33%)	5 (42%)	8 (67%)

Percentage of total granulomas indicated in brackets

6.2.2.1.2 Association between IFNγ, IL-4, TNFα proteins, IL-12p40 mRNA and caseous necrosis in individual granulomas

The data were further analysed for associations between cytokines and necrosis and the results were expressed in contingency tables using Fisher's exact and Mantel Haenszel x2 tests. Fisher's exact test is designed to give robust estimates of significance when

frequencies in the contingency table are low, i.e. scores of zero for some cytokines. The patients were also divided into two groups, those expressing IL-4 in some of their granulomas (patients B3, B4, B5 and B7) and those not expressing IL-4 in any of their granulomas (patients B1, B2 and B6). Dual-parameter associations in the IL-4 positive and negative patients were considered separately.

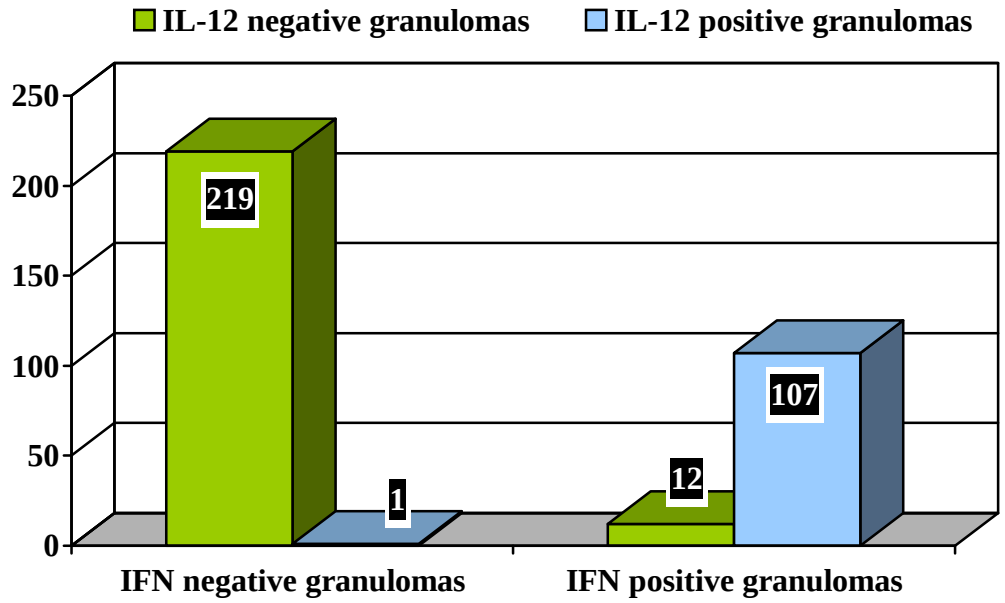
6.2.2.1.2.1 IFNγ AND IL-12P40

Seventy six percent (107 out of 141) of all granulomas were positive for both IFNγ and IL-12. Almost all granulomas staining positive for IL-12p40 were also expressing IFNγ (107 out of 108 granulomas) with only one granuloma positive for IL-12p40 and negative for IFNγ (Table 40). The probability of a granuloma producing IFNγ was significantly greater if the granuloma was also positive for IL-12p40 (fig. 34). However, 12 granulomas were identified which were negative for IL-12p40 but IFNγ positive. Granulomas that were positive for either IFNγ or IL-12 were more likely to be IFNγ positive rather than IL-12p40 positive (P=0.002 McNemar's test). No significant differences between IL-4 positive or negative patients were found with respect to these associations.

Table 40: Correlation between IFNγ protein and IL-12 mRNA in all granulomas
Almost all granulomas staining positive for IL-12p40 were also expressing IFNγ (107 out of 108 granulomas) with only one granuloma positive for IL-12p40 and negative for IFNγ (Chi-square test p = 0.001).

	IFNγ		
IL-12p40	0 (negative)	1 (positive)	Total
0 (negative)	219	12	33
1 (positive)	1	107	108
Total	22	119	141

Figure 34: IFNγ and IL-12p40
The probability of a granuloma producing IFNγ was significantly greater if the granuloma was also IL-12 positive; only 12 IFNγ positive granulomas were IL-12 negative (Chi-square test p=0.001).



6.2.2.1.2.2 IFNγ AND IL-4

In table 41 the correlation between IFNγ and IL-4 in patients with IL-4 positive granulomas demonstrates that no IL-4 positive granulomas were negative for IFNγ, although not all IFNγ positive granulomas were IL-4 positive. IL-4-negative patients also tended to have a higher percentage of IFNγ positive granulomas than the IL-4 positive group (Chi-square test p = 0.001).

Table 41: Correlation between IFNγ and IL-4 in IL-4 positive patients

All granulomas that were IL-4 positive were also IFNγ positive. However, 15 IFNγ positive granulomas were IL-4 negative.

	IFNγ		
IL-4	0 (negative)	1 (positive)	Total
0 (negative)	15	27	42
1 (positive)	0	20	20
Total	15	47	62

6.2.2.1.3 TNFα and caseous necrosis and TNFα and IL-4

It is quite apparent from table 42 that all granulomas with caseous necrosis were TNFα positive. However, in IL-4 negative patients, non-necrotic granulomas were more likely to be TNFα positive than negative. The opposite was true for IL-4 positive patients. In IL-4 positive patients, half of the granulomas were TNFα positive, but only 5 granulomas showed positivity for both IL-4 and TNFα (Table 43).

Table 42: Patients separated into two groups on the basis of the presence or absence of IL-4 and their granulomas scored for TNFα and caseous necrosis.
All granulomas with caseous necrosis were TNFα positive, from both IL-4 positive and negative patients. (P<0.001: Mantel Haenszel).

	Caseous necrosis		
TNFα	0 (negative)	1 (positive)	Total
IL-4 negative patients			
0 (negative)	18	0	18
1 (positive)	43	18	61
Total	61	18	79
IL-4 positive patients			
0 (negative)	31	0	31
1 (positive)	8	25	31
Total	37	25	62

Table 43: Contingency table of TNFα and IL-4 (IL-4 positive patients only).
Half of the granulomas in IL-4 positive patients (B3,B4,B5 and B7) were TNFα positive. However, these TNFα positive granulomas tended to be negative for IL-4. TNFα negative granulomas were equally likely to be IL-4 positive or negative.

	TNFα		
IL-4	0 (negative)	1 (positive)	Total
0 (negative)	16	26	42
1 (positive)	15	5	20
Total	31	31	62

6.3 Controls

Tissue sections taken from 2 patients who underwent surgery for pulmonary malignancies were examined and a histological diagnosis of squamous carcinoma was made in both instances. Sections from the carcinoma were subjected to ISH for IL-12, IL-4, TNFα and IFNγ. The carcinoma cells showed no signs of positivity for any of these cytokines. Due to subsequent technical difficulties photographic records of these controls no longer exist and could not be included in the thesis.

7. Results: A Comparison Between Pleural Tuberculosis, Sarcoidosis and Pulmonary Tuberculosis.

A summary of the results of the various groups in this study can be seen in table 44. Necrotic granulomas were seen in most of the patients with tuberculosis, while no necrosis was present in the sarcoid granulomas. Most granulomas in the pleural tuberculosis group had a Th0 profile, while in sarcoidosis all had a Th1 profile. The pulmonary granulomas had a mixed response.

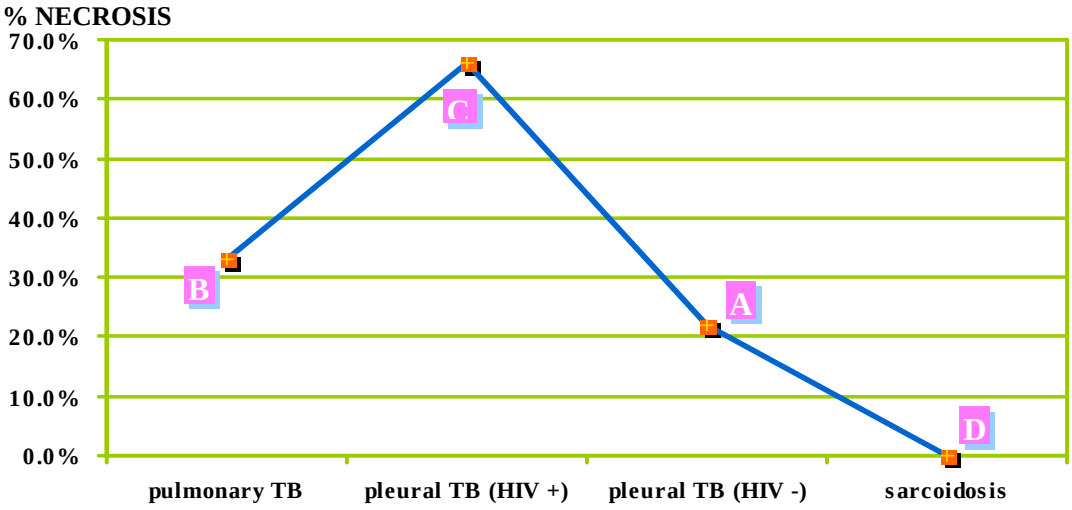
Table 44: Summary of the cytokine profile of individual patients in the pleural tuberculosis, sarcoidosis and pulmonary tuberculosis groups.
The granulomas positive for a specific parameter are expressed as a percentage of the total number of granulomas. All patients with pulmonary tuberculosis had necrotic granulomas, while most of the patients with pleural tuberculosis and none with sarcoidosis had necrotic granulomas. There were clear differences in the Th profile of the various groups.

Group	Nr. of granulomas	Necrosis	IL-12	IFNγ	TNFα	IL-4	Th1	Th0
Pleura +	5	88%	100%	100%	100%	100%	0%	100%
Pleura +	9	30%	100%	100%	100%	67%	33%	67%
Pleura +	10	100%	100%	100%	100%	100%	0%	100%
Pleura +	3	100%	100%	100%	100%	100%	0%	100%
Pleura +	6	63%	100%	100%	100%	100%	0%	100%
Pleura +	8	20%	100%	100%	100%	100%	0%	100%
Pleura -	24	68%	100%	100%	100%	88%	13%	87%
Pleura -	16	6%	100%	100%	100%	100%	0%	100%
Pleura -	16	25%	100%	100%	100%	100%	0%	100%
Pleura -	8	80%	100%	100%	100%	100%	0%	100%
Pleura -	10	0%	100%	100%	100%	100%	0%	100%
Pleura -	51	0%	100%	100%	100%	100%	0%	100%
Sarcoid	103	0%	100%	100%	100%	0%	98%	0%
Sarcoid	1212	0%	100%	100%	100%	0%	100%	0%
Sarcoid	145	0%	100%	100%	100%	0%	100%	0%
Sarcoid	209	0%	100%	100%	100%	0%	100%	0%
Sarcoid	426	0%	100%	100%	100%	0%	100%	0%
Sarcoid	537	0%	100%	100%	100%	0%	100%	0%
Sarcoid	1677	0%	100%	100%	100%	0%	100%	0%
Sarcoid	452	0%	100%	100%	100%	0%	100%	0%
Sarcoid	379	0%	100%	100%	100%	0%	100%	0%
Sarcoid	488	0%	100%	100%	100%	0%	100%	0%
Sarcoid	261	0%	100%	100%	100%	0%	100%	0%
Sarcoid	604	0%	100%	100%	100%	0%	100%	0%
Sarcoid	535	0%	100%	100%	100%	0%	100%	0%
Sarcoid	234	0%	100%	100%	100%	0%	100%	0%
Sarcoid	608	0%	100%	100%	100%	0%	100%	0%
Lung	6	33%	N/A	100%	83%	33%	67%	33%
Lung	7	29%	N/A	86%	71%	43%	43%	43%
Lung	12	50%	N/A	75%	58%	0%	75%	0%
Lung	11	45%	N/A	82%	64%	0%	82%	0%
Lung	9	33%	N/A	67%	78%	44%	22%	44%
Lung	10	20%	90%	90%	100%	0%	90%	0%
Lung	40	23%	73%	93%	75%	0%	93%	0%
Lung	12	33%	75%	92%	33%	33%	58%	44%
Lung	15	53%	73%	67%	53%	40%	27%	55%
Lung	23	39%	74%	74%	65%	22%	52%	29%
Lung	29	24%	86%	90%	72%	0%	90%	0%
Lung	40	33%	67%	75%	33%	42%	33%	63%

Pleura+ = HIV positive group; pleura - - HIV negative group N/A = not applicable

The tissue destruction in granulomatous disease is ascribed to necrosis and in the instance of tuberculosis specifically caseous necrosis. It is clear from the comparison between necrosis in the various groups (fig. 35) that HIV positive pleural tuberculosis is the group with the highest percentage of necrotic granulomas (66%), followed by pulmonary tuberculosis (33%), HIV negative pleural tuberculosis (21%) and sarcoidosis (0%).

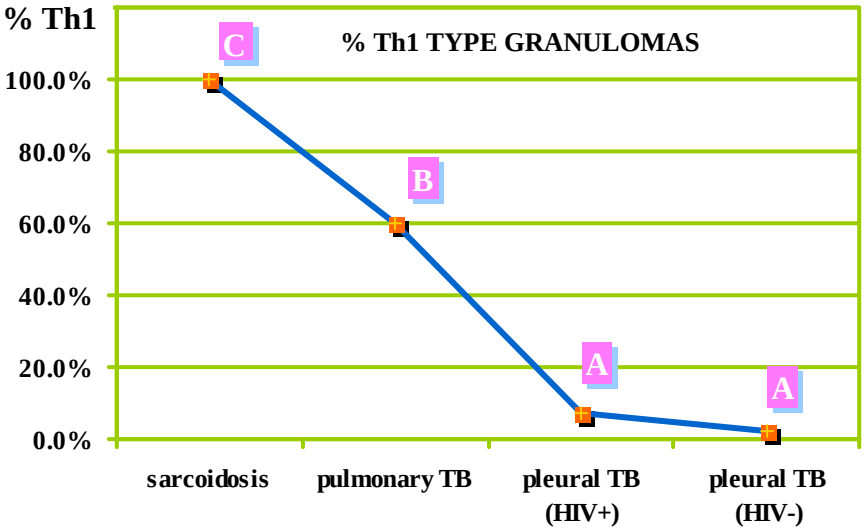
Figure 35: A comparison between necrosis in pleural tuberculosis, pulmonary tuberculosis and sarcoidosis.
Results are expressed as the % granulomas with necrosis. A bootstrap means analysis indicates a statistically significant difference of $p < 0.01$ between the percentage of necrotic granulomas in sarcoidosis and pulmonary tuberculosis, sarcoidosis and pleural (HIV +) tuberculosis, pleural (HIV-) tuberculosis and pulmonary tuberculosis, and between HIV positive and HIV negative granulomas in pleural tuberculosis. A bootstrap analysis illustrates differences by allocating different letters of the alphabet, while similarities are illustrated by allocating the same letter, therefore an A for HIV positive pleural tuberculosis, a C for HIV negative pleural tuberculosis, a B for pulmonary tuberculosis and a D for sarcoidosis indicates a statistically significant difference between each of the individual parameters.

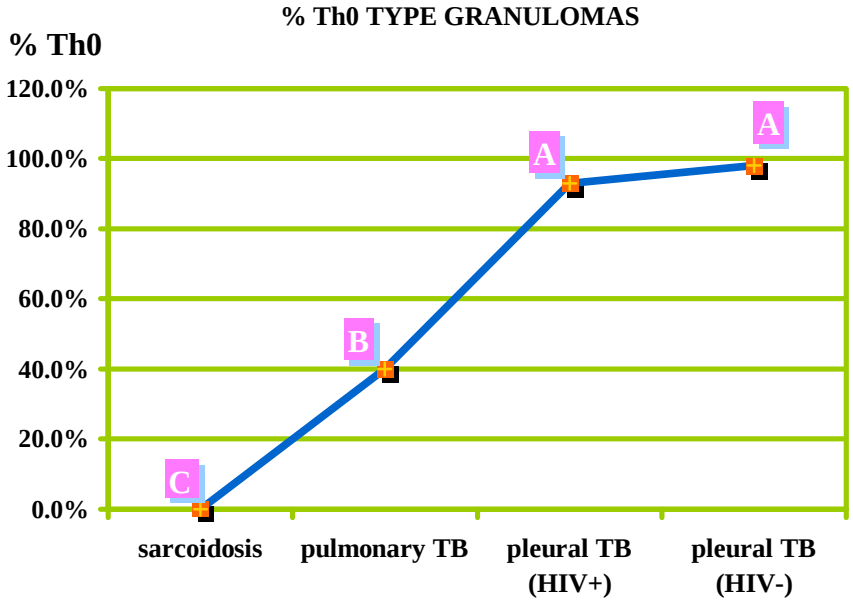


A comparison of the Th profiles of the four groups (fig. 36) indicates that sarcoidosis has the strongest Th1 response (100%), followed by pulmonary tuberculosis, with a 60% Th1 response and lastly pleural tuberculosis as a combined group of HIV positive and HIV negative (average 4,5%). There is a statistically significant difference between

the percentages of Th1 positive granulomas in each of these 3 groups respectively. No statistically significant difference can however be seen between the HIV positive and negative groups of pleural tuberculosis.

Figure 36: A comparison between the Th1 response in pleural tuberculosis, pulmonary tuberculosis and sarcoidosis in the top graph and Th0 in the bottom graph.
Results are expressed as the % granulomas with a specific Th profile. A bootstrap means analysis indicates a statistically significant difference of $p < 0.01$ between the percentage of Th1 positive granulomas in sarcoidosis and pulmonary tuberculosis, sarcoidosis and pleural tuberculosis, pleural tuberculosis and pulmonary tuberculosis, but not between HIV positive and HIV negative granulomas in pleural tuberculosis. A bootstrap analysis illustrates differences by allocating different letters of the alphabet, while similarities are illustrated by allocating the same letter, therefore an A for both HIV positive and negative pleural tuberculosis indicates no statistical significance between the HIV positive and negative groups, but do indicate a statistically significant difference between A (pleural tuberculosis as a group), B (pulmonary tuberculosis) and C (sarcoidosis).





There is however, no statistical difference between the Th1 response in HIV positive (2%) and HIV negative patients (7%). The exact reverse applies to a comparison of the Th0 profiles in the various groups, with sarcoidosis clearly having no Th0 response, pulmonary tuberculosis with a 40% response and pleural tuberculosis with a 98% Th0 response in HIV positive and a 93% Th0 response in HIV negative patients.

8. Discussion

8.1 Pleural Tuberculosis

It has been proposed that pleural tuberculosis in HIV negative patients is characterised by Th1 predominance [244]. This data is usually based on the measurement of cytokine levels in body fluids, or examining cytokine production by immune cells *in vitro*.

This study is the first description of *in situ* hybridisation of cytokine mRNA in pleural biopsies of HIV sero-negative and sero-positive patients. In both groups of patients a Th0 response, characterised by the presence of both Th1 and Th2 cytokines, was noted. Of the 166 granulomas counted in total, only 6 had a Th1 profile, while the remainder all had a Th0 profile. There were however, significant differences in the cytokine profiles between the 2 groups.

All 12 patients with pleural tuberculosis presented with symptoms of pleural effusion, which was confirmed by CXR and a pleural biopsy was performed as a diagnostic procedure. HIV ELISA tests showed that 6 patients were infected with this virus. ISH for IL-12, IFNγ, TNFα and IL-4 was carried out on a total of 166 granulomas in these patients.

It is quite apparent from the results that granulomas in HIV positive patients have a higher incidence of necrosis, as 66% of these granulomas showed necrosis as opposed to 17% in granulomas of HIV negative patients, in keeping with the histological appearance of granulomas in HIV positive patients as described by Lucas[161].

The necrotic granulomas have a tendency towards more IFNγ, TNFα and IL-4 positive cells per granuloma in comparison to the non-necrotic granulomas. TNFα presence in granulomas, especially a Grade 3 (> 75% positive cells) distribution, was associated with a dramatic increase in necrosis. In addition, granulomas with a high number of TNFα positive cells also tend to have a high number of IFNγ positive cells. In the case of IL-12 there is a more equal distribution of the number of cells throughout the granulomas.

In non-necrotic granulomas a significant number have low numbers of cells positive for all of the cytokines tested, and less than 25% of all the non-necrotic granulomas have a high presence of either TNFα or IL-4 mRNA. The granulomas that do have a high number of IL-4 positive cells also have a high number of TNFα positive cells. In the HIV negative group these granulomas tend not to be necrotic. A similar picture can be seen for IL-4 and IFNγ where only 8 non-necrotic granulomas (7%) had high numbers of IL-4 and IFNγ positive cells.

Substantial evidence exists that IFNγ, the hallmark cytokine of the Th1 response, plays a key role in the defence against tuberculosis. IFNγ is a pro-inflammatory cytokine promoting macrophage activation and an inflammatory rich environment that is beneficial for the inhibition and killing of mycobacteria. It is apparent from the literature that IFNγ is crucial in the defence against mycobacteria[71,72]. However, it is also suggested that persistently elevated IFNγ is responsible for the damage caused by granulomatous disease and that moderating cytokines like IL-4 are necessary to down-regulate the IFNγ response to more appropriate levels after effective containment of the

organisms. It is therefore suggested that a dual role exists for IFNγ, where early containment requires sufficient IFNγ levels, but similar levels, without modulation by other cytokines later in disease may lead to tissue destruction.

Recently a virtual model to predict cell mediated immune regulatory mechanisms during human infection with *M tuberculosis* was designed by Wigginton et al[245]. According to this model a high production of IL-4 by Th0 cells is associated with a shift from latency to active disease. On the other hand, a high level of IFNγ is protective and prevents reactivation.

If reactivation does occur, patients may or may not incur tissue damage as a consequence of the immune response. It is not only desirable to suppress bacterial infection, but also to do so in the most efficient and least damaging manner. According to this virtual model[245], an increase in IFNγ beyond the lower limit needed to control infection after reactivation, exacerbates infection. In addition, if the rate of IL-4 decay increases or the production of IL-10 decreases, greater damage will occur.

In the pleural tuberculosis cases, non-necrotic granulomas tend to have lower numbers of IFNγ positive cells in the presence of low numbers of IL-4 positive cells, while the inverse is true for necrotic granulomas. This pattern is in keeping with the virtual model described. An increase in IFNγ correlates with an increase in necrosis, while a decrease of IFNγ positive cells is seen in non-necrotic granulomas.

It is apparent that an intricate balance exists between down-and-up-regulatory immune components. Immune activation is essential for suppression of infection, but once bacterial load is controlled, down-regulation is required to minimise tissue damage. Unfortunately the role of TNFα is not addressed in the model designed by Wigginton[245]. My data suggests, however, together with other studies of TNFα-induced immunopathology[84,85,86,87,88] that this cytokine plays an important role in this balance between essential immune activation on the one hand and immunopathology on the other. The correct timing and amount of TNF production might have a very narrow limit to offer a benefit to the *M. tuberculosis* infected host.

There is a definite correlation between TNFα and necrosis in the pleural tuberculosis granulomas. Although a causative relationship between TNFα and necrosis cannot be deduced from this evidence, it appears likely that TNFα may be involved in necrosis development[277]. The caseous necrosis is a consequence of apoptosis of infected macrophages and activated T cells within the granuloma[278] and TNFα is a known inducer of apoptosis[279].

A strong positive correlation exists between high numbers of TNFα positive cells and HIV positivity. This finding supports the fact that TNF regulates HIV transcription and that higher TNF levels are expected in HIV positive patients[246]. Further support of this finding is the fact that there is a more even distribution of the numbers of TNFα positive cells in HIV negative granulomas. The IL-4 and IFNγ profiles follow a similar pattern to that in necrotic granulomas, with more cells positive for these cytokines in HIV positive than in HIV negative granulomas. IL-12 positivity is once again more

evenly distributed, with 31% granulomas containing <25% positive cells in HIV negative granulomas and 31% containing >75% positive granulomas in HIV positive granulomas.

When exploring the relationship between the various cytokines in HIV negative granulomas it is found that there is a very strong relationship between IFNγ and TNF, IL-4 and TNFα, IL-12 and IFNγ as well as between IL-12 and IL-4, with a predominantly low number of positive cells for these cytokines in HIV negative granulomas. IL-12 and TNFα positivity is also linked, but a more even distribution of the number of positive cells is observed.

On the other hand, the higher number of cytokine positive cells in HIV positive granulomas is reflected in the relationship between all the various cytokines, except for the relationship between IL-12 and TNFα, where most granulomas have a very high number of TNFα positive cells and an intermediate number of IL-12 positive cells.

The observation that more cytokine positive cells are seen in HIV positive granulomas supports the theory that an overproduction of cytokines may be seen in HIV positive patients as a mechanism to compensate for the failure of another immune effector mechanism[122]. The presence of mRNA of course does not assure that the cytokines are produced, secreted or effective. It is therefore possible that the high number of positive cells is an indication of a failed attempt to induce cytokine production.

On exploring the association between HIV status and necrosis in more depth it is found that in HIV negative granulomas without necrosis, a strong positive correlation exists between all the cytokines. Granulomas in this group have a tendency towards fewer positive cells per granuloma than in any of the other groups. The inference from this is that fewer cells positive for cytokines, and possibly therefore less cytokines produced, are beneficial to the patient, as destruction does not occur. The inverse appears to be true for necrotic granulomas in the HIV negative group of patients.

Although the association between the various cytokines in the HIV negative granulomas with necrosis is rarely statistically significant, there is a very clear association between IFNγ and TNFα, with 52% of all granulomas in this group containing high numbers of positive cells for both these cytokines. The trend followed by the other cytokines is similar. On examination of individual cytokines, a statistically significant relationship can be identified between necrotic granulomas and IFNγ as well as necrotic granulomas and TNFα. In both instances more than half of the necrotic granulomas have more than 75% positive cells of each cytokine.

In the group of HIV positive granulomas with necrosis, there is also a strong positive correlation between IFNγ and TNFα, as well as IFNγ and IL-4, but a strong negative correlation between IL-12 and TNFα. On investigation of the HIV positive granulomas the only individual cytokine with a statistically significant result is IFNγ with a low number of positive cells in necrotic granulomas. This is in direct contrast to the HIV negative group where necrotic granulomas contained a high number of IFNγ positive cells. Overall the IFNγ presence is however still higher in HIV positive granulomas

than HIV negative granulomas. This finding concurs with several studies confirming an increase of IFNγ in HIV positive patients with tuberculosis[247,248].

TNFα has a central role to play both in the host immune response to *M.tb* and the immunopathology of tuberculosis[85]. The release of TNFα in response to *M.tb* has several beneficial effects[86], and is required for granuloma formation, which is accepted to be protective and indicates a successful immune response. Other benefits include regulation of chemokines and leukocyte recruitment[88].

Excessive production of TNFα and an increased sensitivity to the cytokine have, however, been implicated in the immunopathology of tuberculosis, for example caseous necrosis[96]. Although TNFα is an essential component of the host immune response against mycobacterial infection, high levels of the cytokine at the site of infection may induce an excessive inflammatory response that overwhelms the beneficial effects of the cytokine.

It may also be possible that Th2 cytokines mediate local tissue inflammation and necrosis through their effect on TNFα induced cytotoxicity[97,98]. The relationship between the various cytokines in the current study supports the theory that high IFNγ levels later in disease may be detrimental, and there is a suggestion that this may be especially important in the presence of high numbers of TNFα positive cells.

Significant immune activation in HIV positive patients is demonstrated by the current findings, and both the Th1 and Th2 arms appear to be excessively stimulated. The

present study and the virtual model by Wigginton suggest that such cytokine overproduction is detrimental to the patient[245].

The presence of numerous IL-12 positive cells appears to have an impact on the presence of other cytokine positive cells in the granuloma. More IFNγ, TNFα and IL-4 positive cells can be seen in the presence of high numbers of IL-12 positive cells, when the patients are HIV negative. This relationship is of course interchangeable and the statement that IL-12 positive cells have an impact on the presence of other cytokine positive cells is just as applicable to any of the other cytokines.

It is apparent that necrotic granulomas contain higher numbers positive cells, especially IL-4 and TNFα, but also IL-12 and IFNγ. On the other hand, high numbers of IL-12 cells are less prevalent in necrotic granulomas. In the non-necrotic granulomas with high numbers of IL-12 positive cells, high numbers of IL-4 positive cells can also often be seen. The high number of IL-4 positive cells does not appear to have an effect on necrosis. It is possible that this may be due to the influence of the IL-12 positive cells. On the other hand, the presence of high numbers of IL-4 positive cells in these granulomas may in itself be a regulatory presence and prevent the development of necrosis by acting as opposition to necrosis inducing factors.

The number of IFNγ positive cells appears to be higher in non-necrotic granulomas when numerous IL-12 cells are present, but lower when less IL-12 positive cells can be seen. This is of course in keeping with the function of IL-12 as an IFNγ regulatory cytokine[16,23]. In necrotic granulomas the opposite is true, with more IFNγ positive cells

in necrotic granulomas where there are less IL-12 positive cells. Very few necrotic granulomas contain high numbers of IFNγ positive cells in the presence of high numbers of IL-12 positive cells. This finding may suggest some breakdown in the regulatory mechanism of IL-12.

High numbers of IL-4 cells are also present in granulomas with a high number of IFN positive cells, but there is an equal distribution between necrotic and non-necrotic granulomas. This suggests that although IL-4 may have a regulatory effect on the possible detrimental impact of high levels of IFNγ at this stage of disease according to the virtual model by Wigginton, this definitely is not the only, or even the most important controlling factor.

In tuberculosis, the first step in host defence is the ingestion and uptake of *M. tb* organisms by alveolar macrophages and other cells. Apoptosis is an important host defence mechanism to contain mycobacterial survival and growth[233,234]. By inducing apoptosis of macrophages containing organisms, the organism is deprived of its intracellular sanctuary and the process of apoptosis therefore favours the host[233,234]. It is possible that dissemination in tuberculosis depends at least in part on a paucity of apoptosis. Caseous necrosis in granulomas is also associated with an increase in apoptotic activity. Within the granuloma, apoptosis is prominent in the epithelioid cells as demonstrated by condensed chromatin viewed by light microscopy or with the *in situ* terminal transferase mediated nick end labelling (TUNEL) technique[249]. The effects of Fas L- mediated or TNFα-induced apoptosis on *M. tuberculosis* viability in human and mouse macrophages is controversial; some studies report reduced bacterial numbers

within macrophages after apoptosis[250] while others indicate this mechanism has little antimycobacterial effect[251]. Insufficient T cell apoptosis may also interfere with clonal deletion and maintenance of tolerance, resulting in inappropriate T cell accumulation, contributing to chronic inflammation. This may lead to maintenance of the granulomas and to consequent fibrosis[235].

Detailed analysis of human tuberculous tissues revealed that apoptotic CD3+, CD45RO+ cells are present in productive tuberculous granulomas, particularly those containing a necrotic centre[252]. Studies carried out by Raja have demonstrated the ability of mycobacterial antigens to bring about apoptosis in animal models[253]. In addition, increased spontaneous apoptosis, which is further enhanced by mycobacterial antigens, has also been shown to occur in pleural fluid cells[254].

In the pleural tuberculosis cases in the current study, a definite increase in the number of TNFα mRNA positive cells was observed in necrotic granulomas. In the pulmonary tuberculosis patients all the necrotic granulomas stained positive for TNFα mRNA or protein. It is apparent that there is an association between TNFα and necrosis in tuberculous granulomas. This association may be ascribed to the increased apoptotic activity of TNFα.

8.1.1 In summary

This study is the first description of *in situ* hybridisation of cytokine mRNA in pleural biopsies of HIV sero-negative and sero-positive patients. In both groups of patients a Th0 response, characterised by the presence of both Th1 and Th2 cytokines, was noted.

Granulomas in HIV positive patients have a higher incidence of necrosis than granulomas in HIV negative patients. There is a clear association between TNFα and necrosis in tuberculous granulomas that may possibly be ascribed to the increased apoptotic activity of TNFα.

The cytokine pattern is in keeping with the virtual model described by Wigginton. An increase in IFNγ correlates with an increase in necrosis, while a decrease of IFNγ positive cells is seen in non-necrotic granulomas. This supports the theory that high IFNγ levels later in disease may be detrimental, and there is a suggestion that this may be especially important in the presence of high numbers of TNFα positive cells.

A strong positive correlation exists between high numbers of TNFα positive cells and HIV positivity. This finding supports the fact that TNF regulates HIV transcription and that higher TNF levels are expected in HIV positive patients, as well as the theory that an overproduction of cytokines may be seen in HIV positive patients as a mechanism to compensate for the failure of another immune effector mechanism.

In the non-necrotic granulomas with high numbers of IL-12 positive cells, high numbers of IL-4 positive cells can also often be seen. The high number of IL-4 positive cells does not appear to have an effect on necrosis. It is possible that this may be due to the influence of the IL-12 positive cells. On the other hand, the presence of high numbers of IL-4 positive cells in these granulomas may in itself be a regulatory presence and prevent the development of necrosis by acting as opposition to necrosis inducing factors.

Very few necrotic granulomas contain high numbers of IFNγ positive cells in the presence of high numbers of IL-12 positive cells. There were also granulomas with high numbers of IFNγ positive cells in the presence of less IL-12 positive cells. This finding may suggest some breakdown in the regulatory mechanism of IL-12.

8.2 Sarcoidosis

Sarcoidosis is a multi-system granulomatous disease of unknown cause, although several organisms have been investigated as a possible cause, amongst these *M. tb*[192,193,194,195]. The disease is characterised by non-caseating granulomas, which either resolve or progress to fibrosis. The granulomas are well formed, consisting of tightly clustered epithelioid macrophages, usually with Langhans or foreign body-type giant cells, and often lacking significant surrounding inflammation. Concentric fibrosis, surrounding the granuloma, is often a feature.

This fibrosis represents the major cause of morbidity and mortality in pulmonary sarcoidosis. Histologically it would appear that granulomas act as a focus for fibrosis and that fibrosis is not an independent process. This leads to fibrosis becoming more diffuse[255,256,257]. Clinical support for this is found in the improvement of symptoms and organ function in patients with sarcoidosis receiving some form of immunosuppressive treatment[257]. It can be concluded therefore that propagation or preservation of granulomas eventually lead to fibrosis and severe disease.

The investigations performed in the current study confirm the presence of a very strong Th1 response in all the cases of sarcoidosis as has been described in other studies[208,209,210]. A prominent IL-12, IFNγ and TNFα signal was observed, but a complete absence of IL-4 was noted. The patients all had at least stage II radiological disease and therefore fall in the group of those with chronic disease according to the findings by Möllers et al[201]. This stratification was based on comprehensive clinical data[258,259] and may differentiate between patients with a high likelihood of spontaneous

remission and those with a more prolonged course of disease. One draws the conclusion that their differentiation between acute (spontaneous remission) and chronic (prolonged disease) is analogous to the terms inactive and active disease in other publications.

In the patients with acute disease and a good prognosis, described by Möllers et al[201] the level of expression of Th1 cytokines, specifically IFNγ, IL-2 and TNFα, were much higher than in the patients with chronic disease, while the Th2 cytokine expression level, including IL-4, IL-10 and IL-5 was very low in both groups. Similar results were seen in other studies[207,211].This suggests that a high level of Th1 cytokines is required for a good prognosis. It has also been shown that patients with high serum levels of IFNγ have better chance of achieving complete resolution after corticosteroid treatment.

The strong expression of IFNγ may be responsible for the absence or suppression of the Th2 cytokines as IFNγ is known to be a Th2 suppressor. In addition, however, IFNγ acts as a promoter for accessory macrophage function, which may lead to the production of more inflammatory cytokines. The result of the IFNγ associated promotion of macrophage activity is the production and release of additional TNFα. There is convincing evidence that overexpression of TNFα is associated with persistence of inflammation and the development of fibrotic lung disease[260,261]. In addition, chronic sarcoidosis patients with corticosteroid resistant disease, studied by Ziegerhagen et al[262], are characterised by a significantly increased release of TNFα by

cultured alveolar macrophages. IFNγ also has a profibrotic effect under certain circumstances[263].

Supporting evidence for the adverse effect of the Th1 subset is provided by the high levels of TGFβ and the negative correlation between IL-2, a Th1 cytokine, and TGFβ found in patients with acute remitting disease[264], where patients who have gone into remissions expressed high levels of TGFβ and low levels of IL-2. TGFβ is known to have a suppressive effect on the Th1 response and an increased release in TGFβ is associated with spontaneous remission. In addition IL-12 levels have been shown to be higher in sarcoid patients who worsened without therapy[230].

Although it appears that a pure Th1 response may have an adverse effect on the disease, primarily through TNFα, a shift to a predominant Th2 response promotes deposition of fibronectin and other collagen matrix products from recruited fibroblasts[265]. It has been shown that IL-4 stimulates collagen production in fibrosing alveolitis, while INFγ inhibits fibrogenesis. Evidence of increased TGFβ expression causing extracellular matrix deposition in fibrotic lung diseases has also been found[266,267]. On the one hand a pure Th1 response appears to be present in patients with with periods of recurrence and remission of disease, but on the other hand IFNγ is also a promoter of TNFα production, which is associated with fibrosis and progressive disease. Suppression of Th1 by TGFβ appears to have a positive effect, whereas a strong Th2 shift has a negative effect.

The critical question is whether the Th1 response is purely beneficial in sarcoidosis or whether the effect of Th1 cytokines depends on the stage of disease. An early pure Th1 response may be beneficial to the patient if it effectively clears the granuloma-inducing antigen. In chronic disease where failure to remove the antigen has resulted in progression of granulomas with subsequent fibrosis, a pro-inflammatory Th1 response may be detrimental to the patient and minimising of this detrimental effect is needed. It therefore appears that a fine balance between excessive pro-inflammatory action (Th1) and a Th2 shift is essential for the best possible prognosis in sarcoidosis.

In the current study strong evidence of a Th1 response and no evidence of a Th2 response was found. All the patients had radiological evidence of pulmonary involvement and all but one had at least a moderate degree of overall disease severity. Although four of the patients had no signs of fibrosis, there was no difference in the cytokine profile between these four and the remainder of the patients. Furthermore no difference in cytokine profile was found between those patients with a favourable and those with an unfavourable clinical course.

Apoptosis results from the activation of an internally encoded suicide program induced by a variety of extrinsic and intrinsic signals. It is a form of cell death that serves to eliminate cells that are no longer needed[232]. Apoptosis is the net result of the balance between pro-apoptotic and anti-apoptotic stimuli, including cytokines, death receptors, caspases, mitochondria bcl-2 proto-oncogenes and certain tumour-suppressor genes. TNFα is an established pro-apoptotic cytokine, released during the inflammatory process.

The composition of the subset of lymphocytes in sarcoidosis depends on the balance of immigration, local proliferation, apoptosis and migration (removal)[268]. The removal of activated lymphocytes by apoptosis is one prerequisite for the resolution of inflammation[269]. The balance of pro-inflammatory cytokines promoting granuloma growth and resolution of tissue inflammation via cell apoptosis in combination with immunosuppressive cytokines may be one of the reasons why spontaneous remission and aggravation occur in sarcoidosis. Failure of cells to die might be one mechanism contributing to the maintenance of immune granulomas, characterised by persistence of inflammation at the site of disease activity and resulting in fibrosis.

It is interesting to note that in patients with chronic, fibrotic disease high levels of TNFα are found. As TNFα is a potent inducer of apoptosis one would expect it to be absent. However, dysregulation of the TNF receptor family may prevent TNFα induced apoptosis. It may be possible that more TNF is produced in an effort to overcome this dysregulation and in the process fibrosis is induced.

The strong presence of Th1 cytokines as well as TNFα in the sarcoidosis patients in this study supports the theory that a strong Th1 presence in conjunction with TNFα may induce fibrosis, as most of these cases showed signs of at least focal fibrosis.

A spectrum of changes was observed in the histological grading. Most of the cases (10) showed changes in keeping with moderate overall severity. Only one cases showed minimal disease and 4 severe disease. Diffuse fibrosis was present in 8 cases and focal

fibrosis in 3. Four cases had no fibrotic change. None of these differences in the histological grading are reflected in the cytokine mRNA profile of these cases.

None of the differences in the histological grading are reflected in the cytokine mRNA profile of these cases. It is possible that the cytokine profile does not reflect the histological grade of disease or that the cytokine profiles may reflect various stages of the disease. Patients with little or focal fibrosis might possibly be in an earlier stage of disease, when a Th1 response is expected and possibly beneficial to the patients. Cases with more established and diffuse fibrosis may represent a much later stage of disease, where the presence of a pure Th1 response may be detrimental to the patient.

8.2.1 In summary

In the current study strong evidence of a Th1 response and no evidence of a Th2 response was found. All the patients had radiological evidence of pulmonary involvement and all but one had at least a moderate degree of overall disease severity. Although four of the patients had no signs of fibrosis, there was no difference in the cytokine profile between these four and the remainder of the patients. Furthermore no difference in cytokine profile was found between those patients with a favourable and those with an unfavourable clinical course.

The strong presence of Th1 cytokines as well as TNFα in the sarcoidosis patients in this study supports the theory that a strong Th1 presence in conjunction with TNFα may induce fibrosis, as most of these cases showed signs of at least focal fibrosis.

8.3 Pulmonary Tuberculosis

The tissue sections of two of the patients contained granulomas staining positive for only for TNFα and IFNγ and negative for IL-4 mRNA. According to the dogma[270], these patients are considered to be launching an appropriate Th1 response[271,272]. In three of the patients the sections contained some granulomas staining positive for only TNFα and IFNγ and other granulomas staining positive for all three cytokine mRNAs. Accordingly these patients are regarded to have a mixed, or Th0 response. The presence of both types of granulomas in one patient casts doubt on the precise distinction between Th1 and Th0 responses. It might be more suitable to assess individual granulomas and confine this classification to individual granulomas.

Previously the presence of IL-4 in patients with TB has been has been regarded as an inappropriate immune response, indicative of a poor prognosis. Based on this the patients with a Th0 pattern should be expected to have a poorer outcome than the patients with a Th1 cytokine pattern. Despite this difference in cyokine patterns however, all five patients were successfully treated for TB and were disease free at last follow-up, which was at least two years post-surgery. This implies a Th0 response may not be an indication of bad prognosis, but may be an important component of granuloma formation in tuberculosis. The possibility exists that it may have a function in the control of tissue damage.

In the second group of patients, immunohistochemistry to determine the cytokine profile and mRNA ISH was performed on lung tissue sections of seven patients with

pulmonary tuberculosis. All seven of the patients stained positive for IFNγ and TNFα protein as well as IL-12p40 mRNA. All patients had necrotic granulomas. Despite a similar clinical and histological appearance, only four patients had granulomas staining positive for IL-4 protein, which is comparable to the results observed in the first group of patients where three of the five had granulomas positive for IL-4 mRNA. It is apparent that two-thirds of the patients had granulomas positive for IL-4. This discrepancy in the cytokine profile was again not reflected in the clinical course of the patients. There was no difference in outcome between the IL-4 positive and IL-4 negative patients. Unfortunately the patient numbers are very small and positive conclusions cannot be drawn from this observation.

The patients with tuberculosis described in the second group can be divided into an IL-4 positive and an IL-4 negative group. This is similar to the immune response described in the two extremes of leprosy, where tuberculoid leprosy, with a Th1 response is regarded as having an effective immune response and lepromatous leprosy with a Th2 response being regarded as having an ineffective response. This is not reflected in the clinical follow-up of these patients. According to the available data these patients have been successfully treated for tuberculosis.

The four patients who had granulomas positive for IL-4 also had a lower number of IFNγ and TNFα positive granulomas than patients who had no IL-4 positive granulomas. This is in direct opposition to the result from the first group of patients, where the IL-4 positive patients had more IFNγ and TNFα positive granulomas than the IL-4 negative group. This phenomenon may serve as an indication that these

patients have less ability to launch an effective Th1 immune response. These granulomas were however always associated with IFNγ positivity in the same granulomas, as well as IL-12 mRNA positivity in most cases. Furthermore, none of these granulomas were necrotic. It is quite apparent that it is virtually impossible to fit the cytokine patterns in these patients into the existing dogma of Th1 versus Th2. It has also been suggested in the literature that IL-4 knockout mice were more susceptible to *M. tuberculosis*, which suggests that IL-4 may have a protective role in tuberculosis[273].

There is a distinct difference in the overall picture between the first and the second group of patients, where in the first group IL-4 positive patients had more IFNγ and TNFα positive granulomas and less necrotic granulomas than the IL-4 negative group, while in the second group IL-4 positive patients had less IFNγ and TNFα positive granulomas and more necrotic granulomas than the IL-4 negative group. However, on an individual granuloma level, the cytokine profile is distinctly similar. In both groups all IL-4 positive granulomas are also IFNγ positive and these granulomas tend to be non-necrotic, as most of the IL-4 positive granulomas are non-necrotic. On the other hand, most of the necrotic granulomas are TNFα positive and negative or weakly positive for IL-4 and IFNγ.

Most adults with active tuberculosis have secondary disease due to reactivation of latent disease or re-infection. One of the burning questions in tuberculosis research concerns the nature of the initial event that triggers reactivation. The virtual model discussed earlier in the pleural tuberculosis section [245], postulates that a high production

of IL-4 by Th0 cells is associated with a shift from latency to active disease, while a high level of IFNγ is protective and prevents reactivation. If reactivation does occur, patients may or may not incur tissue damage as a consequence of the immune response. According to the virtual model, an increase in IFNγ beyond a lower limit that would control infection, exacerbates infection, and if the rate of IL-4 decay increases or the production of IL-10 decreases, greater damage will ensue.

The predictions of this virtual model are supported by evidence in the literature as reviewed by Shelley Rhodes in 2002[74]. Several studies of mouse models suggest that IL-4 has a controlling effect on IFNγ and that this control may be at the level of dendritic cells. IFNγ appears to be crucial for protection against reactivation, but once this has occurred, IFNγ may be active in the immunopathology of tuberculosis.
In comparing the current results with this virtual model, a similar picture emerges. A strong IFNγ response can be seen in smaller non-necrotic granulomas, while IL-4 is absent or weak. Larger granulomas still show an IFNγ presence, but IL-4 expression is now present or strongly positive. In the necrotic granulomas TNFα is the predominant cytokine, with IL-4 and IFNγ either absent or weakly positive.

In a study by Fuller et al [274] on Cynomolgus Macaques, it was found that there was abundant IFNγ and TNFα mRNA expression in both necrotic and non-necrotic granulomas, with no difference in expression between these two groups. No IL-4 mRNA expression was found. This finding illustrate the importance of IFNγ and TNFα presence in driving granuloma formation.

It has been suggested in the literature that the effect of TNFα may be influenced by the cytokine environment in which TNFα is produced.[275,276]. On the background of a Th1 response TNFα may be advantageous to the patient and augment control of the organism. However, when TNFα is produced on a background of in a site with a Th0 or Th2 response it may cause significant damage to the tissue[275,276]. In contrast to this dogma, all the patients described here had signs of major disease pathology. The patients produced TNFα against both aTh1 and a Th0 background. When assessing individual granulomas, the circumstances may be more intricate. While TNFα is positive in most necrotic granulomas, IL-4 and IFNγ are generally negative or weakly positive. There appears to be an inverse relationship between IL-4 and IFNγ on the one side and TNFα on the other. In looking at the evolution of granulomas, the earliest granulomas appear to be predominantly Th1, with a very small amount of IL-4. Once the granuloma grows larger, an increase in IL-4 and TNFα is noted, while there is a decrease in IFNγ. In the necrotic granuloma however, very little IL-4 and IFNγ is present, whereas a strong TNFα presence can be noted.

All necrotic granulomas from all the patients stained positive for TNFα. Although this is not sufficient evidence to prove that there is a direct relationship between TNFα and necrosis, it seems possible that TNFα may be involved in the development of necrosis[277]. The caseous necrosis is likely to be due to apoptosis of infected macrophages and activated T cells in the granuloma[278] and TNFα is known to induce apoptosis[279]. IL-4 positive patients had more necrotic granulomas than IL-4 negative patients and most of the non-necrotic granulomas were TNFα negative. This suggests

that IL-4 positive patients are more inclined to develop necrosis due to induction by TNFα than IL-4 negative patients. The relationship is however unclear, as most of the necrotic granulomas were IL-4 negative.

8.3.1 In summary

The presence of IL-4 may not be an indicator of poor prognosis in pulmonary tuberculosis patients but rather may be an integral feature of tuberculous granuloma formation with a role in controlling tissue damage.

There is a distinct difference in the overall picture between the first and the second group of patients. However, on an individual granuloma level, the cytokine profile is distinctly similar. In both groups all IL-4 positive granulomas are also IFNγ positive and these granulomas tend to be non-necrotic, as most of the IL-4 positive granulomas are non-necrotic. Conversely, most necrotic granulomas are TNFα positive and negative or weakly positive for IL-4 and IFNγ. These results support the virtual model described by Wigginton.

All necrotic granulomas from all the patients stained positive for TNFα. Although we cannot extrapolate from these findings to a causative relationship between TNFα and necrosis, it appears likely that TNFα may be involved in necrosis development. The caseous necrosis appears to be a consequence of apoptosis of infected macrophages and activated T cells within the granuloma and TNFα is a known inducer of apoptosis.

IL-4 positive patients tended to have more necrotic granulomas than IL-4 negative patients and almost all non-necrotic granulomas were TNFα negative. This may imply that in IL-4 positive patients it is more likely that TNFα will induce necrosis in the granuloma that in those patients who are IL-4 negative. However, most of the necrotic granulomas were IL-4 negative.

An essential role for IL-12 and IFNγ in protective immunity to mycobacterial infection has been described. Individuals who are susceptible to disseminated mycobacterial infection have been shown to have defects in their receptors for IL-12 or IFNγ. Also, in patients with tuberculosis, decreased IFNγ production by peripheral blood mononuclear leucocytes has been shown to correlate with decreased IL-12 receptor subunits B1 and B2. In the tuberculous granulomas studied here, 91% of the granulomas gave the same pattern of cytokine expression for IL-12p40 and IFNγ being either positive or negative for both cytokines, as expected from the literature.

8.4 A Comparison between Pleural Tuberculosis, Pulmonary Tuberculosis and Sarcoidosis

A summarised schematic representation of the findings in this study can be seen in fig. 37 at the end of this chapter. The formation of granulomas at the site of antigen presentation in both tuberculosis and sarcoidosis is an essential component of host immunity for controlling infection. This process is dependant on the activation of T lymphocytes, especially IFNγ secreting CD4+ cells. Granuloma formation however, is a complex process that not only requires the activation of lymphocytes, but also their recruitment with monocytes to the site of infection and arrangement into a granuloma.

In tuberculosis the presence of macrophages and lymphocytes facilitate the activation of bactericidal mechanisms in infected macrophages by T-cell derived cytokines. Some bacteria unfortunately survive within the macrophages and persistent antigenic stimulation leads to chronic granuloma formation. These granulomas contain the infection and prevent dissemination to other organs, but they are also responsible for pulmonary immunopathology. In sarcoidosis similar events occur when the unknown antigen cannot be eliminated.

TNF plays an important role in the regulation of granuloma formation. TNF is a highly potent cytokine with a wide range of activities. It is essential for the early induction of chemokines and the subsequent leukocyte recruitment. It is also essential for the

differentiation of macrophages into epithelioid cells and the maintenance of granulomas.

It is apparent that granulomas are essential for the elimination or containment of intracellular antigens. The continuous propagation of granulomas is however, detrimental to the host as immunopathology ensues. In tuberculosis this manifests as caseous necrosis, with destruction of tissue and subsequent fibrosis, whereas in sarcoidosis fibrosis develops directly, without the intervention of necrosis.

In the pleural tuberculosis cases, non-necrotic granulomas tend to have lower numbers of IFNγ positive cells in the presence of low numbers of IL-4 positive cells, while the converse is true for necrotic granulomas. This pattern is in keeping with the virtual model described by Wigginton. An increase in IFNγ correlates with an increase in necrosis, while a decrease of IFNγ positive cells is seen in non-necrotic granulomas. Although the association between the various cytokines in the HIV negative granulomas with necrosis is rarely statistically significant, there is a very clear association between IFNγ and TNFα, with 52% of all granulomas in this group containing high numbers of positive cells for both these cytokines.

High numbers of IL-4 cells are also present in granulomas with a high number of IFN positive cells, but there is an equal distribution between necrotic and non-necrotic granulomas. This suggests that although IL-4 may have a regulatory effect on the possible detrimental impact of high levels of IFNγ at this stage of disease according to

the virtual model by Wigginton, this definitely is not the only, or even the most important controlling factor.

In the pleural tuberculosis cases in the current study, a definite increase in the number of TNFα mRNA positive cells was observed in necrotic granulomas. It is apparent that there is an association between TNFα and necrosis in pleural tuberculous granulomas. This association may possibly be ascribed to the increased apoptotic activity of TNFα.

As has been described earlier in the discussion, in pulmonary tuberculosis there appears to be an inverse relationship between IL-4 and IFNγ on the one side and TNFα on the other. In looking at the evolution of granulomas, the earliest granulomas appear to be predominantly IFNγ positive, with a very small amount of IL-4. Once the granuloma expands, an increase in IL-4 and TNFα is noted, while there is a decrease in IFNγ. In the necrotic granuloma however, very little IL-4 and IFNγ is present, whereas a strong TNFα presence can be noted.

All necrotic granulomas from all the patients were positive for TNFα and it appears likely that TNFα may be involved in necrosis development, due to apoptosis of macrophages and T cells[278]. It also appears that TNFα is more likely to induce necrosis in IL-4 positive patients than in IL-4 negative patients, even though most of the necrotic granulomas were IL-4 negative. Supportive evidence for this phenomenon is also found in the fact that necrosis is not present in the absence of IL-4, as observed in sarcoidosis. It is therefore clear that TNFα, most probably through inducing apoptosis, may have a significant effect on the course of tuberculosis.

Although there are differences between the cytokine profile in pleural and pulmonary tuberculosis, the common thread is that of a high TNF presence in necrotic granulomas, associated with an IL-4 presence. The relationship between IFNγ, IL-4 and necrosis as described by Wigginton, is not as readily apparent in the current study. In pleural tuberculosis this trend is evident, but in pulmonary tuberculosis the changes are more subtle, but still present.

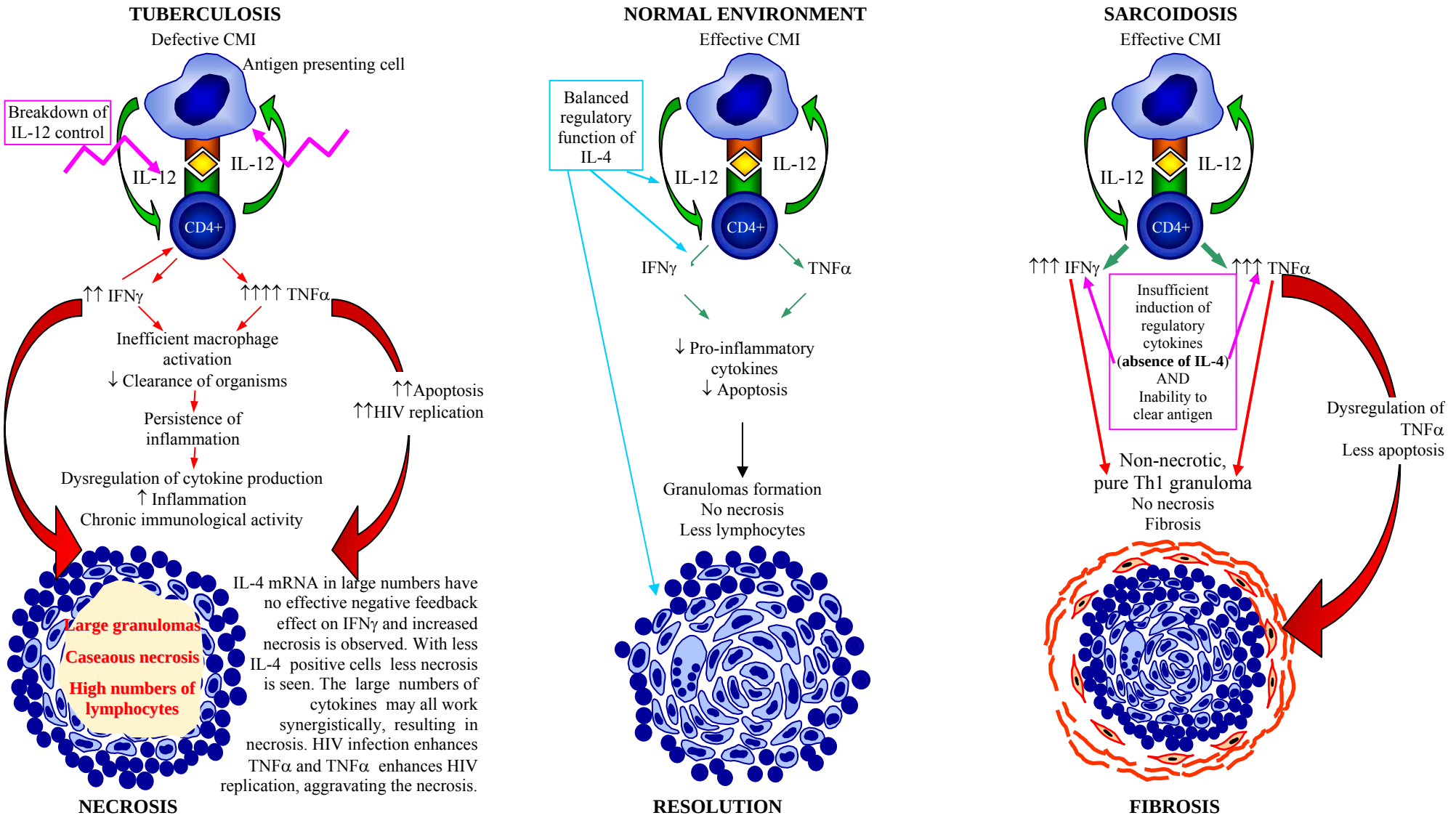
In sarcoidosis on the other hand, there is convincing evidence that overexpression of TNFα is associated with persistence of inflammation and the development of fibrotic lung disease[260,261] as well as with corticosteroid resistant sarcoidosis[262]. From the literature it is clear that a raised TNFα is a strong indicator of poor prognosis in sarcoidosis.

In contrast to the relationship between TNFα and necrosis in tuberculosis the relationship in sarcoidosis is between TNFα and fibrosis. The common denominator in both is however, quite clearly TNFα. It is clear that TNF is needed for granuloma formation and the consequent removal or containment of the causal antigen. However, a continuous presence of TNFα results in the development of immunopathology with subsequent permanent damage to the lungs.

It appears therefore, that the dogma of a pure Th1 response being beneficial is not entirely true in either tuberculosis or sarcoidosis. It is possible that the effect of Th1 cytokines depends entirely on the stage of disease. An early pure Th1 response may be beneficial to the patient if effectively clearing the granuloma inducing antigen. At this

stage a Th2 presence will be harmful to the patients as clearing of the antigen will not be as effective. In chronic disease where failure to remove the antigen results in progression of granulomas with subsequent necrosis and/or fibrosis, a pro-inflammatory Th1 response may be detrimental to the patient and minimising of this detrimental effect is needed. It therefore appears that a fine balance between excessive pro-inflammatory action (Th1) and a Th1/Th2 shift are essential for the best possible prognosis in both these granulomatous diseases.

Figure 37: SUMMARISED SCHEMATIC REPRESENTATION OF THE FINDINGS IN THIS STUDY



9. Conclusions and Future Research

My hypothesis proposed: *"Cytokine profiles determine clinical and histopathological phenotypes of disease. This thesis tests the hypothesis that this will be reflected by cytokine expression profiles in granulomas in different forms of tuberculosis and in sarcoidosis."* To investigate this, *in situ hybridisation* assessment of the cytokine profile of each of these diseases as well as a comparison between the various profiles was performed, with the following conclusions:

In both HIV sero-negative and sero-positive patients with pleural tuberculosis, a Th0 response, characterised by the presence of both Th1 and Th2 cytokines, was noted, while necrotic granulomas were more evident in HIV positive than HIV negative patients. There was a clear association between TNFα and necrosis in tuberculous granulomas that may possibly be ascribed to the increased apoptotic activity of TNFα. An increase in IFNγ correlated with an increase in necrosis, supporting the theory that high IFNγ levels later in disease is detrimental. This effect may be enhanced by the presence of high numbers of TNFα positive cells.

The strong positive correlation between high numbers of TNFα positive cells and HIV positivity supports the fact that TNF regulates HIV transcription and that higher TNF levels are expected in HIV positive patients. An increase in both Th1 and Th2 cytokine mRNA in HIV positive patients supports the theory that an overproduction of cytokines may be a mechanism to compensate for the failure of another immune effector mechanism.

In sarcoidosis strong evidence of a Th1 response and no evidence of a Th2 response was found. No difference in cytokine profile was found between those patients with a favourable and those with an unfavourable clinical course, neither between patients with severe diffuse fibrosis and those without. The strong presence of Th1 cytokines as well as TNFα supports the theory that a strong Th1 presence in conjunction with TNFα may induce fibrosis, as most of these cases showed signs of at least focal fibrosis.

The results in pulmonary tuberculosis patients also support the virtual model described by Wigginton. The presence of IL-4 may not be an indicator of poor prognosis in pulmonary tuberculosis patients but rather may be an integral feature of tuberculous granuloma formation with a role in controlling tissue damage. It again appears likely that TNFα may be involved in necrosis development. The caseous necrosis appears to be a consequence of apoptosis of infected macrophages and activated T cells within the granuloma and TNFα is a known inducer of apoptosis.

Numerous aspects are involved in granulomatous inflammation, of which the T helper response forms only a component. The earlier dogma of good (Th1) versus evil (Th2), stating that a pure Th1 response is beneficial, and that a Th2 response is detrimental, is an oversimplification of a very complex process. When investigating the contribution of the Th response to the disease process, it is clear that the effect of a cytokine depends at least partially on the stage of disease.

The balance between the various cytokines and also the levels of these cytokines contribute to their role in resolution or disease progression. An early pure Th1

response may be beneficial to the patient if effectively clearing the granuloma-inducing antigen. At this stage, a Th2 presence will be harmful to the patients as clearing of the antigen will not be as effective. In chronic disease where failure to remove the antigen results in progression of granulomas with subsequent necrosis and/or fibrosis, a pro-inflammatory Th1 response may be detrimental to the patient and minimising of this detrimental effect is needed. A strong presence of the various cytokines may also be detrimental to the patient, while lower levels will be beneficial. Numerous other cytokines are involved in the process and other representatives of the Th1 and Th2 subsets also exist. An investigation into these cytokines will fill out some of the numerous blank spaces in this equation.

The discrepancy between the presence of cytokines in circulation and at the site of disease is also of interest (Fig 35). This is best demonstrated by the high numbers of IFNγ mRNA positive cells in necrotising granulomas in pulmonary tuberculosis, while a virtual absence is noted in the peripheral blood. Possible explanations for this occurrence include a selective anergy, and a redistribution of cells with preferential treatment of the disease site. It is also possible that the presence of mRNA does not reflect the production, secretion and/or effectiveness of a cytokine. An investigation into the distribution and quantities of cytokines in various compartments, including the site of the disease, will contribute greatly to our understanding if the inflammatory response.

Another area of interest is the interaction between the causative organism and the granuloma. Work in this area by Fenhalls et al and has already resulted in 2

publications[280,281]. Further investigations into the various factors of virulence, latency and other factors, by mRNA will provide invaluable information into the behaviour of both the host and the organism at the site of disease and in context of the cellular components.

Possible areas of future research, some of which are already in progress include:

- Investigation of additional cytokines and chemokines involved in granulomatous disease.
- Investigation of other sites of disease in tuberculosis and a comparison with the sites already studied.
- Investigation of the numerous receptors for organisms, cytokines and chemokines involved in tuberculosis and other granulomatous diseases.
- Expansion of the study of HIV sero-negative and sero-positive tuberculosis
- An investigation into the distribution and quantities of cytokines in various compartments, including the site of the disease.
- Further investigations into the expression by *Mycobacterium tuberculosis* of factors of virulence, latency and other factors and its relationship to cytokine expression by the host.

Studies already in progress, either resulting from this initial work or related to the topic:

Publications:

1. *In situ* detection *of Mycobacterium tuberculosis* transcripts in human lung granulomas reveals differential gene expression in necrotic lesions. Fenhalls G, Stevens L, Moses L, Bezuidenhout J, Betts JC, Helden Pv P, Lukey PT, Duncan K. *Infect Immun.* 2002 Nov;70(11):6330-8.
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5. 2004: "**STATE OF THE ART:** Bacterial Persistence And Human Disease: A Multidisciplinary Approach To Unravel The Association Between Mycobacteria And Sarcoidosis" *Fenhalls G[1], Stevens L[1], Bezuidenhout J[2], Lewis L[1], Warren R[1], van Helden P[1], Walzl G[1]*. 48th Academic Yearday, Faculty of Health Sciences, Stellenbosch University.

PhD studies

"Clinical and Immunological Aspects of Tuberculous Pleurisy. A prospective, randomised study of the role of initial complete drainage in the treatment of tuberculous pleurisy, combined with a vertical and horizontal study of cytokines in tuberculous pleurisy". ***A Diacon**, M Schuurmans, J Theron, F Swart, Z Williams, G Fenhalls, B vd Wal, G Walzl, J Bezuidenhout, E Irusen, P van Helden, C Bolliger.* Promotor: G Walzl.

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