THE HISTOPATHOLOGICAL ANALYSIS OF CELLULAR ELEMENTS, ACCESSORY MOLECULES AND CYTOKINES IN MYCOBACTERIAL GRANULOMAS FROM HIV POSITIVE AND NEGATIVE INDIVIDUALS

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DECLARATION

I, Reubina Wadee declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Anatomical Pathology in the University of the Witwatersrand, Johannesburg. It has not been submitted before any degree or examination at this or any other University.

_____

Signature of candidate

The _____ day of _____ 2013

DEDICATION

I dedicate this research report to my husband, Nitesh, my parents, Ahmed and Zainab, our Scully, and most of all, to Snellie, who sadly was not able to see this project through with me to fruition.

ABSTRACT

The immune response to infection with *Mycobacterium tuberculosis* (Mtb) involves complex interactions between macrophages, T-cells, cytokines and accessory molecules. Mycobacteria evade the host's immune response by interfering with cell mediated immune systems. Granulomas are central to the host's defenses against Mtb. These responses may be modified by immune alterations especially in patients co-infected with Human Immunodeficiency Virus (HIV).

This study investigated the immunohistochemical profile of CD4+, CD8+, CD68+, Th-17 (also known as Interleukin-17 cells) and Forkhead box (FOXP3) cells, accessory molecule expression (HLA Class I and II) and selected cytokines (Interleukin 2, 4, 6 and Interferon-γ) of various cell types within mycobacterial granulomas, in lymph nodes from ten HIV negative and ten HIV positive patients. Tissue from a foreign body granuloma in skin was utilised for comparison.

This study illustrated retention of CD4+ lymphocyte numbers within granulomas from HIV negative (-) patients but documented a reversal in the ratio of CD4+ to CD8+cells in granulomas from HIV positive (+) patients. Similar IL-17 cell counts were noted in mycobacterial granulomas from both HIV (-) and HIV (+) patients. CD68 was identified in all macrophages and HLA Class II stained 100% of cells. Mycobacterial granulomas from HIV (-) patients showed marginally lower numbers of HLA Class I cells when compared to those from HIV (+) patients.

The percentage of FOXP3 positive cells differed significantly between mycobacterial granulomas from HIV (-) and HIV (+) patients. This study highlights the complex interplay between different cell types and cytokines secreted into the microenvironment that ultimately results in containment of the organism or disease progression.

Tuberculosis mono-infection causes variation in the expression of cell markers such as FOXP3 with accompanying noteworthy changes in cytokine production in areas of granuloma formation. The alterations noted in TB and HIV co-infection are even greater and point toward evolution of micro-organism synergism with host demise.

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CHAPTER 1

1.0 INTRODUCTION

The pathological hallmark of *Mycobacterium tuberculosis* (Mtb) is granuloma formation. [1-3] A granuloma is defined as a collection of epithelioid histiocytes. In addition, granulomas tend to be infiltrated peripherally by lymphocytes. [3-4] Granulomas may also contain dendritic cells, varying numbers of foamy macrophages, scattered neutrophils and occasional fibroblastic cells. [4] Multinucleated giant cells may also be identified and central caseous necrosis is often present. [3- 4] Granuloma formation is not restricted to Mtb infection and may be seen in foreign body reactions, sarcoidosis, malignancies, chronic inflammatory bowel disease, as well as in many infective processes such as viral, bacterial, fungal and protozoal infections. [5]

In tuberculosis, the occurrence of granulomatous inflammation is believed to be a delayed type hypersensitivity reaction [2] which ensues subsequent to the detection of mycobacterial bacilli by host responses. [2] Granuloma formation serves to contain the micro-organism and provides a milieu within which cellular mechanisms attempt to limit bacterial replication.[1] The formation of granulomas are however, not adequate to control mycobacterial infection as the infected individuals often have progressive disease with multiple granulomas in multiple sites. [4] Mycobacteria have evolved such that they are able to avoid eradication by the host. As such, granulomas may function as both a haven for bacterial survival as well as a site for host resistance. [4]

Mycobacterial infection resulting in granulomatous inflammation thus occurs as a consequence of a multitude of interactions between the bacteria and host. [5]

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

Mycobacterium tuberculosis (Mtb) is a leading cause of morbidity and mortality. [1, 6] It has been suggested that roughly thirty-three percent of the human population is infected by the organism but that less than one tenth of infected individuals progress to active disease. [3] In Africa alone, more than four (4) million people are affected by active disease. [6] The remainder of infected people act as reservoirs for the bacilli and it is in such individuals that the disease persists in a latent form. [3] Immune suppression in such people may result in reactivation with subsequent spread of infection. [3] The incidence of tuberculosis (TB) has increased significantly with the advent of Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) with Mtb being the leading cause of death in HIV positive individuals. [6-9]

The tuberculin skin test is generally used for the assessment of Mtb infection but has been shown to be unable to differentiate active disease from latent infection [6, 10-11] whilst the benefits of the Bacillus Calmette Guerin (BCG) vaccine has been questioned. [6, 10] It has been suggested that the BCG affords protection to young children from disseminated disease but it has not necessarily been protective against adult lung infections. [5, 11] The current chemotherapeutic regimen is protracted and patient compliance may be problematic. [12] In addition, the emergence of multidrug resistance strains (MDR) and extensively drug resistant strains (XDR) is disconcerting. [6, 12] These considerations necessitate the search for new

treatment strategies and as such, a better understanding of the disease pathogenesis is required.

Human tuberculosis exhibits a spectrum of clinical and immunologic features. These range from patients with good cellular immunity to those with extensive or disseminated lesions which show poor cellular reactivity. [13, 14] The efficacy with which the host is able to eradicate bacilli determines the outcome. [3] In most cases of sputum positive and many sputum negative patients, Mtb has been shown to inhabit the lung. [6 and 15] Mtb is a respiratory pathogen that passes from an individual with active disease [16] to invade uninfected individuals. The bacilli gain entry to the alveoli in the lung where they are engulfed by alveolar macrophages and interstitial dendritic cells. [17] Bacteria may drain to local lymph nodes or may reside within the lung interstitium. [17] Macrophages drawn to areas of bacterial deposition reside within the parenchyma; where they accumulate, forming loose aggregates. [6] Within local lymph nodes, antigen presenting cells have been demonstrated to process and present bacterial antigens together with Human Leukocyte Antigen (HLA) Class II antigens to T-lymphocytes. [19, 20] These T-cells have been found to recognise Mtb infected macrophages and dendritic cells, such that these antigen presenting cells that had engulfed Mycobacteria produce Interleukin (IL)-12, which has been shown to initiate the differentiation of T-helper (Th)1 cells via the pro-inflammatory cytokine network. [19]

2.2 CELL TYPES

The different combinations of cytokines produced by cells in the area of infection have been shown to attract different populations of leukocytes to the site. [6, 21-23] Activated T-cells include CD4+ and CD8+ lymphocytes, together with CD1 restricted T-cells and γδ

lymphocytes. [6, 16] CD4+ cells expressing HLA Class II antigens have been shown to play a key role in protecting against Mtb. [24] T-helper cells have been divided into two groups based on their cytokine production; and are termed Th1 and Th2 cells as subsets of CD4+ lymphocytes. [6] Th1 cells have been shown to produce interferon-gamma (IFN-γ), IL-2 and other pro-inflammatory cytokines. [25, 26] IFN-γ has been demonstrated to activate macrophages resulting in the control of bacterial replication. [27] However, through a series of evasive mechanisms mycobacteria have been shown to effectively overcome destruction by host defences. [24, 28-30] These include interference with the production of reactive oxygen species, inhibition of degranulation and phagosome-lysosome fusion and an interference with macrophage activation. [31-34] Due to the inhibitory effects on macrophages and subsequent down-regulated microbicidal activity, [35] it has been suggested that mycobacteria may negatively influence antigen presentation by interfering with the expression of accessory molecules as well as HLA Class II antigens. [17] This ultimately implies a decrease in Th1 cell activation which results in reduction in the host's cytokine response. [17] This has been suggested to be due to a decrease in availability of mycobacterial antigen/HLA Class II or perhaps due to suppression of cytokines required for T-cell function [17] Decreased HLA Class II expression on cells infected with Mycobacterium *leprae* or M. *kansasii* or Mtb components have been previously reported. [34, 36]

Furthermore, it has been demonstrated that macrophages infected with large numbers of Mtb are defective in serving as accessory cells by being unable to present soluble antigen. [37] Foamy macrophages within granulomas have been demonstrated to have foregone their phagocytic and killing abilities such that Mtb may continue to exist uncontrolled in these cells. [3]

It is well documented that macrophages present soluble antigens to Th1 cells by expressing Class II antigens and accessory molecules. [6, 18, 19] Antigen presentation to CD8+ cells has been demonstrated to be mediated via the expression of soluble antigens together with HLA Class I antigens.

A subgroup of CD8+ cells, also known as Cytolytic T-Lymphocytes (CTL) has been shown to have direct cytolytic activity by means of granzymes, granulysins and perforins. [6, 17, 64] Other CD8+ cells produce interferon-γ which activates macrophages [6, 39] thus contributing to the immediate host response. More recently, a subgroup of CD4+ regulatory cells (T-regs) have been identified. These cells are broadly characterised by a CD4+ CD25+ FOXP3 immunophenotype [17, 40] and are known as FOXP3 regulatory cells, which express the forkhead-winged-helix transcription factor. [41, 42] FOXP3 has however been recently identified on CD8+ cells. [41, 43] Forkhead box transcription factor P3 (which is denoted as "FOXP3 in humans, but Foxp3 in animals" [41]) is currently thought to be the most reliable marker to highlight regulatory T-cells. [41] FOXP3 cells are essential for immune modulation. [40, 41, 44] Naturally occurring Tregs are activated in the thymus. [44, 45] There are two types of induced Tregs. These are IL-10 producing T regulatory 1 (Tr1 cells) and transforming growth factor-beta (TGF-β) producing Th3 cells. [57] T regulatory cells are required to ensure peripheral tolerance and thus decrease the likelihood of auto-immunity. [40, 45, 46] Tregs have been shown to decrease CD4+ helper cell function by way of cytokines such as IL-10, chemokine (C-C motif) ligand 4 (CCL4) or TGF-β. [17] Tregs have also demonstrated immune modulation by cell-cell contact mediated suppression. [17, 41] They are able to inactivate macrophages [17] and thus may prevent antigen presentation to T-cells. Moreover, they have been described to prevent CD4+ cells from proliferating and therefore are able to prevent the adaptive immune response to Mtb from occurring. [47]

It has been found that up to five percent (5%) of circulating CD4+ cells are CD4+, CD25+.[51, 58,65] Of these cells, it is only the subpopulations that express the IL-12 receptor alpha chain that have a prominent regulatory function. [51, 52, 58] FOXP3 expression has been shown to differentiate activated effector cells from Tregs, all of which are CD25+. [51] As such, mRNA expression of FOXP3 is believed to be the best marker of Treg function. [51, 53, 54]

It has been demonstrated that Tregs inhibit antimicrobial responses with particular reference to organisms causing chronic infections. [40, 48] Simultaneously, Tregs protect the host during infections by preventing excess damage which may be brought about as a consequence of the inflammatory response (by T effector cells) to the infective pathogen. [17, 40, 57] Thus, T-regs may be seen to regulate the balance between host tissue destruction (anti-inflammatory effects) and survival of microbes. [17, 40, 50, 51] Some pathogens use this to their advantage and evade immune mechanisms. [54] Down modulation of Th1 responses by means of immunosuppressive molecules and the cell surface molecule CTCL4 as well as TGF-β and IL-10 have been described. [54-56]

It has been suggested that increased levels of FOXP3 mRNA levels noted in Mtb patients are likely to be due to an increase in CD4+ CD25+ T-cells instead of being a consequence of up-regulated gene expression. [51, 53] However, other studies, [53] suggest that over-expression of the FOXP3 gene occurs in Mtb. [54] The raised levels of FOXP3 are believed to inhibit T-cell responses to protective antigens which may be a mechanism adopted by Mtb to protect itself from host defences. [54]

γδ Lymphocytes have been demonstrated to play a part in host defences in Mtb. [38] These lymphocytes have been shown to recognise phosphorylated ligands in the absence of

restriction elements such as HLA Class I and II. γδ Lymphocytes are capable of causing destruction of mycobacteria engorged histiocytes. [38] Furthermore, they have been identified to produce IFN-γ in the early stages of the host response. [38, 59] These cells are also believed to release cytokines which may contribute to granuloma formation. [3, 15, 60]

CD1 restricted T-cells, which in addition to CD4+, CD8+ and γδ lymphocytes, are activated T-cells [6 and16] that have been demonstrated to be specific for mycobacterial glycolipids such as mycolic acids and lipoarabinomannan. It is thought that these cells may produce cytokines similar to the Th1 and CD17 cells but their precise role in protective immunity has not been fully elucidated. [6]

Th1 cells are believed to have a prominent role in intracellular infections. [15, 44] Th1 cells and macrophages have been shown to produce IL-2 which serves as a growth factor for Th1 cells and CTL's .[6, 35] In addition, secretion of tumour necrosis factor-alpha (TNF-α) [6, 15] by Th1 cells and macrophages, together with IFN-γ has been found to improve the bactericidal activity of macrophages. [6, 35, 61, 62] TNF-α is well known to have a multitude of effects; which include: cell activation, cell recruitment, cell differentiation and apoptosis. [63] TNF-α, as a result of increasing chemokine production elicits an influx of inflammatory cells. [63] In some macrophages, TNF-α may cause apoptosis thus bringing about destruction of mycobacteria contained by the affected macrophages. [63] Collectively, TNF-α and IFN-γ have been demonstrated to be integral to the promotion of granuloma formation. [3] Thus, it may be seen that TNF-α is an essential part of the host response.

It has recently been proposed that a subgroup of Th cells producing Interleukin 17 (IL-17), also known as Th17 cells may act as a first line of defence against Mtb. [6] IL-17 has been

demonstrated to be a cytokine that may initiate inflammation as well as facilitate macrophage accumulation. [38] Thus, IL-17 may have a role in granuloma formation. [6] Activated Th17 cells establish chemokine gradients [38] and have been shown to produce pro-inflammatory IL-6 and IL-22 which then stimulate production of defensin in epithelial cells. [6, 25, 61] Of note is that IL-6 has both pro-inflammatory as well as anti-inflammatory properties [15] and this cytokine may display either of such properties depending on the target population. [29]

Whilst Th2 cells have been shown to produce B-cell growth and differentiating factors (such as IL-4 and IL-5) [44] promoting antibody production, these responses by the host do not have a significant role in host protection against Mtb infection. [6, 48] There is evidence indicating that depressed cellular immunity seen in mycobacterial diseases is in some instances due to over-reactivity of suppressor cell systems .[13, 14] One study, [6] demonstrated the presence of suppressor adherent cells in certain patients with TB and that suppressor cell activity has been observed in BCG-immunised individuals. [66] Cellular unresponsiveness in tuberculosis has been shown to be associated with lesions containing enormous numbers of viable mycobacteria. [6, 35] IL-4 and TFG-β produced by regulatory T-cells have been implicated in suppression of immunity, [6, 35], with IL-4 acting to suppress Th1cytokines namely IFN-γ, TNF-α and IL-12 production. [2] In other systems, TNF-α and IL-10 have been shown to have opposing functions in Mtb; where TNF-α has been demonstrated to activate macrophages whilst IL-10 results in inhibition of macrophage function with subsequent mycobacterial proliferation. [6, 62] TGF-β has been seen to down-regulate TNF-α levels resulting in deactivation of macrophage function. [6] In addition, TGF-β has been proven to induce the production of FOXP3 and generate Tregs from resting naïve T-cells [44] which then has downstream effects on Th2 cells as FOXP3 is able to directly inhibit Th2 cell

development. [44] It has thus been suggested that inappropriate secretion of IL-10 and TGF-β may be a cause of failure of the immune response in Mtb. [6]

2.3 PATHOGENESIS

Once mycobacteria reach lung parenchyma, the infected macrophages attract other macrophages and dendritic cells to the site. Some organisms are carried to regional lymph nodes whilst others remain in the lung. Inflammatory cells such as lymphocytes, granulocytes, dendritic cells and macrophages accumulate in areas of infected macrophages, forming loose aggregates [6] which progress to granulomas. Thus granulomas may contain bacilli [18] but paradoxically also facilitate mycobacterial survival by attracting more host cells to the site which then also become infected. [9] The constant influx of inflammatory cells allows for uninfected macrophages to phagocytose the contents of necrotic, infected macrophages. [19] Whilst this may be seen as amplification of the immune response, it simultaneously benefits mycobacteria as more macrophages become infected. [5]

Once mycobacteria are within macrophages, the organisms prevent phagosome-lysosome fusion. [15, 19, 67] Approximately three weeks post-infection, a Th1 response and cell mediated reaction occurs. [19] This causes macrophages to become activated and therefore increases bactericidal activity. [19] The Th1 cells are stimulated by antigen presentation together with HLA Class II molecules by antigen presenting cells. [19, 38] It has been well documented that Th1 cells then produce IFN-γ which stimulates macrophage activation and allows for expression of inducible nitric oxide synthase (iNOS) which then produces nitric oxide. [19] Nitric oxide in turn produces reactive nitrogen intermediates as well as other free radicals which culminate in destruction of mycobacterial constituents. Simultaneously, activated

macrophages produce TNF-α which attracts more macrophages to the site. These cells undergo morphological changes imparting an epithelioid appearance to the cells. [19] In TB, central areas of granulomas often show caseation which is due to cellular degradation of necrotic cells. [5, 16]

2.4 HIV AND TB CO-INFECTION

Infection with Human Immunodeficiency Virus (HIV) increases the risk of mycobacterial infection; with subsequent increased likelihood of disease progression. [9] It has been noted that HIV positive patients have an increased risk of acquiring TB throughout the course of their infection and that this risk also exists in the first few years following infection when CD4 counts are still high. [9, 68, 69] Whilst antiretroviral therapy has been shown to elevate CD4 counts, the effects of HIV on TB are however, not completely opposed. [9] It has been shown that patients infected with both HIV and TB have a higher likelihood of acquiring other opportunistic infections than HIV positive patients without TB who have similar CD4 counts. [9, 70]

In HIV, the spectrum of Mtb infection may be varied with subclinical infection in patients who are sputum culture positive, but without clinical manifestations on the one hand; and on the other, Mtb may have extensive cavitatory disease. [9] There may also be patients who have a large number of bacilli but who have a mild inflammatory response. [9] It has been shown that in patients with high CD4 counts, usual granuloma formation is present. [9] Patients who have low CD4 counts have been shown to have ill-defined, vague granuloma formation. [9] Some authors have demonstrated an increase in neutrophils [9, 35,71] in granulomas of HIV positive patients with TB together with a decrease in TNF-α staining. [9]

It has been suggested that infection by tuberculosis may occur early in HIV infection [2, 74] due to the presence of dysfunctional HIV infected cells which are incorporated into granulomas. These dysfunctional cells may contribute to the formation of poorly formed granulomas or disruption of granulomas [4, 9]. It has been proposed by some investigators that HIV infection of macrophages encourages Mtb growth and that dual infection decreases the viability of macrophages and may be associated with increased secretion of IL-10. [9, 72, 73] It has been suggested that HIV-1 infection prevents apoptosis of macrophages infected by Mtb due to increasing IL-10 levels which thus reduce TNF-α levels and therefore affect the pro-apoptotic tendencies of TNF-α. [4, 9].

Patients with HIV have an increased risk of acquiring Mtb. [9] This suggests that CD4+ cells have a protective role against Mtb. [9] HIV results in T-cell destruction with rapid T-cell turn-over. In addition, there is constant antigenic stimulation as a consequence of genetic variation of the virus which allows for survival of the virus. These are all believed to play a role in HIV progression. [1] Some studies suggest that early on in HIV infection, Mtb specific CD4+ cells are destroyed. This destruction may be due to expression of the chemokine receptor CCR5 molecule which these cells express. [1] It has been documented that HIV gains entry into cells via the cellular CCR5 receptor. [9] Furthermore, these authors [9] have shown that in latently Mtb infected patients who were HIV negative, the levels of CCR5 expressing cells were twice that of the total Mtb specific CD4+ T-cells. [9] From this, the converse suggests that the increased number of Mtb specific CD4+ cells (which express CCR5) result in a raised susceptibility to HIV in these patients. This therefore suggests that Mtb infection renders susceptibility to HIV infection and that HIV infection promotes mycobacterial disease. In vitro studies have also suggested that IL-2 production by Mtb specific T-cells raises susceptibility to infection by HIV, [4] which adds to the suggestion of a dual susceptibility

model. HIV has been shown to destroy T-cells within mucosae of the gastrointestinal tract as well as the periphery and as such it has been proposed that destruction of CD4+ cells within granulomas implies a failure of the host's immune mechanism to contain Mtb. [4]

2.5 HIV, TB AND CYTOKINE ALTERATIONS

HIV has been shown to alter cytokine expression in Mtb granulomas. [1, 4, 9] One such study [2] illustrated an increase in IFN-γ, IL-12 and IL-4 mRNA production in HIV positive patients compared to HIV negative patients. The increase in TNF-α corresponded to an increase in necrosis within granulomas. [2] Whilst TNF has been shown to control bacterial proliferation, [1] TNF-α is believed to initiate HIV replication within macrophages. [1] As such, it may be seen that in an attempt to contain one organism, this may inadvertently stimulate proliferation of another. There are however, authors who have reported lower TNF-α expression in HIV positive patients with extensive necrosis. [71] The lowered TNF-α may have been attributed to lowered numbers of T-cells and macrophages within granulomas. These conflicting reports on TNF-α levels may reflect the various stages of HIV disease such that early on in HIV infection, the TB granulomas may contain significant numbers of T-lymphocytes and macrophages whereas in chronic stages of HIV infection, CD4+ cell levels are diminished and macrophage viability is compromised.

HIV results in persistent cellular activation and "increases proportions of intermediately differentiated CD8+ T-cells". [1] Furthermore, the finely balanced scale between Tregs and IL-17 is thought to be thrown out of synchrony and has been hypothesized to increase the permeability of the gastrointestinal tract [1] which allows for additional pathogens to invade

the host. [1] The continuous antigenic mediated immune stimulation eventually exhausts the host's capabilities to contain either TB or HIV with subsequent uncontrolled disease.

It has been previously suggested in vitro that in the presence of mycobacteria, macrophages exhibit a decrease in accessory and co-stimulatory molecules. [36, 75] It may therefore be deduced that accessory molecule function in the presence of mycobacteria is locally suppressed. This reduction in accessory molecule function or expression of co-stimulatory signals by human monocytes may result in decreased T-cell responses to antigens with a consequent evasion of the host's response to Mycobacteria. [36, 76, 77] In addition, it has been demonstrated that in the presence of Mtb, there is an increase in the expression of HLA Class I antigens which are required for the presentation of foreign antigens to CD8+ suppressor cells. [36] This may then result in further immune suppression. [36, 66]

To date, there have been only a few studies detailing the exact production of cytokines in human granulomatous inflammation. Little is known of the expression of accessory molecules in tuberculous granulomas. The present study therefore aims to identify some of these components of the immune response in human tissue granulomas in both HIV positive and negative patients. In addition, the study aims to assess localised cell mediated effects as would be seen in mycobacterial granulomas. The present study therefore is an attempt to confirm previous suggestions made on in vitro studies of imbalances observed in localised mycobacterial infections. The study involves the use of archived histological tissue with proven mycobacterial infection to identify and confirm the cell mediated processes exhibited in HIV positive and negative mycobacterial positive granulomas with specific reference to Mtb.

CHAPTER 3

3.0 AIMS

1. To characterise the immunohistochemical profile of cell types within TB granulomas using CD4+, CD8+, CD68, Th17 and Forkhead box (FOX) P3 in HIV negative and HIV positive patients.
2. To identify the immunohistochemical staining of accessory molecules HLA Class I and II in TB granulomas from HIV negative and HIV positive patients.
3. To study the presence of cytokine production namely IL-2, IL-4, IL-6 and IFN-γ in mycobacterial granulomas in HIV negative and positive patients.

CHAPTER 4

4.0 MATERIALS AND METHODS

4.1 BIOPSY SPECIMENS

The material for this study has been drawn, following ethics clearance, (Appendix, Chapter 8.2) from archived tissue blocks of surgically excised lymph nodes that were previously diagnosed as granulomatous inflammation with positive Ziehl-Neelsen stain. Mycobacterial culture studies had not been performed in all the selected cases. The exact topographical site of derivation of the biopsy was not stated in the clinical information received from clinicians in the majority of cases, and as such for those cases in which a topographical site of origin was stated, this was excluded for the purpose of uniformity in the study. The archived tissue blocks were retrieved from the Division of Anatomical Pathology, University of the Witwatersrand/National Health Laboratory Services (NHLS). Ten lymph nodes (n=10) containing granulomatous inflammation from HIV negative patients and another ten lymph nodes (n=10), from HIV positive patients, containing granulomatous inflammation were retrieved. The age and sex of each of the patients from whom the biopsies were derived, has not been documented. The initial sections, stained with Haematoxylin and Eosin (H&E) were reviewed (figures 1 and 2) and the diagnosis confirmed. In all cases, well formed granulomas were documented.

Control tissue in the form of a foreign body granuloma from skin was used for comparative purposes in this study. The aetiology of foreign material in foreign body granulomas may elicit variable immune responses. In order to prevent the possibility of this occurring in

multiple foreign body granulomas which would alter the parameters evaluated in this study, only a single foreign body granuloma was utilised as control tissue for comparative purposes only.

The HIV status of the patient from whom this tissue was derived, is unknown, as is the age and sex of the patient.

The paraffin embedded tissue blocks were initially fixed in 10% neutral buffered formalin for a period of 12-48hrs. These tissue blocks had multiple serial sections cut, in order to be stained by the various immunohistochemical stains required for this study.

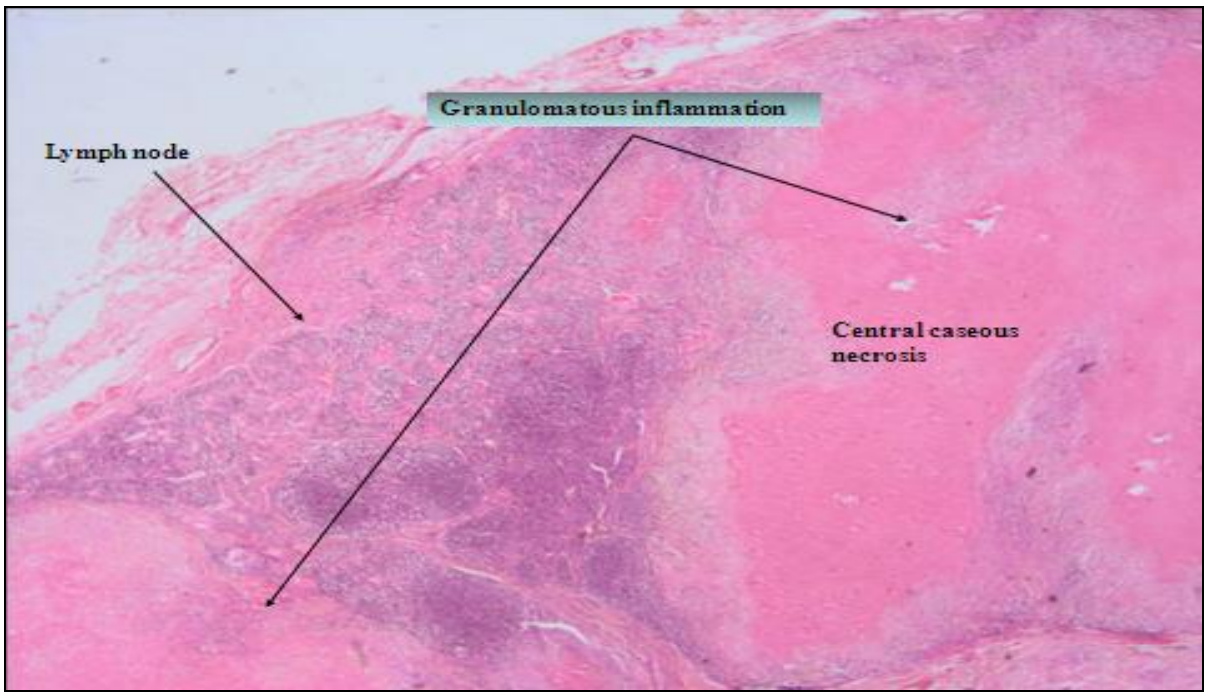

Figure 1: The histological features of a lymph node showing granulomatous inflammation in an HIV negative patient. Central areas of caseous necrosis are apparent. (H&E, 2 μm section; Original magnification: 20X)

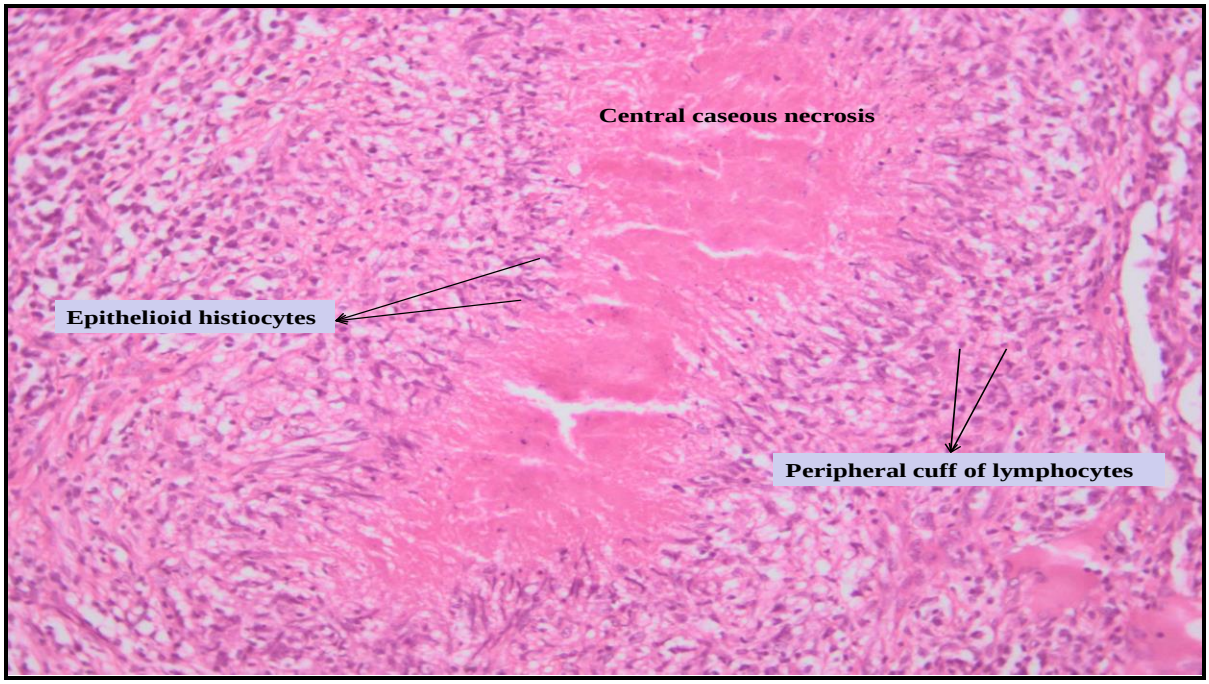

Figure 2: Histological features of a granuloma are depicted in the high magnification photomicrograph above. The central caseous necrosis, epithelioid histiocytes together with lymphocytes at the periphery are highlighted. (H&E, 2μm section; Original magnification:400X)

4.2 IMMUNOHISTOCHEMISTRY.

Immunohistochemistry was performed on 4μm deparaffinised sections using the following immunohistochemical stains:

1. CD4 (BioGenex, CA)
2. CD8 (Leica Microsystems, UK)
3. CD68 (DAKO, Denmark)
4. Th-17/ IL-17 (R&D Systems, UK)
5. FOXP3 (R&D Systems, UK)
6. HLA Class I (Santa Cruz Biotechnology, Inc. CA)
7. HLA Class II (Santa Cruz Biotechnology, Inc. CA)
8. IL-2 (Santa Cruz Biotechnology, Inc. CA)
9. INF-γ(Santa Cruz Biotechnology, Inc. CA)
10. IL-4 (Abcam, UK)
11. IL-6 (Santa Cruz Biotechnology, Inc. CA)

4μm consecutive tissue sections were cut from the paraffin blocks and were floated onto slides. The slides were dried overnight at 60^{o} C. CD4, CD8 and CD68 immunohistochemical stains had already been optimised according to the standard operating procedure within the department. For the remainder of the antibodies used in this study, the concentration for each stain was deemed optimal when lymphocytes and histiocytes showed crisp cytoplasmic and membranous staining with minimal staining noted in the background. A similar approach was utilised for FOXP3 until desired intranuclear staining was observed. Optimisation was obtained following dose kinetic evaluation of each antibody. The avidin-biotin method of immunohistochemical staining was utilised to stain the tissue sections. This involved the application of primary antibody serum, for which each section was stained with the following immunohistochemical stains CD 4 (Clone 1F6, Neat), CD 8 (Clone 4B11, I:100), CD 68 (Clone PG-M1 , 1:100), IL-17 (1:5), HLA Class I (Clone B-D11, 1:10), HLA Class II (Clone TAL 1B5, I:300), IL-2 (Clone N7.48A, 1:50), IFN-γ (Clone H-145, 1:75), IL-4 (Clone 1:50), IL-6 (Clone 1, 1:400) and FOXP3 (1:50). Thereafter, the sections were washed with Tris buffered saline (TBS at pH 7.6). Immunohistochemistry was performed with an automated staining machine (DAKO Autostainer Link 48, Denmark); and using the The EnVision™ FLEX Target Retrieval Solution, High pH (a ready-made solution including EDTA) for antigen retrieval. 3,3' Diaminobenzidine hydrochloride solution (DAB, Sigma) was used as the chromogen, with a resultant brown reaction product. The tissue sections were counterstained with Meyer's haematoxylin.

Negative control sections were processed and stained in a similar manner as for the experimental tissue, except that peroxidase labelled secondary anti-sera was used without the primary cytokine or cell specific antibody. Positive tissue controls were used for all the

immunohistochemical stains. These were drawn from the departmental stock of reactive lymph nodes.

4.3 IMMUNOHISTOCHEMICAL ASSESSMENT

Weak, pale to intense dark membrane and cytoplasmic staining of lymphocytes and histiocytes was considered positive for all of the immunohistochemical stains except FOXP3. FOXP3 was deemed positive if any degree of nuclear staining was identified. Positive staining for all markers was assessed in the relevant cells contained within and in areas surrounding granulomas.

Representative areas surrounding granulomas were identified by scanning on a low power 4x objective. Within the representative areas, the number of positive cells stained with each of the immunohistochemical stains was counted. This was performed by utilising an eyepiece graticule containing one hundred (100) squares fitted to an Olympus BX41 microscope at 10x ocular and 40x objective. Five of the most representative high power fields were selected from each tissue section. In each field using high magnification (40x objective), five areas with the most intense staining were identified. In each of these areas, groups of ten lymphocytes and histiocytes each were counted and the number of positive staining cells from each group of ten was then recorded. Thus each field provided a total of fifty cells. As five such fields were examined per slide, this provided evaluation of a total of two hundred and fifty cells in total. The grid ensured that foci counted in each area were not repeated.

The number of positive cells out of a total of two hundred and fifty (250) was recorded and percentages obtained by dividing the number of positive cells by the total number of cells

counted (250) and then multiplying by one hundred (100). The data obtained from this has been statistically examined.

The fields that were evaluated were marked on the slides and the area matched on each slide to ensure the same area was evaluated for each of the various stains (figure 3).

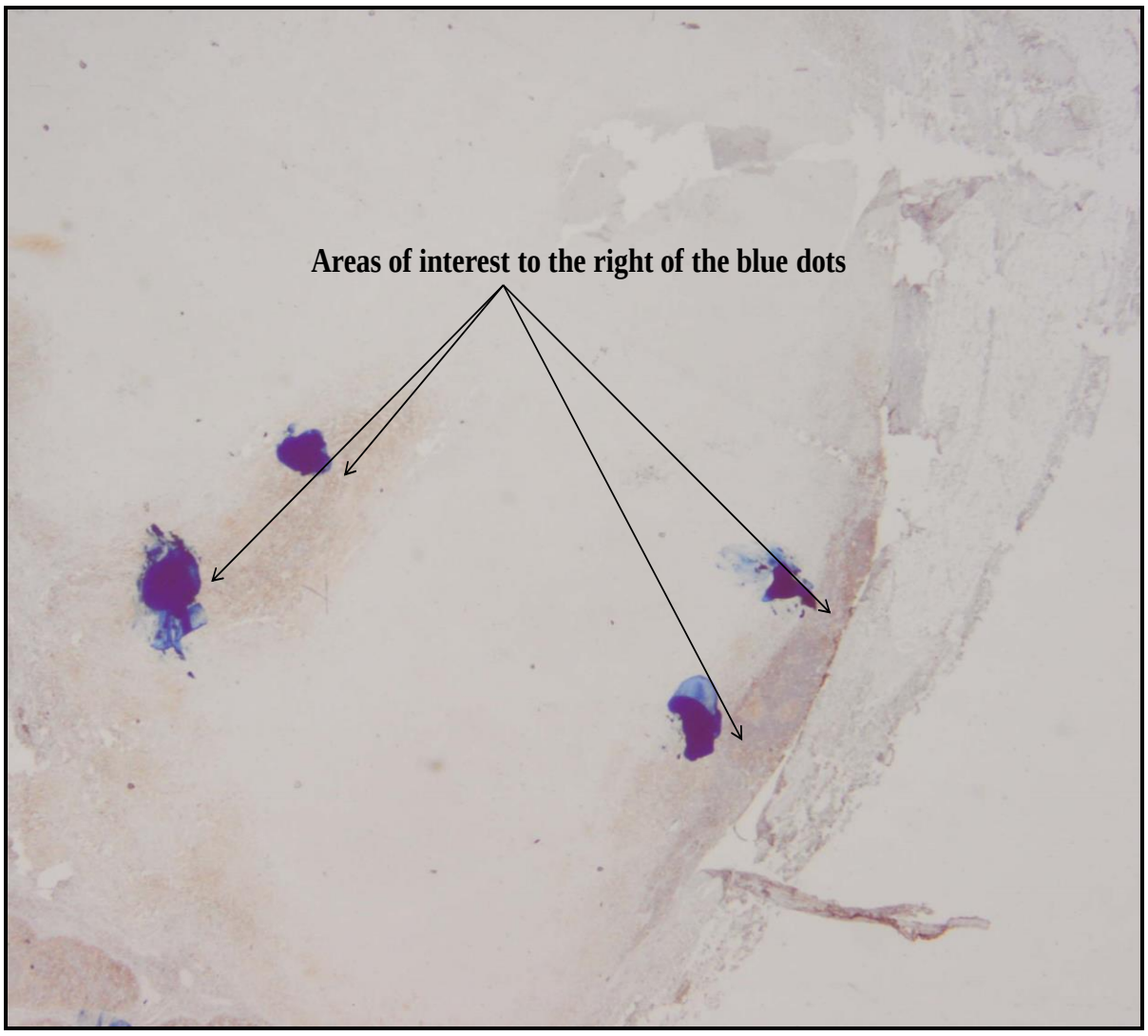

Figure 3: The photomicrograph above highlights areas of greatest immunoreactivity; which are identified to the right of the marked blue dots on the slide. This lymph node with TB granuloma formation is from an HIV negative patient. (HLA Class I, 4μm; Original magnification: 40X.)

CHAPTER 5

5.0 RESULTS

5.1 STATISTICAL ANALYSIS

Data obtained from various observations were entered into a Microsoft Excel 2007 programme. The mean, standard deviation, range and median of positivity for the various stains were obtained for each set of observations. Statistical significance was ascertained using the Student's two-tailed t-test (unpaired t-test).

5.2 IDENTIFICATION OF CD4+ AND CD8+ T-CELLS IN GRANULOMAS.

Figures 4, 5 and 6 demonstrate CD4, 8 and 68 positive cells. Evaluation of CD4+ staining cells in TB granulomas from HIV negative and HIV positive patients showed a distinct difference between the two groups. The HIV negative group exhibited approximately 1.5 times more CD4+ cells than the mean of the HIV positive group. The difference between the two groups was significant ($p<0.001$; $p = 0.000011119$).

The mean percentage of positive CD8+ T-cells in TB granulomas from HIV negative patients in this study was found to be marginally lower than the mean percentage from the HIV positive group. As depicted in figure 7, there was a moderate but insignificant increase in the numbers of CD8+ cells in the HIV positive group ($p = 0.39658$). The CD8+ cells in the granulomas from HIV negative patients showed a more peripheral localisation (shown in

Figure 5), in contrast to the HIV positive group in which the CD8+ cells were scattered throughout the granulomas.

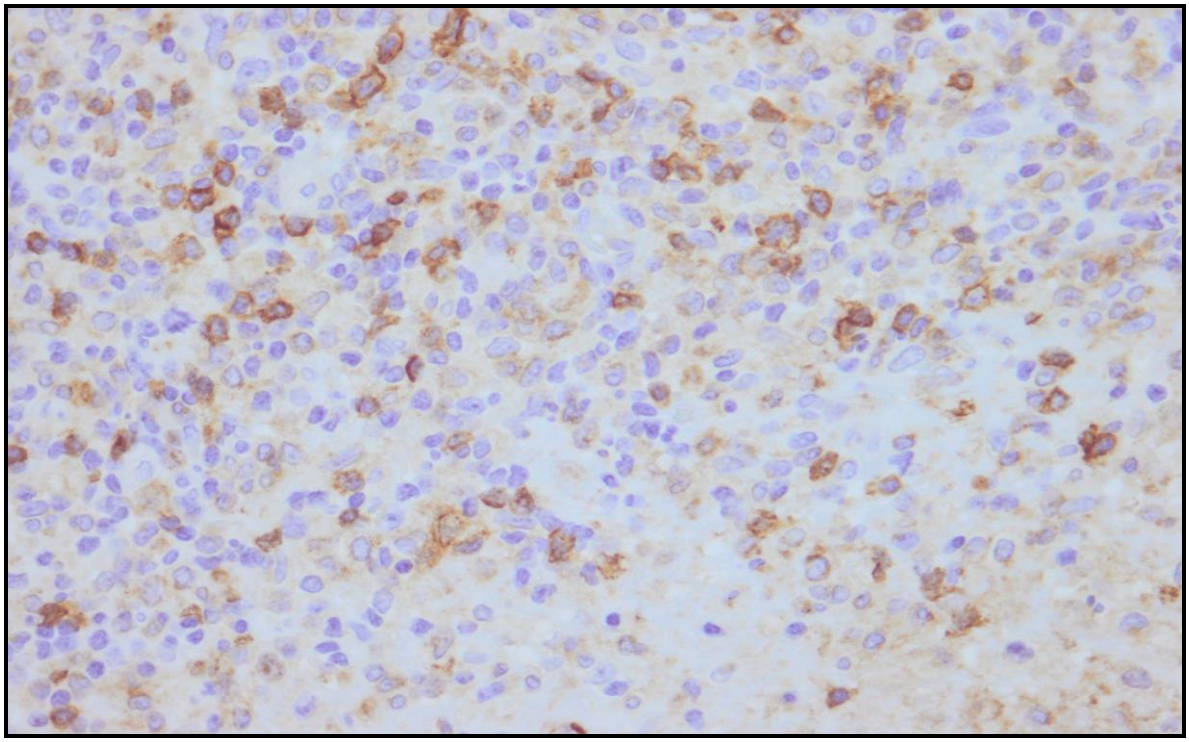

Figure 4: The photomicrograph above highlights CD4+ cells in a TB granuloma from an HIV negative patient. (CD4, 4μm section; Original magnification: 400X.)

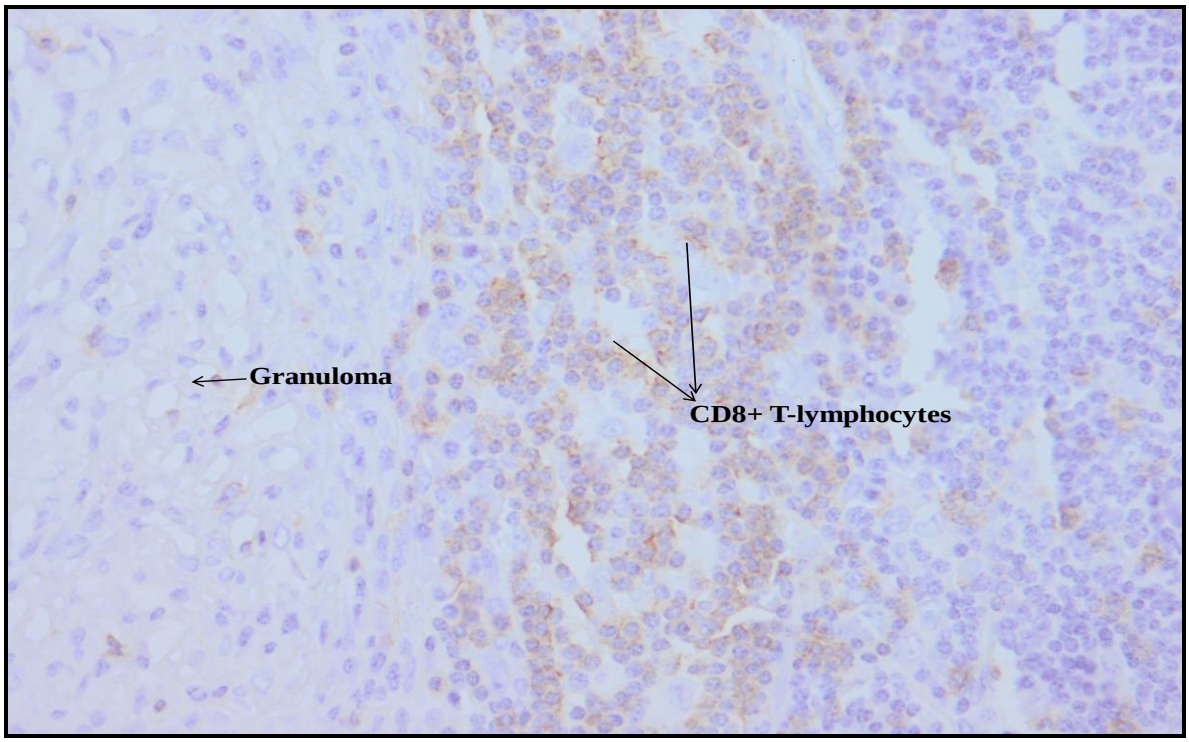

Figure 5: The photomicrograph above highlights CD8+ cells in a TB granuloma from an HIV negative patient. The peripheral localisation of the CD8+ cells is well demonstrated. (CD8, 4μm section; Original magnification: 400X).

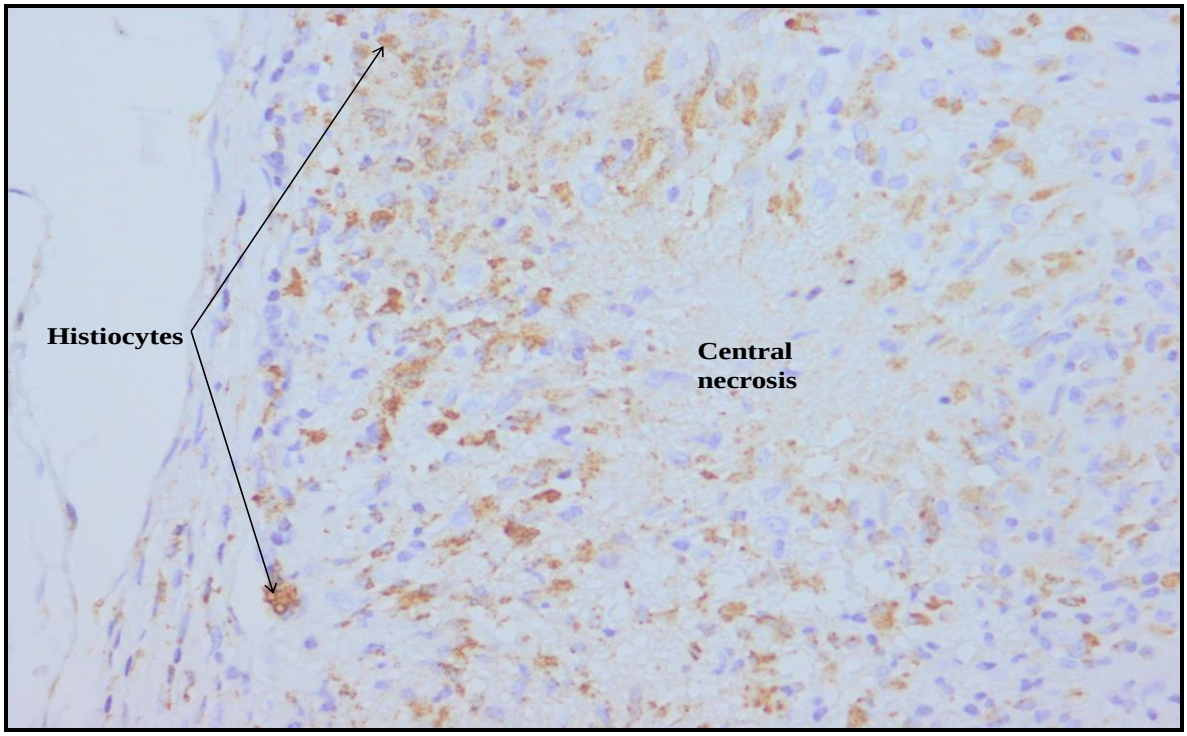

Figure 6: The photomicrograph above shows CD68+ histiocytes in a TB granuloma from an HIV negative patient. The coarse granular staining is well demonstrated. (CD68, 4μm section; Original magnification: 400X).

	HIV NEGATIVE			*HIV POSITIVE*			*FOREIGN BODY GRANULOMA*	
	CD4	**CD8**	**CD68**	**CD4**	**CD8**	**CD68**	**CD8**	**CD68**
	62	72	100	38	60	100		
	58	70	100	34	74	100		
	80	90	100	36	66	100		
	60	40	100	42	64	100		
	42	50	100	40	58	100		
	56	56	100	44	66	100		
	72	62	100	32	68	100		
	72	42	100	30	62	100		
	68	50	100	48	50	100		
	68	56	100	38	66	100		
							29	**100**
Mean	**63.8**	**58.8**	**100**	**38.2**	**63.4**	**100**	**29**	**100**
SD	**10.0975**	**14.4554**	**0.0000**	**5.2498**	**6.1351**	**0.0000**		
Min	**42**	**40**	**100**	**30**	**50**	**100**		
Max	**80**	**90**	**100**	**48**	**74**	**100**		
Median	**65**	**56**	**100**	**38**	**65**	**100**		

Table 1: <u>CD4, CD8 and CD68 staining in HIV negative, HIV positive TB granulomas and control tissue.</u>

The table above displays the statistical parameters as well as the percentage of CD4, CD8 and CD68 positive staining cells in TB granulomas from HIV negative, HIV positive and foreign body granuloma (control tissue).

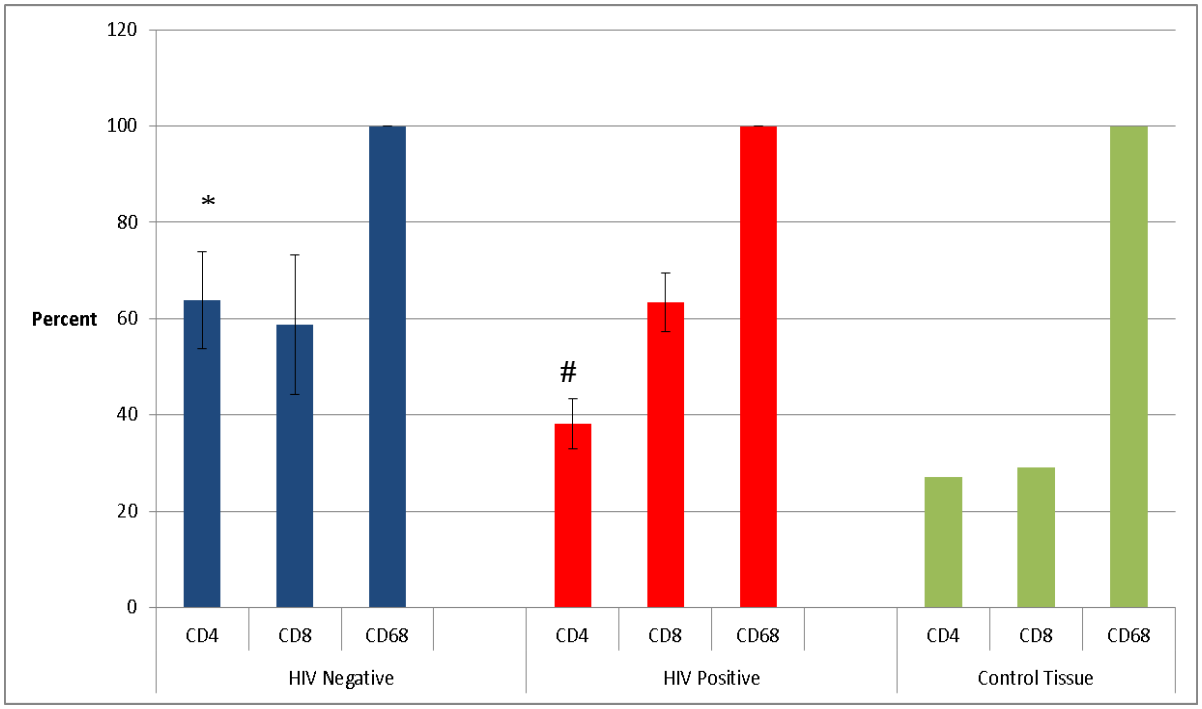

Figure 7: <u>CD4, CD8 and CD68 staining in granulomas.</u>
The figure compares CD4, CD8 and CD68 staining cells in TB granulomas from HIV negative, HIV positive patients and the foreign body granuloma (control tissue).
* $p<0.001$: CD4 HIV negative versus HIV positive patients
# $p<0.001$: CD4 compared to CD8 in HIV positive patients

The percentage of CD4+ and CD8+ staining cells in TB granulomas from HIV negative patients in this study is similar (figure 7). Comparison between CD4+ and CD8+ cell staining revealed a marked and significant decrease in the numbers of CD4+ cells identified ($p < 0.001$; $p = 0.000000030021$) in TB granulomas in the HIV positive group. Whilst it is recognised that the combined percentages may exceed 100%, this can be explained on the basis of variation in numbers that could be expected as a result of the cell counting being performed on different tissue sections.

Figure 7 additionally demonstrates the percentage of cells expressing CD4+ or CD8+ on their surfaces in the foreign body granulomas (used as control granulomas for comparison). These results showed a lower percentage of CD4+positive cells in the foreign body granuloma in comparison to TB granulomas in the HIV negative patients. Marginally lower numbers of

cells expressed CD4 in the foreign body granuloma compared to granulomas from HIV positive patients.

There was a negligible difference in the percentage of cells expressing CD4 or CD8 in the foreign body (control) granuloma (Figure 7). These granulomas expressed a lower percentage of CD8+ T cells, in contrast to the average cell count noted in TB granulomas from HIV negative and positive patients.

5.3 IDENTIFICATION OF CD68+ CELLS IN GRANULOMAS.

All macrophages examined in granulomas from both the HIV negative and HIV positive groups stained positively with the CD68 marker. The foreign body granulomas also demonstrated 100% positivity with the CD68 stain (figure 7).

5.4 IDENTIFICATION OF Th17 CELLS IN GRANULOMAS.

Figure 8 demonstrates IL-17 (a marker of Th17 cells) positive staining. TB granulomas of the HIV negative group showed similar numbers (percentages) of IL-17 positive staining cells to that recorded in the HIV positive group, (difference not statistically significant; $p = 0.963427$; figure 9). The percentage of Th17 cells identified in the TB granulomas was far greater than those identified in the foreign body granuloma.

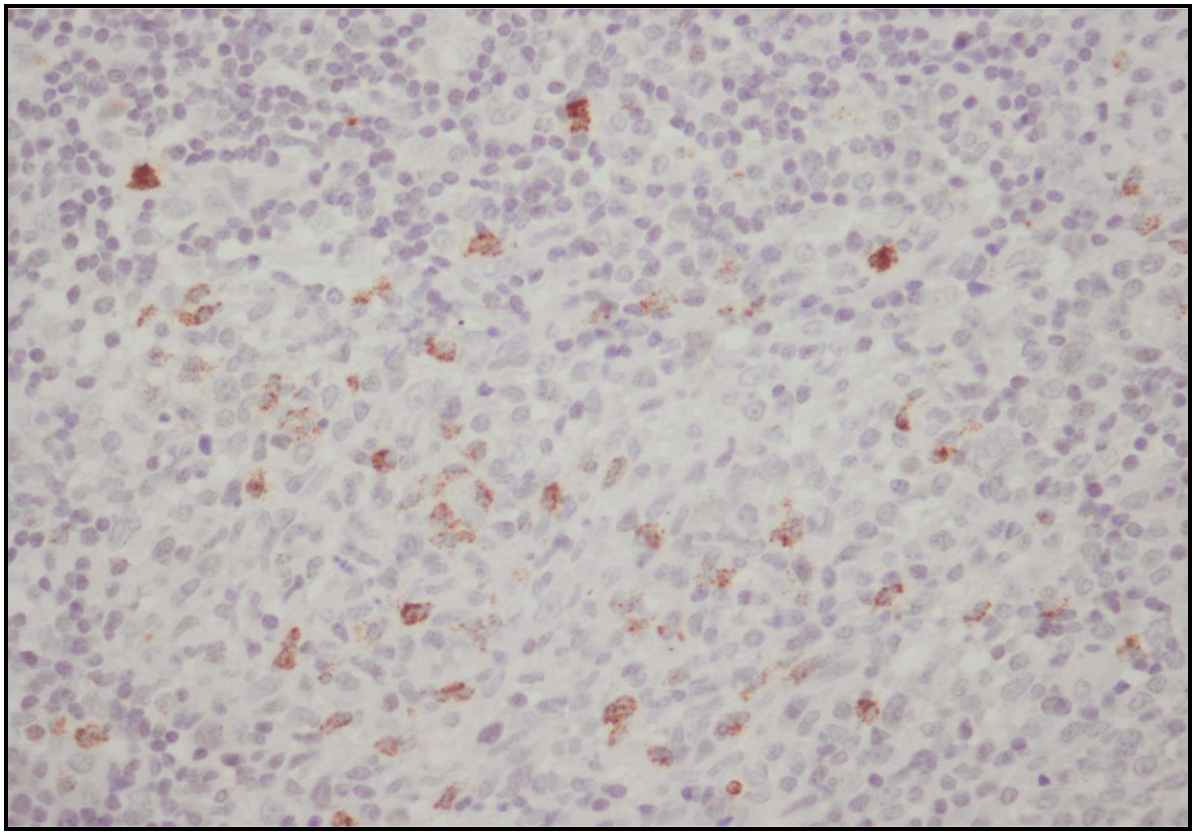

Figure 8: The photomicrograph above demonstrates IL-17 expression in a TB granuloma from an HIV negative patient. (IL-17, 4μm section; Original magnification: 400X).

	HIV NEGATIVE	*HIV POSITIVE*	*FOREIGN BODY GRANULOMA*
	26	26	
	31	9	
	22	26	
	19	27	
	15	12	
	15	8	
	17	24	
	26	27	
	9	22	
	11	9	
			8
Mean	19	**19**	**8**
SD	**7**	**8**	
Min	**9**	**8**	
Max	**31**	**27**	
Median	**18**	**23**	

Table 2: IL-17 staining in HIV negative, HIV positive TB granulomas and control tissue.
The table above displays the statistical parameters and percentage of positive IL-17 staining in TB granulomas from HIV negative, HIV positive patients and foreign body granuloma (control tissue).

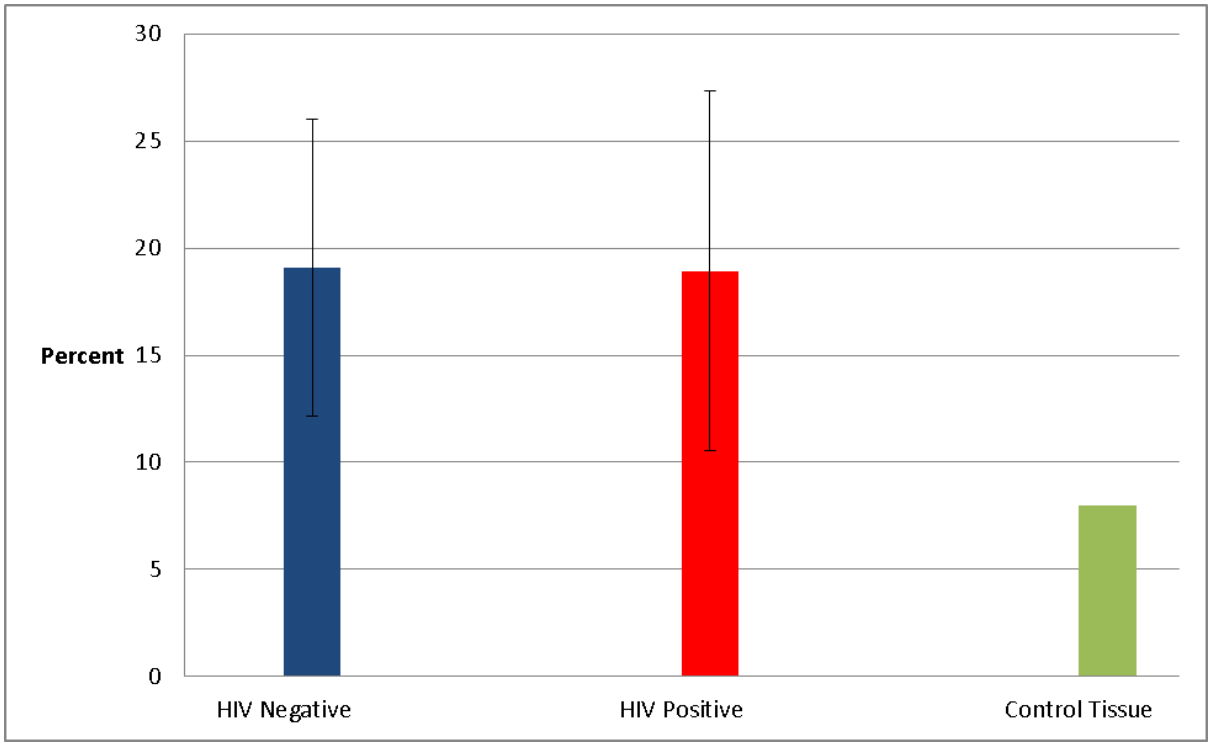

Figure 9: <u>IL-17 Staining in granulomas</u>.
The figure compares IL-17 staining cells in TB granulomas from HIV negative, HIV positive patients and the foreign body granuloma (control tissue).

5.5 IDENTIFICATION OF FOXP3 T-CELLS IN GRANULOMAS.

Figure 10 demonstrates nuclear FOXP3 staining of lymphocytes in granulomas. Nuclear staining was noted in a small percentage of cells from the TB granulomas in the HIV negative group in contrast to a far greater and statistically significant ($p<0.001$; $p = 0.00011523$) percentage of cells in the HIV positive group (figure 11).

Evaluation of the percentage of nuclear staining in the foreign body granuloma was higher than the documented average in the TB granulomas in the HIV negative group but was however much lower than the average identified in the TB granulomas from the HIV positive group.

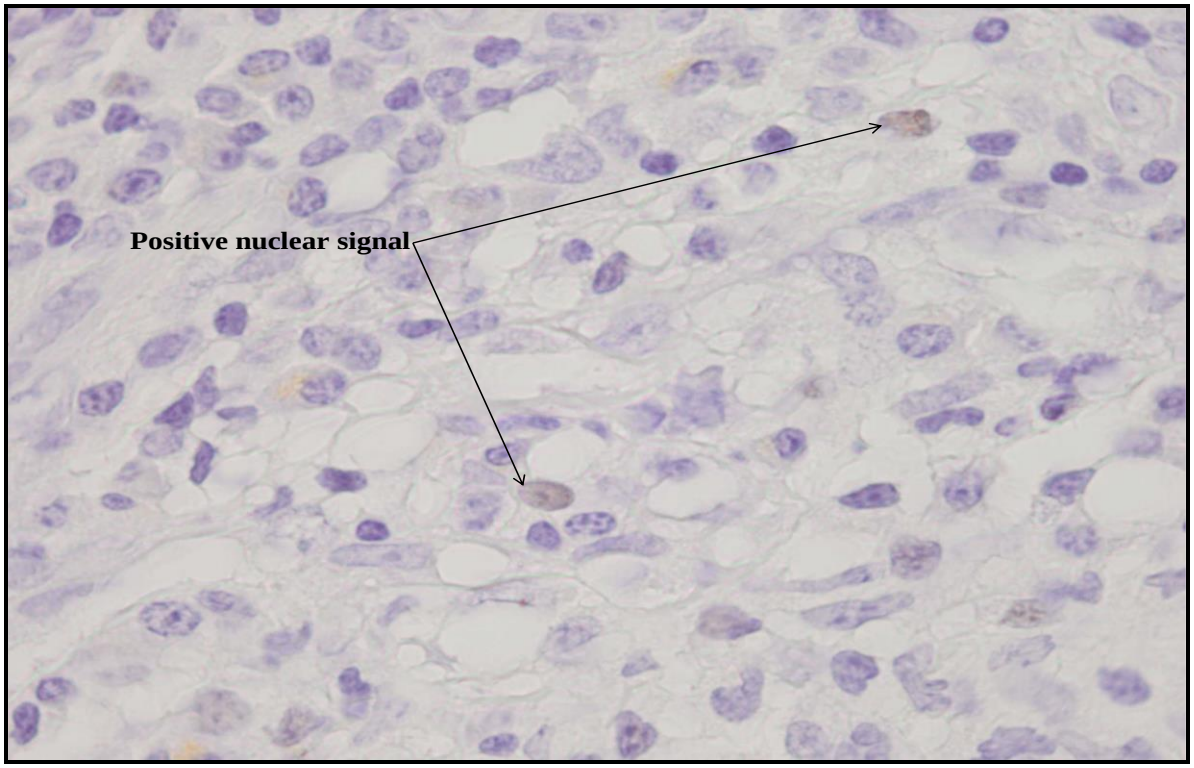

Figure 10: FOXP3 nuclear positivity is highlighted in very few cells in the photomicrograph above derived from a TB granuloma from an HIV negative patient. (FOXP3, 4μm section; Original magnification: 1000X, Oil immersion).

	HIV NEGATIVE	*HIV POSITIVE*	*FOREIGN BODY GRANULOMA*
	2	24	
	2	34	
	2	36	
	2	30	
	18	16	
	4	18	
	4	14	
	2	12	
	2	22	
	2	2	
			14
Mean	**4**	**20.8**	**14**
SD	**4.7329**	**10.0479**	
Min	**2**	**2**	
Max	**18**	**36**	
Median	**2**	**20**	

Table 3: FOXP3 staining in HIV negative, HIV positive TB granulomas and control tissue. ***The table above displays the statistical parameters and percentage of positive FOXP3 staining in TB granulomas from HIV negative, HIV positive patients and foreign body granuloma (control tissue).***

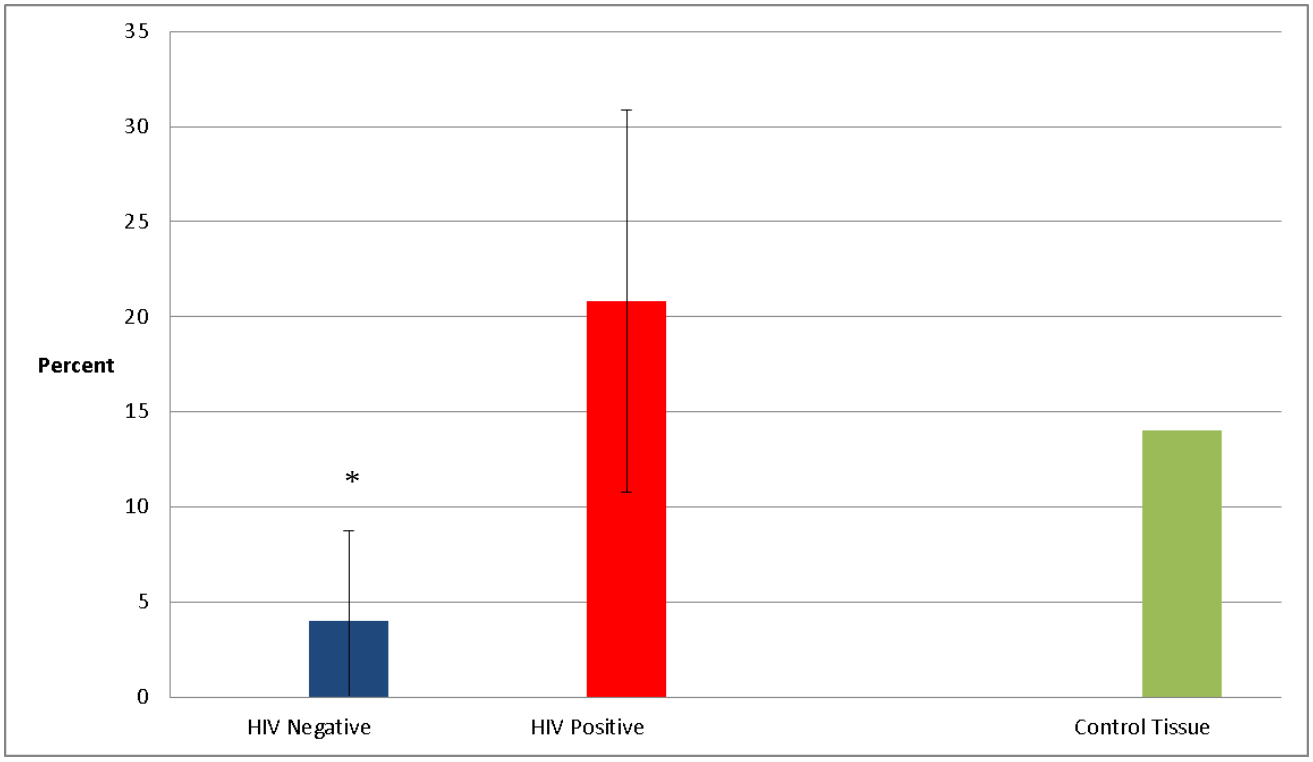

Figure 11: ***<u>FOXP3 staining in granulomas.</u>***
Comparison of FOXP3 nuclear positivity in TB granulomas from HIV negative, HIV positive patients and the foreign body granuloma (control tissue).
* $p<0.001$: FOXP3 HIV negative compared to HIV positive patients.

5.6 IDENTIFICATION OF HLA CLASS I AND II IN GRANULOMAS.

Identification of HLA Class I and II expression on cells in granulomas is demonstrated in figures 12 and 13. HLA Class I positive cells in TB granulomas from HIV negative patients showed an average that was approximately 6% lower than the percentage documented for the average of HLA Class I positive cells in TB granulomas from HIV positive patients. Figure 14 demonstrates the minor difference in percentage of HLA I positivity between the two groups ($p = 0.395124736$).

HLA Class I cell positivity in the foreign body granuloma showed a markedly decreased percentage of staining in contrast to the TB granulomas from the HIV negative group. The TB granulomas in the HIV positive group, was found to have twice the percentage of positive cells compared to the foreign body granuloma (figure 14).

HLA Class II cell positivity in TB granulomas from HIV negative patients showed positivity in 100% of cells. The results obtained for this stain also applied to the TB granulomas from the HIV positive group and to the foreign body granuloma.

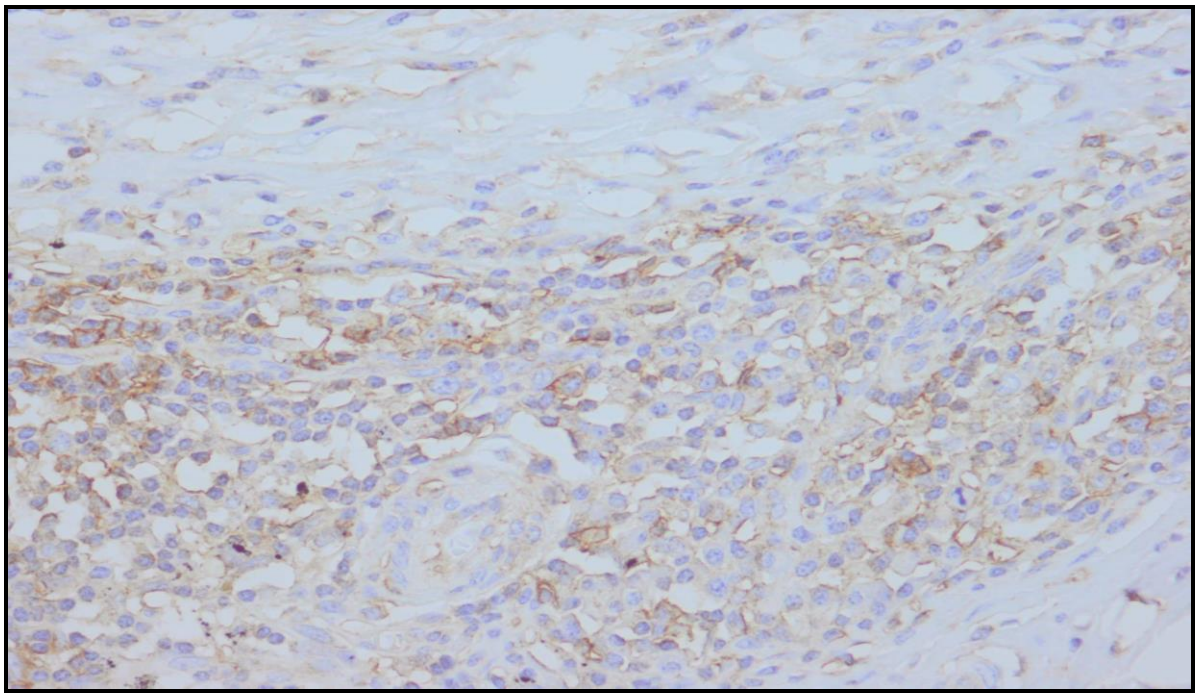

Figure 12: The photomicrograph above highlights HLA Class I positive cells in a TB granuloma from an HIV negative patient. (HLA Class I, 4μm section; Original magnification: 400X.)

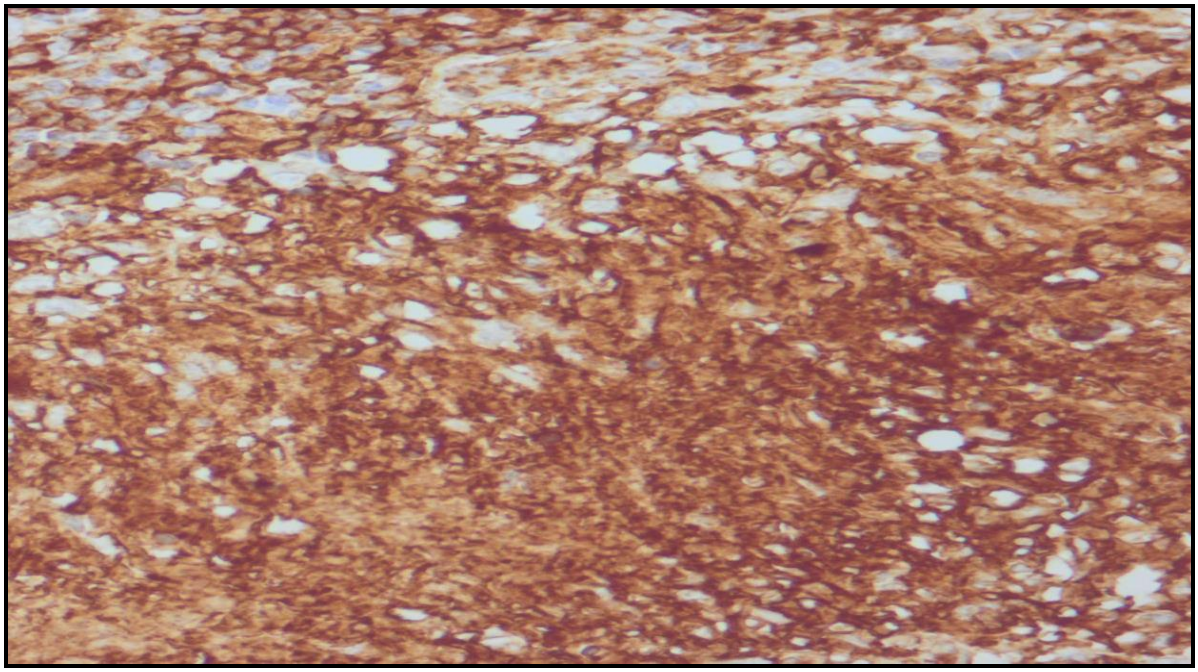

Figure 13: Diffuse, intense positive HLA Class II staining of cells within a TB granuloma from an HIV negative patient. (HLA Class II, 4μm section; Original magnification: 400X)

	HIV NEGATIVE		*HIV POSITIVE*		*FOREIGN BODY GRANULOMA*	
	HLA I	**HLA II**	**HLA I**	**HLA II**	**HLA I**	**HLA II**
	44	100	60	100		
	30	100	66	100		
	44	100	56	100		
	22	100	58	100		
	68	100	54	100		
	56	100	70	100		
	68	100	54	100		
	64	100	50	100		
	70	100	54	100		
	48	100	54	100		
					28	**100**
Mean	**51.4**	**100**	**57.6**	**100**	**28**	**100**
SD	**15.8758**	**0.0000**	**5.8515**	**0.0000**		
Min	**22**	**100**	**50**	**100**		
Max	**70**	**100**	**70**	**100**		
Median	**52**	**100**	**55**	**100**		

Table 4: <u>HLA Class I and II staining in HIV negative, HIV positive TB granulomas and control tissue.</u>
The above table demonstrates the statistical parameters and percentage of positive HLA Class I and II staining in TB granulomas from HIV negative, HIV positive patients and foreign body granuloma (control tissue).

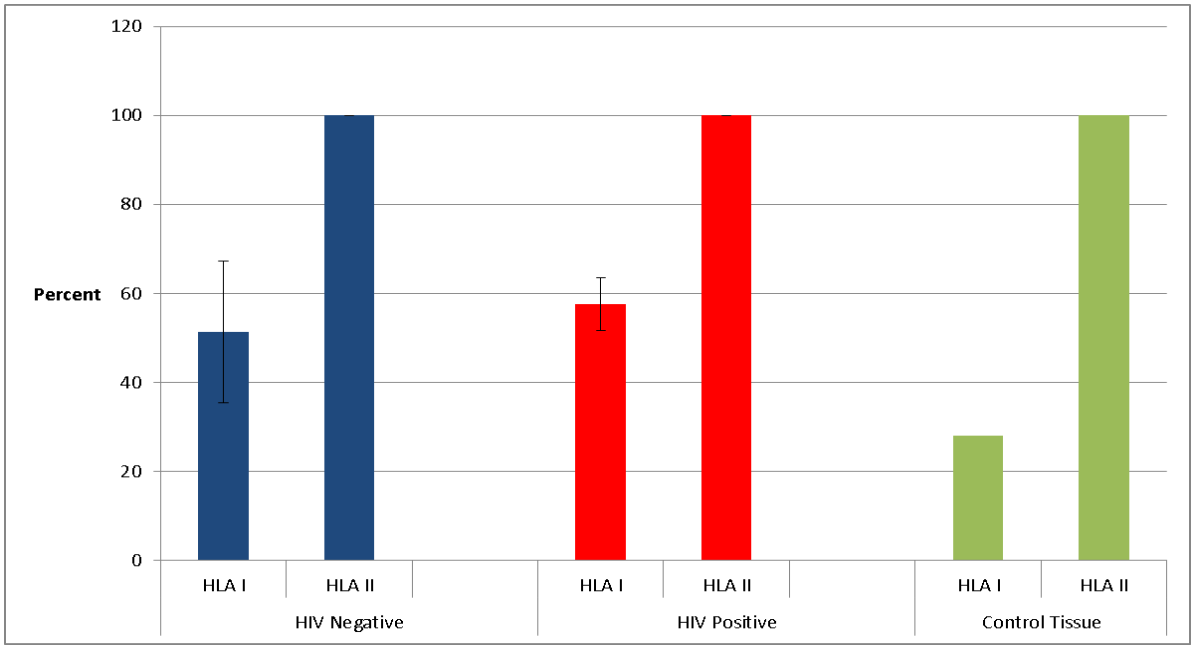

Figure14: <u>HLA Class I and II staining in granulomas.</u>
Comparison of HLA Class I and II staining cells in TB granulomas from HIV negative, HIV positive patients and the foreign body granuloma (control tissue).

5.7 IDENTIFICATION OF SELECTED CYTOKINES IN GRANULOMAS.

As discussed in the literature review, IL-2 and IFN-γ are generally regarded as pro-inflammatory cytokines whereas IL-4 is considered as an anti-inflammatory cytokine; with IL-6 belonging to either group depending on the target cell population. In the present study, and based on the studies of Sussman and Wadee [29], IL-6 was placed in the group of the anti-inflammatory cytokines. IL-2, IFN-γ, IL-4 and IL-6 staining in cells (macrophages and lymphocytes) are depicted in figures 15, 16, 17 and 18 respectively.

5.7.1 Pro-inflammatory Cytokines.

IL-2 cell positivity in the TB granulomas of the HIV negative group of patients showed minimal, insignificant ($p = 0.58256$) differences compared to those from TB granulomas from HIV positive patients (figure 19).

IL-2 positive cells in the foreign body granuloma were elevated in comparison to TB granulomas from the HIV negative group. The percentage of positive cells in the foreign body granuloma was also much greater than that noted in TB granulomas from the HIV positive group as depicted in figure 19.

IFN-γ in TB granulomas from HIV negative patients showed a mean that was approximately 10% higher, but not statistically significant ($p = 0.097693$) when compared to the average percentage of staining seen in TB granulomas from HIV positive patients.

Evaluation of IL-2 percentage cell positivity and percentage of IFN-γ cell positivity in TB granulomas from the HIV negative group demonstrated that the percentage of IFN-γ cells was approximately twice the percentage, and thus significantly raised ($p<0.05$; $p = 0.016842665$) in comparison to the percentage of IL-2 positive cells. A comparison of the percentage positivity between these two stains in the HIV positive group showed a non-significant ($p = 0.11218$) increase of 1.2 times more cells staining with IFN-γ compared to those that stained for IL-2.

Evaluation of percentage cell positivity of IFN-γ staining in the foreign body granuloma showed a prominent increase when compared to TB granulomas derived from the HIV negative group. This difference was even greater when comparisons were made to the TB granulomas of the HIV positive group.

The percentage of cells staining positive with IFN-γ in the foreign body granuloma was seen to be much higher than the percentage cells staining with IL-2.

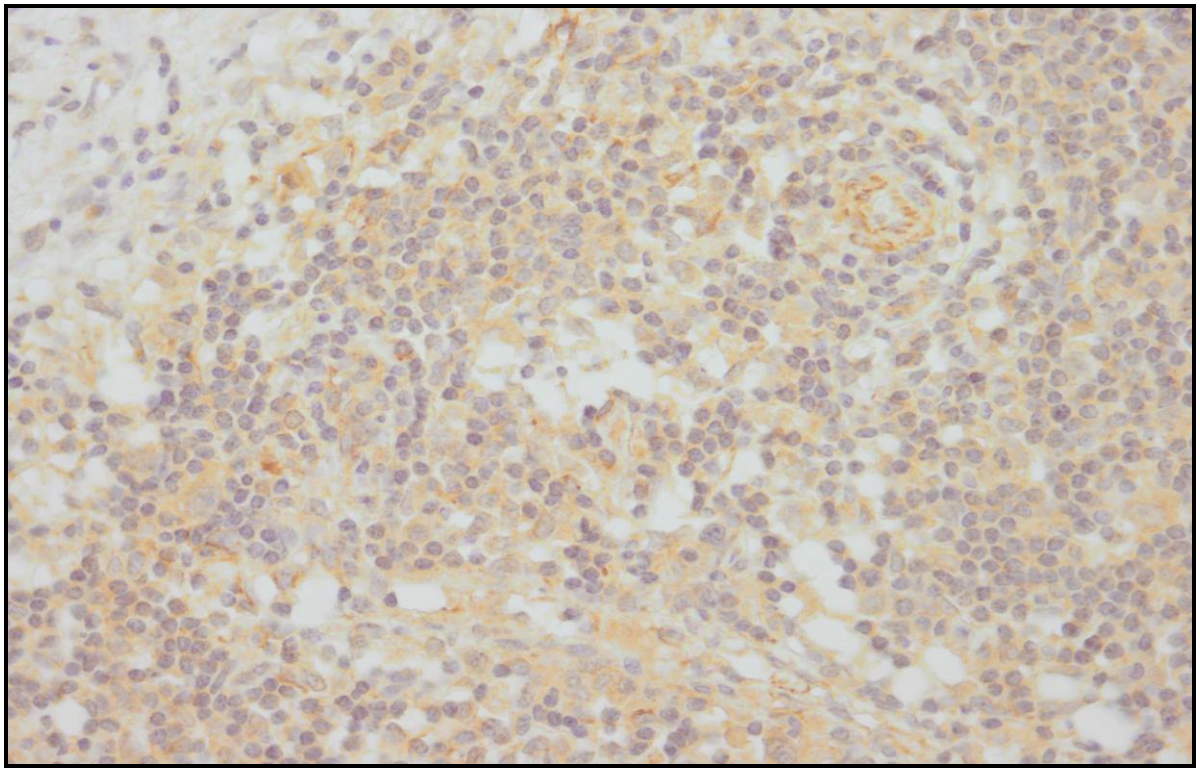

Figure 15: IL-2 positive cells in a TB granuloma from an HIV negative patient are highlighted in the photomicrograph above. (IL-2, 4μm section; Original magnification: 400X.)

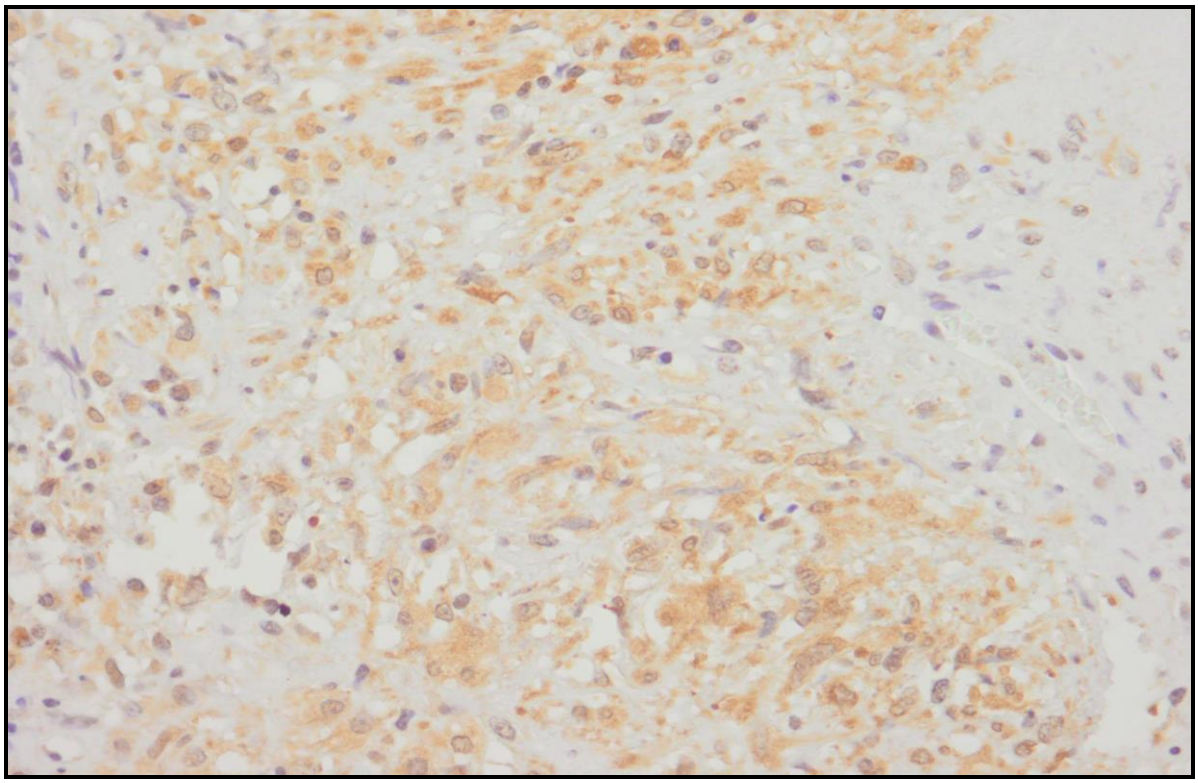

Figure 16: IFN-γ positive cells in a TB granuloma from an HIV negative patient are shown above. (IFN-γ, 4μm section; Original magnification: 400X.)

5.7.2 Anti-inflammatory Cytokines.

The percentage of cells staining with IL-4 in TB granulomas from the HIV negative group was approximately 13% lower, but not statistically significant ($p = 0.064799$), when compared to IL-4 staining cells noted in TB granulomas from the HIV positive group.

Evaluation of IL-4 within the foreign body granuloma showed a much a lower percentage of cells stained compared to the average percentage cell staining in the TB granulomas from the HIV negative group. The percentage of IL-4 positivity in the foreign body granulomas was however much lower in contrast to the TB granulomas from the HIV positive group.

The mean percentage of IL-6 staining in TB granulomas from the HIV negative group was far higher and markedly significant ($p<0.001$; $p = 0.00000046158$) than that documented in TB granulomas from the HIV positive group.

Staining of IL-6 positive cells in the foreign body granuloma was approximately 5 times lower than the average percentage of positive cells in TB granulomas from HIV negative patients. Cell staining with this cytokine in the foreign body granuloma, was 1.8 times lower than the average percentage of positive cells in TB granulomas from HIV positive patients.

Comparison of the percentage of positive IL-4 cells to the percentage of IL-6 positive cells in TB granulomas from HIV negative patients showed that IL-6 was much higher and that the differences between the two were significant ($p<0.05$; $p = 0.010010786$).

The percentage of IL-4 positive cells in comparison to IL-6 positive cells in TB granulomas from HIV positive patients showed that IL-4 was markedly and significantly elevated ($p<0.001$; $p= 0.00000609701$).

When the individual cytokines were compared to one another as regards correlation co-efficients, there was no significant correlation noted (results not shown).

An example of the immunohistochemical stains performed on a TB granuloma from an HIV positive patient; and on the foreign body granuloma, is presented in chapter 5.8 and 5.9 respectively. Similarly, an example of stains performed on a TB granuloma from an HIV negative patient are presented in the current results chapter. Tables containing absolute cell counts of the stains performed on granulomas are included in Chapter 8.1.

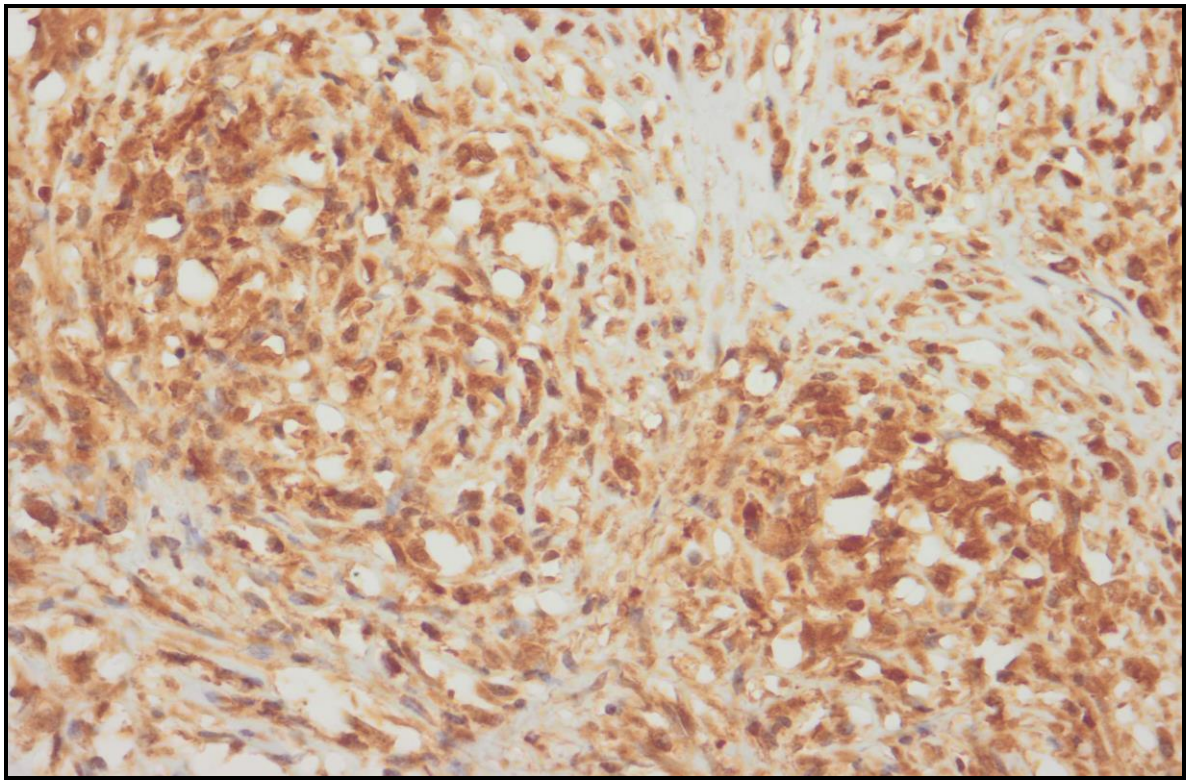

Figure 17: IL-4 positive cells in a TB granuloma from an HIV negative patient are shown in the photomicrograph above. (IL-4, 4μm section; Original magnification: 400X.)

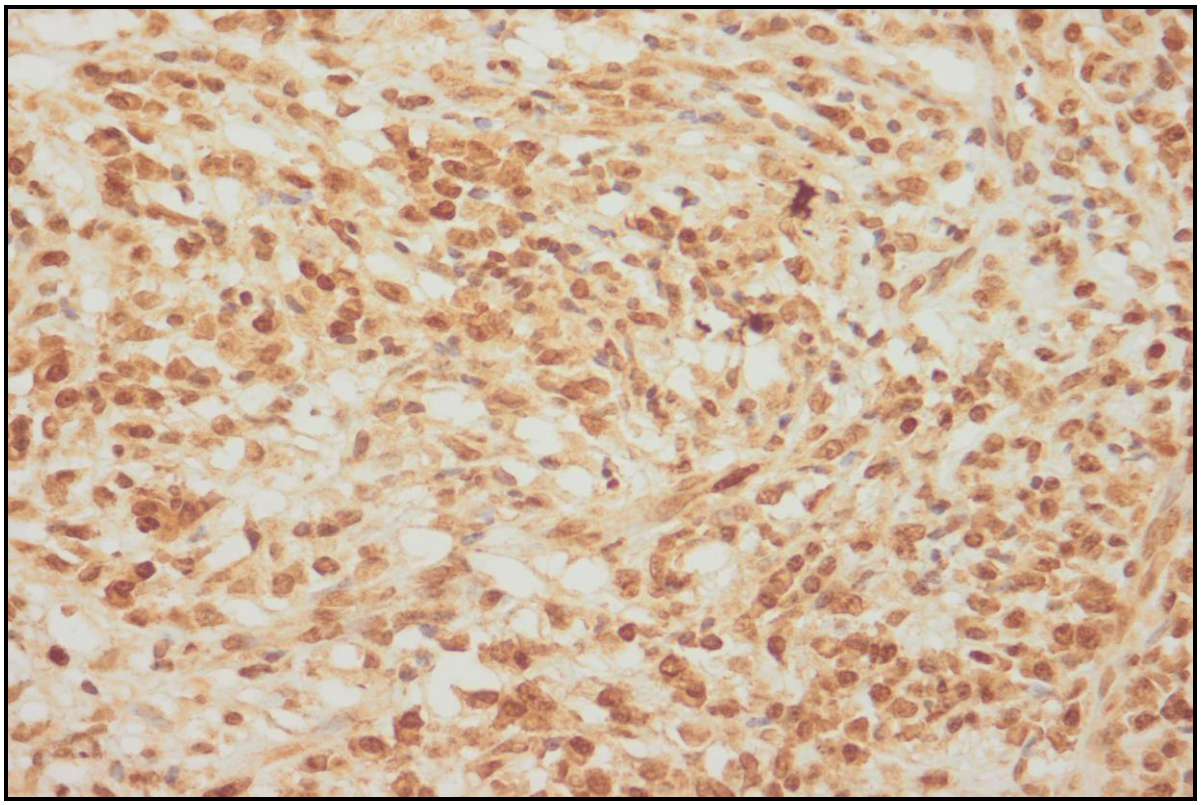

Figure 18: The above photomicrograph shows IL-6 positive cells in a TB granuloma from an HIV negative patient. (IL-6, 4μm section; Original magnification: 400X.)

	HIV NEGATIVE				*HIV POSITIVE*				*FOREIGN BODY GRANULOMA*			
	IL2	IFNγ	IL4	IL6	IL2	IFNγ	IL4	IL6	IL2	IFNγ	IL4	IL-6
	60	54	74	80	36	38	70	48				
	10	54	70	86	30	48	70	62				
	30	26	30	90	26	28	72	6				
	20	68	28	80	22	30	70	24				
	20	30	30	78	32	34	78	28				
	30	34	70	80	24	36	78	28				
	34	46	76	84	34	32	64	20				
	2	12	70	62	38	34	78	18				
	20	54	68	86	16	20	62	22				
	14	60	70	62	14	30	70	14				
									38	**62**	**48**	**15**
Mean	**24**	**43.8**	**58.6**	**78.8**	**27.2**	**33**	**71.2**	**27**	**38**	**62**	**48**	**15**
SD	**15.1526**	**16.6721**	**19.2883**	**9.0863**	**7.8077**	**6.8848**	**5.3066**	**15.6269**				
Min	**2**	**12**	**28**	**62**	**14**	**20**	**62**	**6**				
Max	**60**	**68**	**76**	**90**	**38**	**48**	**78**	**62**				
Median	**20**	**50**	**70**	**80**	**28**	**33**	**70**	**23**				

Table 5: Cytokine staining in HIV negative, HIV positive TB granulomas and control tissue.

The table above displays the statistical parameters and percentage of positive cytokine staining in TB granulomas from HIV negative, HIV positive patients and foreign body granuloma (control tissue).

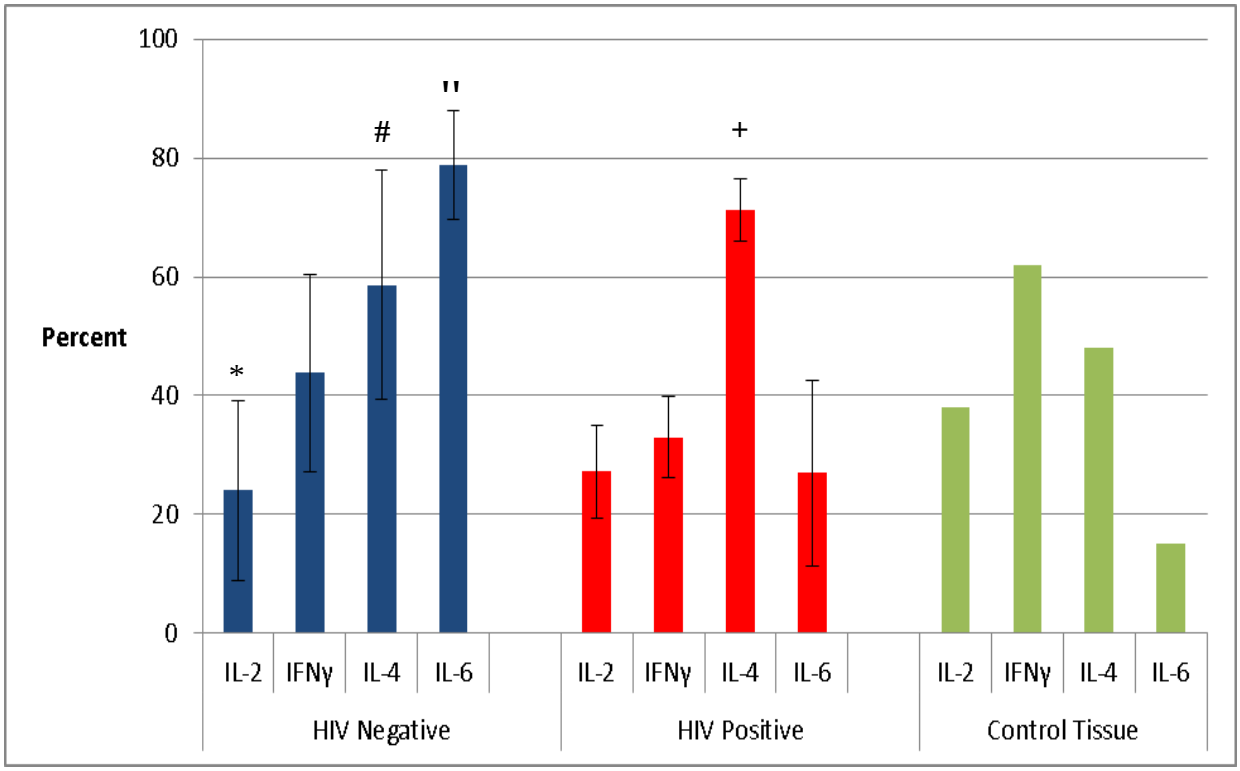

Figure 19: <u>Cytokine staining in granulomas.</u>

Comparison of the various cytokine staining cells in TB granulomas from HIV negative, HIV positive patients and the foreign body granuloma (control tissue).

* $p<0.05$ IL-2 compared to IFN-γ in HIV negative patients.
# $p<0.05$ IL-4 compared to IL-6 in HIV negative patients.
!! $p<0.001$ IL-6 in HIV negative compared to HIV positive patients.
+ $p<0.001$ IL-4 compared to IL-6 in HIV positive patients.

	HIV NEGATIVE		*HIV POSITIVE*		*FOREIGN BODY GRANULOMA*
	Mean (%) absolute value	**SD(+/-)**	**Mean (%) absolute value**	**SD(+/-)**	**Mean (%) absolute value**
CD4	**159.5 (63.8)**	**25.2438**	**95.5 (38.2)**	**13.1244**	**68 (27)**
CD8	**147 (58.8)**	**36.1386**	**158.5 (63.4)**	**15.3379**	**73 (29)**
CD68	**250 (100)**	**0**	**250 (100)**	**0**	**250 (100)**
Th17	**47.7 (19)**	**17.3144**	**43.7 (19)**	**20.97**	**22 (8)**
FOXP3	**10 (4)**	**11.8322**	**54 (20.8)**	**21.8861**	**35 (14)**
HLA Class I	**133.5 (51.4)**	**32.6382**	**144 (57.6)**	**14.6287**	**70 (28)**
HLA Class II	**250 (100)**	**0**	**250 (100)**	**0**	**250 (100)**
IL-2	**60 (24)**	**37.8814**	**68 (27.2)**	**19.5192**	**95 (38)**
IFN-γ	**109.5 (43.8)**	**41.6803**	**82.5 (33)**	**17.2119**	**155 (62)**
IL-4	**144 (58.6)**	**47.5815**	**178 (71.2)**	**13.2665**	**120 (48)**
IL-6	**197 (78.8)**	**22.7156**	**67.5 (27)**	**39.0672**	**38 (15)**

Table 6: <u>Summary of the immunohistochemical staining in HIV negative, HIV positive TB granulomas and control tissue.</u>

The table above summarises staining in the various granulomas.

5.8 PHOTOMICROGRAPHS OF TB GRANULOMAS IN AN HIV POSITIVE PATIENT

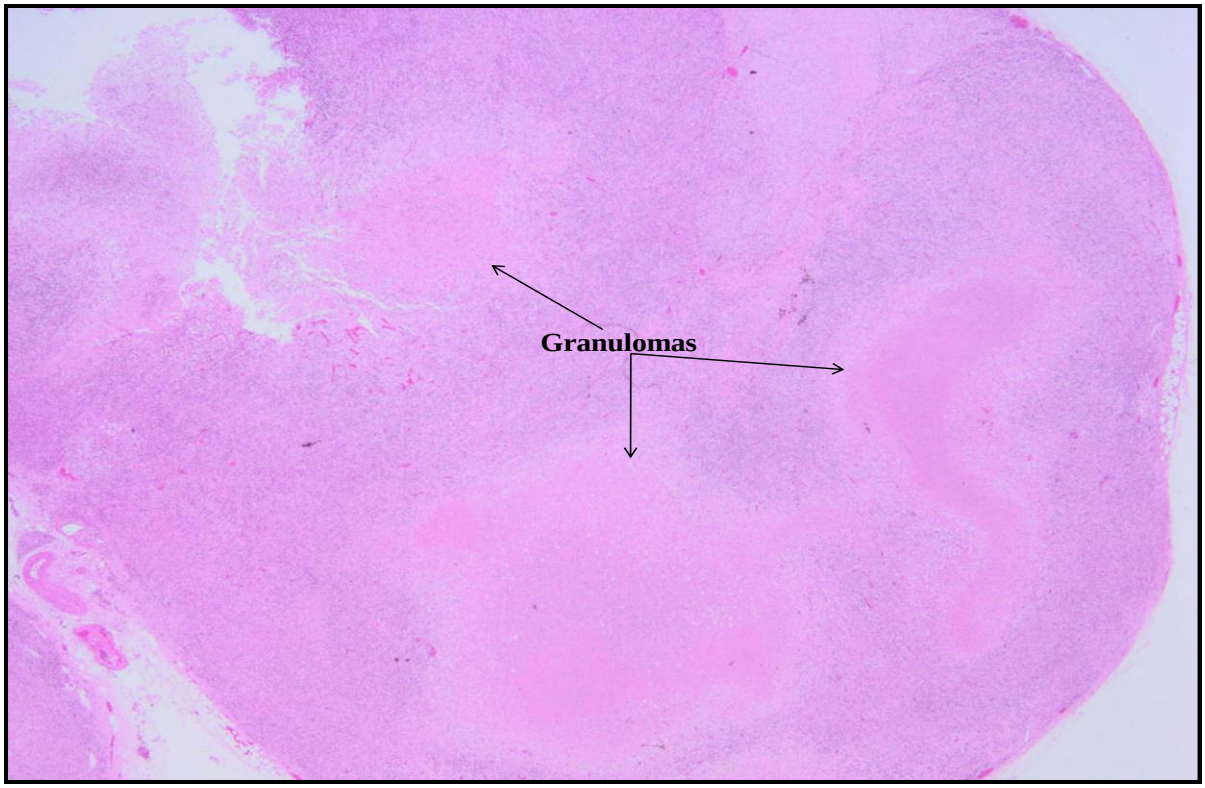

Figure 20: The photomicrograph above depicts TB granulomas in a lymph node from an HIV positive patient. (H&E, 2μm section; Original magnification: 20X).

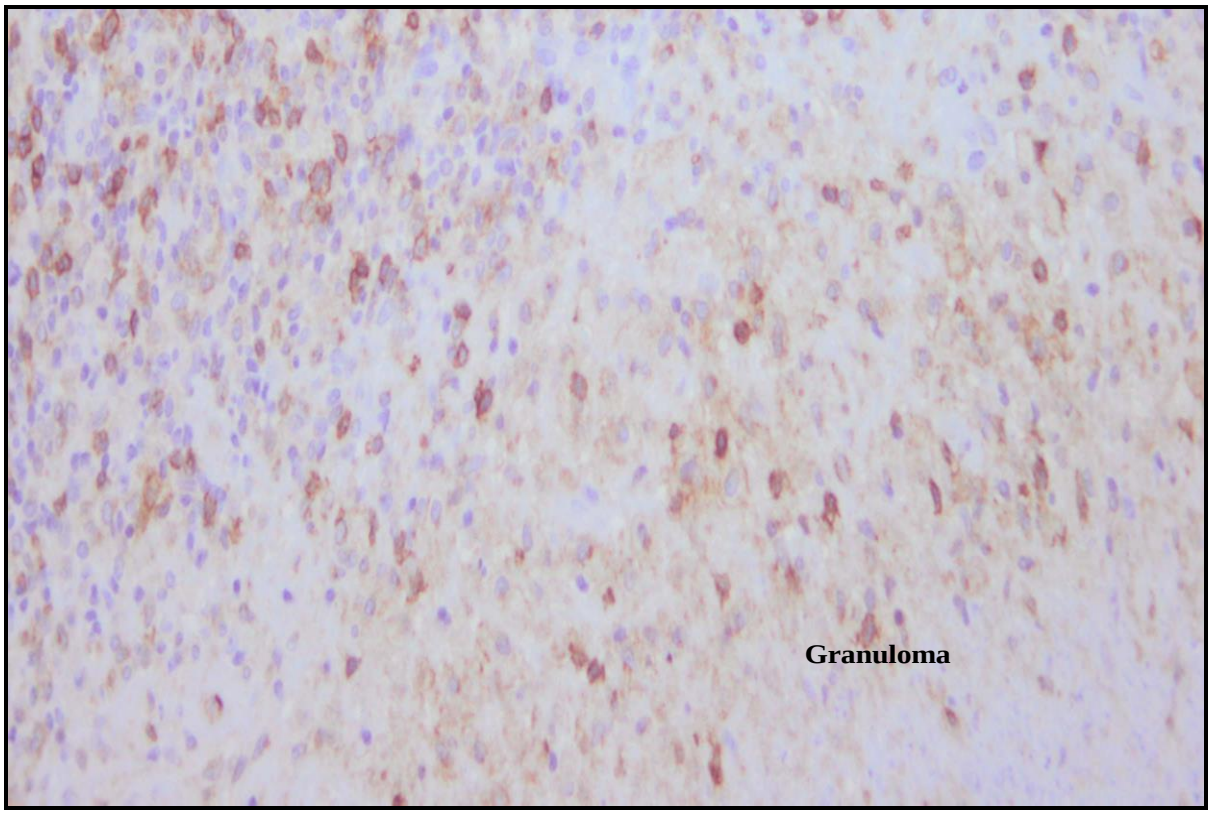

Figure 21: CD4 staining in a TB granuloma from an HIV positive patient. (CD4, 4μm section; Original magnification: 400X.)

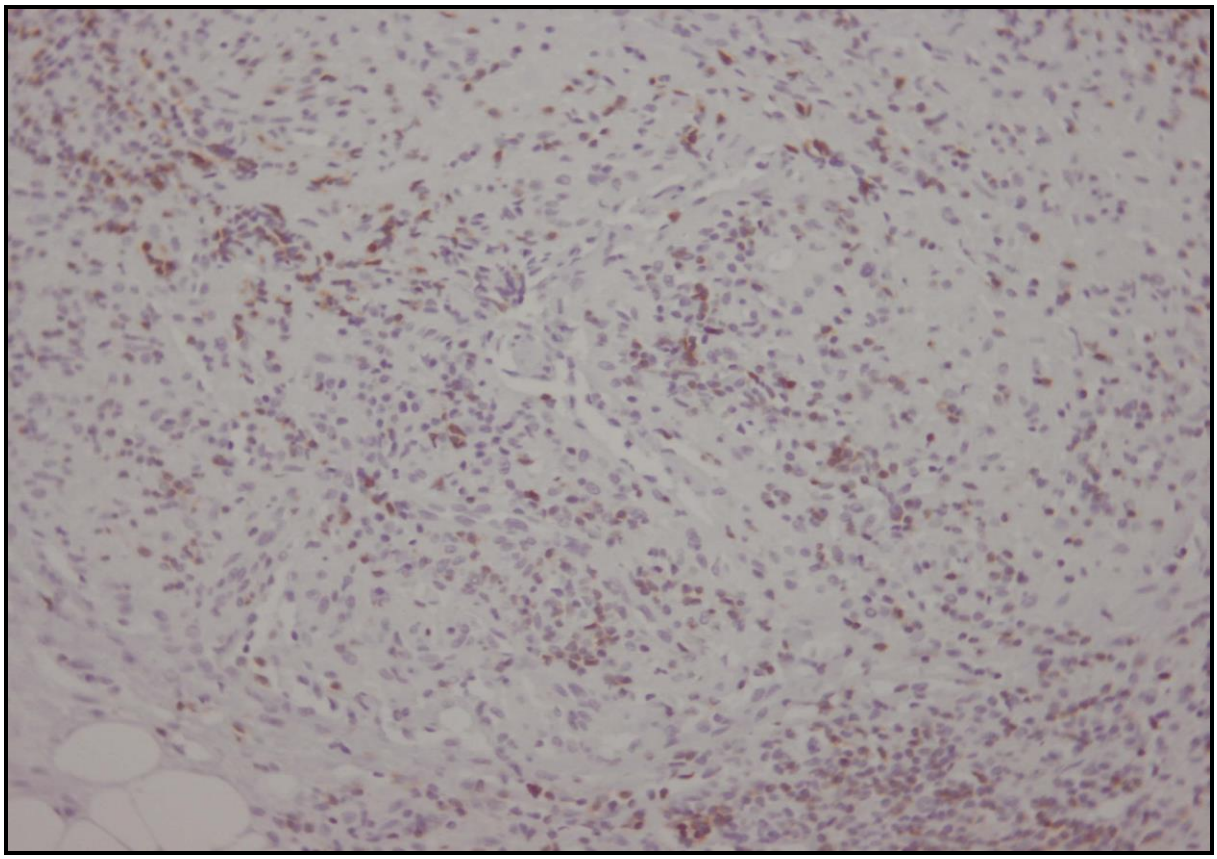

Figure 22: CD8+ cells are seen at the periphery as well as in a central location in a TB granuloma from an HIV positive patient. (CD8, 4μ section; Original magnification: 400X.)

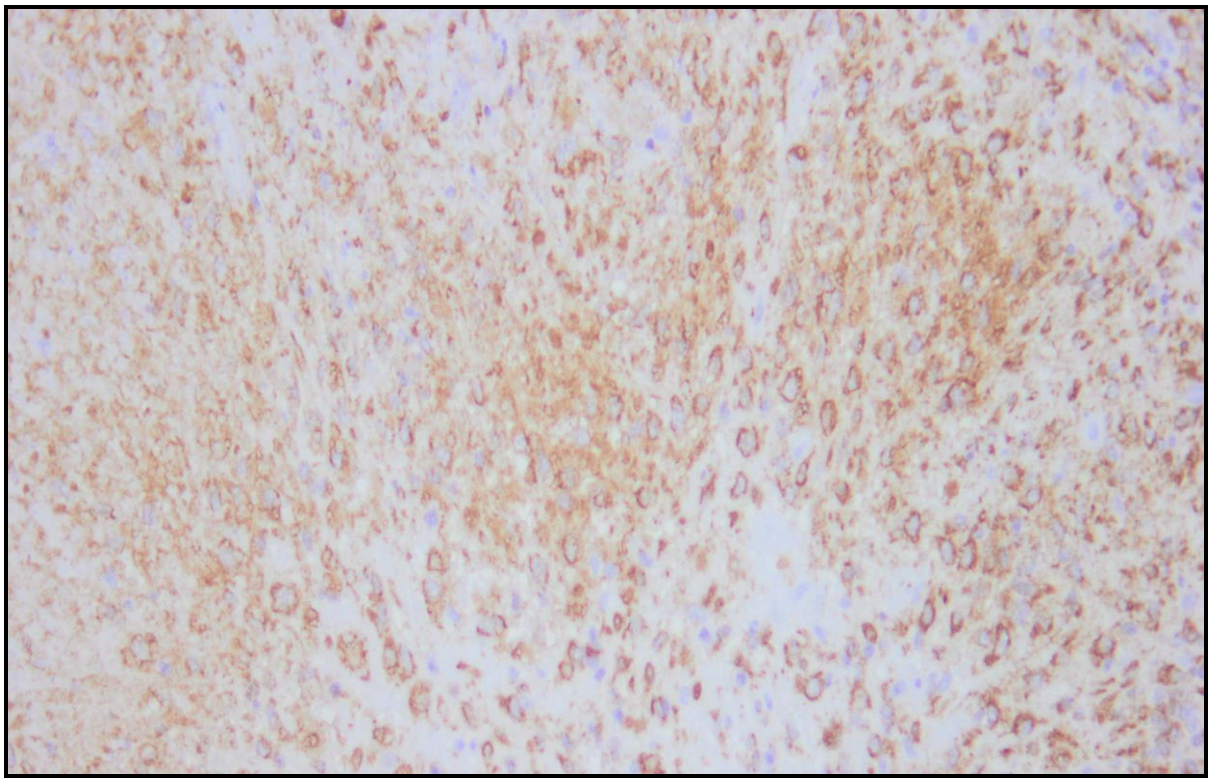

Figure 23: Diffuse CD68 positivity is identified in a TB granuloma from an HIV positive patient. (CD68, 4μm section; Original magnification: 200X.)

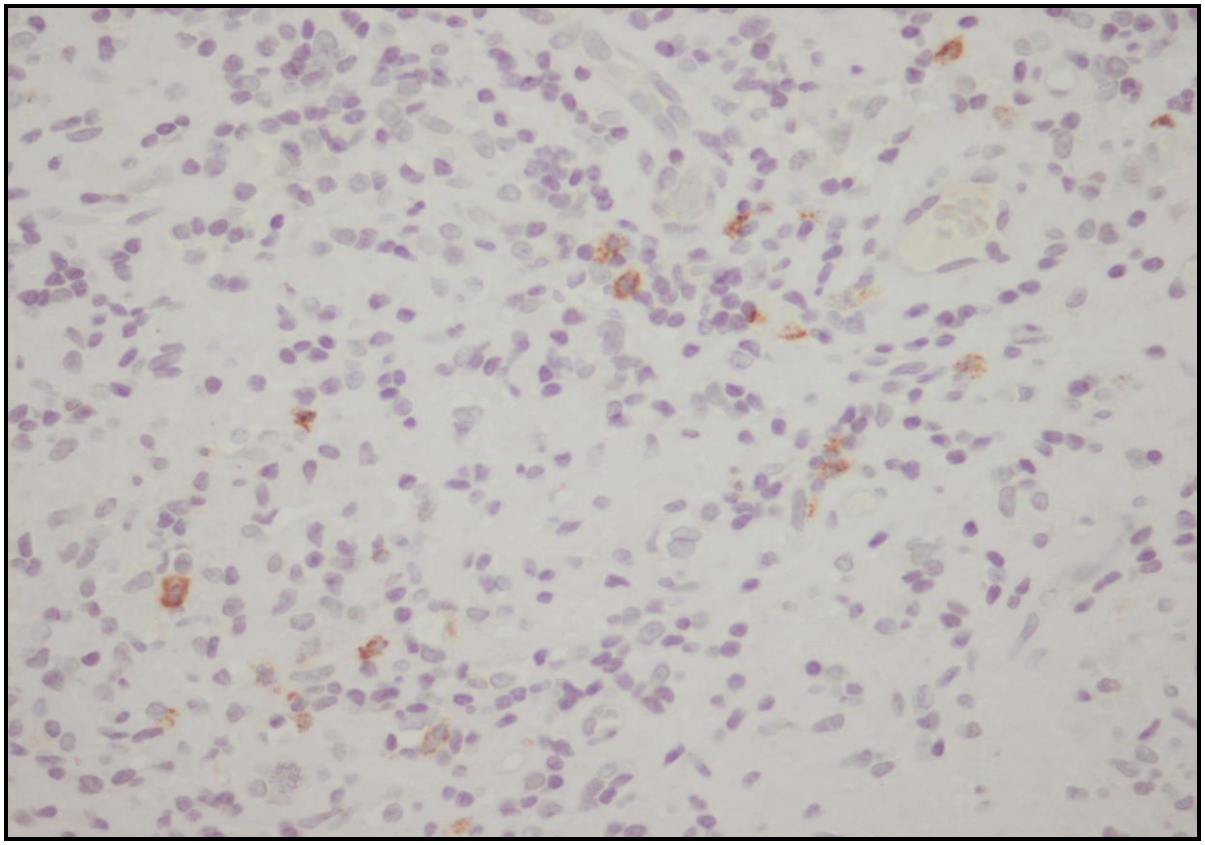

Figure 24: IL-17 cells are highlighted above in a TB granuloma from an HIV positive patient. (IL-17, 4μm section; Original magnification: 400X.)

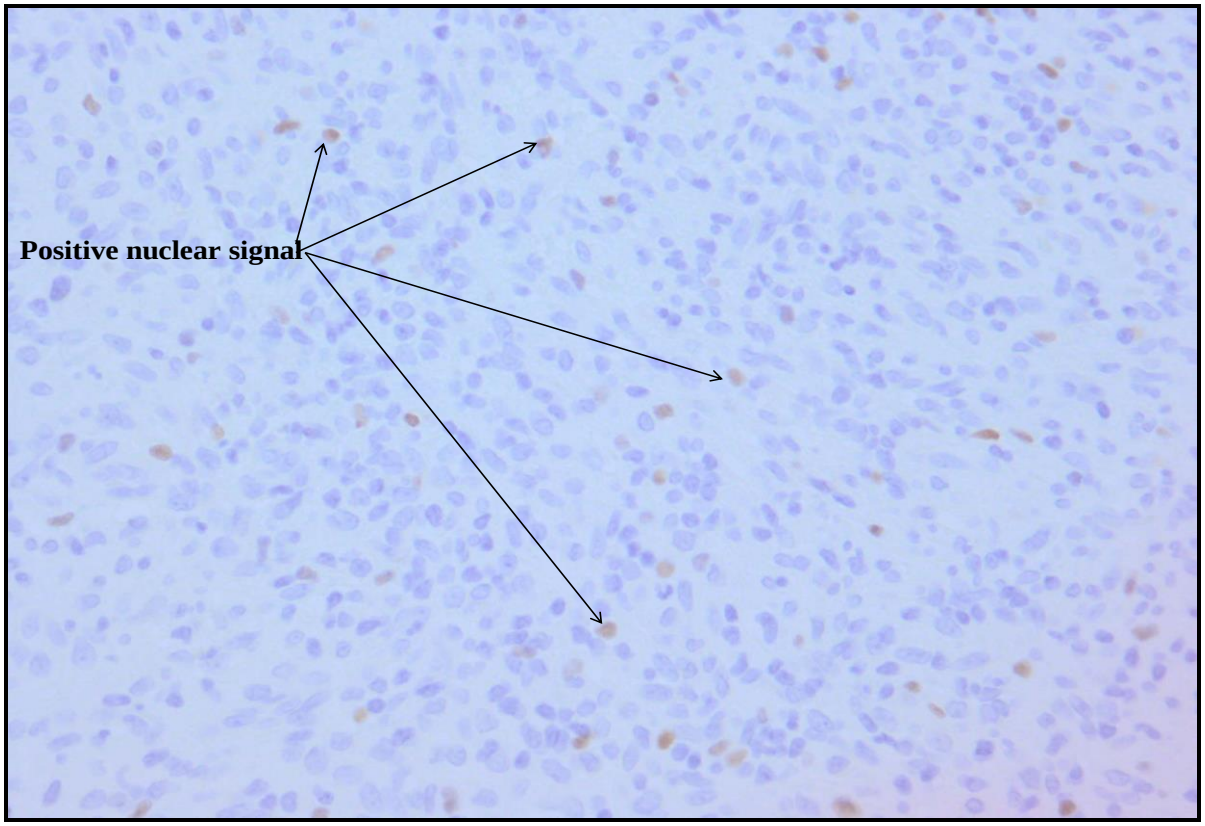

Figure 25: Numerous FOXP3 nuclei are demonstrated in a TB granuloma from an HIV positive patient. (FOXP3, 4μm section; Original magnification 1000X Oil immersion.)

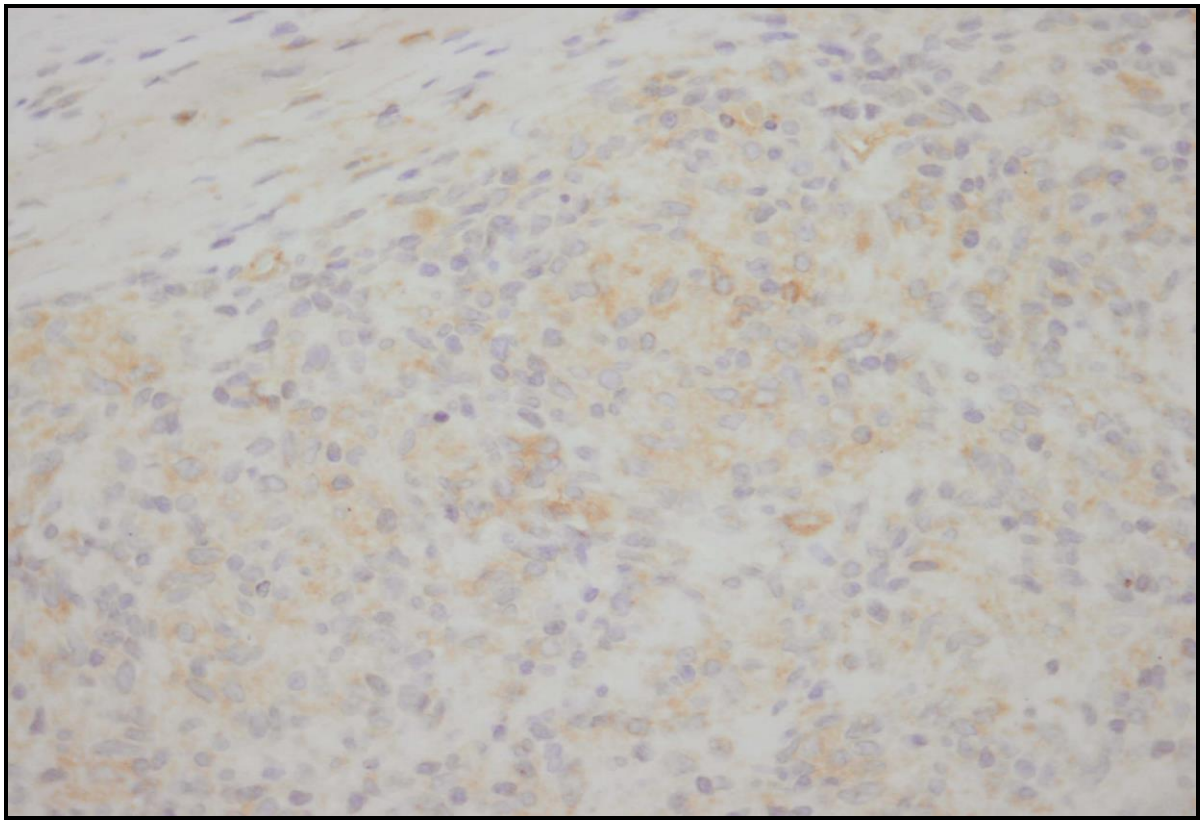

Figure 26: HLA Class I cells are highlighted in a TB granuloma from an HIV positive patient. (HLA Class I, 4μm section; Original magnification: 400X.)

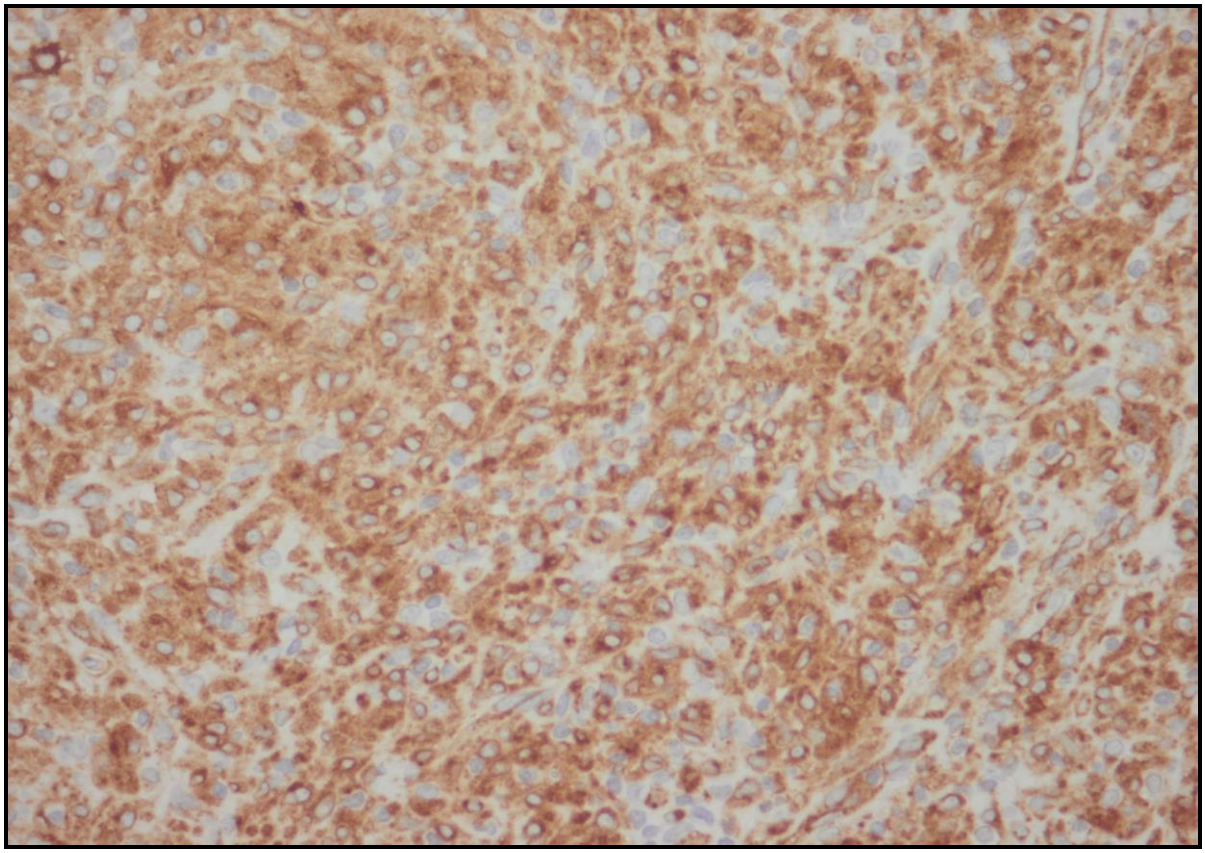

Figure 27: Diffuse HLA Class II staining is identified in a TB granuloma from an HIV positive patient. (HLA Class II, 4μm section: Original magnification: 400X)

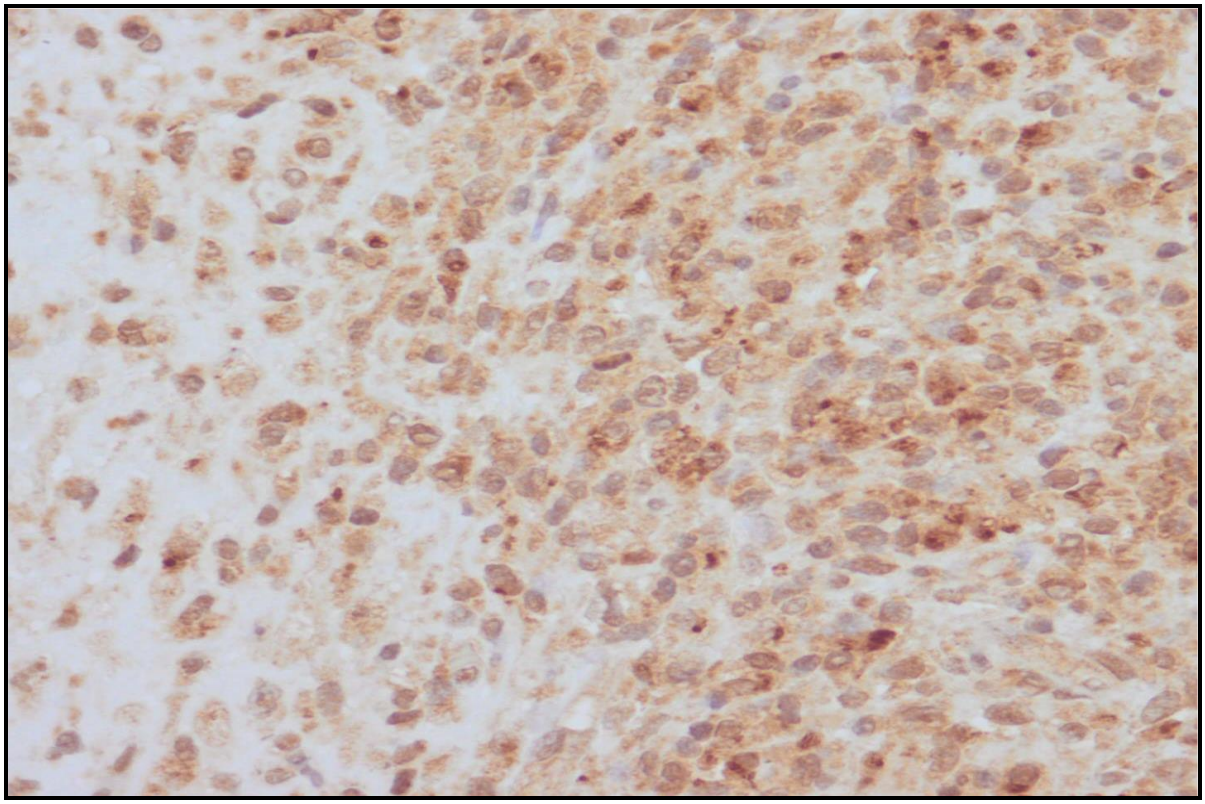

Figure 28: IL-2 cells are demonstrated in a TB granuloma from an HIV positive patient. (IL-2, 4μm section: Original magnification: 400X.)

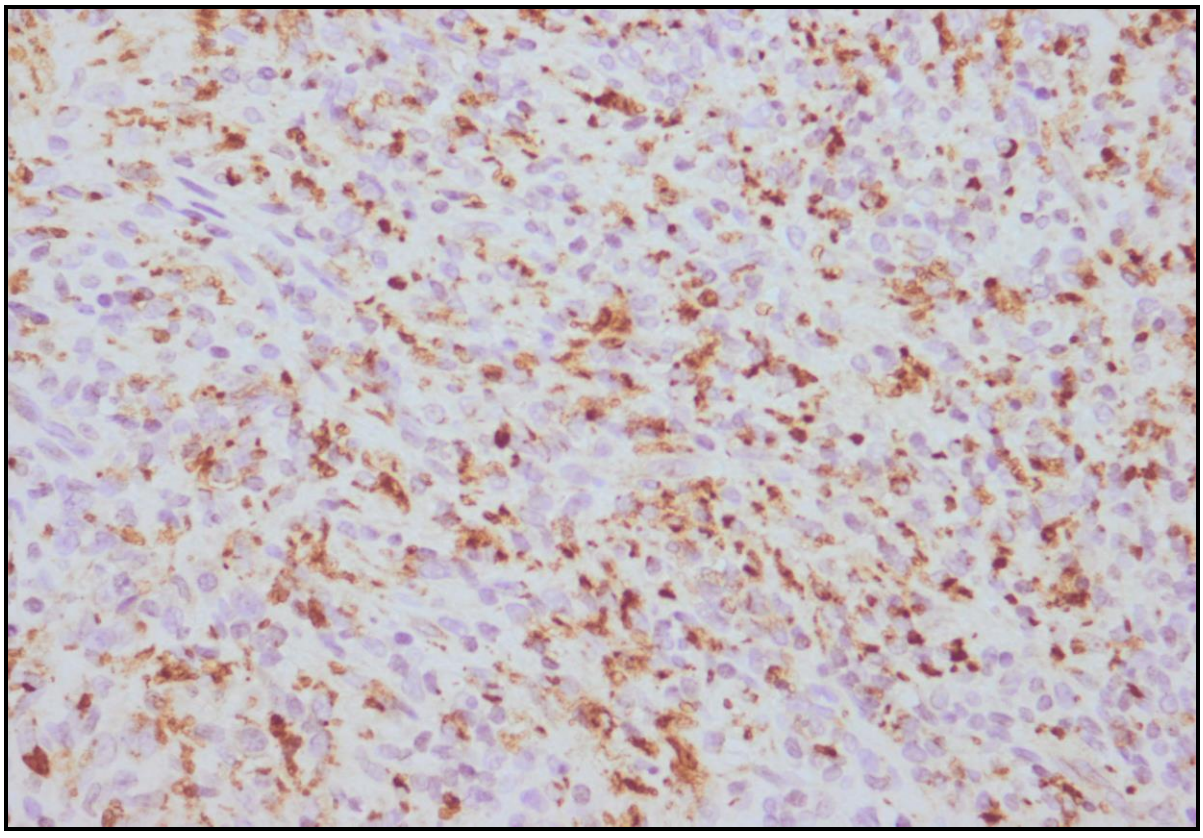

Figure 29: The photomicrograph above illustrates IFN-γ staining in a TB granuloma from an HIV positive patient. (IFN-γ, 4μm section; Original magnification: 400X.)

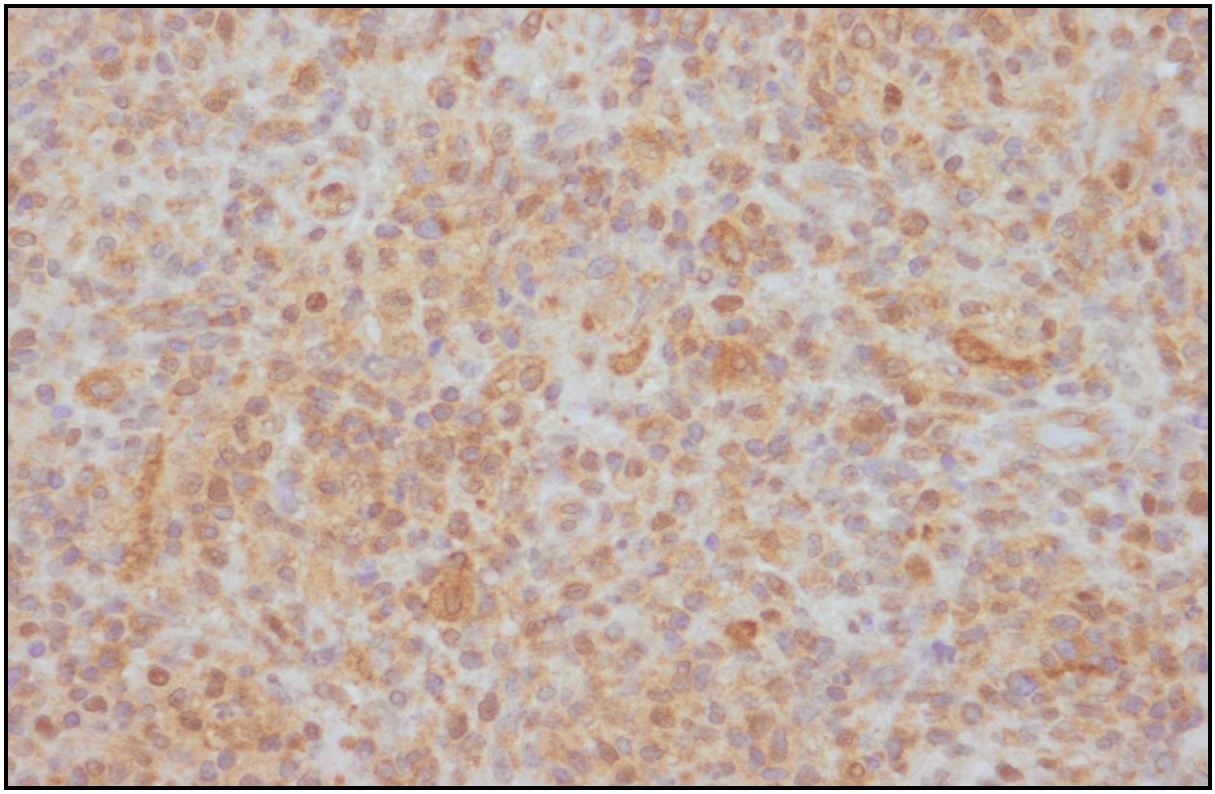

Figure 30: IL-4 staining is identified in a TB granuloma from an HIV positive patient. (IL-4, 4μm section; Original magnification: 400X.)

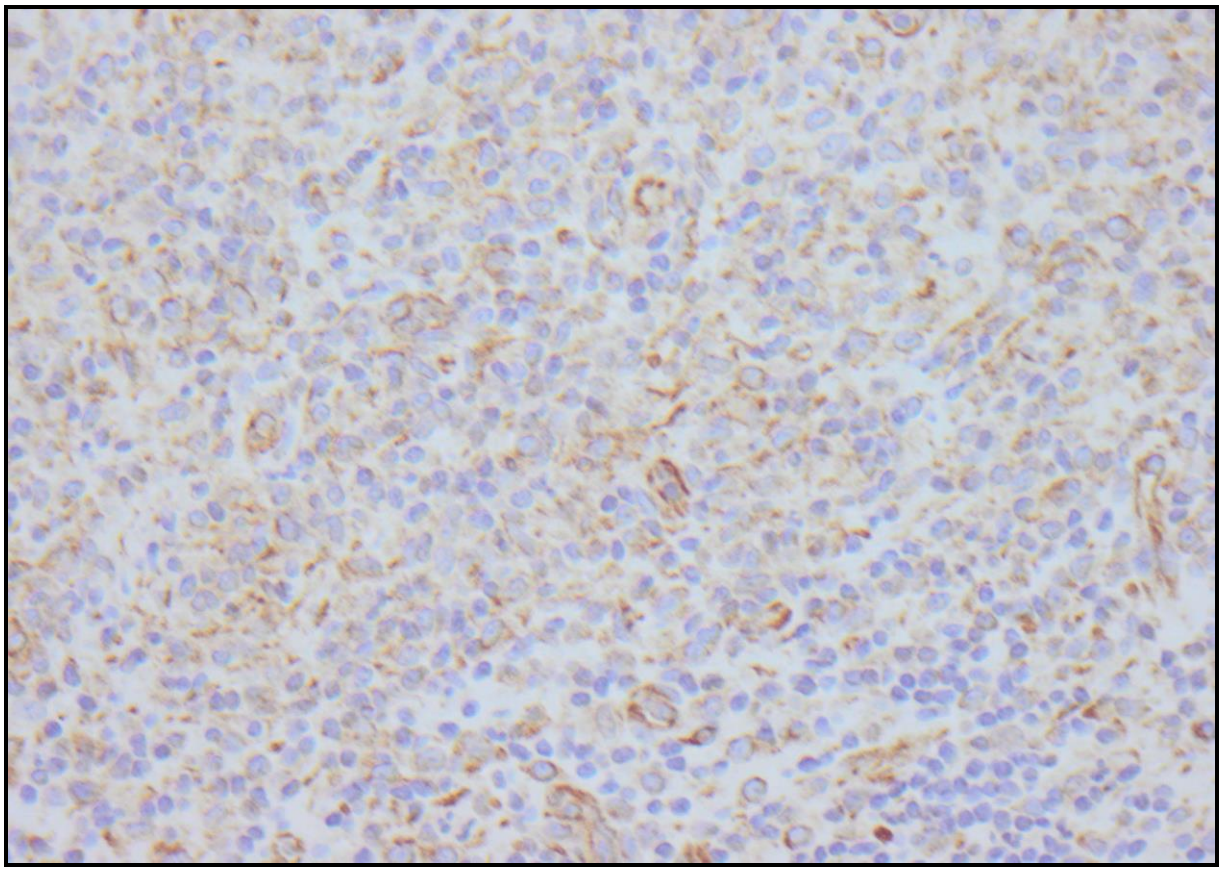

Figure 31: IL-6 staining in a TB granuloma from an HIV positive patient. (IL-6, 4μm section; Original magnification: 400X.

5.9 PHOTOMICROGRAPHS FROM FOREIGN BODY GRANULOMA (CONTROL TISSUE)

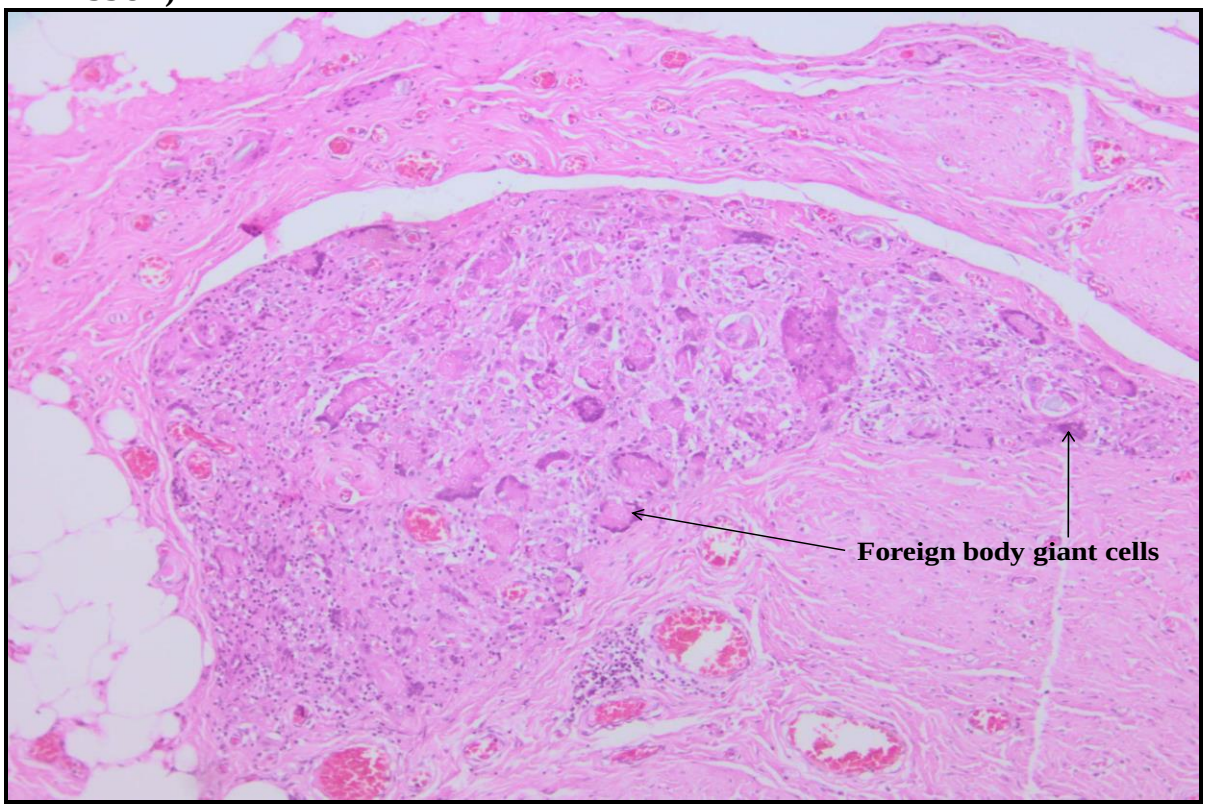

Figure 32: The photomicrograph above shows a foreign body granuloma containing foreign body giant cells. (H&E, 2 μm section; Original magnification: 100X.)

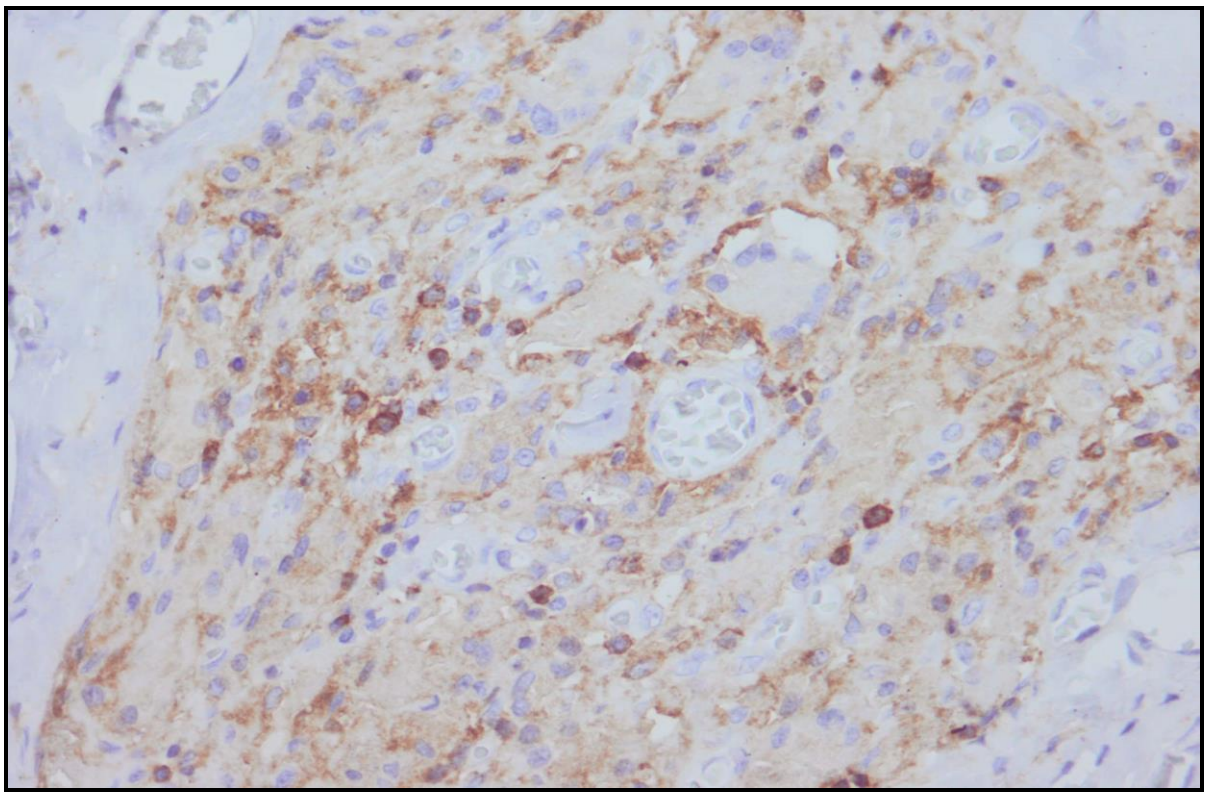

Figure 33: CD4 staining in the foreign body granuloma control tissue. (CD4, 4μm section; Original magnification: 400X.)

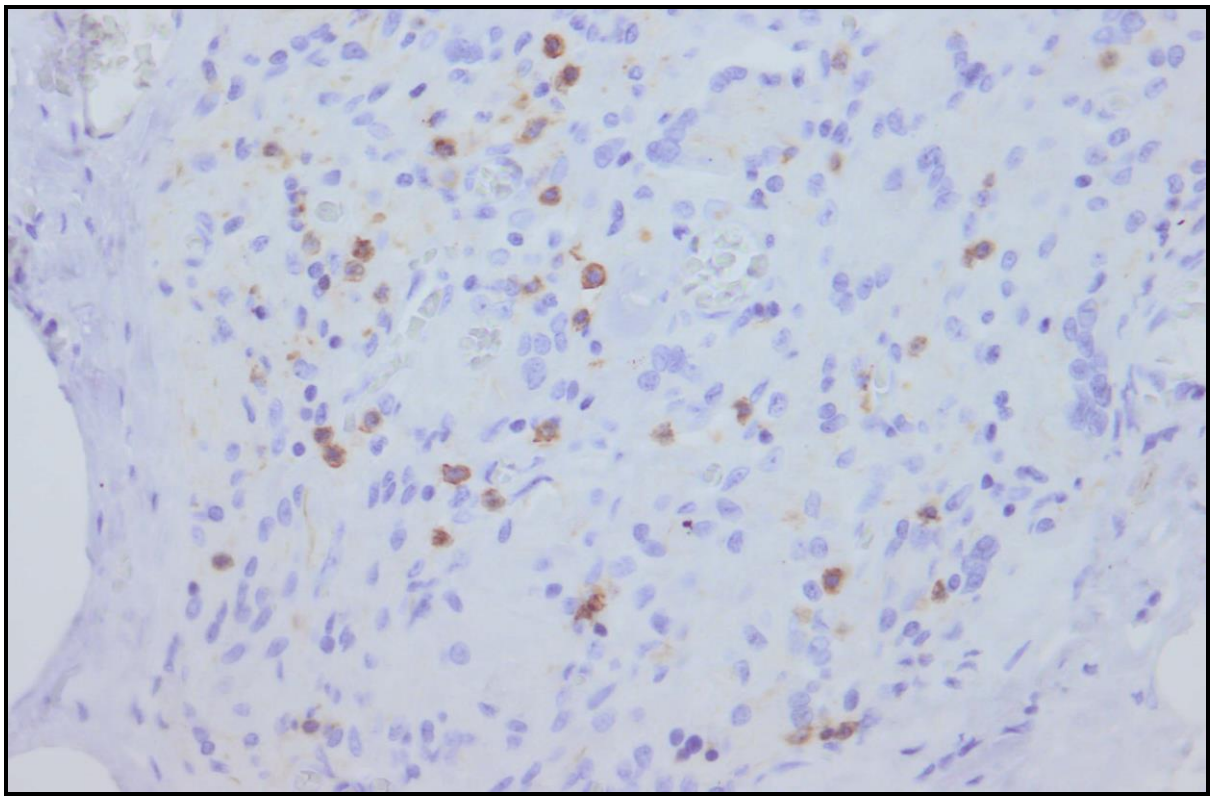

Figure 34: CD8 staining is identified in the foreign body granuloma. (CD8, 4μm section; Original magnification: 400X.)

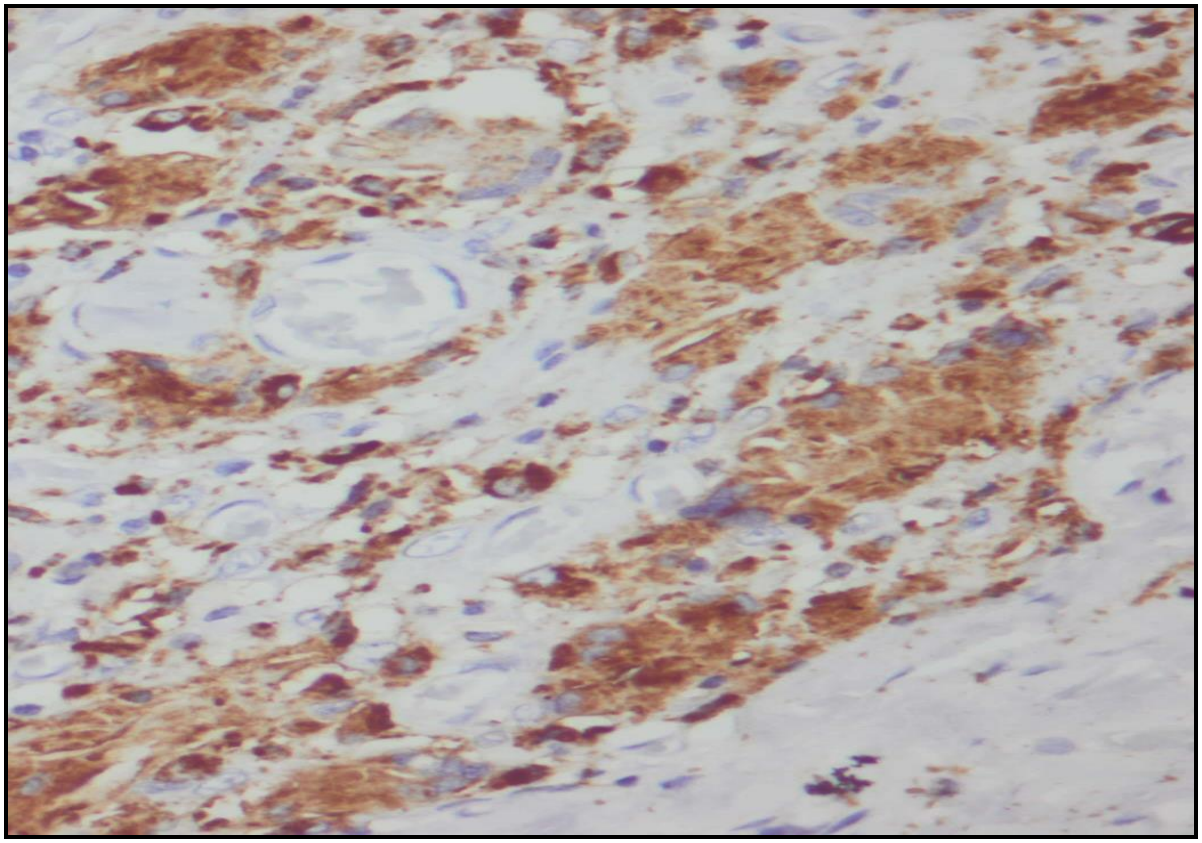

Figure 35: CD68 stains histiocytes in the foreign body granuloma. (CD68, 4μm section: Original magnification 400X.)

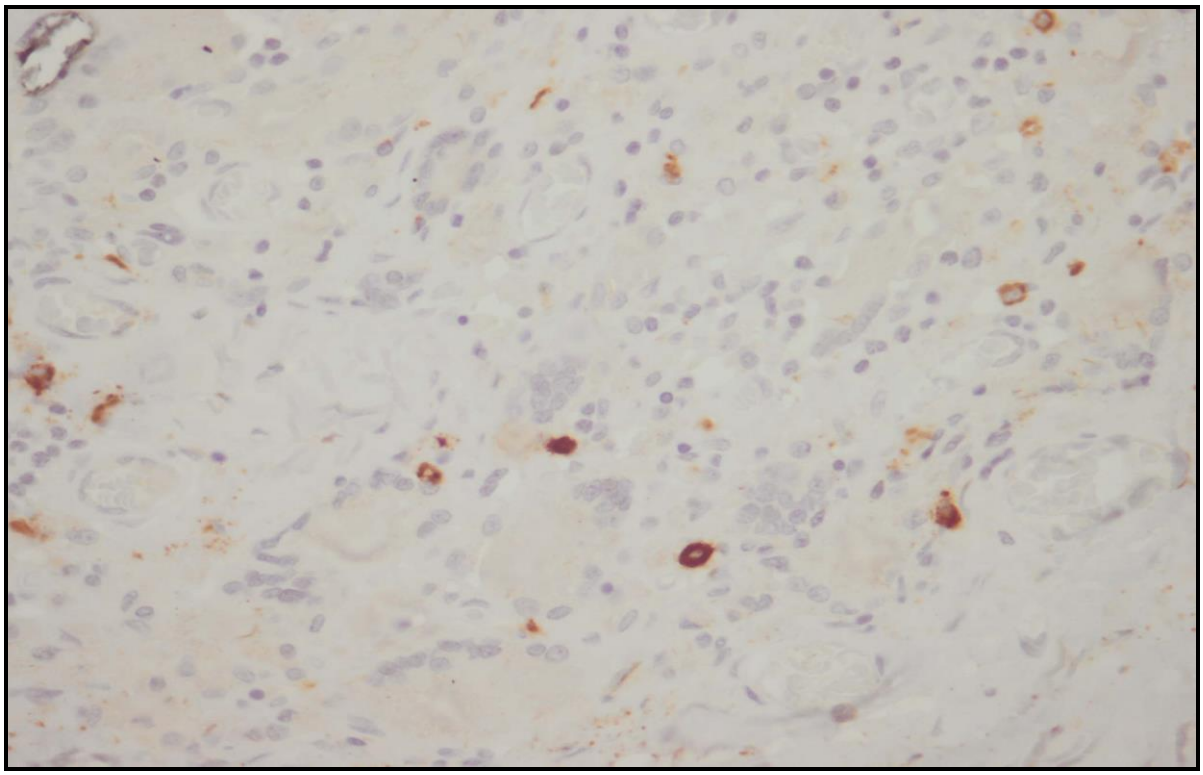

Figure 36: IL-17 staining is shown above in the foreign body granuloma. (IL-17, 4μm section; Original magnification: 400X.)

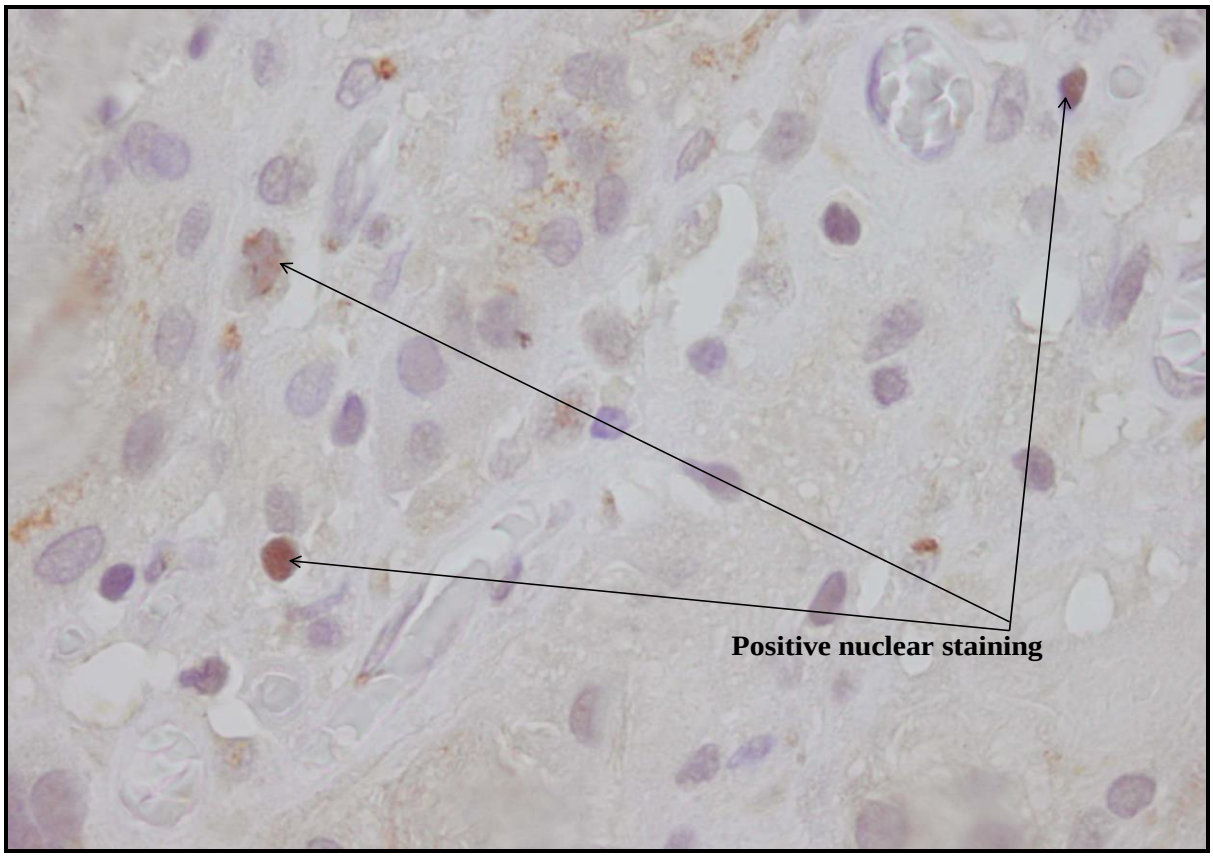

Figure 37: FOXP3 nuclear staining is identified in the foreign body granuloma. (FOXP3, 4μm section; Original magnification: 1000X Oil immersion.)

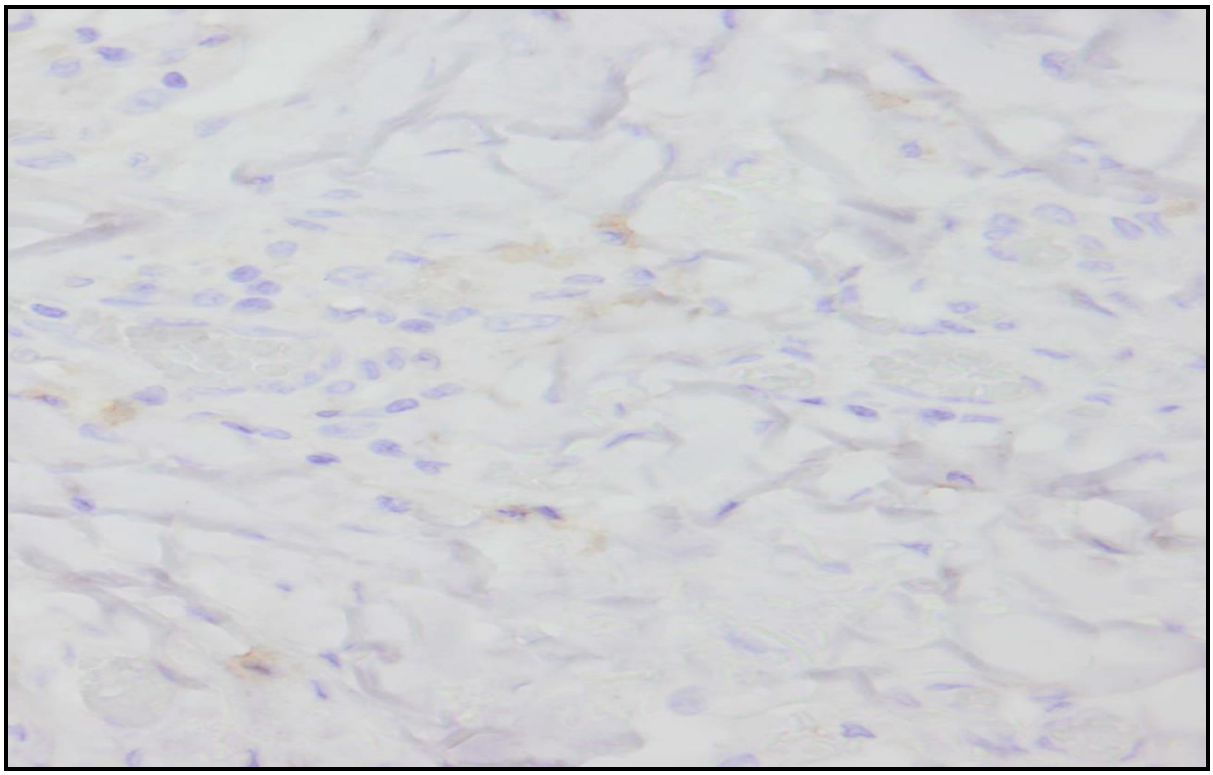

Figure 38: Faint HLA Class I staining in the foreign body granuloma. (HLA Class I, 4μm section: Original magnification: 400X.)

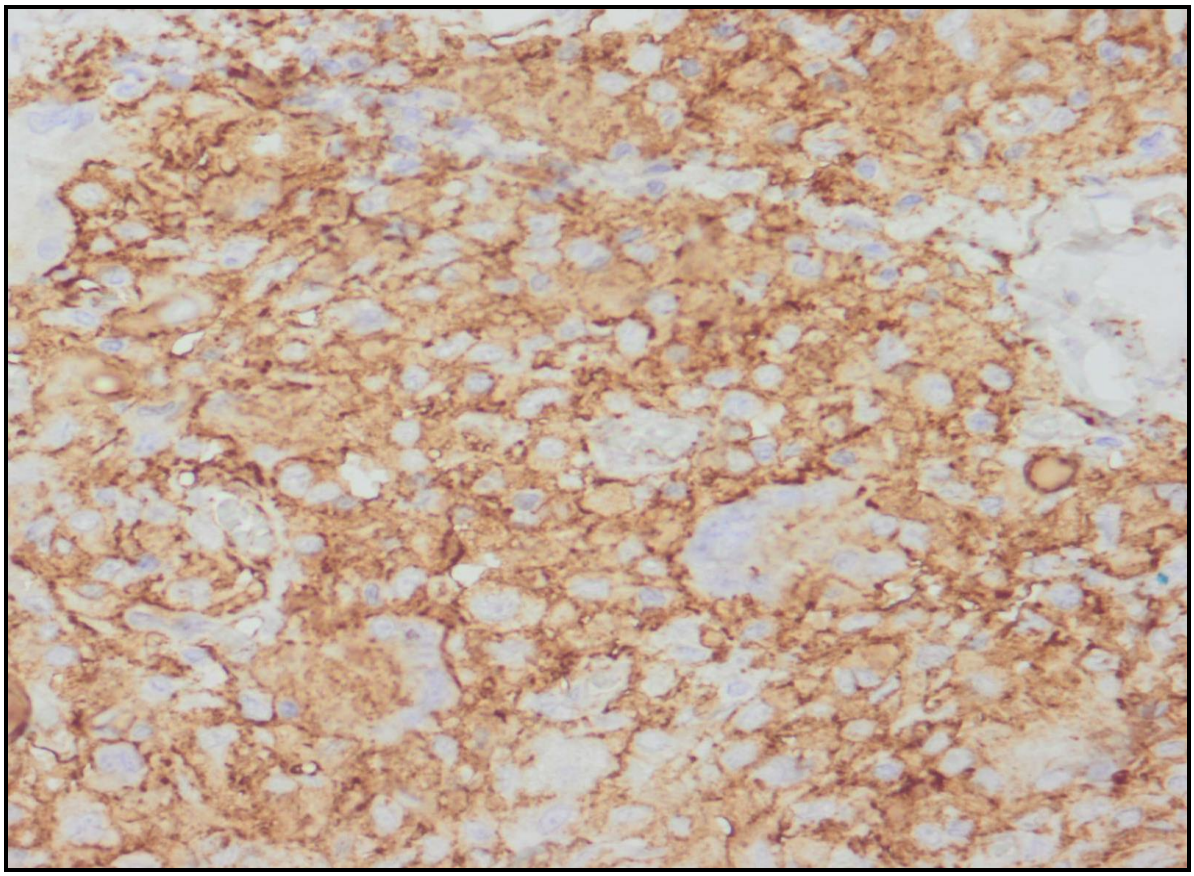

Figure 39: Diffuse HLA Class II staining in the foreign body granuloma. (HLA Class II, 4μm section; Original magnification: 400X.)

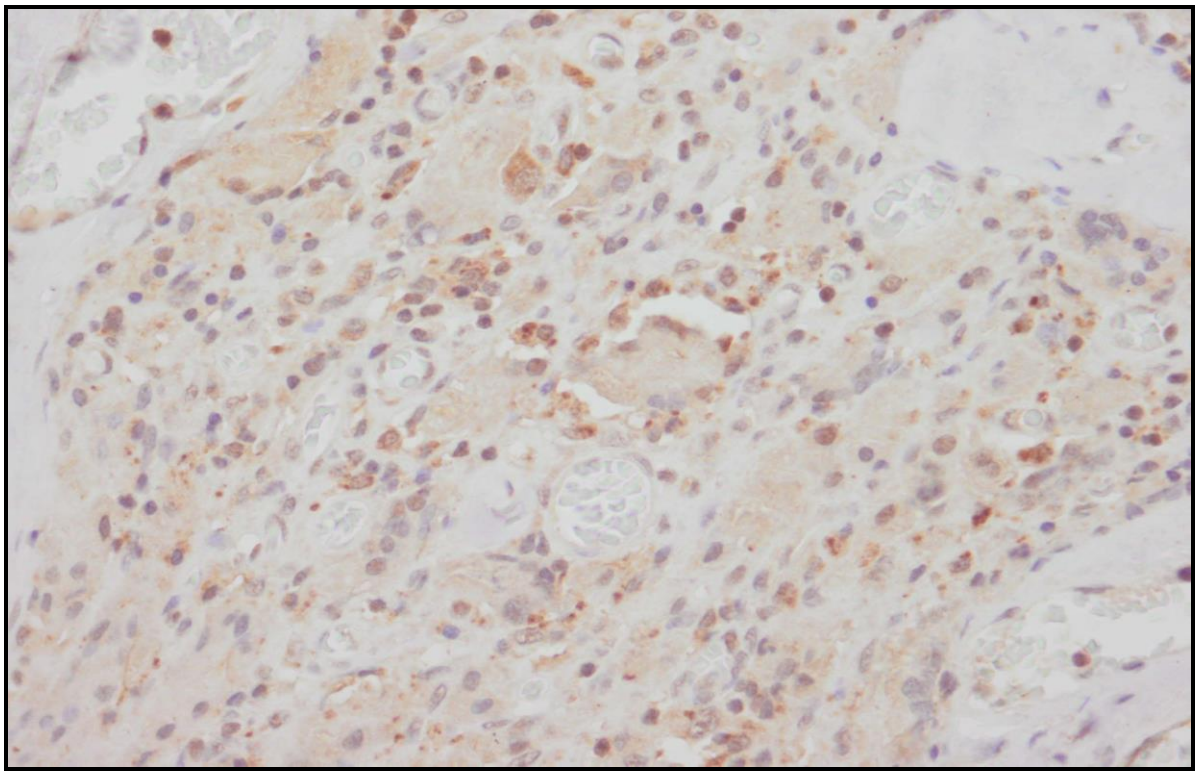

Figure 40: The photomicrograph above highlights IL-2 staining in a foreign body granuloma. (IL-2, 4μm; Original magnification: 400X.)

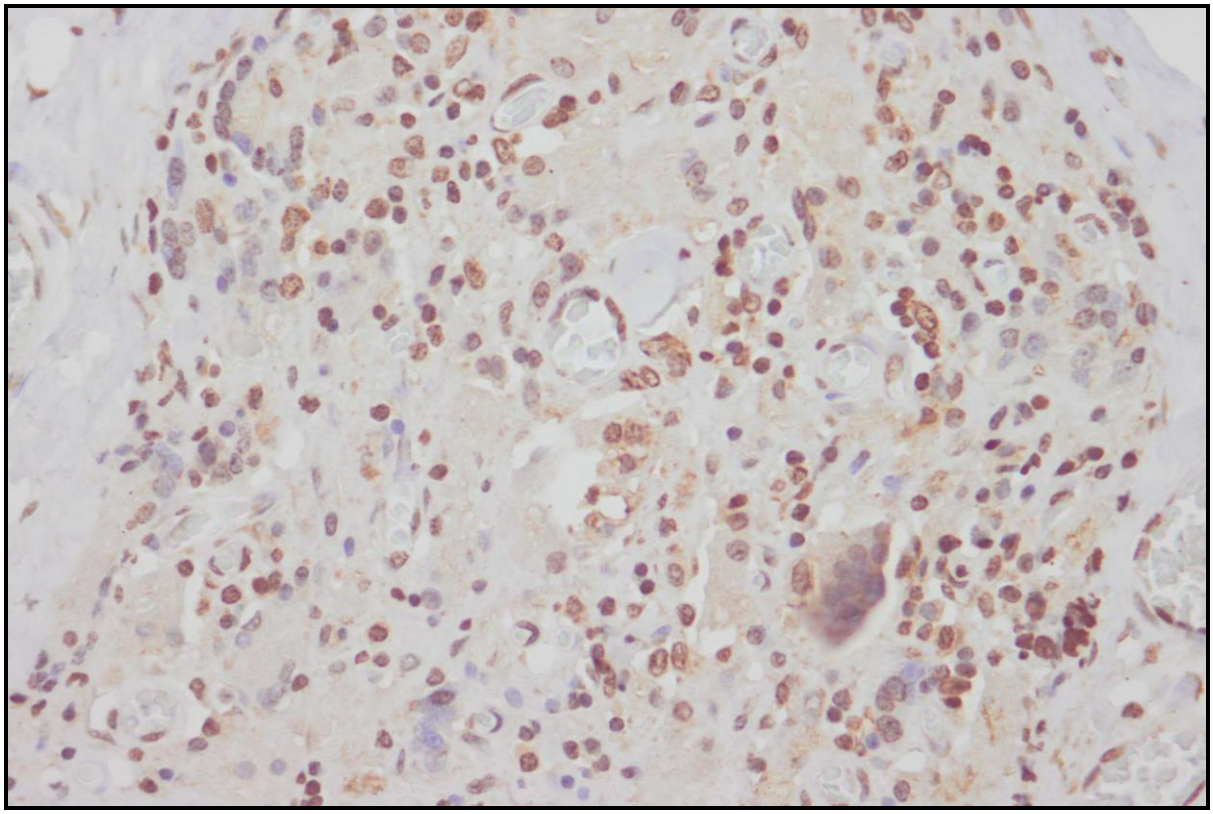

Figure 41: Numerous IFN-γ staining cells are noted in the foreign body granuloma above (IFN-γ, 4μm section; Original magnification: 400X.)

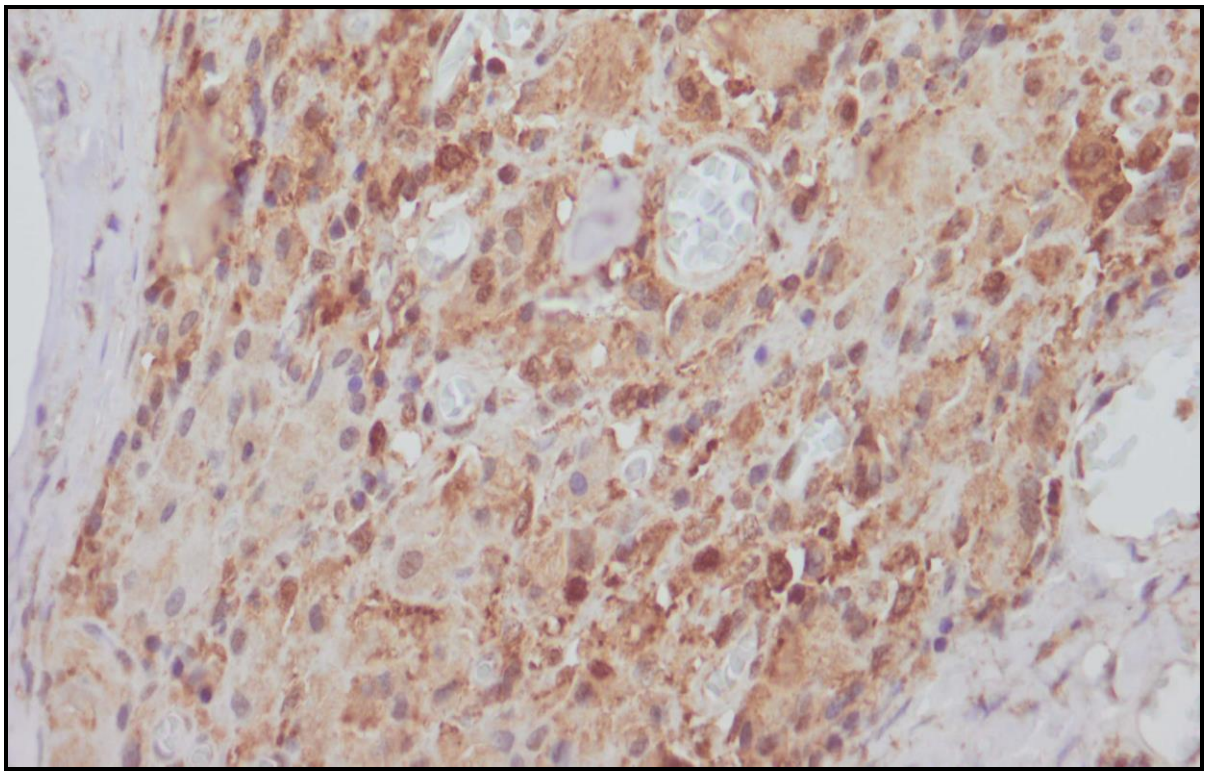

Figure 42: IL-4 staining in the foreign body granuloma. (IL-4, 4μm section: Original magnification: 400X.)

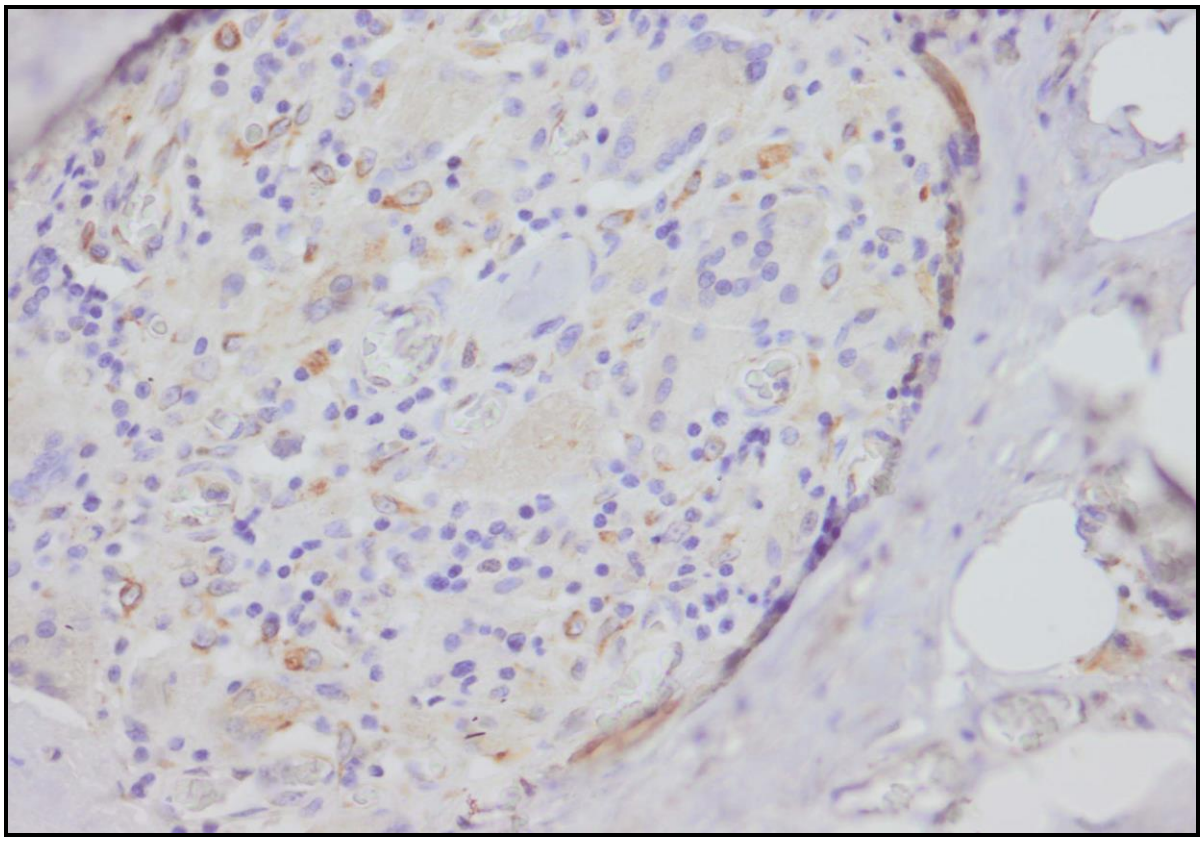

Figure 43: IL-6 staining in the foreign body granuloma is depicted above. (IL-6, 4μm section; Original magnification: 400X.)

CHAPTER 6

6.0 DISCUSSION

6.1 SUMMARY OF RESULTS.

The results illustrate preservation of CD4+ cell counts and retention of CD4+: CD8+ T-cell ratios in TB granulomas from HIV negative patients when compared to TB granulomas from HIV positive cases. In TB granulomas from HIV positive cases, the CD8+ cell count was much higher than CD4+ cell counts. The increase in CD8+ cell counts in the TB granulomas from the HIV positive group together with the marked decrease in CD4+ cell levels contributed to a striking reversal in the CD4+: CD8+ T-cell ratio. The foreign body granuloma showed lower percentages of CD4+ and CD8+ T-cells relative to TB granulomas in both HIV positive and negative groups.

CD68 positivity was identified in each histiocyte in all the TB granulomas, in addition to those within the foreign body granuloma.

The percentage of IL-17 positive cells in TB granulomas from both HIV negative and positive groups was almost identical. However, the Th17 counts in these two groups were markedly raised in comparison to those noted in the foreign body granuloma.

The percentage of cells that exhibited FOXP3 nuclear expression was found to be greatest in TB granulomas from HIV positive patients, followed by the foreign body granuloma tissue.

Immunoreactivity for this marker was lowest in TB granulomas from the HIV negative group.

The percentage of HLA Class I cell positivity in TB granulomas from HIV negative patients mirrors the CD8+ levels in this group, in comparison to the TB granulomas from the HIV positive group. The percentage of HLA Class I positive cells is much higher in the TB granulomas from both HIV negative and positive patients compared to the foreign body granuloma.

HLA Class II positivity was identified in all 250 cells examined in the three groups of granulomas. This immunohistochemical stain showed diffuse positivity of inflammatory cells, endothelial cells and perinodal adipocytes, but there was no uptake of the stain in smooth muscle cells of blood vessels.

Marked variations were noted in the cytokine expression in the three groups of granulomas. The TB granulomas from the HIV negative group showed both a pro-inflammatory and an anti-inflammatory cytokine profile. There was evidence of high percentages of positive staining with IFN-γ antibodies in this group relative to the TB granulomas in the HIV positive group. TB granulomas from the HIV negative group showed a marginally lower percentage of IL-2 positivity than that noted in TB granulomas from HIV positive group. In addition, the TB granulomas in the HIV negative group showed marked elevation in the percentage of IL-6 positive cells in comparison to the TB granulomas from HIV positive patients. The percentage of IL-4 positive cells was approximately 10% lower in TB granulomas from HIV negative patients in contrast to TB granulomas from HIV positive patients. The percentage of

IL-6 cell positive cells was however, much higher than those of IL-4 in TB granulomas from the HIV negative groups.

In the TB granulomas from the HIV positive group, the cytokine profile showed high expression of the anti-inflammatory marker IL-4. In this group, the percentage of IL-4 positive cells far exceeded that of IL-6. In stark contrast, the foreign body granuloma demonstrated a striking pro-inflammatory profile in which the percentage of IL-2 and IFN-γ positive cells far exceed those recorded amongst the other two groups.

6.2 LIMITATIONS.

Only a few cytokine markers from the pro-inflammatory and anti-inflammatory groups were chosen. This is due to the fact that some cytokine stains are deemed only suitable for flow cytometry, western blotting or immunofluorescence microscopy which is not the method of evaluation chosen for this study. In addition, it was decided that only a few stains would be selected as this would ensure that the study did not become an insurmountable task. Furthermore, this helped curtail costs of the study.

This study utilised immunohistochemical examination of tissue which involved the evaluation of a protein product. In contrast, many studies in the current literature have assessed various cytokines, cell markers and accessory molecules by various means such as PCR or in situ hybridisation. Results obtained by use of such methods may be entirely different to those seen on immunohistochemical stains. This is because the mRNA may be assembled in various quantities implying that a certain gene has been transcribed, but the protein (as identified on immunohistochemistry) may or may not be expressed to the same

degree. Moreover, the positivity noted under light microscopic examination depends on the affinity of the antibody for the antigens in the cells concerned.

The exact site of derivation of each of the lymph nodes retrieved in this study is not known. This has limitations for exact comparison of FOXP3 staining in the current study to those found in the literature. This is due to the different staining patterns in lymph nodes from different anatomical sites.

It is not known whether or not the HIV positive patients were on antiretroviral therapy at the time of tissue biopsy. However, the present study was not aimed at assessing the effects of antiretroviral therapy, but rather comparing granulomas from HIV positive and negative patients, in addition to comparing the presence of immunological markers in appropriate control tissue.

The aetiology of foreign material in foreign body granulomas may elicit variable immune responses. In order to prevent the possibility of this occurring in multiple foreign body granulomas which would alter the parameters evaluated in this study, only a single foreign body granuloma was utilised as comparative control tissue. Furthermore, the aim of this study was to compare the immune pathology of TB granulomas from HIV negative and positive patients. The foreign body granuloma was used to confirm histopathological staining and identification and to establish parameters for the various markers utilised.

The HIV status of the patient from whom the foreign body granuloma was derived was unknown. This may have an impact on the local immune response that is observed in the control tissue. This tissue was utilised as a comparison of granuloma tissue and not

necessarily comparing foreign body granulomas against TB granulomas. It was also of necessity to use another granuloma of a different sort to determine laboratory standardisation techniques.

6.3 DISCUSSION OF RESULTS.

Granuloma formation is the pathological hallmark of Mtb infection. However, granulomas are not unique to TB. Histologically, granulomas consist of a collection of tissue histiocytes which upon activation acquire an epithelioid morphology [3, 4, 19]. The peripheries of granulomas are often infiltrated by a cuff of both CD4+ and CD8+ lymphocytes. Within the innermost zone of the granuloma, central caseous necrosis may be identified.

TB granulomas in the HIV negative group of patients in the present study show preservation of CD4+ cell counts as well as maintenance of the CD4+: CD8+ ratio (figure 7, table 1). This concurs with previous reports in which pulmonary TB infection shows infiltration by CD4+ cells [9]. Whilst CD4+ cell counts are preserved, it has been reported that there is a lag in the influx of effector T-cells in addition to a delay in the commencement of T-cell activation [5, 78] in TB when compared to other pulmonary infections. [78] It has been postulated that the delay in entry of T-cells may be due to Mtb preventing apoptosis of infected macrophages [1, 4, 5, 78] whilst simultaneously impeding presentation of antigens by antigen presenting cells in the draining lymph nodes. [5, 78] The impediment to activation may be attributed to a decrease in responsiveness by infected macrophages to T-cells [5] or may be due to inhibition of IFN-γ function, [5] which may ultimately impair the host's protective antimicrobial response. The present study identifies that granulomas from HIV negative patients demonstrated CD4:CD8 ratios of approximately 1:1 (mean CD4 percentage – 63.4 versus mean CD8 – 58%). The

retention of CD4+ cells in these patients results in good granuloma formation albeit at a slower rate as suggested above. However, as the current study did not examine time based granuloma formation, this theory could not be assessed. Nevertheless, well formed granulomas were identified with CD8+ cells, although high in number, noted at the peripheries. In addition, the presence of FOXP3 regulatory cells has been demonstrated to result in delayed recruitment of T–cells to the site of infection as well as causing impairment of activity of effector CD4+ cells. [5, 78] However in the HIV negative group, (discussed in greater detail below), FOXP3 numbers were on average less than five percent, which lends support to the hypothesis of impairment of CD4 activity as a consequence of FOXP3 cells. [78]

Decreased numbers and thus percentages of CD4+ T-cells in TB granulomas of HIV positive patients are well demonstrated in this study and highlight a raised CD8+ : CD4+ count in the tissue granulomas which mirrors such changes seen in blood. [80] The findings in this study are in concordance with results from a study which examined TB granulomas of the spine in both HIV negative and HIV positive patients. [80] The findings of the current study also corroborate results obtained from reports [4, 81] which evaluated CD4 positivity in lymph nodes in HIV and TB patients and compared these to findings in patients with TB mono-infection. The CD4+ cell counts in the present study correlate with current knowledge of CD4+ destruction and depletion in HIV. [1, 4, 5, 9, 17, 79] Furthermore, the results of the present study recapitulate those seen in autopsy findings in cynomolgus macaque models, in which some of the non-human primates were infected with either Mtb; or Simian Immunodeficiency Virus (SIV), whilst other monkeys were infected with both Mtb and SIV. [1, 9, 82] The granulomas identified microscopically in the co-infected monkeys showed less staining of CD4+ T-cells than those infected with only Mtb and less CD4+ cells were noted in

pulmonary nodal parenchyma of dual-infected monkeys when compared to SIV or Mtb mono-infected mokeys. [9] The majority of CD4+ cells that are destroyed by HIV are effector memory T-cells, which predominate in topographical sites such as mucosae, the gastrointestinal tract and the periphery. [1, 4]

It has been shown that the loss of CD4+ cells in HIV infection substantially increases the risk of acquiring primary or reactivation TB [4, 78, 83] and that this may be due to inability to control the organism [4] with subsequent spread of infection. It has been suggested that the favoured loss of Mtb specific CD4+ cells in the periphery early on in HIV infection is due to the raised propensity of IL-2 producing cells to be infected by HIV. [78] This has been substantiated by identifying lower numbers of polyfunctional CD4+ T-cells which secrete pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ in pulmonary specimens of HIV positive patients exposed to TB, in comparison to HIV negative patients. [78] It has been demonstrated that in regions of active TB as well as HIV proliferation, that Mtb specific CD4+ T-cells alter their immunophenotype such that they become polyfunctional [78] with an effector phenotype. [9] Whilst these cells are less well differentiated, they have been shown to possess a robust antigen-specific response. [9] This is advantageous and may possibly be due to the fact that at regions of active disease, terminally differentiated T-cells express the chemokine receptor CCR5 on their cell surface that HIV utilises to infect cells, [78, 83] replicate within them and eventually lyse the CD4+ cells. [83] There is often an initial rapid cell turn-over [83] with an increase in apoptosis of HIV infected and uninfected CD4+ cells within the gastrointestinal tract, lymph nodes and in the periphery. [79] In addition, there is an increase in maturation of effector memory cells from central memory cells which is hypothesised to amplify viral proliferation.[83] However, with time, the constant requirement for replenishment of CD4+ effector cells cannot be met and this is thought to be a contributor to HIV progression. [83]

Anergic effects in the form of a decrease in IL-2 production and thus a decrease in pro-inflammatory mediators, may also contribute to the impaired immune response in HIV [79]. This serves to highlight the numerous levels at which HIV cripples the immune system.

Results from the present study illustrate lower percentages of CD4+ cells in the foreign body granuloma compared to the percentages observed in TB granulomas from both HIV negative and positive patients. The current literature in the context of Mtb examines TB mono-infection and compares this to healthy controls as well as results obtained in the context of TB and HIV co-infection. As such, there is a dearth of information comparing TB granulomas to foreign body granulomas and this may be a focus of interest for future studies. Whilst it is known [19] that foreign body granulomas form in response to material that cannot be easily phagocytosed and that the granulomas contain numerous histiocytes, it is believed that the foreign material does not elicit a marked inflammatory cell response [19]. To date, studies detailing the numbers of CD4+ cells in foreign body granulomas are lacking.

The results of CD8+ cell counts in the TB granulomas from the HIV negative group in this study were marginally lower than those documented in TB granulomas from the HIV positive group (figure 7, table 1). This reflects findings in a study [80] which demonstrated fairly similar numbers of CD8+ cells in both HIV negative and HIV positive patients. Whilst the precise function of CD8+ cells in TB is yet to be entirely clarified, [17] it has been shown [15, 38] that CD8+ cells secrete IFN-γ which results in macrophage activation. CD8+ cells may also secrete IL-4 and thus may act to equilibrate a Th1 and Th2 response. [15] Furthermore, it has been demonstrated [64] that CD8+ cells are important in the recognition of macrophages containing Mtb. These CD8+ cells require perforin to penetrate the infected cell, following

which the CD8+ cells are then able to directly destroy the intracellular organisms by means of granulysin. [2, 38, 64] It has been illustrated that granulysins are able to elicit activation of apoptosis of Mtb infected cells. [79] As these granulysins may be released into the surrounding tissue, there is a risk of tissue damage. [79] It has been suggested that perforins enhance the uptake of granzyme A and B which results in apoptosis of affected cells. [79] Other authors [83] propose that CD8+ cells in granulomas is the produce XCL1 (lymphotactin) which down-regulates IFN-γ by CD4+ cells. It is also thought that synchronized phases of perforin and granulysin or perforin and granzyme activity are required so that effective microbicidal activity may occur without extensive tissue damage. [79]

Studies have shown [79] that HIV patients have a disproportionately high number of "intermediately differentiated" CD8+ cells. [9, 79] These immature cells produce insufficient quantities of perforin thus reducing their cytolytic capabilities. [79] It has been also been shown [79] that in TB the decreased numbers of mature Mtb-specific CD8+ cells consequently produce decreased quantities of perforin and IFN-γ. [79] As such, it may be deduced that patients with HIV and TB co-infection have impaired CTL cytolytic activity which ultimately implies reduced destruction of infected cells. In addition, it has been demonstrated that HIV positive patients with raised expression of FOXP3 mRNA, were found to harbour reduced perforin in CD8+ CTL's. [79] Moreover, it has been illustrated that TB granulomas in HIV possess raised expression of IL-13 and TGF-β both of which antagonise a Th17 and Th1 pro-inflammatory immune response. [79] From these studies, it may be surmised that the FOXP3 suppresses the cytolytic activities of CTL's.

A study in a murine model, [3] and another that assessed human TB granulomas [81] showed that many of the CD8+ cells were scattered throughout the granulomas instead of the usual

location at the periphery of the granulomas. In the present study, whilst occasional CD8+ cells were identified in a more central location within granulomas in the HIV negative group, the majority were noted at the periphery. In contrast, the HIV positive TB granulomas were found to have many more CD8+ cells dispersed throughout granulomas. This suggests that retention of CD4+ cells may be central to organisation of granulomas.

The foreign body granulomas in the present study showed a lower CD8+ cell count in comparison to the TB granulomas in HIV negative and HIV positive groups. As discussed previously for CD4+ cell counts in foreign body granulomas, there is deficient literature documenting T-cell subsets in foreign body granulomas. It may be surmised from this study that Mtb has a different and perhaps unique effect on the formation of TB granulomas as compared to foreign body granulomas. This is compounded by the presence of HIV that alters the distribution of CD8+ cells in these TB granulomas.

Positive staining of CD68 was identified in all histiocytes in TB granulomas from both HIV positive and negative patients in addition to the foreign body granuloma (figure 7, table 1). These results parallel those identified in granulomas from HIV positive and negative patients in a study in 2002 [85] and one more recently in 2013 [80] which illustrated that all tissue granulomas in TB patients were immunoreactive with a CD68 stain. Macrophages in the blood and histiocytes (tissue macrophages), in contrast to other haemopoietic cell lines, have surface markers that are not well typed. [86] Nevertheless, both macrophages and histiocytes are immunoreactive for CD68. [86] CD68 is believed to function in lysosomal transfer and is an essential part of cellular maintenance of all cells. [86] As such, whilst various cell types may express CD68, this occurs at extremely low levels in comparison to the strong and diffuse staining identified on macrophages and histiocytes. [86] The staining of histiocytes in

the TB granulomas and the foreign body granuloma in the present study showed intense, coarsely granular staining indicative of the increased lysosomal activity in these cells. Furthermore, the morphological appearance of histiocytes, including epithelioid histiocytes, ensured that it was only these cells that were counted when assessing the CD68 stain and not another cell type, for example lymphocytes; that were examined. As is often seen on tissue sections stained with the CD68 antibody, there may be "background" staining which may occur as a consequence of cellular debris and/or insufficient blocking antibody. The "background" staining usually has a fainter appearance than the coarse, granular strong intensity staining in histiocytes. When evaluating this stain in the experimental tissue, the "background" faint non-specific staining was ignored and accounted for.

Detection of IL-17 positive cells in the present study demonstrated similar numbers in both groups of TB granulomas but lower numbers of IL-17 cells were noted in the foreign body granuloma (figure 9, table 2). Whilst the precise role of IL-17 is not entirely clear, [17] it is thought that these cells do not have a direct function, and are not the orchestrators of the immune response to intracellular organisms such as Mtb. [87] Cells identified as being IL-17 positive, however, are believed to play a role, albeit a dispensable one, against this organism. [87] Il-17 has also been shown to increase the influx of neutrophils to the site of infection. [3, 5] In addition, it has been demonstrated that IL-17 production relies on IL-23 expression, as reduced levels of the latter result in lower levels of the former. [87] IL-23 stimulates an increase in the levels of IL-17 as well as raised IFN-γ in Mtb infection [87, 88] thus augmenting the Th1 response. [78] It has been suggested, based on a murine model that IL-17 is required for well formed granulomas to be produced [88] and that lower levels of IL-17 consequently imply vague, poorly formed granulomas. A recent study [88] showed decreased IL-17 cell numbers detected by flow cytometry in patients with active TB. Others [79] have demonstrated

that early in HIV infection there is an increase in Tregs which inhibit the differentiation and induction of IL-17 cells. It could therefore be expected that lower levels of IL-17 are present at this stage of the disease. It has also been illustrated that in patients who are able to control HIV, that an IL-17 response is also stimulated at the same time as decreased Treg levels. This insinuates an inverse relationship between the two cell types. [79]

The current study compares the TB granulomas in each group to one another and to a foreign body granuloma. It is not possible to state whether the IL-17 cell counts are increased or decreased in comparison to normal, non-granulomatous tissue from these findings. It is however clear that the IL-17 cell count is raised in contrast to that noted in the foreign body granulomas. The IL-17 cell counts in the granulomas of both HIV negative and positive TB groups are similar to one another. The FOXP3 cell counts in the TB granulomas in HIV positive patients are much higher than IL-17 cells counts but, in light of the HIV negative TB granulomas having similar IL-17 cell counts, it is unlikely; given the current understanding of the role of the Tregs [79] that these could have had a prominent part in suppressing IL-17 in this study.

To date, insufficient data exists regarding the presence or absence of IL-17 positive cells in foreign body granulomas. As such it is not possible to compare the IL-17 staining of the foreign body granuloma in this study to that noted in other reports. Suffice to state that higher numbers of IL-17 cells were identified in the TB granulomas from both HIV negative and positive groups than in the foreign body granuloma.

In the current study, the number of FOXP3 positive cell nuclei in the granulomas from the HIV negative group, relative to the foreign body granuloma, is low (figure 11, table 3).

However, in this study, the control tissue used is a foreign body granuloma, and not normal nodal parenchyma. Therefore, it is not possible to state definitively, whether levels of FOXP3 nuclear expression in the TB granulomas are raised in comparison to normal lymph node parenchyma. A recent report [40] investigated foxp3 levels in a mouse-model of Mtb infection. In that study, the percentage of foxp3 cells increased in both Mtb infected and uninfected mice as they aged. [40] Of note, the study [40] also demonstrated a higher percentage of foxp3 positivity in pulmonary lymph nodes and splenic parenchyma prior to infection, in addition to following infection, when compared to lung tissue in the same experimental animal. The study reported that in sites distal to infection, such as mesenteric lymph nodes, an increase in foxp3 cells was not noted. [40]

The low levels of FOXP3 staining in the HIV negative TB granulomas of the present study, relative to the control tissue may possibly be attributed to the fact that lymph nodes harbouring the granulomas were not necessarily, and unlikely to have been derived from lung in contrast to the cases identified in the literature, [40] in which foxp3 cells were reported to accumulate in pulmonary lymph nodes following Mtb infection. Furthermore, another possibility may be that foreign body granulomas do in fact show raised levels of FOXP3 positivity. The age of the patient from whom the control tissue was derived in the current study, is not known. If the patient was, at the time of biopsy, over the age of 60 years, this may account for the high levels of FOXP3 positivity, as it has been documented that patients older than this have a threefold increase in numbers of Tregs. [50] Unfortunately, and yet again, there is presently a paucity of information regarding the staining of FOXP3 in foreign body granulomas which will require further investigation in the future.

Previous studies have identified increased FOXP3 mRNA expression at disease sites [43, 51] and have illustrated a role for Tregs in TB, where such a role of Tregs is to dampen down immune responses. [16, 43, 51, 90] From this, is may be deduced that an increase in FOXP3 results in TB disease progression whilst low levels of FOXP3 suggests raised T-cell responses [43, 51, 57] with containment of disease. Guyot-Reval *et al* [51] elegantly demonstrated an increase in T-cells producing IFN-γ following elimination of Tregs, thus illustrating the fundamental function of Tregs in the host's immune response to Mtb. This study [51] advocates that whilst Tregs may hinder the host's response to infection, they simultaneously control the extent of immune–mediated pathology. The study [51] also brings to the fore, the proliferation of Tregs which may occur concurrently with effector T-cells directed against a specific pathogen [51]. Roberts and colleagues [57], in their study, demonstrated raised mRNA levels of IL-4 and TGF-β which, [57] together with increased FOXP3, served to diminish the pro-inflammatory Th1 response. [57, 91] This highlights the homeostatic role of Tregs [57] as confirmed in other studies [51, 58] and illustrates the tight control that is needed to maintain a fine balance between disease containment on one hand, and prevention of excessive tissue destruction on the other.

Guyot-Reval *et al* [51] documented greater FOXP3 levels in patients afflicted by extrapulmonary TB in comparison to those with lung infection alone. It is currently uncertain whether the raised FOXP3 levels in extrapulmonary disease increases the risk of spread of the organism or whether the increased FOXP3 levels occur due to disseminated disease in these patients. [51]

The present study shows markedly elevated levels of FOXP3 in the HIV positive TB granulomas in contrast to the HIV negative group. This is in concordance with findings in

other studies, [1, 41, 43, 79] pointing to the identification of marked increases of FOXP3 T-cells within haematolymphoid tissue. [79] This is confirmed in the lymph nodes examined in the current study. It has been suggested that Tregs have a prominent effect in immune suppression [92] and may repress HIV specific CTL's. [91] In HIV positive patients, altered levels of suppressor of cytokine signalling (SOCS) 1 have been identified. [1, 93] These proteins regulate cytokine signalling and have been demonstrated to affect CD4+ cell differentiation [93] and are involved in FOXP3 development. [93] Thus it may be deduced that high SOCS1 levels will have direct effects on functionality of FOXP3 cells with subsequent down-regulation of cellular immune responses at sites of inflammation, whilst containing tissue damage. Furthermore, it has been demonstrated [1] that SOCS1 is associated with down-regulation of IFN-γ and IL-12 production. This then results in the inability of macrophages to effectively contain Mtb bacilli resulting in a situation in which the organisms flourish. As there are raised levels of FOXP3 in TB alone, the occurrence of both HIV and TB are likely to have an additive suppressive effect. [79] In addition, it has been suggested [79] that TB and HIV target a specific C-type lectin binding protein on the surface of dendritic cells. Once this C-type lectin binds to the cell wall of Mtb and to the envelope proteins of HIV, the dendritic cell will no longer mature and immune suppression may ensue. [79] In addition, the interaction of Mtb and HIV with the C-type lectin causes an increase in IL-10 which has anti-inflammatory properties and acts to decrease IFN-γ activity. [79] This undoubtedly alters the inflammatory milieu in favour of disease progression.

Whilst some authors [94] have demonstrated that FOXP3 results in increased gene expression of HIV, others [92] have shown that FOXP3 cells may resist infection by HIV [91] and may inhibit infection of surrounding CD4+ cells. The present study however corroborates the role that FOXP3 may play in that granulomas from HIV positive patients contain higher

percentages of these cells compared to the HIV negative group. Further studies in this field may bring clarity on this matter.

The granulomas in the HIV positive group of patients were well formed despite having contained higher percentages of FOXP3 cells than the HIV negative groups. Whilst the granulomas in this group of patients contained CD8+ cells at the periphery, they did however contain scattered CD8+ cells within the inner regions. This then supports the notion that granuloma integrity is maintained by CD4+ cells (as discussed above). The role of FOXP3 nevertheless is indicated by identifying these cells in the relevant granulomas.

Cell counts of HLA Class I were only marginally lower in the TB granulomas of HIV negative patients in comparison to TB granulomas in the HIV positive patients (figure 14, table 4). Cell counts for this immunohistochemical stain in the foreign body granulomas were however much lower than those documented in the TB granulomas of both HIV negative and HIV positive patients. HLA Class II cell counts showed 100% staining in all three groups. The percentage of positive cells stained with the HLA Class I antibody in TB granulomas of HIV negative patients parallels the results identified for CD8+ cells in the same subset of patients. As antigen presenting cells present soluble antigen in conjunction with HLA Class I molecules to CD8+ T-cells; an increase in percentage of HLA Class I cells would suggest an increase in the number of CD8+ cells, which is illustrated in the present study. The slight increase in HLA Class I cell counts in the HIV positive group may be attributed to the constant antigenic stimulation that occurs in HIV infection. This is supported by the identification of two markers that highlight T-cell collapse, namely Programmed Death-1 and T-cell immunoglobulin and mucin domain 3. [1] Both markers function to dampen-down the host's inflammatory response. [1] It has been demonstrated that T-cell immunoglobulin and

mucin domain 3 was markedly increased on CD8+ cells in HIV as well as in TB. Collectively these studies suggest that in these infections there is excessive consumption of these cells resulting in eventual exhaustion in the host's ability to replenish such cells.

It has been postulated that Mtb may impair identification of macrophages by CD4+ cells by decreasing surface expression of HLA Class II molecules [83] or by reducing cytokines needed for T-cell priming. [17] However, some authors [83] have experimentally found that infection by Mtb of dendritic cells did not cause a reduction in HLA Class II expression. This was confirmed by a marked increase in CD4+ cells in response to Mtb infection. Various studies [96-98] have been undertaken in which different allele subtypes within HLA Class I and II molecules have been examined. The present study differs in this regard as only a cell count was performed and allele subtypes were not examined. A study undertaken in Harare, Zimbabwe [96] showed that HLA Class II DRB1 homozygosity had a higher prevalence in HIV positive patients with pleural effusion TB when compared to pulmonary TB. The study [96] suggested that the decreased CD4+ stimulation by HLA Class II DRB1 may impair the cytokine responses required for the induction of active TB. This would then further suppress expression of pulmonary TB. [96] Other studies, [97] have however illustrated that expression of a specific allele on either Class I or II, depends on the various ethnic groups and races of individuals, or as suggested by others, [98] certain alleles on HLA Class I affect an individual's risk of acquiring TB and may also affect the severity of disease. A study undertaken in KwaZulu/Natal, [99] South Africa, indicates that HLA-B, a Class I locus, functions to control HIV replication. This lends support to knowledge of CD8+ cells playing a part in eradication of primary viral infection as well as in controlling viral proliferation. [99]

Whilst different studies reveal varying results for accessory molecules HLA Class I and II, the variability may be attributed to the stage in the disease. [1] Early on in HIV, at a point in time when CD4+ levels are high or relatively normal, HLA Class II levels are greater than those of Class I with the reverse possibly occurring with chronic HIV when CD8+ cells are believed to limit the rate of HIV replication. [1]

The results from the present study illustrate HLA Class II positivity in 100% of cells counted. The inflammatory cells examined all showed diffuse positivity. In addition, HLA Class II positivity was noted in adipocytes as well as in endothelial cells of blood vessels. Identical degrees of staining intensity were noted despite various concentrations having been attempted on the sample tissue. The concentrations varied from 1:50 to 1:300 (results not shown) and were part of the optimisation for the antibodies utilised in the immunohistochemical staining of tissue. The only cells that were not immunoreactive in all the tissue sections examined, were smooth muscle cells of blood vessel walls. This served as an internal negative control, thus eliminating the possibility of non-specific staining. As such, the staining pattern for this antibody is deemed suitable for inclusion in this study.

There has been little research undertaken in the study of accessory molecule expression in foreign body granulomas, however, one study in rats [100] showed that following inhibition of IFN-γ by inhibitory antibodies there was decreased expression of HLA Class II molecules. The current study did not utilise inhibitory antibodies, but the percentage of positive staining with HLA Class II antibody in the foreign body granuloma was identical to that noted in the TB granulomas from both HIV negative and positive patients. Thus while the aetiology of TB granulomas and the foreign body granuloma are entirely different, and the number of CD4+ cells (to which macrophages present soluble antigens in conjunction with HLA Class II

molecules) are different between these granulomas, the number of HLA Class II positive cells have remained identical.

The number of positive IL-2 positive cells in the present study was marginally lower in the HIV negative TB granulomas compared to the HIV positive TB granulomas (figure 19, table 5). The number of IL-2 positive cells in the TB granulomas is also much lower in comparison to the IL-2 cell count in the foreign body granuloma. IL-2 has been shown to increase the number of antigen specific T-cells [15] and is considered to be an integral part of the host's immune response [101]. In addition, there have been reports suggesting that IL-2 may have a direct bearing on the path of infection with Mtb. [15] It has been suggested [4] that in HIV positive cases the rapid turn-over of T-cells may result in a reduction of cytokines such as IL-2. Consequently, there is diminution of Mtb specific T-cells as well as a decrease in the cytokines produced by these cells. [4] This suggests that survival of both HIV and Mtb is thus favoured.

A study examining IL-2 cell positivity (in addition to other cytokines) in murine TB granulomas [102] showed high levels of IL-2 in mice with active TB in comparison to mice with latent disease. With time, the levels of IL-2 in the active disease group of mice decreased minimally. [102] In the present study IL-2 was evaluated at an isolated point in time and it is thus not possible to determine whether or not cytokine immunoexpression would be altered with time.

The results from the current study suggest that the foreign body granuloma is capable of eliciting a stronger pro-inflammatory response than that seen in TB granulomas from both HIV negative and HIV positive patients. This is contrast to the notion that inert substances in

foreign body granulomas do not raise an inflammatory response to the foreign substance resulting in their induction. [19] Luttikhuizen *et al* [100] suggest that in foreign body reactions, IL-2 serves to induce production of IFN-γ, TNF-α and IL-1. However these authors [100] have not undertaken quantitative analysis of the expression of these cytokines. Thus it is not appropriate for results of cytokine expression in the foreign body granulomas in the current study to be compared to findings by Luttikhuizen *et al*. [100]

The percentages of IFN-γ immunoreactive cells in the current study are higher in TB granulomas from HIV negative patients when compared to the HIV positive group of TB granulomas (figure 19, table 5). The raised number of IFN-γ staining cells in the current study, corroborate the high number of IFN-γ positive cells identified in a study [85] which evaluated the distribution of various cytokines in human lung TB granulomas. Similar results have been documented in TB granulomas derived from lung parenchyma in comparison to normal lung tissue. [103] Furthermore, the findings in the current study mirror those identified in a report [104] in which serum levels of various cytokines including IFN-γ and IL-4 were measured. These authors [104] reported a rise in IFN-γ in cases of mild to moderate Mtb infection. It was however found that patients with progressive disease were eventually unable to produce IFN-γ to Mtb. Whilst IFN-γ production may not be adequate at controlling Mtb infection, [85, 105] it is a key cytokine in the Th1 host response. It has been shown that patients with chronic HIV are unable to secrete sufficient quantities of IFN-γ. [79] This undoubtedly suggests that HIV positive patients are unable to produce an effective Th1 response to either HIV or TB. In contradistinction, one study [2] demonstrated a clear positive correlation between IFN-γ expression and HIV. This lends weight to the premise that an increase in cytokine secretion in HIV positive patients may be an endeavour to counterbalance the reduction in CD4+ effector cell activities [2]. The findings in the present study are therefore

suggestive of the host immune system in the HIV negative TB granulomas being able to mount a far stronger Th1 response with a marked pro-inflammatory reaction than that noted in the HIV positive TB granulomas. This therefore suggests that the HIV negative patients are able to contain Mycobacteria more efficiently than HIV positive patients.

The percentage of positive cells in the foreign body granuloma in the present study, are far higher than those identified in both groups of TB granulomas. A study published in 2005 [35] documented moderately increased numbers of positive cells in giant cells in TB granulomas compared to foreign body granulomas. Luttikhuizen *et al* [100] suggest that IFN-γ is produced in foreign body reactions by T-cells and results in activation of macrophages. However, these authors [100] did not evaluate the degree of expression of this cytokine. From the present study, however, it may be deduced that a far stronger pro-inflammatory response is raised in response to foreign material than the pro-inflammatory response elicited to Mtb in granulomas from HIV negative and positive patients. This however contradicts beliefs that foreign material is inert and consequently does not illicit an inflammatory reaction. [19]

Results from the existing study demonstrate that the mean cell count of IL-4 was lower in TB granulomas from the HIV negative group in contrast to the HIV positive TB granulomas (figure 19, table 5). In addition, the IL-4 positive cell count in the foreign body granuloma was much lower than that noted in the TB granulomas from both HIV negative and positive groups. One study [103] showed lower levels of IL-4 positive cells in TB granulomas in HIV negative patients in contrast to normal lung parenchyma. This suggests that the experimental group in this study [103] had a lower Th2 response than the patients in the control group.[103] Contradictory findings, were however, noted in a murine model [101] which documented a rise in IL-4 expression in TB granulomas with an increase in duration of disease. The authors of

the latter study [101] discussed findings of raised IL-4 mRNA in humans and proposed that with disease progression, there is a change from a Th1 to a Th2 response. Other authors [85] identified low IL-4 positive cell counts by immunohistochemistry on TB granulomas, illustrating that cases with IL-4 immunoreactivity was suggestive of an increase in caseous necrosis within the granulomas. Currently, the Th2 response in TB is a cause of much debate as there are authors [15, 104,105] who have documented the conflicting IL-4 results in various studies. Regardless of the debate, the role of the Th2 response in TB granulomas may not be ignored.

It is well known that IL-4 is required for the differentiation of monocytes into dendritic cells which are required for antigen presentation [103] and that IL-4 functions to oppose Th1 produced cytokines such as TNF-α [103, 106]. One study has shown that although serum levels of IL-4 were similar in healthy controls as well as in patients with TB, [103] it was more common for patients with cavitatory disease (which is considered to be a severe form of disease) to have increased IL-4 expression in CD4+ as well as CD8+ T-cells. It has also been demonstrated that in latent Mtb infection a Th1 response prevails and that IL-4 expression is maintained at low levels [102] which is thought to be a driving factor in ensuring latent infection. [102]Some authors [4] have illustrated an increase in IFN-γ, TNF-α and IL-4 mRNA in TB granulomas from HIV positive patients. This may be a result of an increase in cytokine production by Mtb specific T-cells or may be a consequence of an increased number of Mtb specific cells. [4]

Luttikhuizen *et al* [100] proposed that IL-4 functions in a manner to oppose IL-1 and TNF-α production and that IL-4 facilitates the formation of multinucleated giant cells. There is however no mention of the extent of expression and whether IL-4 is increased or decreased in

relation to other cytokines. The foreign body granulomas in the current study demonstrated a lower percentage of positive IL-4 staining cells than that documented in the TB granulomas from HIV negative and positive patients.

The present study showed a marked and very significant difference ($p = 0.00000046158$) between the percentage of IL-6 positive cells in TB granulomas of the HIV negative group compared to TB granulomas in the HIV positive group (figure 19, table 5).

IL-6 has been demonstrated to have numerous functions, some of which include having a pro-inflammatory role, whilst IL-6 may also result in T-cell suppression with a resultant reduction in TNF-α. [15, 29, 100, 105-107] It has been suggested that inhibition of TNF-α by IL-6 may promote spread of disease. [107] Furthermore, it has also been demonstrated that IL-6 opposes IFN-γ activity [108], hence the proposal that inhibition of IFN-γ by IL-6 may be yet another mechanism that Mtb takes advantage of in its attempt at escaping host cell defences. [108] Evidence suggests [15] that IL-4 facilitates increased production of IL-6 such that these cytokines have synergistic effects in abrogating a Th1 response in TB granulomas.

The number of IL-6 positive cells in the foreign body granuloma is markedly decreased in comparison to the TB granulomas from both HIV negative and positive groups in the present study. It has been shown that IL-6 expression in foreign body granulomas down-regulates pro-inflammatory cytokines, [100] although the degree of expression has not been measured by these authors. When both anti-inflammatory markers are examined with respect to the pro-inflammatory markers in the foreign body granuloma in the present study, it may be seen that the pro-inflammatory response outweighs the anti-inflammatory response.

Overall evaluation of the cytokines in the TB granulomas from HIV negative patients shows a fairly robust pro-inflammatory response. However, the percentages of both the anti-inflammatory cytokines, IL-4 and IL-6 in TB granulomas from this group far exceed the percentages of both pro-inflammatory cytokines, IL-2 and IFN-γ. It is therefore postulated that whilst the HIV negative patients have been able to raise a cellular response to contain Mtb, the concomitant anti-inflammatory response likely served to prevent excessive tissue damage and also curtailed the host's ability to eradicate infection.

It is important to bear in mind that the impaired Th1 response may be further decreased in progressive HIV disease due to an increase in Tregs. [79] With regard to TB granulomas in the HIV positive group of patients in the current study, the anti-inflammatory response is greater than the pro-inflammatory response. This implies that a Th2 response prevails with eventual progression of disease.

CHAPTER 7

7.0 CONCLUSION

It is evident from this study that depletion of CD4+ cells in HIV positive patients, impacts on granuloma formation with subsequent down-stream effects on cytokines expressed and produced in the granulomas. The present study has illustrated the preservation of CD4+ cells in TB granulomas from HIV negative patients as well as demonstrating the reversal of CD4+:CD8+ cell counts in TB granulomas from HIV positive patients. This confirms other studies undertaken on blood specimens in HIV positive patients. [1, 4, 80]

The cytokine profile in TB granulomas from both HIV negative and positive groups, show an overwhelming predominance of anti-inflammatory cytokines which is more striking in the HIV negative group of patients. This suggests that whilst the HIV negative host may not be able to effectively eradicate Mtb, the immune system still has sufficient resources to produce a large amount of cytokines. The HIV positive group of patients in this study however, have not produced large quantities of pro- or anti-inflammatory cytokines in TB granulomas. This may be attributed to the decreased number of CD4+ cells. However, a contradictory argument may be put forth, with the notion that HIV positive patients secrete greater quantities of cytokines in an attempt to counterbalance the decreased numbers of CD4+ cells. This however is not borne out by the results in the present study.

The interplay between Tregs and T-cells in granulomas undoubtedly tips the balance in favour of an environment conducive to Mtb and HIV survival, replication and proliferation.

This is likely to result in disease progression with further morbidity for the affected individual.

The high percentage of FOXP3 positive cells in granulomas from HIV positive patients together with the elevated expression of Th2 markers points toward a predominantly anti-inflammatory response. The results suggest that while Tregs limit localised tissue destruction, failure to contain Mtb facilitates spread of the organism. This therefore entails granuloma formation at another site with concomitant tissue destruction there. Thus whilst at a localised site, tissue destruction may be limited, disseminated spread thus implies disease progression at multiple sites.

This study has therefore shown that TB mono-infection causes alterations in the expression of cell markers such as FOXP3 with accompanying noteworthy changes in cytokine production in areas of granuloma formation. The alterations noted in TB and HIV co-infection are even greater and point toward evolution of micro-organism synergism with host demise.

The percentage of IL-17 positive cells in this study has shown that it does not have a major impact in affecting TB granulomas in either HIV negative or HIV positive patients; as similar results were noted in TB granulomas from both groups

Whilst there is currently limited information on the staining of various cytokines, cell markers and accessory molecules in foreign body granulomas, perhaps future studies may focus on this area of histopathology.

CHAPTER 8

8.0 APPENDIX

8.1 TABLES CONTAINING ORIGINAL DATA

	HIV NEGATIVE			*HIV POSITIVE*			*FOREIGN BODY GRANULOMA*		
	CD4	**CD8**	**CD68**	**CD4**	**CD8**	**CD68**	**CD4**	**CD8**	**CD68**
	155	180	250	95	150	250			
	145	175	250	85	185	250			
	200	225	250	90	165	250			
	150	100	250	105	160	250			
	105	125	250	100	145	250			
	140	140	250	110	165	250			
	180	155	250	80	170	250			
	180	105	250	75	155	250			
	170	125	250	120	125	250			
	170	140	250	95	165	250			
							68	**73**	**250**
Mean	**159.5**	**147**	**250**	**95.5**	**158.5**	**250**	**68**	**73**	**250**
SD	**25.2438**	**36.1386**	**0.0000**	**13.1244**	**15.3379**	**0.0000**			
Min	**105**	**100**	**250**	**75**	**125**	**250**			
Max	**200**	**225**	**250**	**120**	**185**	**250**			
Median	**162.5**	**140**	**250**	**95**	**162.5**	**250**			

Table 7: Absolute cell counts of CD4, CD8 and CD68 in granulomas.

Comparison of CD4, CD8 and CD68 absolute cell counts (out of a possible total of 250 cells) in the TB granulomas from HIV negative, HIV positive patients and foreign body granuloma (control tissue). (CD4 in HIV negative compared to HIV positive patients p=0.000011119; CD4 compared to CD8 in HIV positive patients p=0.000000030021)

	HIV NEGATIVE	*HIV POSITIVE*	*FOREIGN BODY GRANULOMA*
	64	64	
	77	22	
	56	66	
	48	67	
	37	30	
	38	19	
	42	60	
	64	67	
	23	55	
	28	23	
			22
Mean	**47.7**	**47.3**	22
SD	**17.31441**	**20.97**	
Min	**23**	**19**	
Max	**77**	**67**	
Median	**45**	**57.5**	

Table 8: Absolute cell counts of IL-17 staining cells in granulomas.
Comparison of IL-17 absolute cell counts, (out of a possible 250 cells) in TB granulomas from HIV negative, HIV positive patients and foreign body granuloma (control tissue).

	HIV NEGATIVE	*HIV POSITIVE*	*FOREIGN BODY GRANULOMA*
	5	60	
	5	85	
	5	90	
	5	75	
	45	40	
	10	45	
	10	35	
	5	30	
	5	55	
	5	25	
			35
Mean	**10**	**54**	**35**
SD	**11.8322**	**21.8861**	
Min	**5**	**25**	
Max	**45**	**90**	
Median	**5**	**50**	

Table 9: <u>Absolute cell counts of FOXP3 staining cells in granulomas.</u>
Comparison of FOXP3 absolute cell counts, (out of a possible 250 cells) in TB granulomas from HIV negative, HIV positive patients, and foreign body granuloma (control tissue). ($p<0.001$ $p = 0.00011523$)

	HIV NEGATIVE		*HIV POSITIVE*		*FOREIGN BODY GRANULOMA*	
	HLA I	**HLA II**	**HLA I**	**HLA II**	**HLA I**	**HLA II**
	110	**250**	150	250		
	75	**250**	165	250		
	110	**250**	140	250		
	105	**250**	145	250		
	170	**250**	135	250		
	140	**250**	175	250		
	170	**250**	135	250		
	160	**250**	125	250		
	175	**250**	135	250		
	120	**250**	135	250		
					70	**250**
Mean	**133.5**	**250**	**144**	**250**	**70**	**250**
SD	**32.6382**	**0.0000**	**14.6287**	**0.0000**		
Min	**75**	**250**	**125**	**250**		
Max	**175**	**250**	**175**	**250**		
Median	**130**	**250**	**137.5**	**250**		

Table 10: <u>Absolute cell counts of HLA Class I and II staining in granulomas.</u>
Comparison of HLA Class I and II absolute cell counts, (out of a possible 250 cells) in TB granulomas from HIV negative, HIV positive patients and foreign body granuloma (control tissue).

	HIV NEGATIVE				*HIV POSITIVE*				*FOREIGN BODY GRANULOMA*			
	IL-2	**IFNγ**	**IL-4**	**IL-6**	**IL-2**	**IFNγ**	**IL-4**	**IL-6**	**IL-2**	**IFNγ**	**IL-4**	**IL6**
	150	135	185	200	90	95	175	120				
	25	135	175	215	75	120	175	155				
	75	65	75	225	65	70	180	15				
	50	170	70	200	55	75	175	60				
	50	75	75	195	80	85	195	70				
	75	85	175	200	60	90	195	70				
	85	115	190	210	85	80	160	50				
	5	30	175	155	95	85	195	45				
	50	135	145	215	40	50	155	55				
	35	150	175	155	35	75	175	35				
									95	**155**	**120**	**38**
Mean	**60**	**109.5**	**144**	**197**	**68**	**82.5**	**178**	**67.5**	**95**	**155**	**120**	**38**
SD	**37.8814**	**41.6803**	**47.5815**	**22.7156**	**19.5192**	**17.2119**	**13.2665**	**39.0672**				
Min	**5**	**30**	**70**	**155**	**35**	**50**	**155**	**15**				
Max	**150**	**170**	**190**	**225**	**95**	**120**	**195**	**155**				
Median	**50**	**125**	**175**	**200**	**70**	**82.5**	**175**	**57.5**				

Table 11: <u>Absolute cell counts of cytokine staining in granulomas.</u>
Comparison of cytokine absolute cell counts (out of a possible 250 cells) in the TB granulomas from HIV negative, HIV positive patients and foreign body granuloma (control tissue). ($p<0.05$; IL-2 compared to IFN-γ in HIV negative patients $p=0.016842665$; IL-4 compared to IL-6 in HIV negative patients $p= 0.010010786$ and $p<0.001$; IL-6 in HIV negative compared to HIV positive patients $p= 0.00000046158$; IL-4 compared to IL-6 in HIV positive patients $p= 0.00000609701$)

8.2 ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Reubina Wadee

CLEARANCE CERTIFICATE	**M091020**
PROJECT	The Hisopathological analysis of Cellular Elements, Accessory Molecules and Cytokines in Mycobacterial Granulomas from HIV Positive and Negative Individuals
INVESTIGATORS	Dr Reubina Wadee.
DEPARTMENT	Division of Anatomical Pathology
DATE CONSIDERED	2009/10/30
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 2009/11/02 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof MJ Hale

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

.....

8.3 TURNITIN ANAYLSIS AND REPORT

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CHAPTER 9

9. 0 REFERENCES

1. Pawlowski A, Jansson M, Skold M, Rottenberg M, Kallenius G. Tuberculosis and HIV Co– Infection. PLoS Pathog. 2012 February; 8 (2): e1002464. doi:10.1371/journal.ppat.1002464. Epub 2012 Feb 16.
2. Bezuidenhout J, Roberts T, Muller L, van Helden P, Walzl G. Pleural tuberculosis in patients with early HIV infection is associated with increased TNF –α expression and necrosis in granulomas. PLoS One. 2009 January; 4 (1): e4228. doi: 10.1371/ journal.pone.0004228. Epub 2009 Jan 19.
3. Miranda MS, Breiman A, Allain S, Deknuydt F, Altare F. The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for bacteria. Clin Dev Immunol. 2012;2012:139127. doi: 10.1155/2012/139127. Epub 2012 Jul 3.
4. Diedrich CR, Flynne JL. HIV- 1/*Mycobacterium tuberculosis* coinfection immunology: how does HIV- 1exacerbate tuberculosis? Infect Immun. 2011; 79 (4):1407-17.
5. Ramakrishnan L. Revisiting the role of the granuloma in tuberculosis. Nat Rev Immunol. 2012; 12(5):352–66.
6. Kaufmann SHE and Parida SK. Tuberculosis in Africa: learning from pathogenesis for biomarker identification. Cell Host Microbe. 2008; 4(3):219-28.
7. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC *et al*. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. 2003; 163(9):1009–21.

8. Kaufmann SHE, McMichael AJ. Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. Nat Med. 2005 (4 Suppl): S33-44.

9. Walker NF, Meintjes G, Wilkinson RJ. HIV-1 and the immune response to TB. Future Virol. 2013; 8(1): 57-80.

10. Lalvani A. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. Chest. 2007; 131(6):1898–906.

11. Fine PE. Variation in infection by BCG: implications of and for heterologous immunity. Lancet. 1995; 346(8986):1339–45.

12. Schaible UE, Kauffmann SHE. Malnutrition and infection: complex mechanisms and global impacts. PLoS Med. 2007 4(5):e115.

13. Ellner JJ, Wallis RS. Immunologic aspects of mycobacterial infections. Rev Infect Dis. 1989; 11 Suppl 2:S455-9.

14. Wadee AA, Sher R and Rabson AR. Production of suppressor factor by human adherent cells treated with mycobacteria. J Immunol. 1980; 125(3):1380–86.

15. Raja A. Immunology of tuberculosis. Indian J Med Res. 2004; 120(4):213–32.

16. Flynn JL, Chan J, Lin PL. Macrophages and control of granulomatous inflammation in tuberculosis. Mucosal Immunol. 2011; 4(3):271–8.

17. Ottenhoff TH. The knowns and unknowns of the immunopathogenesis of tuberculosis. Int J Tuberc Lung Dis. 2012; 16(11):1424–32.

18. Ulrichs T, Kauffmann SH. New insights into the function of granulomas in human tuberculosis. J Pathol. 2006; 208(2):261–9.

19. Kumar V, Abbas AK, Fausto N, Aster JC. Robbins and Cotran Pathologic basis of disease. 8th edition: Elsevier Inc; 2010.

20. Russel DG. Who puts the tubercle into tuberculosis? Nat Rev Microbiol. 2007; 5(1)39–47.

21. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Eng J Med. 2006; 354(6): 610–21.

22. Li MO, Flavell RA. Contextual regulation of inflammation: a duet by transforming growth factor – beta and interleukin–10. Immunity. 2008; 28(4):468–76.

23. Wolf M, Albrecht S, Marki C. Proteolytic processing of chemokines: implications in physiological and pathological conditions. Int J Biochem Cell Biol. 2008; 40(6-7):1185-98.

24. Smit van Dixhoon MG, Munir R, Sussman G, Stad R, de Haan M, van der Hoeven *et al*. Gene expression profiling of suppressor mechanisms in tuberculosis. Mol Immunol. 2008; 45(6): 1573–86.

25. McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. Immunity. 2008; 28(4):445-53.

26. Ouyang W, Kolls JK, Zheng Y. The biological functions of T-helper 17 cell effector cytokines in inflammation. Immunity. 2008; 28(4):458–67.

27. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Blooms BR. An essential role for interferon-γ in resistance to *Mycobacterium tuberculosis* infection. J Exp Med. 1993; 178(6):2249–54.

28. Wadee AA. A 25 kDa fraction from *Mycobacterium tuberculosis* that inhibits leukocyte bactericidal activity reversal by gamma interferon. Res Microbiol. 1990 Feb; 141(2):249-52.

29. Sussman G, Wadee AA. Supernatants derived from CD 8+ lymphocytes activated by mycobacterial fractions inhibit cytokine production. The role of interleukin–6. Biotherapy. 1992; 4(2):87-95.

30. Nathan CF, Hibbs JB Jr. Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr Opin Immunol. 1991; 3(1):65-70.

31. Wadee AA, Anderson R, Rabson AR. Clofazamine reverses the inhibitory effect of *Mycobacterium tuberculosis* derived factors on phagocyte intracellular killing mechanisms. J Antimicrob Chemother. 1988; 21(1):65–74.

32. Wadee AA, Clara AM. A 25–kilodalton fraction from *Mycobacterium tuberculosis* that inhibits hexose monophosphate shunt activity by lysozyme release and H_2O_2 production reversal by gamma interferon. Infect Immun. 1989; 57(3):864-9.

33. Wadee AA, Cohen JD, Rabson AR. Gamma interferon reverses inhibition of leukocyte bactericidal activity by a 25–kilodalton fraction from *Mycobacterium tuberculosis*. Infect Immun. 1987; 55(11):2777–82.

34. Tsuyuguchi I, Kawasumi H, Takashima T, Tsuyugudi T, Kishimoto S. *Mycobacterium avium Intracellulare* complex induced suppression of T–cell proliferation in vitro by regulation of monocyte accessory cell activity. Infect Immun. 1990; 58(5):1369–78.

35. Mustafa T, Mogga SJ, Mfinanga SG, Morkve D, Sviland L. Immunohistochemical analysis of cytokines and apoptosis in tuberculous lymphadenitis. Immunology. 2005; 117(4):454–62.

36. Wadee AA, Kuschke RH. Expression of accessory molecules in the presence of *Mycobacterium tuberculosis* extracts on unstimulated and activated monocytes. South Afr J Epidemiol Infect. 2003; 18(4):89–95.

37. Gercken J, Pryjma J, Ernst M, Flad HD. Defective antigen presentation by *Mycobacterium tuberculosis*– infected monocytes. Infect Immun. 1994; 62(8):3472–8.

38. Mortaz E, Varahram M, Farnia P, Bahadori M, Masjedi MR. New aspects in immunopathology of *Mycobacterium tuberculosis*. ISRN immunology. 2012, Article ID 963879, 11 pages. Doi10.5402/2012/963879.

39. Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med 1983. 158(3):670–89.

40. Scott–Browne JP, Shafiani S, Tucker–Heard G, Ishida –Tsubota K, Fontenot JD, Rudensky AY, *et al*. Expansion and function of Foxp3–expressing T–regulatory cells during tuberculosis. J Exp Med. 2007; 204(9):2159-69.

41. Suchard MS, Mayne E, Green VA, Shalekoff S, Donniger SL, Stevens WS *et al*. FOXP3 expression is upregulated in CD 4+ T-cells in progressive HIV–1 infection and is a marker of disease severity. PLoS ONE 2010; 5(7):e11762. doi: 10.1371/journal.pone.0011762.

42. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self–tolerance maintained by activated T-cells expressing IL–2 receptor alpha chains (CD25). Breakdown of a single mechanism of self–tolerance causes various autoimmune diseases. J Immunol. 1995; 155(3):1151-64.

43. Burl S, Hill PC, Jeffries DJ, Holland MJ, Fox A, Lugos MD *et al*. FOXP3 gene expression in a tuberculosis case contact study. Clin Exp Immunol. 2007; 149(1):117-22.

44. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sofel RA *et al*. IL–4 inhibits TGF-β induced Foxp3+ T cells and, together with TGF-β, generates IL–9+ and IL–10+ Foxp3- effector T cells. Nat Immunol. 2008; 9(12):1347–55.

45. Buckner JH, Ziegler SF. Functional analysis of FOXP3. Ann NY Acad Sci. 2008; 1143: 151–69.

46. Campbell DJ, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T-cells. Nat Rev Immunol. 2007; 7(4):305-10.

47. Shafiani S, Tucker–Heard G, Kariyone A, Takatsu K, Urdahl KB. Pathogen–specific regulatory T – cells delay the arrival of effector T–cells in the lung during early tuberculosis. J Exp Med. 2010; 207(7):1409-20.

48. Belkaid Y, Rouse BT. Natural regulatory T–cells in infectious disease. Nat Immunol. 2005; 6(4):353–60.

49. Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006; 24:209–26.

50. Rowe JH, Ertelt JM, Way SS. FOXP3+ regulatory T cells, immune stimulation and host defence against infection. Immunology 2012; 136(1):1–10.

51. Guyot – Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A. Regulatory T – cells are expanded in blood and disease sites in patients with tuberculosis. Am J Respir Crit Care Med. 2006; 173(7):803-10.

52. Baecher –Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+ CD25 high regulatory cells in human peripheral blood. J Immunol 2001; 167(3):1245–53.

53. Gazzola L, Tincati C, Gori A, Sarasella M, Marventano I, Zanini F. FoxP3 mRNA expression in regulatory T cells from patients with tuberculosis. Am J Respir Crit Care Med. 2006; 174(3):356; author reply 357.

54. Hougardy JM, Place S, Hilderbrand M, Drowart A, Debrie AS, Locht C *et al*. Regulatory T Cells Depress Immune Responses to Protective Antigens in Active Tuberculosis. Am J Respir Crit Care Med 2007; l 176(4): 409-16.

55. Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, Dascher CC *et al*. IL–10–inducing T-cells suppress immune responses in anergic tuberculosis patients. J Clin Invest. 2000; 105(9):1317–25.

56. Hirsch CS, Hussain R, Toossi Z, Dawood G, Shahid F, Ellner JJ. Cross–modulation by transforming growth factor beta in human tuberculosis: suppression of antigen–

driven blastogenesis and interferon gamma production. Proc Natl Acad Sci USA 1996; 93(8):3193–98.

57. Roberts T, Beyers N, Aguirre A, Walzl G. Immunosuppression during active tuberculosis is characterized by decreased interferon- gamma production and CD25 expression with elevated forkhead box P3, transforming growth factor- beta , and interleukin-4 mRNA levels. J Infect Dis. 2007; 195(6):870-8.

58. Kusar M, Koch M, Mittrucker HW, Nouailles G, Bonhagen K, Kamradt T *et al*. Cutting edge: regulatory T-cells prevent efficient clearance of *Mycobacterium tuberculosis*. J Immunol. 2007; 178(5): 2661–5.

59. Dieli F, Troye-Blomberg M, Ivanyi J, Fournié JJ, Bonneville M, Peyrat MA *et al*. Vγ9/VΔ2 T lymphocytes reduce the viability of intracellular *Mycobacterium tuberculosis*. Eur J Immunol. 2000; 30(5):1512-9.

60. Munk ME, Gatrill AJ, Kaufmann SH. Target cell lysis and IL–2 secretion by gamma/delta T–lymphocytes after activation with bacteria. J Immunol. 1990; 145(8):2434–9.

61. Pearl JE, Khader SA, Solarche A, Gilmartin L, Ghilardi N, de Saurage F *et al*. IL–27 signalling comprises control of bacterial growth in mycobacteria– infected mice. J Immunol. 2004; 173(12):7490–6.

62. Vanham G, Toossi Z, Hirsch CS, Wallis RS, Schwarder SK, Rich EA *et al*. Examining a paradox in the pathogenesis of human pulmonary tuberculosis: immune activation and suppression/anergy. Tuber Lung Dis. 1997; 78(3-4):145–158.

63. Ehlers S. Role of tumour necrosis factor (TNF) in host defense against tuberculosis: implications for immunotherapies targeting TNF. Ann Rheum Dis. 2003; 62 (suppl 2): ii37-42.

64. Lazarevic V, Flynn J. CD8+ T-cells in tuberculosis. Am J Respir Crit Care Med. 2002; 166(8):1116-21.

65. Ray JC, Flynn JL, Kirschner DE. Synergy between individual TNF dependent functions determines granuloma performance for controlling Mycobacterium tuberculosis. J Immunol. 2009; 182(6):3706-17.

66. Elner JJ. Suppressor adherent cells in human tuberculosis. J Immunol. 1978; 121(6): 2573-9.

67. Hart PD, Armstrong JA, Brown CA, Draper P. Ultrastructural study of the behaviour of macrophage toward parasitic mycobacteria. Infect Immun. 1972; 5(5):803–7.

68. Sonnenberg P, Glynn JR, Fielding K, Murray J, Godfery–Fausset P, Shearer S. How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African miners. J Infect Dis. 2005; 191(2): 150-8.

69. Sonnenberg P, Murray J, Glynn JR, Shearer S, Kambashi B, Godfrey-Faussett P. HIV–1 and recurrence, relapse and re–infection of tuberculosis after cure: a cohort study in South African miners. Lancet. 2001; 358(9294):1687–93.

70. Badri M, Ehrlich R, Wood R, Pulerwitz T, Maartens G. Association between tuberculosis and HIV disease progression in a high tuberculosis prevalence area. Int J Tuberc Lung Dis. 2001; 5(3): 225–32

71. de Noronha AL, Báfica A, Nogueira L, Barral A, Barral-Netto M. Lung granulomas from *Mycobacterium tuberculosis* /HIV-1 coinfected patients display decreased in situ TNF production. Pathol Res Pract. 2008; 204(3):155–61.

72. Pathak S, Wentzel-Larsen T, Asjö B. Effects of *in vitro* HIV–1 infection on mycobacterial growth in peripheral blood monocyte-derived macrophages. Infect Immun. 2010; 78(9): 4022–32.

73. Imperiali FG, Zaninoni A, La Maestra L, Tarsia P, Blasi F, Barcellini W. Increased *Mycobacterium tuberculosis* growth in HIV-1-infected human macrophages: role of tumour necrosis factor-α. Clin Exp Immunol 2001; 123(3):435-42.

74. Mukadi Y, Perriëns JH, St Louis ME, Brown C, Prignot J, Willame JC, et al. Spectrum of immunodeficiency in HIV–1 infected patients with pulmonary tuberculosis in Zaire. Lancet. 1993; 342(8864):143–6.

75. López Ramírez GM, Rom WN, Ciotoli C, Talbot A, Martiniuk F, Cronstein B, *et al*. *Mycobacterium tuberculosis* alters expression of adhesion molecules on monocytic cells. Infect Immun. 1994; 62(6):2515–20.

76. Mohagheghpour N, Gammon D, Van Vollenhoven A, Horrig Y, Bermudez LE, Young LS. *Mycobacterium avium* reduces expression of co-stimulatory/adhesion molecules by human monocytes. Cell Immunol. 1997; 176(1)82-91.

77. Sullivan L, Sano S, Primez C, Salgame P, Mueller C, Hofman F *et al*. Expression of adhesion molecules in leprosy lesions. Clin Exp Immun. 1991; 59(11):4154–60.

78. O 'Garra A, Redford PS, McNab FW, Blom CL, Wilkinson RJ, Berry MP. The immune response in tuberculosis. Annu Rev Immunol. 2013; 31:475–527.

79. Brighenti S, Andersson J. Induction and regulation of CD8+ cytolytic T–cells in human TB and HIV infection. Biochem Biophys Res Commun. 2010; 396(1):50–7.

80. Danaviah S, Sacks JA, Kumar KP, Taylor LM, Fallows DA, Naicker T *et al*. Immunohistological characterization of spinal TB granulomas from HIV-negative and -positive patients. Tuberculosis. (Edinb) 2013 Mar 27. pii: S1472-9792(13)00017-6. doi: 10.1016/j.tube.2013.02.009. [Epub ahead of print].

81. Shen JY, Barnes PF, Rea TH, Meyer PR. Immunohistology of tuberculous adenitis in symptomatic HIV infection. Clin Exp Immunol. 1987; 72(2):186–9.

82. Diedrich CR, Mattila JT, Klein E, Janssen C, Phuah J, Sturgeon TJ *et al*. Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV in association with early peripheral T–cell depletion and not virus load. PLoSOne 2010 March; 5(3)e9611. doi: 10.1371/journal.pone.0009611

83. Geldmacher C, Zumla A, Hoelscher M. Interaction between HIV and *Mycobacterium tuberculosis*: HIV-1 induced CD4 depletion and the development of active tuberculosis. Curr Opin HIV AIDS. 2012; 7(3):268–75

84. Ordway D, Higgins DM, Sanchez-Campillo J, Spencer JS, Henao-Tamayo M, Harton M *et al*. XCL1 (lymphotactin) chemokine produced by activated CD8 T cells during the chronic stage of infection with *Mycobacterium tuberculosis* negatively affects production of IFN-gamma by CD4 T cells and participates in granuloma stability. J Leukoc Biol. 2007; 82(5):1221-9

85. Fenhalls G, Stevens L, Bezuidenhout J, Amphlett GE, Duncan K, Bardin P *et al*. Distribution of IFN-gamma, IL-4 and TNF-alpha protein and CD8 T cells producing IL-12p40 mRNA in human lung tuberculous granulomas. Immunology. 2002; 105(3):325–35

86. Holmes CL, Simmons DL. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood. 1993; 81(6):1607-13.

87. Curtis M, Way SS. Interleukin-17 in host defense against bacterial, mycobacterial and fungal pathogens. Immunology. 2009; 126(2):177–85.

88. Abebe F. Is interferon-gamma the right marker for bacille Calmette-Guérin-induced immune protection? The missing link in our understanding of tuberculosis immunology. Clin Exp Immunol. 2012; 169(3):213–9.

89. Chen X, Zhang M, Liao M, Graner MW, Wu C, Yang Q, *et al*. Reduced Th17 responses in patients with tuberculosis correlates with IL–6R expression on CD4+ T–cells. Am J Respir Crit Care Med. 2010; 181(7):734–42.

90. Gavin MA, Torgerson TR, Houston E, deRoos P, Ho WY, Stray-Pedersen A. Single cell analysis of normal and FOXP3-mutant human T–cells: FOXP3 expression without regulatory T–cell development. Proc Natl Acad Sci USA. 2006; 103(17): 6659–64.

91. Masood KI, Rottenberg ME, Salahuddin N, Irfan M, Rao N, Carow B et al. Expression of *Mycobacterium tuberculosis*-induced suppressor of cytokine signalling (SOCS) 1, SOCS 3, FoxP 3 and secretion of IL–6 associates with differing clinical severity of tuberculosis. BMC Infect Dis. 2013; 13: 13.

92. Selliah N, Zhang M, White S, Zoltick P, Sawaya BE, Finkel TH *et al*. FOXP3 inhibits HIV–1 infection of CD4 T-cells via inhibition of LTR transcriptional activity. Virology. 2008; 381(2): 161–7.

93. Knosp CA, Schiering C, Spence S, Carroll HP, Nel HJ, Osbourn M *et al*. Regulations of Foxp3 inducible regulatory T–cell stability by SOCS 2. J Immunol. 2013; 190(7): 3235–45.

94. Holmes D, Knudsen G, Mackey-Cushman S, Su L. FOXP3 enhances HIV–1 gene expression by modulating NFκB occupancy at the long terminal repeat in human T cells. J Biol Chem. 2007; 282(22):15973-80.

95. Jiang Q, Zhang L, Wang R, Jeffrey J, Washburn ML, Brouwer D *et al*. FoxP3+CD4+ regulatory T cells play an important role in acute HIV-1 infection in humanized Rag2−/−γC−/− mice *in vivo*. Blood. 2008; 112(7):2858–68.

96. Louie LG, Hartogensis WE, Jackman RP, Schultz KA, Zijenah LS, Yiu CH, *et al*. *Mycobacterium tuberculosis*/HIV–1 Co-infection: role of human leukocyte antigen variation. J Infect Dis. 2004; 189(6):1084–90.

97. Dubaniewicz A and Dubaniewicz A. Human leukocyte antigens class I and class II: Associations and distribution in different ethnic groups of patients with pulmonary tuberculosis. Curr Respir Med Reviews 2005; 1(2):117–21.

98. Balamurugan A, Sharma SK, Mehra NK. Human leukocyte antigen Class I supertypes influence susceptibility and severity of tuberculosis. J Infect Dis. 2004; 189(5):805–11.

99. Leslie A, Matthews PC, Listgarten J, Carlson JM, Kadie C, Ndung'u T *et al*. Additive contribution of HLA class 1alleles in the immune control of HIV–1 infection. J Virol. 2010; 84(19):9879–88.

100. Luttikhuizen DT, Harmsen MC, van Luyn JA. Cellular and molecular dynamics in the foreign body reaction. Tissue Eng. 2006; 12(7):1955-70.

101. Almeida AS, Lago PM, Boechat N, Huard RC, Lazzarini LC, Santos AR *et al*. Tuberculosis is associated with a down modulatory lung immune response that impairs Th1-type immunity. J Immunol. 2009; 183(1):718–31.

102. Arriaga AK, Orozco EH, Aguilar LD, Rook GA, Hernandez Pando R. Immunological and pathological comparative analysis between experimental latent tuberculous infection and progressive pulmonary tuberculosis. Clin Exp Immunol. 2002; 128(2):229–37.

103. Bai X, Wilson SE, Chmura K, Feldman NE, Chan ED. Morphometric analysis of Th(1) and Th(2) cytokine expression in human pulmonary tuberculosis. Tuberculosis (Edinb). 2004; 84(6):375–85.

104. Deveci F, Akbulut HH, Turgut T, Muz MH. Changes in serum cytokine levels in active tuberculosis with treatment. Mediators Inflamm. 2005; (5):256–62.

105. Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol. 2001; 19:93–129.

106. Rook GA, Hernandez–Pando R, Dheda K, Teng Seah GT. IL–4 in tuberculosis: implications for vaccine design. Trends Immunol. 2004; 25(9):483–8.

107. Zhang Y, Broser M, Rom WN. Activation of the interleukin-6 gene by *Mycobacterium tuberculosis* or lipopolysaccharide is mediated by nuclear factors NF-IL6 and NFκB. Proc Natl Acad Sci USA. Immunology 1994; 91(6):2225-9.

108. Nagabhushanam V, Solarche A, Ting LM, Escarou CJ, Zhang JY, Ernst JD. Innate inhibition of adaptive immunity: *Mycobacterium tuberculosis* induced IL-6 inhibits macrophage responses to IFN-gamma. J Immunol. 2003; 171(9):4750-7.