# Defining mechanisms of tissue destruction in tuberculosis-HIV co-infection and tuberculosis immune reconstitution inflammatory syndrome

Thesis presented for the degree of Doctor of Philosophy

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**Declaration of Originality**

I declare that this work is my own and that all else is appropriately referenced.

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**Abstract**

Tuberculosis (TB) is the leading cause of death in HIV-infected patients. The paradoxical TB-immune reconstitution inflammatory syndrome (TB-IRIS) complicates antiretroviral therapy (ART). Matrix metalloproteinases (MMPs) are implicated in TB immunopathology, causing pulmonary matrix degradation. I explored the hypothesis that MMPs drive immunopathology in TB-HIV co-infection. I also investigated invariant Natural Killer T (iNKT) cells, cytotoxic T cells with innate immune functions.

In a cross-sectional study in Cape Town, South Africa, HIV-infected and uninfected TB patients were compared to respiratory symptomatic and healthy controls. In a longitudinal study of HIV-infected ART naïve TB patients from TB diagnosis, for the first 3 months of ART, patients who developed TB-IRIS were compared with those who did not. Clinical features of disease severity were recorded, induced sputum and plasma collected for MMP and cytokine quantification by multiplex, and plasma for procollagen III N-terminal propeptide (PIIINP) quantification. Peripheral blood iNKT cells were enumerated by flow cytometry, using CD1d tetramers.

In the cross-sectional study, 227 patients were enrolled. TB and TB-HIV co-infection were associated with elevated sputum MMP-1, -3, and -8. MMP-1 was 33-fold higher in HIV-uninfected and 4-fold higher in HIV-infected TB patients, than corresponding controls. MMP-1 correlated with radiographic inflammation. Plasma PIIINP was increased in TB and TB-HIV co-infection and correlated with sputum MMPs and radiographic inflammation.

In the longitudinal study, 49 HIV-infected TB patients were enrolled and followed up. 29 (59%) developed TB-IRIS. In TB-IRIS, sputum MMP-7 and MMP-10 were elevated, prior to and during ART, in addition to plasma MMP-1, -3, and -8 and PIIINP. TB and HIV infection were associated with reduced iNKT cell frequencies. In TB-IRIS iNKT cell frequencies were elevated compared to non-IRIS controls.

Elevated MMP activity in TB-HIV co-infection is a novel target for host-directed therapy. INKT cells may have a pathological or protective role in TB immunopathology. Further study is required.

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# Chapter 1 Introduction

Tuberculosis (TB) causes 1.5 million deaths annually (1). Despite increasing political and financial commitment to reduce the impact of this disease over recent decades, morbidity due to TB has escalated. In South Africa, a country with one of the highest TB burdens, the incidence of TB quadrupled from a case notification rate of 163 per 100 0000 population in 1986 to 628 per 100 000 population in 2006 (2). This increase was driven principally by the emergence of the human immunodeficiency virus (HIV)-1 epidemic. HIV-1 is now recognized as the strongest risk factor for TB. A recent study demonstrated substantially higher TB rates in HIV-infected individuals despite antiretroviral therapy (ART), with 2.70-25.49 TB cases per 100 person-years in HIV-infected individuals on ART, compared to 0.62 cases per 100 person-years in HIV-uninfected individuals living in the same community (3). TB is the commonest cause of death in HIV-infected individuals in Africa and a third of all TB deaths are in HIV-infected patients (4, 5).

A compromised immune response increases the risk of TB disease. Extremes of age, malnutrition, alcohol excess, diabetes and vitamin D deficiency are all associated with some degree of impaired immune function (6-11). In many populations affected significantly by TB, HIV coexists with these socioeconomic and environmental factors, affecting TB exposure, risk of infection and progression to disease, potentially exacerbating the problem (7, 8, 12, 13).

The epidemiological and immunological relationships between TB and HIV are complex. HIV-induced progressive CD4 depletion is associated with increased risk of TB, disseminated TB and death (14). However, unlike other opportunistic infections, the risk of TB is elevated in HIV-infected individuals throughout the course of HIV infection, even in the first few years after acquisition, when the CD4 cell count remains high (15, 16). ART, whilst restoring absolute CD4 cell numbers, does not completely reverse the effect of HIV on TB risk (17). In addition, TB infection is associated with clinical progression of HIV-1 infection (18). TB-HIV co-infected patients are found to be at higher risk of new, additional opportunistic infections and mortality than HIV-infected patients without TB, with the same CD4 count (19-21). All patients with active TB are now recommended to start ART, irrespective of CD4 cell count (22).

## The spectrum of immune responses to TB

Classically, *Mycobacterium tuberculosis* (M.tb.) exposure is considered to result in one of three outcomes: uninfected, latently (quiescently) infected or actively infected (diseased). Examining TB in the context of HIV-1 infection has contributed to a new paradigm of TB infection, where a continuum of immune responses in an infected individual correlate with disease progression risk, rather than binary latently infected or diseased state concepts (see Figure 1-1) (23-25). At one end of the spectrum, it is proposed that an effective innate immune response can clear the host of M.tb. without T cell priming and without causing clinical symptoms. At the other end of the spectrum, failure of both innate and acquired immune responses leads to mycobacterial replication and

symptomatic disease. “Latent” infection, rather than simply describing the presence of non-replicating bacteria, is considered to more accurately reflect a range of immune responses providing relative control of M.tb. It is now recognized that subclinical TB can occur, particularly in HIV-infected individuals, who may be sputum culture positive for M.tb. without manifesting signs or symptoms of disease (25-27).

In addition, symptomatic active TB presents with a spectrum of pathology (Figure 1-1). At one end, severe inflammatory TB disease occurs, attributed to exaggerated immune responses, with severe destructive pulmonary pathology and cavitation. This is typically paucibacillary in the early stages before cavitation occurs and is less commonly seen in advanced HIV infection. At the other end of the spectrum, disseminated TB infection may occur, with a high M.tb. burden but only a limited inflammatory response. The latter is more frequently seen in advanced HIV. However, during the TB-immune reconstitution inflammatory syndrome (TB-IRIS) when HIV-infected patients start ART, inflammatory TB pathology may rapidly develop despite anti-tuberculosis therapy (paradoxical TB-IRIS) or develop acutely in the absence of a previous TB diagnosis (unmasking TB-IRIS) (28).

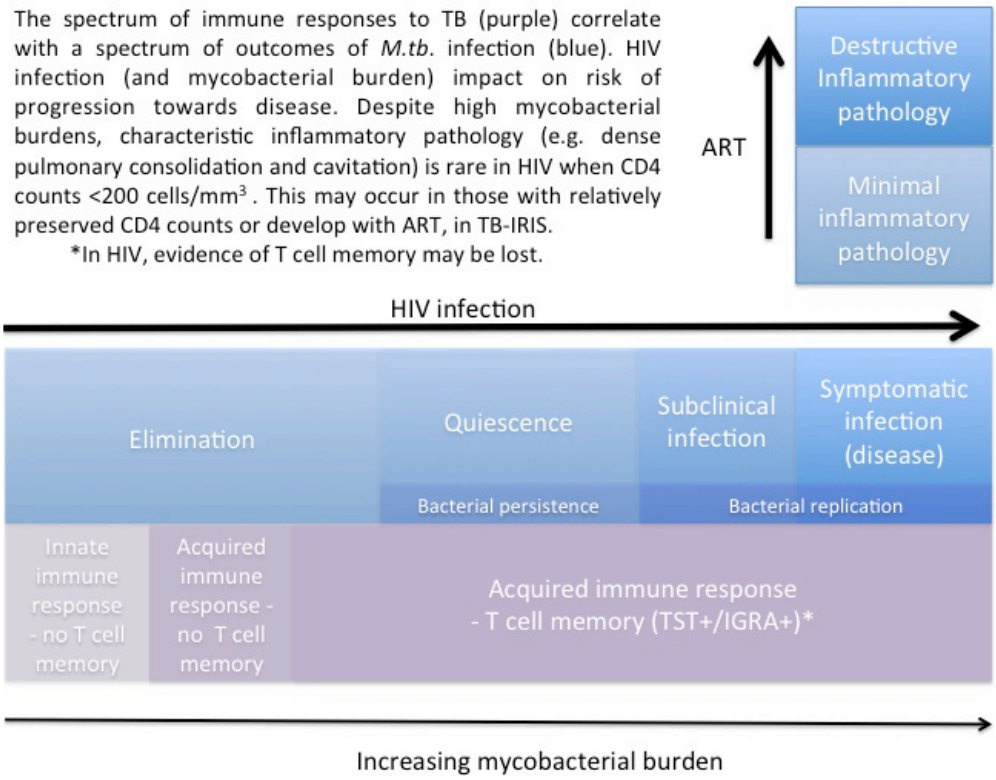



**Figure 1-1 The spectrum of immune responses to TB**

In this introductory chapter, I begin by reviewing literature on the immune interactions between TB and HIV infections, firstly by examining features of the immune response that are important to TB infection that are modulated by HIV infection, and then by examining how TB infection impacts on the host immune response to HIV-1 infection. This is adapted from a previously published work (Walker et al, **HIV-1 and the immune response to TB**, *Future Virology,* 2013) (29).

## What features of the immune response are important in TB infection?

M.tb., the causative organism of TB infection, is spread by respiratory droplets, aerosolized from a pulmonary TB patient when coughing, and inhaled by an uninfected exposed person. Not all exposed individuals develop lasting immunological evidence of infection. The reported incidence of "latent" infection following exposure is wide (0-89%) depending on the exposure, environment and population of study (30-32). The immune response to TB comprises two major components. The "innate" immune barriers include the physical components of the airway cilia, mucus, cells resident in the lung (e.g. alveolar macrophages) and cells recruited rapidly to the site of infection that can mount an immediate response (e.g. neutrophils). The "acquired" cellular responses require prior sensitization or priming to effect immunity, such as CD4 and CD8 T lymphocytes. Th1 cytokines including tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are critical to TB immunity, as demonstrated by mouse knock out models. Increased susceptibility to TB occurs in humans treated with anti-TNF-α agents and with genetic disorders associated with defective IFN-γ function (33-38). IFN-γ is produced largely by lymphocytes. It promotes macrophage activation and plays a key role in granuloma formation (33, 34, 39). B lymphocytes are present within granulomas but their precise role in TB immunity is unclear (40).

### *The central role of the TB granuloma*

The histopathological hallmark of M.tb. infection, and the focal point of the immune response to M.tb., is the granuloma, a structure characterized by

epithelioid macrophages and Langhans giant cells, around which T lymphocytes cluster. A centre of caseous necrotic material distinguishes TB from other granulomatous diseases, such as sarcoid, although it is not always present. M.tb. granulomas may be non-necrotic or fibrotic. Typically, scanty if any M.tb. bacilli are detectable at the centre of caseous granulomas. However, whether a granuloma provides an "effective" immune response is controversial. Initially, granulomas were believed to facilitate containment of M.tb. bacilli and mycobacterial killing. However, some studies suggest that the granuloma may facilitate microbial persistence by recruitment of potential uninfected host cells to the disease site, and even favour mycobacterial transmission by promoting host tissue damage (41-44).

A limited number of human studies have compared histopathological features of granulomas found in HIV-uninfected TB patients, with those found in TB patients with advanced HIV. Early post-mortem studies of TB in advanced HIV infection in sub-Saharan Africa demonstrated that the density of Langhans giant cells in TB lesions correlated with pre-mortem blood CD4 counts (45, 46). In patients with relatively preserved CD4 counts, typical granuloma architecture was seen. In advanced HIV infection, M.tb. lesions were typically found to be multi-bacillary, highly necrotic, with ill-formed or absent granulomas, and lacking in epithelioid and Langhans giant cells. Similar findings were reported in a post-mortem study from Brazil examining pulmonary lesions in TB-HIV co-infection and histopathological analysis of biopsy specimens from cases of TB lymphadenitis (47-49). The Brazilian study also reported increased

polymorphonuclear cell infiltration in TB-HIV co-infected granulomas and reduced TNF-α staining (47).

More detailed analysis of the influence of HIV-1 infection on the TB granuloma has been impaired by limitations of widely used animal models of TB, including the mouse model, where granulomas lack similar structure and caseation seen in human M.tb. Infection (50).

## TB, HIV and innate immunity

The majority of research into the mechanisms of susceptibility to TB in HIV-infected patients has focused on defects in cell-mediated immunity, particularly relating to CD4 T cell number and function (discussed in subsequent sections). However, the first immune cells to encounter M.tb. are innate immune cells in the lung, including alveolar macrophages and dendritic cells. In recent years, as questions remained unanswered about the susceptibility of HIV-infected patients to TB, more attention has been paid to defects of the innate immune system and interactions between innate and acquired immune responses.

### *Interference with macrophage function*

Following pulmonary M.tb. infection, alveolar macrophages phagocytose M.tb. bacilli. They are able to present peptide antigen to T cells via MHC class II molecules and secrete cytokines, e.g. IFN-γ and TNF-α, which influence T cell differentiation and cytotoxic responses. An important feature of mycobacterial virulence is resistance to the bactericidal mechanisms of phagocytic cells. M.tb.

interferes with phagosomal maturation, can resist lysosomal acidification, and potentially inhibits macrophage apoptosis (a process which facilitates mycobacterial killing), therefore promoting intracellular persistence (51-53).

Evidence suggests that TB-HIV co-infection may potentiate some of these mycobacterial virulence factors. A study of alveolar macrophages from TB-HIV co-infected patients in Malawi demonstrated intact phagocytosis in TB-HIV co-infection (54). However, intracellular M.tb. bacilli were found to reside in vacuoles at a higher pH than that of neighbouring M.tb. uninfected vacuoles and these vacuoles failed to fuse with lysosomes and acidify, suggesting that these vacuoles were functionally impaired. There is conflicting evidence as to whether M.tb. replication in macrophages is increased in HIV-1 co-infection. Some *in vitro* studies suggest HIV-1 infection of human monocyte-derived macrophages (MDMs) increases M.tb. growth (55, 56). Other studies have not found a significant effect of HIV-1 infection on M.tb. growth in human MDMs (57, 58).

There is an increasing body of evidence that suggests that TNF-α dependent macrophage apoptosis is reduced in TB-HIV co-infection. Patel *et al* demonstrated reduced M.tb.-induced apoptosis of human alveolar macrophages from HIV-infected donors compared to HIV-uninfected donors (59). Their studies suggest that HIV-1 infection inhibits macrophage apoptosis in response to M.tb. infection by inducing IL-10, which via a BCL-3 mediated pathway reduces TNF-α production and its pro-apoptotic effects. Kumawat *et al* have also demonstrated that HIV nef protein directly reduces M.tb.-induced macrophage apoptosis via inhibition of TNF-α promoter activation and interference with

TNF-α mRNA stability (60). Apoptosis of infected cells is thought to be a protective response, and therefore inhibition of apoptosis will prevent the development of an effective immune response.

Previous work has demonstrated that HIV-1 infection of macrophages resulted in attenuation of early IL-10 responses to M.tb. infection, up to 72 hours after M.tb. infection, augmenting later pro-inflammatory responses, including IL-1β and TNF-α (61). An attenuation of early regulatory immune responses, that is permissive of later exaggerated pro-inflammatory responses, may be favourable for both HIV replication (discussed below) and potentially promote M.tb. dissemination by recruitment of immune cells to the site of infection (42).

The process of capture and degradation of cytoplasmic contents by autophagosomes (double membrane organelles), known as autophagy, is an important mechanism of macrophage resistance to bacterial and viral infections and is likely to have a role in clearance of mycobacteria (51). HIV proteins interfere with autophagy in a variety of cells types including CD4 T cells and macrophages, both by promoting early stages of autophagy (which may facilitate HIV replication in some cells) and by arresting maturation of the autophagocytic process (62-64). The active metabolite of Vitamin D, 1α,25-dihydroxycholecalciferol, has recently been shown to induce autophagy in macrophages co-infected with M.tb. and HIV-1, leading to reduced M.tb. growth and HIV replication (58). This is interesting in light of epidemiological evidence that vitamin D deficiency is associated with an increased risk of TB and

progression of HIV (13, 65-68). This suggests impaired autophagy may contribute to reduced innate responses to TB.

Neutrophils are innate immune cells that have been potentially underestimated in TB protection and pathogenesis, despite their ability to phagocytose (although not necessarily kill) mycobacteria and their presence at sites of TB disease (69-71). Human neutrophil peptides 1-3 (HNP 1-3) have antimycobacterial activity *in vitro* (72). Neutrophil activity in TB has been associated both with increased severity of disease and with protection (72, 73). The presence of suppurative necrosis, rather than caseating granulomas, in human TB lesions in advanced HIV infection suggests a prominent role for the neutrophil in TB-HIV pathology, but this is yet to be defined (46).

### HIV, TB and Acquired Immunity

The acquired immune response is a complex and highly orchestrated system that has evolved to permit the eradication of pathogens without the development of autoimmunity. Components include CD4 and CD8 T cells, which have highly variable receptors, and the invariant T cells, including invariant Natural Killer T (iNKT) cells. I will discuss the role of each group of cells in the host immune response to TB, and how HIV co-infection modulates this, with evidence from both human and animal studies.

### *CD4 T cells and susceptibility to TB infection: animal studies*

The heightened susceptibility of HIV-infected persons to TB strongly implicates the CD4 T cell in a protective immune response to TB, as the characteristic immunological effect of HIV is on CD4 cell frequency and CD4 T cells are the main cell type infected by HIV. CD4 T cells are present on the periphery of TB granulomas and when M.tb. is phagocytosed, antigen presenting cells present mycobacterial peptide to CD4 T cells via MHC class II. The T cells may then produce cytokine and chemokine signals to activate innate immune cells and recruit cytotoxic lymphocytes and other cells to the disease site (74) .

Mogues *et al* attempted to tease out the relative importance of different aspects of cell-mediated immunity in a mouse model of M.tb. infection (75). Mice devoid of any functional T cells (thymectomy followed by monoclonal antibody infusions to CD4 and CD8) succumbed rapidly to M.tb. infection, by comparison with wild type mice, who appeared to control the infection within 20 days and survived over 250 days. Mutant mice with defective CD4 T cell function were unable to control M.tb. infection, whereas those with CD8 T cell dysfunction (targeted disruption of the β2-microglobulin gene) controlled infection, although notably with a higher bacillary burden than the wild type mice. Similar results were achieved using blocking antibody to CD4 and CD8 (75).

The mouse model of TB, whilst regularly used to understand immune mechanisms, fails to mimic human pathology. Mouse M.tb. granulomas lack the organization seen in humans and mice do not develop caseous necrosis or

cavitation (76). Therefore, more recently cynomolgus macaque models have been used to better mimic the spectrum of TB pathology found in humans (77-80). In macaques challenged with low dose aerosol M.tb. infection, a latently infected state occurs in around 50% animals (defined as tuberculin skin test (TST) positive and no clinical symptoms/signs of disease 8-10 months post-infection), which is then maintained for many years (77, 80). Subsequent Simian immunodeficiency virus (SIV) infection has been used to model the effect of HIV on latent TB (77, 81). After a dip in CD4 and CD8 cell numbers immediately after SIV infection, T cell numbers recover to pre-SIV levels in all macaques by 8 weeks. All SIV-TB co-infected monkeys developed reactivation of latent TB within 47 weeks, with some reactivating "early" (12-17 weeks post SIV infection), and some reactivating "late" (26-47 weeks) (77). There was a significant correlation between the extent of initial peripheral CD4 and CD8 T cell depletion in primary SIV infection and the time to reactivation of latent TB. However, peripheral blood CD4 or CD8 T cell numbers at the time of reactivation and just prior to necropsy were similar in early and late reactivating groups.

Another study using macaques examined the effect of CD4 cell depletion by using huOKT4A antibodies to deplete CD4 T cells in blood and tissues, rather than SIV infection (78). In CD4 depleted monkeys, an increased incidence of active TB following acute TB infection was seen compared to non-depleted monkeys (80% vs 17% respectively). In monkeys with established latent M.tb. infection observed over 14 weeks, huOKT4A antibody treatment resulted in reactivation in 50%, compared to 0% control monkeys. Although all huOKT4A monkeys had lower frequencies of CD4 T cells in peripheral blood mononuclear cells (PBMC),

bronchoalveolar lavage (BAL) and hilar lymph nodes than non-CD4 depleted monkeys at necropsy, significantly lower frequencies of CD4 T cells were found in hilar lymph nodes of huOKT4A monkeys that reactivated TB compared to huOKT4A monkeys that did not (3.7 vs 16.7%, $p<0.05$).

A benefit of animal studies is the ability to control the timing of HIV/SIV and M.tb. infection, allowing observation of early immunological events. Together these studies highlight the importance of CD4 T cells in susceptibility to TB and suggest that consideration of cell dynamics at the site of mycobacterial infection and local lymph nodes, in addition to absolute peripheral blood CD4 T cell numbers, may be valuable.

### *CD4 T cells and susceptibility to TB infection: human studies*

M.tb. is an obligate pathogen of man and therefore human studies are the most important in understanding the host-pathogen interaction. Human studies have examined immune responses at the site of active TB, as well as in peripheral blood. Pulmonary and pleural TB are typically characterized by a CD4 lymphocyte infiltration at the site of disease in HIV-uninfected populations. In HIV-infected patients with TB, a comparatively reduced number of CD4 T cells are seen in BAL, despite the total number of lymphocytes being increased relative to healthy controls (82, 83). Studies of human disease have also attempted to characterize functional (in addition to quantitative) defects in CD4 T cells in TB-HIV co-infection. This has been demonstrated in 2 ways: firstly, by examining production of key cytokines (e.g. IFN-γ) by CD4 lymphocytes in

response to stimulation by TB antigens (84-87); secondly, by examining the differences in differentiation phenotype of prevalent T lymphocytes. This phenotyping includes antigen naïve cells, antigen-specific memory cells mainly found in lymphoid tissue, and terminally differentiated effector memory cells found in the periphery and at disease sites (84, 88, 89).

In an example of the former approach, Geldmacher *et al* performed a cross-sectional study of PBMC from asymptomatic HIV-infected and -uninfected Tanzanian patients with no signs of TB disease, and a small number of patients with active TB. CD4 T cell IFN-γ responses were measured by ELISpot (Enzyme linked immunosorbent spot) and intracellular cytokine staining was performed, after overnight stimulation with purified protein derivative (PPD) and the M.tb.-specific antigen, early secreted antigenic target 6 (ESAT6) (84). Chronic HIV-1 infection, in the absence of TB disease, was associated with significantly decreased detectable IFN-γ responses. In active TB, these responses were detectable in the majority of both HIV-infected and -uninfected patients. There was no linear correlation between IFN-γ response and total CD4 cell count in M.tb.-infected or -uninfected HIV-infected patients. In a longitudinal study, they examined 5 "latently" M.tb.-infected HIV-uninfected subjects who acquired HIV infection. In 4 out of 5 of these patients, detectable responses to M.tb. antigens diminished completely within 1 year of seroconversion (84). None of these 4 patients developed active TB within 4 years of subsequent follow up. However, one patient developed dramatically increased responses 1 year after seroconversion. This patient was concurrently diagnosed with active TB (84).

Similarly, Rangaka *et al* found reduced IFN-γ responses to PPD in a whole blood assay, comparing HIV-infected with HIV-uninfected asymptomatic patients in a highly TB exposed population from Khayelitsha, South Africa (87). However, in this study, ELISpot responses to ESAT-6 (and additionally culture filtrate protein 10 (CFP10)) were not different between HIV-infected and HIV-uninfected individuals. PPD responses significantly correlated with CD4 count but ESAT-6 and CFP-10 responses did not.

These studies suggest that M.tb.-specific CD4 T cells are depleted early in HIV infection. Why should this be the case? CCR5 expression on the surface of these cells may have a role. CCR5, a disease site homing receptor, is the most common co-receptor used by HIV virus to enter cells (90). M.tb.-specific memory CD4 T cells from latently M.tb.-infected HIV-uninfected individuals have been shown to frequently co-express CCR5 (at twice the frequency of the total memory CD4 T cell population), implying that M.tb.-specific CD4 T cells may be particularly susceptible to HIV-1 infection. However, cytomegalovirus (CMV)-specific CD4 T cells are depleted less rapidly after acute HIV infection than M.tb.-specific CD4 T cells, despite similar CCR5 expression (84).

Other phenotypic features of these M.tb.-specific T cells have been implicated in their susceptibility, such as production of IL-2, which promotes CD4 T cell proliferation and increases susceptibility to direct HIV infection in cellular models (91). CMV-specific CD4 T cells produce less IL-2 and more macrophage inflammatory protein 1β, (MIP-1β), a CCR5 ligand, which may have a protective role (91, 92). Geldmacher and Koup (2012) suggest that in the absence of clinical

disease, M.tb.-specific CD4 T cells more commonly express CD27, and lack CD57 (a senescence marker), indicating a transitional or early differentiated phenotype, more commonly associated with IL-2 production, whereas circulating CMV-specific CD4 T cells are more commonly CD27- CD57+, a terminally differentiated phenotype. This difference may correspond to a difference between the two pathogens in the degree to which CD4 T cells are exposed to antigen during latent or subclinical infection, with more persistent low level antigen exposure after infection with CMV, compared to that following M.tb. infection.

In summary, the ideal target cell for HIV infection and depletion appears to be a CD4, CCR5 expressing T cell, producing abundant IL-2, and little MIP-1β. Increasing evidence suggests that these features are particularly characteristic of M.tb.-specific CD4 T cell populations and may explain their selective depletion early in the course of HIV infection and the consequent increase in active TB disease.

### *The role of polyfunctional T cells*

The differentiation phenotype of antigen-specific CD4 T cells influences predominant cytokine production and susceptibility to infection and is central to an emerging debate on the role of polyfunctional CD4 T cells in TB-HIV co-infection. Polyfunctional CD4 T cells are antigen-specific CD4 T cells that co-express IFN-γ, TNF-α and IL-2 and are considered to be an effector memory population, less well differentiated but with a more durable antigen-specific response, than terminally differentiated effector cells (93). Mansoor *et al* studied

infant immune responses following Bacillus Calmette-Guerin (BCG) vaccination at birth (86). In peripheral blood of HIV-infected infants, they noted a significant reduction in Th1 cytokine production (IFN-γ, TNF-α, IL-2) in response to restimulation with BCG, compared to HIV-uninfected infants, and a significantly reduced number of "polyfunctional" CD4 T cells. Kalsdorf *et al* showed that polyfunctional CD4 T cells were reduced in BAL samples from HIV-infected compared to HIV-uninfected healthy but highly TB-exposed patients (85).

In a study of pericardial TB, the phenotypic and functional qualities of CD4 T cells, including polyfunctionality, were examined at the site of TB disease (88). Measurement of antigen-induced IFN-γ responses in peripheral blood and pericardial fluid, comparing HIV-infected with -uninfected patients, revealed that pericardial fluid responses were elevated compared to blood in both HIV-infected and -uninfected patients. However, in HIV infection, pericardial fluid contained more polyfunctional and less terminally differentiated CD4 T cells. A possible explanation for this is that terminally differentiated effector cells, which typically express more CCR5, are susceptible to lytic HIV infection, and are rapidly depleted in HIV, leading to migration of less well differentiated, polyfunctional T cells with a central or effector memory phenotype, to the site of disease. In addition, central memory T cells are believed to serve as a reservoir of HIV-infection, and may harbor virus due to their long half-life (94, 95). It is also possible that migration of these cells to sites of M.tb. replication leads to progression of HIV infection, as TB infection drives HIV replication.

Polyfunctionality has been associated with TB protection in recent novel TB vaccine studies (96). However, this association is controversial. A correlation with protection from TB disease in humans has not yet been shown (93, 96). In Italian and Gambian patients, higher proportions of polyfunctional CD4 T cells are found in peripheral blood of active pulmonary TB patients, following restimulation with M.tb. antigens, compared to latently infected controls, and in the Italian study, these cell populations decreased with TB treatment, suggesting a correlation with viable M.tb. burden (97, 98). However, a similar study in Cape Town, South Africa reported conflicting results (99). In this study, decreased peripheral blood polyfunctional CD4 T cell responses to restimulation with M.tb. antigens were found in smear-positive, compared to smear-negative, pulmonary TB and latently infected patients, with an increase in these cell populations with TB treatment. Interestingly, in this study a much higher proportion of the overall CD4 T cell response was attributed to polyfunctional T cells for all studied groups, compared with findings in the previous studies. These differences may reflect differences in methodology between studies in analyzing cell number or variability related to the specific patient populations studied. However, in all cases it is unclear how peripheral blood findings are reflective of cell populations at the site of disease and the role of polyfunctional T cells remains uncertain.

### Cytotoxic lymphocytes

$CD8^+$ T lymphocytes are important in the human immune response to a number of intracellular pathogens, especially chronic viral infections including HIV, and are dependent on CD4 T cell help (100-102). Although less critical than CD4 T

cells for protection from M.tb. in animal models, mouse and primate models have demonstrated a role for $CD8^+$ cytotoxic T lymphocytes in TB immunity (40, 75, 77, 103). CD8-depleted mice developed chronic pulmonary TB with higher mycobacterial burdens, but similar survival, to wild type mice (75). A model of latent TB infection in mice has implicated CD8 T cells particularly in control of chronic rather than acute infection (104).

The mouse model may not be appropriate for fully delineating CD8 T cell responses, as mice lack comparable expression of group 1 CD1 proteins that may present mycobacterial lipid antigens to cytotoxic T cells. Also, mice lack expression of the cytotoxic molecule granulysin, which is released by cytotoxic T lymphocytes and has direct antimycobacterial activity (105). Indeed, studies using human cells have shown that perforin and granulysin are important for CD8 T cell-mediated target cell lysis in TB infection (105-107). $CD8^+$ effector memory T cells, which express cell surface TNF-α, are a major source of granulysin (108). Anti-TNF-α therapy causes a reduction in this cell population, which may contribute to the increased susceptibility of patients on this treatment to TB disease (108-110). HIV infection has a number of effects on CD8 T cell function, including causing persistent cellular activation and increasing proportions of intermediately differentiated CD8 T cells (94, 111, 112).

Granulysin production by CD8 T cells has not been studied in TB-HIV co-infection. However, HIV-1 infection of CD8 T lymphocytes suppresses the induction of granulysin in response to IL-21 and IL-15 (113). Additionally, defective granulysin-mediated cytotoxicity has been shown in HIV-associated

cryptococcal infections (114). Reduced IL-21 may potentiate defective cytolytic T cell responses in HIV infection, which could impact on anti-mycobacterial immunity. CD4 T cell-derived IL-21 is essential to sustain CD8 T cell responses to chronic viral infections in mice and may have a role in control of human chronic infections, such as hepatitis B and C (115-117). Low levels of serum IL-21 are found in HIV infection correlating with CD4 count (118). Taken together, the evidence for a central role for CD4 T cells in human protection is strong. The evidence for CD8 T cells is more circumstantial, but the implication is that CD8 T cells contribute to protection.

### *NK cells*

Natural Killer (NK) cells have innate and cytotoxic activity but their importance in TB-HIV pathogenesis is not well defined. NK cells can lyse M.tb.-infected cells, via the activating receptor NKp46 (119, 120). NK cells isolated from HIV-infected patients have reduced expression of activating receptors, including NKp46, increased expression of inhibitory receptors and are functionally impaired, compared to NK cells from healthy controls (121, 122). Recent studies have implicated NK cell dysfunction in TB-IRIS (see below) suggesting further study in this area is warranted (123, 124).

### *INKT cells*

INKT cells (also known as Type 1 NKT cells) are a T cell subset expressing an invariant T cell receptor comprised of Vα24 coupled with Vβ11 in humans (125). This T cell receptor binds specifically to antigen bound to CD1d molecules on antigen presenting cells, although the nature of the physiological antigen

remains unclear (126). *In vitro* studies have identified that the glycolipid α-galactosylceramide (α-galcer), from the marine sponge, when bound to CD1d potently activates iNKT cells, leading to rapid production of both Th1 and Th2 type cytokines (127). This potent response, coupled with lack of immune memory have led to their classification as innate lymphocytes, displaying phenotypic features of NK cells, including the presence of NK cell surface markers. Therefore, iNKT cells have been proposed to have potential to bridge the gap between innate and acquired immunity (128).

Different phenotypic characteristics have been ascribed to iNKT cells expressing CD4, or CD8 receptors, double positive (CD4 and CD8) or double negative cells (neither CD4 or CD8) (127). Tetramers containing CD1d bound to α-galcer are used in experimental models, both to identify iNKT cell subsets from PBMC populations using flow cytometry, and to activate cells. More recently attempts to identify the endogenous antigen of iNKT cells have identified that β-d-glucopyranosylceramide (β-GlcCer) present in human tissue is capable of activating iNKT cells during infections (129). iNKT cells have been implicated in pathophysiology of several infectious and autoimmune conditions (130-132).

### iNKT cells in TB

iNKT cells can be predicted to have a critical role in TB as they respond to antigen presented by the CD1d molecule (133). CD1 molecules are able to present lipid antigens due to their deep hydrophobic properties. The cell wall of M.tb. is very rich in lipids and therefore CD1-presented molecules are likely to be critical to the host immune response to infection (134). CD1d expression at the

centre of TB granulomas has been demonstrated in human lymph node specimens (135). Presentation of mycobacterial lipid antigens phosphatidylinositol mannoside (PIM) and lipoarabinomannan (LAM) by CD1b has been shown (136). However, presentation of an M.tb. lipid antigen by CD1d has yet to be conclusively demonstrated. Mycobacterial $PIM_4$ from *Mycobacterium bovis* BCG was identified as a candidate in early studies but this finding has not been confirmed in subsequent studies (134, 137). iNKT cells have been studied in animal models and human TB but the results have not all supported a central role for iNKT cells in protection from TB. The following paragraphs summarise the relevant studies to date.

iNKT cells were shown to directly restrict M.tb. growth and have bactericidal activity *in vitro* in a mouse peritoneal macrophage culture model (138). This effect was CD1d-dependent. A role for iNKT cells in mycobacterial control was confirmed *in vivo* in a mouse depletion model (138). However, in other studies CD1d deficiency did not result in a difference in M.t.b. colony forming units (CFU) or survival in mice (139-141).

Activation of iNKT cells in mouse macrophage co-culture models of TB infection was IL-12 and IL-18-dependent and resulted in IFN-γ production (138). Activation of iNKT cells by therapeutic administration of α-galcer augmented responses to TB treatment in mice (142, 143). Additionally, incorporation of α-galcer into BCG vaccine enhanced vaccine efficacy against M.tb. in mice (143). However, in a recent *in vitro* study, iNKT cell antimycobacterial activity was not dependent on IL-12/IL-18 mediated activation, nor IFN-γ production. Rather,

restriction of mycobacterial growth was mediated by iNKT cell-derived Granulocyte macrophage colony-stimulating growth factor (GM-CSF) secretion, and was CD1d dependent.

In health, iNKT frequencies in humans are remarkably stable (144). In human studies of active TB, iNKT cell frequencies were found to be reduced (145-147). In a study of TB patients, TST positive contacts and TST negative contacts, TST positive contacts had lower iNKT percentages than TST negative contacts. In patients with active TB, iNKT cell frequencies were further reduced. Treatment of TB patients with antituberculosis therapy for six months restored iNKT cell numbers to that of TST positive TB contacts, suggesting that low iNKT frequencies were an effect rather than a cause of TB disease (146). In a more recent study, both pulmonary TB and extra-pulmonary TB patients were found to have significantly reduced iNKT cell frequencies compared to healthy TST positive TB contacts and healthy TST negative controls (147). Pulmonary TB was associated with lower iNKT cell frequencies than extra-pulmonary TB. iNKT cells isolated from pulmonary TB patients were found to have reduced proliferative responses to α-galcer, suggesting iNKT cell dysfunction. Increased expression of Programmed cell death protein-1 (PD-1) on iNKT cells from TB and extra-pulmonary TB patients suggested that increased iNKT cell anergy may be responsible for this functional impairment (147, 148). One study found only a slight reduction in total iNKT cell frequency in TB patients compared to healthy controls. However there was a significant reduction in the double negative (CD4-CD8-) iNKT cell subset in both TB and leprosy patients (132). This subset is thought to have a particularly pro-inflammatory, cytolytic phenotype (132, 144).

Few studies have examined iNKT cells in HIV infection. In a study of human iNKT cells from healthy donors, iNKT cells were found to express the HIV-1 co-receptor CCR5 and could be productively infected by HIV-1. The CD4+ subset was to be particularly susceptible to HIV-1 infection, as were activated iNKT cells, which were more susceptible to HIV-1 infection than conventional CD4 T cells (149). In human HIV infection, both CD4+ and CD4- iNKT cell subsets were depleted (145, 150). One study has demonstrated rapid increases in the proportion of iNKT cells present in peripheral blood in response to ART (151). In HIV-leprosy co-infection, iNKT cell populations were found to be reduced more profoundly than in leprosy or HIV infection alone (152).

## What features of the immune response are important in HIV infection?

HIV-1 predominantly infects CD4 T cells by binding to the CD4 receptor on the cell surface, in addition to a co-receptor, usually the chemokine receptor CCR5 (predominantly expressed by effector T cells, but also present on monocytes and macrophages) or CXCR4 (in later stage infections, usually expressed by naïve T cells). This interaction allows fusion of the viral envelope with the host cellular membrane and permits viral entry. Once inside the cell, HIV viral RNA is transcribed into pro-viral DNA by HIV reverse transcriptase enzyme. Proviral DNA is integrated into cellular DNA and transcribed by cellular RNA polymerase II (153).

During primary HIV-1 infection, absolute CD4 T cell numbers decline acutely as viral titers in peripheral blood peak. This is largely due to infection and depletion of transitional effector memory and fully differentiated memory CD4 and CCR5-expressing T lymphocytes (154, 155). However, with the onset of a HIV-specific cell-mediated immune response, in the form of HIV-1-specific cytotoxic CD8 T lymphocytes, and with the production of HIV-1-specific antibodies, some degree of control over viral replication occurs. Additionally, cytotoxic CD4 T cell responses may be important in controlling infection at an early stage (156-158).

During the "chronic" or asymptomatic period of HIV infection, persistent replication of HIV occurs and the CD4 count gradually declines. During this period, stronger HIV-specific CD8 T cell responses and preservation of central memory CD4 T cell subsets are linked to slower progression (100). A persistent decline in CD4 cell numbers correlates with increasing immunocompromise, susceptibility to opportunistic infections, and eventual death, in the absence of ART. In the next section, I will discuss how the immune response to TB infection may accelerate or potentiate the detrimental immune effects of HIV.

### Immune activation due to TB drives HIV replication

Pro-inflammatory cytokine production by innate immune cells in response to M.tb. may facilitate progression of HIV infection (159). Peripheral blood monocytes from active pulmonary TB patients infected with HIV-1 support higher levels of HIV replication than monocytes from PPD positive healthy control donors (160). Activated monocytes, in addition to CD4 T lymphocytes,

contribute significantly to increased plasma HIV viral loads during TB infection (161). M.tb. infection drives HIV-1 viral replication by transcriptional activation in alveolar and pleural macrophages in respiratory TB, and is dependent on macrophage-lymphocyte contact and M.tb. induced inflammatory cytokines and chemokines, such as TNF-α and monocyte chemotactic protein 1 (MCP1, CCL2) (162-165). Recent work has identified increased expression of positive transcription elongation factor (P-TEFb), activation of nuclear factor kappa beta (NFκβ) and loss of an inhibitory CCAAT enhancer binding protein β (C/EBPβ) as transcription factors involved in this process (69, 162, 166). Increased viral replication is associated with development of increased viral heterogeneity at the site of TB disease and in blood and may persist following completion of anti-tuberculosis therapy (18, 167).

### TB infection promotes cellular susceptibility to HIV

Chemokines induced by M.tb. infection of phagocytic cells may also be instrumental in recruiting target cells susceptible to HIV (CCR5 expressing CD4 T cells and monocytes) to the site of HIV-1 replication, further potentiating the increased HIV viral replication at TB infection sites (168). In addition, *ex vivo* studies of human CD4 T cells suggest that TB infection may increase the susceptibility of CD4 T cells to new HIV infection, by upregulation of Toll-like receptor 2 (TLR-2), through which activation of HIV-long terminal repeat may occur, promoting HIV replication (169, 170). This potentially provides a contributory explanation for the epidemiological associations between TB and HIV disease progression, although further studies are required.

In summary, TB and HIV-1 infection have reciprocal detrimental immune effects. HIV-1 appears to have significant effects on cells of the innate immune system particularly macrophages, although evidence of this largely comes from *in vitro* experimental models of TB-HIV co-infection. Cytotoxic lymphocyte function, particularly affecting cells that bridge innate and acquired immune systems, specifically iNKT cells, appears to be impaired. The key role of CD4 T cells has been demonstrated in both animal and human studies. Whilst improved animal models, such as the macaque model, and the addition of new cellular models, will be valuable to investigate the interactions between immune cells involved in the granulomatous response to M.tb. and the impact of HIV-1 infection on these interactions, studies in humans ultimately provide the best system to understand this interaction.

## Impact of the immune response on clinical presentation and pathology in HIV-associated TB

HIV infection has exemplified how the state of the immune system impacts not only on the risk of TB disease and HIV progression, but also on clinical presentation, diagnosis and treatment of TB in HIV-1 infected individuals (171). In the next section, I will examine how the clinical features of TB implicate host immune dysfunction in TB immunopathology.

### Divergent pulmonary pathology in TB and HIV-associated TB

Pulmonary TB is the most common form of active TB, classically presenting with chronic productive cough, fever, weight loss and night sweats, and may be accompanied by haemoptysis and pleuritic chest pain. Localised pulmonary

inflammation is visible on chest radiograph, typically affecting lung apices, and frequently involves cavitation, the hallmark of pulmonary TB. Although TB may infect any organ and miliary TB (disseminated TB) is sometimes reported, these manifestations are less frequent in immunocompetent patients apart from very young infants.

Since the early days of the HIV epidemic, it has been clear that active TB in advanced HIV infection does not follow this typical pattern (171). Pulmonary disease is more likely to be widespread, often causing lower zone infiltration and rarely causing cavitary lung disease in patients with CD4 counts below 200 cells/$mm^3$ (74). Chest radiographic changes may be absent in sputum culture positive TB. In addition, TB is more likely to present with extra-pulmonary forms, particularly lymphadenitis, meningitis, gastrointestinal and disseminated TB. Clinical manifestations of TB in HIV-infected patients with relatively preserved CD4 counts follow a much more typical pattern of predominantly classical pulmonary disease, as described above.

### Diagnostic challenges in HIV-associated TB

Despite increased mycobacterial burdens and more widespread disease in HIV-infected TB patients, confirmation of diagnosis is more elusive (171). Considering that the mainstay of TB diagnosis for many decades has been smear microscopy for acid-fast bacilli (AFB) in sputum, this is not surprising. Cavitation correlates well with smear positivity, as M.tb. bacilli enter the airways through pulmonary parenchymal destruction, and such destructive pulmonary TB lesions are rare in advanced HIV. New PCR based diagnostic tests (GeneXpert) have

improved sensitivity over sputum smear microscopy for pulmonary TB in HIV-infected patients (172). However, many patients with clinical symptoms of TB fail to have a diagnosis confirmed by smear (and in some instances even culture) and clinicians often treat empirically (173).

Assessment of the immune response to mycobacterial antigens has been historically utilized in diagnosis of latent TB. The TST measures a delayed-type hypersensitivity reaction to mycobacterial PPD injected subcutaneously. A positive response indicates immune sensitization and correlates with M.tb. exposure. However, HIV-infected patients with latent and active TB may have an absent response. Therefore, reliability amongst HIV-infected individuals is reduced (174, 175).

Improved understanding of the immune response to TB has heralded the development of new immunodiagnostics, relying on detection of IFN-γ produced by PBMC or whole blood stimulated with M.tb. antigens, by interferon-γ release assay (IGRA), (e.g. ELISpot, QuantiFERON-Gold). There was some initial optimism that these may be more reliable than skin tests in HIV-infected individuals for diagnosing "latent" or "active" infection (176). Studies have not fully substantiated this suggestion (177). Little additive value is evident over standard TB diagnostics in high HIV incidence areas (27). Meta-analyses of test performance as a predictor of future active TB have demonstrated a weak to moderate association, with a relative risk of 2-3 in a mixed HIV-infected and -uninfected population and conclude that a lack of sufficient data exists in HIV-infected patients (178, 179). A recent longitudinal study of South African

adolescents demonstrated an 8-fold increase in risk of TB disease within 2 years in QuantiFERON TB Gold in-tube converters (180). However, this study was in a largely HIV-uninfected population.

In a recent longitudinal study of chronically HIV-infected individuals in Durban, South Africa, region of deletion 1 (RD-1) antigen responses were monitored using ELISpot assays 3-monthly for up to 2 years. There was a high degree of individual variability in T cell antigen response over time, without a correlation with stage of HIV disease, ART status or development of active TB (181). Only just over half the ELISpot responses were considered to be consistently positive or negative and individually in these patients there was a large degree of variability in the number of spot forming cells, despite not meeting criteria for a change in result from positive to negative. This study demonstrates the complexity of antigen-specific IFN-γ responses to M.tb. in HIV infection and cautions against over-interpretation of IFN-γ responses in cross-sectional studies. Improved understanding of immunity in TB-HIV co-infection is required to aid development of TB diagnostics suitable for HIV-infected patients and therapeutics to target TB tissue destruction.

### Mechanisms of divergent immunopathology in HIV-associated TB

In the absence of HIV infection, the risk of developing active TB following infection is lower, and when it does occur M.tb. bacilli tend to be locally contained (mainly at pulmonary sites). The ensuing immune response may be highly inflammatory, damaging local tissue, with devastating physiological effects and promotion of TB transmission through formation of cavities. This

allows for diagnosis as AFB tend to be present in sputum. In advanced HIV, the balance appears to be tipped in favour of M.tb. growth and dissemination, whilst resulting in less inflammatory pathology and reduced cavitation, reduced transmissibility but more challenging for diagnosis.

The wide spectrum of TB pathology in HIV-infected patients and the relationship of these manifestations to CD4 count implicates divergent immune responses. Studies in immunocompromised animals have failed to adequately model clinical and pathological features of human TB-HIV co-infection. Disruption of T cell function in murine models of TB produces some similar features, with fulminant disseminated infection, rather than controlled contained disease seen in wild type mice (75). However, it is hard to interpret these findings, as pulmonary disease in immunocompetent mice does not replicate human pulmonary TB, particularly due to lack of cavitation in most strains (182).

CD4 depletion in the macaque model of acute TB infection causes more widespread dissemination of primary infection than in wild type monkeys, with greater bacterial burden and increased pathology (78). Similarly to advanced HIV infection in humans, no chest radiographic abnormalities were found in these macaques, despite elevated erythrocyte sedimentation rates, higher mycobacterial burdens and macroscopically greater pathology at necropsy, although cellular architecture of the TB granuloma in CD4-depleted and control monkeys was similar.

CD8-depleted macaques exhibited more extensive TB lesions, compared to non-CD8-depleted macaques, following high dose M.tb. infection. In the CD8-depleted macaques, histopathology was similar to advanced TB-HIV in humans: granulomas were less well-organized and more necrotic, with reduced lymphocytic infiltration (45, 183).

In the SIV-TB model of TB reactivation described above, gross pathology scores, bacterial number scores and percent positive samples on mycobacterial culture were similar comparing active infection in SIV-TB co-infected macaques and TB mono-infected macaques. However, it is worth noting that these monkeys had similar peripheral CD4 counts to TB mono-infected monkeys and therefore were not representative of advanced HIV infection. A wide range of granuloma types were present in these co-infected monkeys, ranging from necrotic to fibrotic, with strikingly more fibrotic granulomas in late reactivating SIV-infected monkeys compared to early reactivating co-infected monkeys or mono-infected monkeys (77).

### *MMPs in TB-HIV co-infection*

Whilst the principal focus of the TB-HIV field has been innate and adaptive immune responses, one component driving pulmonary pathology must be proteases that can degrade extracellular matrix, in addition to excessive cytokine and cytotoxic responses causing excessive cellular infiltration and necrosis (184). Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases, including collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9) and elastases (MMP-12). MMPs are important in a diverse range of normal immune

functions and are immunomodulatory (185, 186). They activate and are activated by a range of inflammatory cytokines and chemokines, including IL-1β and TNF-α, and can affect leukocyte migration (187-190). The catalytic activity of MMPs is tightly regulated at the level of gene expression, by compartmentalization, by the requirement for proenzyme activation and by enzyme inactivation. They are produced by a range of innate immune cells, epithelial cells and fibroblasts and are upregulated in response to TB infection, without compensatory upregulation of the endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) (191, 192). Together, MMPs are capable of degrading all components of the extracellular matrix.

MMPs are implicated in TB immunopathology due to their unique ability to degrade collagen fibrils at neutral pH, and therefore are likely to play a central role in TB-driven matrix destruction. This has been demonstrated in animal and human studies of M.tb. virulence, correlating with severity of disease in central nervous system (CNS) TB, severity of pulmonary inflammatory pathology and the presence of cavitation in pulmonary TB (184, 193-195). In a zebrafish model of TB employing *Mycobacterium marinum*, ESAT-6 dependent production of MMP-9 was shown to promote macrophage recruitment to granulomas, which facilitated bacterial dissemination and increased virulence (42). This provides a mechanism by which mycobacterial dissemination from the lung bases, following inhalation, to the lung apices, may occur.

Mice do not express an ortholog of human MMP-1 and do not develop caseous necrosis or cavitation in response to M.tb. infection, and therefore are not an

ideal model for TB tissue destruction. However, we have demonstrated that transgenic mice that expressed MMP-1 in activated macrophages had increased pulmonary tissue destruction in response to M.tb. infection, in comparison to wild type mice, directly implicating MMP-1 in TB immunopathology (193). Additionally, in M.tb.-infected MMP-1 transgenic mice there was evidence of caseation in the centre of granulomas (196). A role for MMPs in cavitary TB has been supported by studies of *M. bovis* in rabbits and M.tb*.* in non-human primates, both models of destructive pulmonary pathology with greater similarity to human pulmonary disease (197-200). Recent data from 3-dimensional culture models of pulmonary architecture suggest that collagen regulates M.tb. survival (196). Taken together these data imply that unopposed MMP activity effects tissue destruction in TB as an early process in TB immunopathology driving the formation of caseous necrosis, rather than as a late consequence of inflammatory processes.

Extracellular matrix turnover releases matrix degradation products (MDPs) into the circulation. This is well described for a number of pulmonary conditions, but surprisingly has been relatively overlooked on TB (201-203). In a previous study, we analysed MDPs in patients with TB and demonstrated that they were elevated both in induced sputum and plasma (204). In addition, sputum MMP-1 correlated with plasma procollagen III N-terminal propeptide (PIIINP), released from type III collagen turnover, demonstrating that it can be used as a peripheral marker of pulmonary pathology, driven by excessive protease activity. However, these MDPs have not been studied longitudinally in TB, nor in TB-IRIS.

MMP dysregulation may provide one mechanism for divergent pathology in TB-HIV co-infection. HIV infection is known to modulate MMP production in cellular models and in some HIV-associated neurological conditions (205-207). In a cellular study of human monocyte-derived macrophages, we have demonstrated that TB-HIV co-infection was associated with increased MMP-1 secretion (61). In a clinical study, I have shown that elevated concentrations of MMP-1 and -2 in induced sputum samples from TB patients correlated with the severity of tissue destruction, mycobacterial load in sputum, and the presence of cavitation in a mixed HIV-infected and uninfected population (195). Reduced levels of MMP -1, -2, -8 and -9 were found in induced sputum of TB patients with advanced HIV, compared to HIV-uninfected TB patients, suggesting that modulation of TB-driven MMP activity by HIV infection may explain the reduced focal inflammatory pathology and cavitary lesions seen in advanced HIV. Furthermore, the interaction between matrix turnover and HIV is implied by the observation that elevated hyaluronic acid, which is released during matrix turnover, before the initiation of ART was associated with higher risk of AIDS events, IRIS and death within twelve months following ART initiation (208).

In the next section I address the effects of ART on the immune response to TB. As ART is now recommended for all HIV-infected patients with TB and as ART coverage improves globally, this becomes an increasingly important area for consideration.

## The effect of ART on the immune response to TB

ART has dramatically reduced HIV-associated mortality, which decreased from a peak of 2.3 million per year in 2005 to 1.6 million in 2012 (209-211). This reflects improvement in access to ART in the last decade, especially for HIV-infected patients in low and middle income countries, where the number of patients receiving ART has increased more than 30-fold (from 300 000 in 2002 to 9.7 million in 2012) and life expectancy is increasing (211, 212). It is now recognized that commencement of ART earlier in HIV infection improves outcomes, and international guidelines have been updated to reflect this (213). TB is now considered to be an indication for ART irrespective of CD4 count, as ART reduces mortality in TB patients (214).

ART suppresses HIV-1 viral replication, increases CD4 T cell numbers and in some studies has been shown to reduce CD8 T cell activation (215-217). The past decade has seen great advances in ART coverage amongst HIV-infected individuals worldwide. Clear mortality and morbidity benefits have been demonstrated by starting ART following a TB diagnosis. In patients with CD4 counts less than 50 cells/$mm^3$, there is a reduction in mortality and progression to AIDS if ART is started at 2 weeks of TB treatment compared with later timepoints (218, 219). A new diagnosis of TB is now an indication for ART initiation (218-220). However, in TB endemic settings, despite ART-mediated CD4 T cell reconstitution and HIV viral suppression, an increased risk of active TB remains (3). How does ART affect the immune response to TB and what is lacking?

Several studies have suggested that early reconstitution of CD4 T cell numbers is comprised largely of memory cells rather than naïve cells, suggesting redistribution from lymphoid sites (89, 215, 217). This is followed by reconstitution of the naïve T cell population. A study of T cell subsets in ART-experienced patients in Denmark demonstrated persistent deficits in absolute CD4 T cell numbers, and subsets (particularly naïve CD4 T cells) compared to healthy blood donors, despite up to 14 years of ART with HIV viral suppression (94). The same study demonstrated differences in the CD8 T cell maturation pathway, with increased frequency and proportion of intermediately differentiated ($CD45RA^-$, $CD27^+$, $CD28^-$, $CCR7^-$) CD8 T cells and increased frequency and proportion of activated CD8 T cells ($CD38^+$, $HLA\text{-}DR^+$) in ART-treated patients compared to HIV–uninfected controls (94).

ART improves antigen-specific CD4 T cell responses to a number of antigens including PPD, compared to pre-ART responses (216, 221). However, despite total CD4 T cell reconstitution and suppression of HIV-1 viral replication, ART-treated patients have significantly depleted M.tb.-stimulated IFN-γ responses and frequency of PPD-specific IFN-γ-secreting CD4 T cells, compared to HIV-uninfected individuals (222, 223). Wilkinson *et al* studied CD4 T cell reconstitution in detail over the first 48 weeks of ART (89). In this study, central memory CD4 T cells expanded significantly by 12 weeks. By 36 weeks of ART, naïve T cells had also expanded. However, the frequency of terminally differentiated CD4 T cells, including PPD-responsive cells, was less dynamic, and by 12 weeks represented a significantly reduced proportion of total CD4 T cells

that was sustained for 48 weeks (although absolute numbers did increase). At week 48, the proportion of $CD4^+$ T cells producing IFN-γ was lower than in healthy HIV-uninfected controls (89).

A study of ART treated patients with a previous history of treated TB found profoundly reduced ESAT-6 and Ag85B responses in these patients, but strong non-specific mycobacterial (PPD) CD4 T cell IFN-γ and proliferative responses, when compared to HIV-uninfected patients with previous TB (224). This suggests that repeated exposure to environmental, non-pathogenic mycobacteria boosts the non-specific mycobacterial immune response, without causing infection. However, in the context of repeat exposure to the more virulent M.tb., HIV-infected patients succumb to TB disease, rather than develop immunity.

The effect of ART on cells of the innate immune system, cytotoxic T cells and regulatory cells is less well defined than on CD4-mediated acquired immunity (see Table 1-1) (63, 112, 225-227). Despite successful ART, tissue macrophages contain a reservoir of HIV-1 virus that is considered important in failure of viral eradication by ART and in the pathogenesis of HIV-related neurological conditions that may manifest despite ART (226, 228). The impact of this reservoir on TB-immunity in ART-treated patients is not clear.

## Table 1-1 The effect of ART on immune cell function

| Immune Cell | Response in TB | Effect of HIV | Effect of ART |
|---|---|---|---|
| **Macrophage** | Phagocytose mycobacteria. Produce IFN- γ & TNF-α. Secrete MMPs.<br><br>Key role in granuloma formation: epitheliod cell/ Langhans giant cell formation.<br><br>Failure of apoptosis linked to intracellular mycobacterial survival. | Increased cellular activation.<br><br>Disruption of autophagy.<br><br>**In TB-HIV co-infection**:<br><br>Reduced IL-10 dependent macrophage apoptosis & reduced TNF–α production.<br><br>Increased *M.tb.* replication (not evident in all studies).<br><br>Increased HIV-1 replication. | Intracellular persistence of HIV-1 in macrophages despite ART.<br><br>Activation markers reduced by ART but not to normal.<br><br>Effect of ART on role of macrophages in *M. tb.* immunity is not well defined. |
| **Neutrophil** | Phagocytose mycobacteria. Possible role in *M. tb.* killing via neutrophil peptides (HNP 1-3, cathelicidin LL-37 & lipocalin 2). | Impaired phagocytic ability & chemotaxis.<br><br>Increased apoptosis. | Improved chemotaxis, but impairment of phagocytosis persists despite ART.<br><br>Protease inhibitors reduce neutrophil apoptosis. |
| **NK cell** | Lysis of *M.tb.* infected cells in vitro. | Reduced functional NK cell populations. Reduced activation, more inhibitory receptor expression. | Reconstitution with functional deficits. Increased activation and degranulation associated with TB-IRIS. |
| **CD4 T cell** | Recruited to disease site in response to cytokine & chemokine signals from infected antigen presenting cells; recognize peptide antigens bound to MHC Class II molecules<br><br>Role in activation of phagocytic & cytotoxic cells: produce Th1 cytokines e.g. IFN- γ which are critical to immune response but may also lead to immunopathology. | Infected & depleted: transiently by acute HIV infection; progressively and irreversibly in chronic HIV infection<br><br>CCR5 expressing, IL-2 producing *M. tb.*-specific CD4 T cells are particularly susceptible to HIV infection & depletion.<br><br>**In TB-HIV co-infection**:<br><br>Reduced at disease sites in TB-HIV co-infection.<br><br>Functionally impaired: reduced IFN-γ response to mycobacterial antigens shown in some studies.<br><br>Reduced IL-21 production may influence CD8 T cell function. | Early reconstitution of memory T cells (possibly from lymphoid sites); later restoration of naïve cells.<br><br>Persistent deficit in CD4 T cell subsets despite long term ART.<br><br>Improvement in frequency and function of *M.tb.*-specific CD4 T cells, although reduced in comparison to HIV-uninfected populations. |
| **CD8 T cell** | Lyse *M. tb.* infected cells via release of granular contents e.g. granulysin, and restrict mycobacterial growth. | Increased cellular activation.<br><br>Defective (granulysin mediated and IL-21 dependent) cytotoxic responses. | Reduced cellular activation in some studies.<br><br>Other studies suggest persistent cellular activation & disruption of cellular maturation pathway. |
| **Regulatory T cell (T reg)** | Expanded populations at TB disease sites; produce IL-10. | Reduced in total number by HIV but increased in proportion.<br><br>**In TB-HIV co-infection**:<br><br>Compartmentalisation evident in pleural fluid in HIV-associated pleural TB. | Normalisation of Treg numbers & proportions with CD4 T cell reconstitution.<br><br>Reduced IL-10 production in one study of *M. avium IRIS* which may indicate a defect in regulatory responses to mycobacteria. Other studies suggest no role in TB-IRIS. |
| **NKT cell** | Can restrict TB growth *in vitro.* Reduced frequency in active TB infection with functional impairment. | Activated, productively infected & depleted. | Reconstituted rapidly. |

In the next section, I discuss inflammatory pathology in TB-HIV co-infection caused by TB-IRIS, an important early complication of ART. This is adapted from a recently published work (Walker *et al* **HIV-associated immune reconstitution inflammatory syndrome**. *HIV/AIDS Research and Palliative Care*, 2015) (229).

## What is IRIS?

HIV-associated IRIS has emerged as an important early complication of ART initiation, associated with considerable morbidity and mortality, particularly in patients who commence ART with advanced immunosuppression (230). In this condition, immune recovery following ART initiation associates with a pathological inflammatory response, usually directed towards microbial antigens. Although there is considerable clinical and pathophysiological heterogeneity, key features include clinical deterioration in the first weeks to months of ART, with evidence of localized tissue inflammation with or without a systemic inflammatory response.

### *Historical perspective and case definitions*

Amongst the first accounts of IRIS-type phenomena were reports of zidovudine-induced fever associated with lymphadenitis in patients with non-tuberculous mycobacterial infections (231, 232). French *et al* reported a case series of unusually localized *Mycobacterium avium intracelullare* (MAI) infections presenting with fevers and lymphadenitis without mycobacteraemia, which developed soon after commencement of zidovudine monotherapy (233). These presentations were accompanied by the emergence of delayed-type

hypersensitivity reactivity to PPD in patients who had previously been PPD unresponsive.

IRIS has emerged as a highly heterogeneous condition, with features differing according to the associated pathogen. However, two distinct temporal patterns of disease were commonly described and are now recognized as "paradoxical IRIS" and "unmasking IRIS" (see Figure 1-2) (234-236). In paradoxical forms of IRIS, symptoms and signs associated with a known opportunistic infection (OI), for which treatment is underway, recur or become acutely worse, despite an earlier favourable response to therapy prior to ART. In unmasking IRIS, a new OI presents with a pronounced inflammatory component following ART initiation. Responding to clinical and research needs, the International Network for the Study of HIV-associated IRIS (INSHI) published consensus case definitions for TB-IRIS and Cryptococcal IRIS (235, 236). Such definitions are suitable for use in low resource settings, as CD4 count and HIV viral load responses are not included as criteria. Consensus case definitions for other forms of IRIS are lacking, although much needed (237). The main challenge with all proposed definitions of paradoxical IRIS is the requirement to adequately exclude other causes of clinical deterioration. There is no definitive diagnostic test for IRIS. In resource-constrained settings, clinicians may find themselves treating multiple conditions concurrently in a sick patient, being uncertain of the definitive diagnosis due to limited laboratory support, with IRIS as a diagnosis of exclusion following unsuccessful treatment of other conditions.

The concept of unmasking IRIS is less well defined than paradoxical IRIS. The broader term of "ART-associated OI" has been proposed to encompass all OI diagnosed during early ART, as unmasking IRIS may be difficult to differentiate from development of an OI in a patient who is still immunocompromised during early ART and which progresses along a typical clinical course (see Figure 1-2).

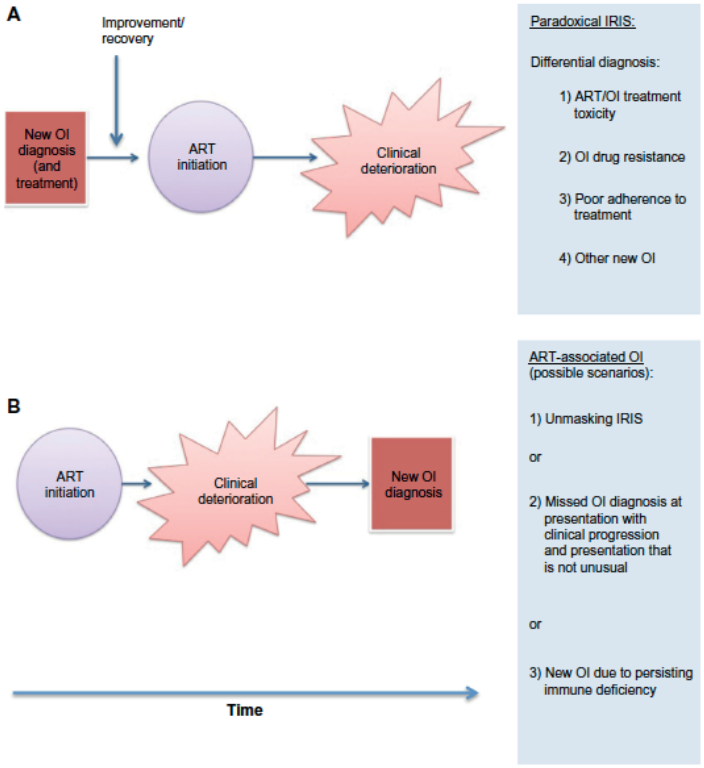



**Figure 1-2 Paradoxical and unmasking IRIS**

Schematic demonstrating the sequence of key events in paradoxical IRIS (A), and unmasking IRIS (B), which may complicate ART initiation in HIV-associated TB and other opportunistic infections (OI). Paradoxical IRIS occurs after the diagnosis of an OI has been made. In paradoxical IRIS, patients who had been recovering from an OI on treatment present with an acute deterioration, following ART initiation. Unmasking IRIS is one possible presentation of an ART-associated OI, and is characterized by an atypically inflammatory or localized presentation, unlike other forms of ART-associated OI.

### *Epidemiology and Risk Factors*

The epidemiology of IRIS reflects the epidemiological distribution of HIV-associated OI and the prevalence of various key risk factors in a given population. Table 1-2 reports the proportion of IRIS attributable to different OI/inflammatory conditions in recently published studies of unselected patients commencing ART, demonstrating that reported incidence varies according to geographic region and by study design (238-243). Risk factors for IRIS include an advanced state of immunosuppression (low CD4 count) and high infective antigen burden/ disseminated OI at ART initiation (244, 245). These characterize a substantial proportion of patients with newly diagnosed HIV in developing countries where suboptimal access to HIV care and health services in general, and stigma associated with HIV, combine to contribute to late presentation (246). Whilst advanced immunosuppression and late presentation are also encountered in patients presenting to ART services in higher resource settings, in low resource settings IRIS incidence and associated mortality appear to be higher (247, 248).

**Table 1-2 Incidence of IRIS in HIV-associated opportunistic infections in unselected patients**

| Population/ Reference | Overall incidence, % | IRIS associated condition/ opportunistic infection, proportion of total IRIS cases (%) |
|---|---|---|
| US[a] n=196 | 11 (Reported on paradoxical IRIS only) | KS, 57<br>TB, 19<br>PCP, 14<br>Disseminated cryptococcus, 5<br>MAC, 5 |
| US[b] n=2610 | 10.6 (Reported on unmasking IRIS only) | Candidiasis, 23<br>Folliculitis, 13.8<br>HSV, 12.4<br>Warts, 12.2<br>Tinea, 11.1 |
| Mexico n=390 | 27 | VZV, 32<br>TB, 11<br>MAC, 9<br>PCP, 6<br>*Cryptococcus neoformans*, 5 |
| India n=97 | 35 | Mucocutaneous (combined), 35.2<br>TB, 29.4<br>PCP, 17.6<br>CMV retinitis 5.9<br>CM 5.9<br>MAC 5.9 |
| Mozambique n=136 | 26.5 | Tinea, 25<br>KS, 22.2<br>TB, 16.7<br>PCP/pneumonitis 8.3<br>HSV, 8.3 |
| South Africa n=498 | 22.9 | Folliculitis, 27.3<br>TB, 24.4<br>HSV, 9.4<br>VZV, 6.5<br>Warts, 7.2 |

§Abbreviations:
CM – cryptococcal meningitis
CMV – Cytomegalovirus
HSV – Herpes simplex virus
KS – Kaposi's sarcoma
MAI – *Mycobacterium avium intracellulare*
PCP – *Pneumocystis jirovecii* pneumonia
TB – tuberculosis
VZV – Varicella zoster virus

References
USA[a] [238], USA[b] (239), Mexico (240), India (241), Mozambique (242), South Africa (243)

IRIS has been described following reversal of other forms of immunosuppression, including after reversal of iatrogenic immunosuppression in transplant recipients, following bone marrow recovery after chemotherapy for hematological malignancies, and following discontinuation of anti-TNF-α therapy for rheumatoid arthritis or treatment with the monoclonal antibody natalizumab for multiple sclerosis (249-252). In the subsequent sections, discussion is focused on TB-IRIS with reference to other forms of HIV-associated IRIS where relevant.

### *TB-IRIS*

TB-IRIS is amongst the commonest forms of IRIS given the global distribution of TB infection (see Table 1-2). Paradoxical TB-IRIS was reported to occur in 15.7% (95% credibility interval 9.7-24.5%) of TB patients starting ART in a meta-analysis reporting on 16 studies of TB-IRIS, although higher rates are reported in some settings (28, 230). For example, in a more recent Indian study an incidence of 54.2% was reported in patients with culture-confirmed pulmonary TB and a South African study reported 47% incidence of paradoxical TB-IRIS in patients with TB meningitis (253, 254). In South Africa, where 65% of TB patients are HIV co-infected this translates into a considerable disease burden (255). Mortality rates between 0-38% have been reported (28, 254, 256-258).

Both paradoxical and unmasking forms of TB-IRIS are now widely reported, although paradoxical IRIS has been more extensively studied (28, 259, 260). Paradoxical TB-IRIS typically presents in pulmonary TB cases as a recurrence or

worsening of respiratory symptoms (cough or shortness of breath), associated with a recurrence or worsening of constitutional symptoms (weight loss, night sweats, fever), and not uncommonly new or expanding infiltrates on chest radiograph (261). Paradoxical IRIS may occur at any disease site, including lymph nodes, which typically manifests as rapid enlargement followed by suppuration (262). CNS TB-IRIS typically presents with new or worsening meningitis and/or features of raised intracranial pressure, due to enlarging cerebral tuberculomas, with a high mortality (256, 257, 263). It may also present with epidural abscesses, spondylitis and radiculomyelopathy (254, 256). Hepatosplenic (typically granulomatous disease leading to hepatitis and/or appearance of microabscesses), abdominal (e.g. lymphadenopathy, peritonitis) and musculoskeletal (e.g. mono- or polyarthritis) features are not infrequent (28). Extrapulmonary TB-IRIS manifestations may occur in patients who originally presented with pulmonary TB, and vice versa. Accompanying laboratory features usually include a raised C-reactive protein, and may include worsening anaemia at the time of TB-IRIS onset.

The differential diagnosis of TB-IRIS includes other new infections (e.g. pneumonia, influenza), unmasking of other OI (e.g. PCP), drug reactions (e.g. pyrazinamide arthropathy) and multi-drug resistant TB (MDR-TB) (264). In resource-limited settings, where M.tb. drug-susceptibility testing is not routinely available, the latter is difficult to exclude and critical in patient management. Additionally, TB-IRIS may occur in cases of drug-resistant TB (264). There is no laboratory test for TB-IRIS and exclusion of differential diagnoses with certainty can be very challenging in practice.

The only randomized placebo-controlled trial of treatment of IRIS was conducted in patients with paradoxical TB-IRIS in South Africa (265). 110 patients were enrolled with a median CD4 count of 116 cells/$mm^3$ and paradoxical TB-IRIS diagnosed according to INSHI criteria, limited to those with increasing infiltrates on chest radiograph, enlarging lymph nodes, serous effusion or cold abscess. Patients with immediately life threatening manifestations of TB-IRIS (respiratory failure, altered level of consciousness, new focal neurological sign/s or compression of a vital structure) were excluded from the study. The intervention arm (n=55) were treated with prednisone 1.5mg/kg/day for 14 days, reduced to 0.75mg/kg/day for a further 14 days. This lead to a reduction in the composite primary endpoint of days of hospitalization and outpatient therapeutic procedures (median per patient 0 vs 3 in placebo arm, p=0.04). There was also more rapid improvement in symptom scores and chest radiographs in prednisone, compared to placebo-treated patients. There were more mild infections (e.g. oral candidiasis) in the prednisone treated arm, but no excess of severe infections. There were no significant differences in possible drug-related side effects reported in each arm. These data support use of prednisone in TB-IRIS, for moderate and severe cases. Unfortunately, TB-IRIS symptoms may recur following steroid withdrawal, requiring longer courses of treatment.(265, 266) Other immunomodulatory therapies have been considered in management of TB-IRIS, with case reports of favourable outcomes with thalidomide and montelukast, but none has been tested in randomized-controlled trials (RCT) (245, 262, 267, 268).

Strategies for TB-IRIS prevention have focused on optimizing the timing of ART initiation, after it was observed that a shorter time between TB treatment initiation and ART initiation increased TB-IRIS risk. Three RCT that have studied the optimal timing of ART in TB patients have informed practice (219, 220, 269). These trials demonstrated that in patients with a CD4 count <50 cells/mm$^3$, starting ART around 2 weeks after TB treatment reduced mortality or a combined endpoint of mortality and AIDS progression. Thus, whilst commencing ART earlier is associated with increased IRIS risk, the survival benefit in these patients with low CD4 counts overrides this. However, in patients with CD4 >50 cells/mm$^3$ ART can be commenced between 2 and 8 weeks post TB treatment, and delaying to 8 weeks is not associated with excess mortality, but may reduce IRIS risk. A recent placebo-controlled study of ART timing in smear-positive pulmonary TB patients with CD4 counts >220 cells/mm$^3$, demonstrated no benefit in a combined end-point of TB treatment failure, TB recurrence and mortality, when ART was commenced at two-weeks post-TB treatment initiation, compared to delaying until after TB treatment was completed. IRIS rates were similar in the early and late arms (10%) (270). A single RCT of ART timing in TBM demonstrated no survival benefit from starting ART within 7 days of commencing TB therapy, compared to following 2 months TB treatment, and an increased probability of serious adverse events (271). As a result, it is recommended that ART initiation be delayed until 8 weeks after TB therapy is commenced in TBM patients (272).

There have been no successful trials of preventive strategies for paradoxical TB-IRIS, although two randomized placebo-controlled trials for paradoxical TB-IRIS

prevention are currently underway in South Africa. A randomized placebo-controlled trial of prednisone for TB-IRIS prevention in high-risk patients is recruiting patients in Cape Town (http://www.clinicaltrials.gov/ct2/show/NCT01924286?term=meintjes&rank=1). The TB-IRIS NSAID Cox-2 Inhibitor Prevention Trial is investigating meloxicam for TB-IRIS prevention (http://www.clinicaltrials.gov/ct2/show/NCT02060006?term=Meloxicam+tuberculosis&rank=1). The results of these studies are awaited.

### Pathophysiology of TB-IRIS

In TB-IRIS, at the time of IRIS onset, elevated concentrations of pro-inflammatory mediators, including C-reactive protein and cytokines (e.g. interleukin (IL)-6, IL-12 p40/p70 , TNF-α) are detectable in serum and may also be elevated in cerebrospinal fluid (CSF) in CM-IRIS and TBM IRIS (273-277). A pro-inflammatory cytokine cascade may be a final common pathway by which IRIS inflammation occurs (273, 278).

The increased incidence of IRIS in patients with lower pre-ART CD4 counts and disseminated OI suggests that more advanced immunodeficiency prior to ART initiation may lead to a higher pathogen load, resulting in excessive inflammation, once the immune system starts to recover. In TB meningitis (TBM)-IRIS, CSF culture positivity for M.tb. at TBM diagnosis confers a 9-fold greater risk of IRIS, compared to those with culture negative TBM, also suggesting that antigen load at OI diagnosis is important (254). However in TBM, prior to ART initiation, higher TNF-α levels and raised CSF neutrophil counts

were observed in IRIS patients compared to those that did not develop IRIS, suggesting a pro- rather than anti-inflammatory milieu precedes TBM IRIS onset (254). Several other studies have reported elevated pro-inflammatory cytokine and chemokine concentrations in peripheral blood of TB-IRIS patients prior to ART initiation, including IL-6 and TNF-α (253) (208, 279). However, reports are not consistent (258, 280). In a recently reported study of TB patients initiating ART in Botswana, reduced pro-inflammatory mediators at baseline were associated with TB-IRIS, including GM-CSF, IL3, IL6, IL-12p40, IL-12p70 and IL-17 (258). Significantly greater increases in Granulocyte colony-stimulating factor (G-CSF), IL-6, IL-8 and TNFα were observed between ART initiation and week 4 of ART in patients who developed TB-IRIS, than those who did not. Increased pro-inflammatory markers were associated with death in the study cohort, but the majority of these deaths were not attributed to TB-IRIS (258).

Studies to date suggest that the adverse inflammatory response in IRIS is contributed to by pathogen-specific acquired immune responses, by restoration of innate immune pathways and perhaps by defective or delayed regulatory responses. As ART initiation leads to a rapid increase in peripheral blood CD4 T lymphocyte count in most patients, the recovery of pathogen-specific cell-mediated immune responses has been studied in TB and other OI in HIV-infected patients following ART initiation. In HIV-infected patients, increased CD4 Th1 responses to mycobacterial antigens have been reported following ART initiation (233, 281-284). Studies indicate these increased responses associate with IRIS (233, 281, 282, 284). However, a detailed longitudinal study of CD4 T cell responses to a range of M.tb. recombinant antigens found that highly dynamic

IFN-γ responses occurred in both TB-IRIS patients and patients who did not develop IRIS and did not clearly differentiate the two groups (283). This finding has been supported by the results of two further studies of Th1 responses to mycobacterial antigens in TB-IRIS, which also call into question a causal link (285, 286).

Whilst a disturbance of regulatory T cell number or function could explain excessive inflammation, this has not been convincingly demonstrated. A few studies have demonstrated an increased rather than decreased number of regulatory CD4 T cells in mycobacterial and C-IRIS (283, 287, 288). Reduced IL-10 has been associated with IRIS in some studies, suggesting that regulatory function may be impaired (287, 289). However, a recent comparison of 20 TB-IRIS patients and 20 non-IRIS control patients found increased IL-10 concentrations in serum of TB-IRIS patients, and increased IL-10 transcript in PBMC of TB-IRIS patients compared to controls after restimulation with M.tb. (290). One study demonstrated reduced numbers of inhibitory NK receptors on mycobacteria-specific Vδ2 TCRγδ T cells (282). Therefore, further studies of regulatory cell types and function in IRIS are required.

Barber *et al* (studying a murine model of MAI-IRIS) argue that because IRIS is not specific to CD4 T cell depletion in HIV (and occurs following reversal of HIV-unrelated immunosuppression, e.g. post-TNF-α treatment), it is unlikely that CD4 T cell responses are the central contributory factor (291). Rather they propose that an uncoupling of innate and adaptive immunity is responsible. They hypothesize that in HIV infection, CD4 deficiency and thus deficiency of CD4 co-

stimulation impairs full activation of innate immune cells, particularly macrophages in TB and MAI infection. The resultant antigen accumulation and excessive priming of innate immune cells leads to an excessive inflammatory response, once activation does occur following immune restoration (291).

Given their importance in antigen processing and pathogen trafficking, cells of the innate immune system such as monocytes, macrophages and neutrophils are of increasing interest in IRIS pathophysiology. Favouring a role for innate immunity is the formation of organized tissue granulomas in IRIS (such as granulomatous hepatitis in TB-IRIS). Granulomatous inflammation, which is notably absent in untreated advanced HIV, suggests enhanced macrophage activity during immune reconstitution (29). A fatal case of unmasking TB-IRIS associated with a pronounced macrophage-dominated pulmonary infiltrate on post-mortem has been reported (292). Suppurative inflammation may develop in IRIS, which suggests heightened neutrophil activity (289, 293). Several studies have reported increased inflammatory cytokines and chemokines of myeloid origin present at and prior to IRIS development, evident in serum and on re-stimulation of cells *ex vivo* with antigen (208, 273, 294).

### *MMPs in TB-IRIS*

In a previous cross-sectional study of PBMC responses, we reported elevated MMP transcripts in TB-IRIS patients compared to controls, accompanied by elevated serum concentrations of MMP-7 (295). In this study, PBMC from 22 TB-IRIS patients and 22 non-IRIS controls, collected at two weeks post-ART initiation, were stimulated with heat-killed H37Rv M.tb. and gene transcripts

measured by reverse transcription-polymerase chain reaction (RT-PCR). M.tb. stimulation increased transcripts of MMP-1, -3, -7 and -10 in both TB-IRIS PBMC and non-IRIS controls compared to unstimulated cultures. After 24 hours, transcripts for MMP-1, -7 and -10 were increased in TB-IRIS compared to non-IRIS controls. MMP-1, -3, -7 and -10 were also found to be higher in the culture supernatants of TB-IRIS patients compared to controls. Only MMP-7 was significantly higher in serum from TB-IRIS patients compared to controls. In a second patient cohort, we assessed the effect of corticosteroid therapy on gene expression and serum levels of MMPs in TB-IRIS patients compared to controls. Corticosteroid therapy was associated with reduced MMP-7 gene transcript and circulating concentration in serum (295).

### *Cytotoxic T cells in TB-IRIS*

Two studies have examined NK cell function in TB-IRIS. In a longitudinal study, NK cells isolated from paradoxical TB-IRIS patients had higher expression of CD107a, a degranulation marker, than non-IRIS controls, prior to IRIS onset and in unmasking IRIS, these cells were found to express increased activation markers (123). The authors hypothesized that increased NK cell-mediated lysis of M.tb-infected cells may increase antigen load.

In a recent study, we identified the importance of cytotoxic cells in TB-IRIS, implicating iNKT cells in pathophysiology (296). In this study, PBMC from 7 TB-IRIS patients and 7 non-IRIS controls, collected at 2 weeks post-ART initiation, were stimulated with heat-killed H37Rv M.tb. and gene expression evaluated by microarray analysis. The most significantly upregulated gene in TB-IRIS patients

compared to non-IRIS controls was perforin 1. Perforin is a cytolytic protein found in granules of innate lymphocytes and neutrophils that has a key role in delivery of granule contents to the target cell cytoplasm. Granzyme B was amongst the top five most upregulated genes. Granzyme B, a serine protease, is a key component of cytotoxic granules and activates apoptosis once in the cytoplasm of target cells. Granzyme B and perforin transcript abundance in TB-IRIS PBMC was confirmed by RT-PCR, ELISA (enzyme-linked immunosorbent assay) and ELISpot. Investigation of the cellular source of increased perforin revealed that both CD4 and CD8 cells from TB-IRIS patients collected at two weeks post-ART initiation (the most common time of IRIS presentation) contained perforin and that this decreased on stimulation with heat-killed H37Rv M.tb. implying antigen-specific degranulation. We found no difference in the proportion of NK and γδ T cells in these patients. However, we did find an increased number of CD3+Vα24+ cells in TB-IRIS patients, likely to be invariant NKT cells, containing perforin on intracellular staining. This was the first study to investigate this cell population in TB-IRIS patients.

In summary, recent evidence suggests that innate immune dysfunction in the context of high antigen load plays a role in driving pathological pro-inflammatory responses in TB-IRIS. Studies of patients are frequently limited by small sample size and examination of peripheral blood rather than tissue immune responses, and have largely investigated cell-mediated immunity (297). Innate mediators of tissue destruction and inflammation, the MMPs and iNKT cells, have not been thoroughly examined. Improved understanding of TB-IRIS

pathophysiology is key to development of diagnostic tests and specific treatment strategies, which are currently lacking.

## Study hypotheses

Based on the evidence outlined above and our preliminary studies, this work aims to define mechanisms of tissue destruction in TB-HIV co-infection and TB-IRIS by addressing the following hypotheses:

1) HIV infection modulates TB-driven MMP activity to cause divergent clinical presentation of TB infection.

2) Circulating iNKT cell populations are reduced more profoundly in TB-HIV co-infection than TB infection or HIV infection alone, but are associated with TB-related inflammatory pathology in TB-IRIS.

3) In TB-HIV co-infected patients, the development of inflammatory pathology during TB-IRIS is associated with increased MMP activity and iNKT cell populations.

## Study objectives

To address these hypotheses, The "Tissue destruction in Tuberculosis 2" (TDTB2) study was designed. This study comprised a cross-sectional and a longitudinal component with the following objectives:

### Cross-sectional study

*Primary objectives:*

1) Characterize inflammatory pathology in HIV-infected and HIV-uninfected TB patients and controls using clinical (symptoms, signs and peripheral blood inflammatory markers), microbiological (sputum bacillary burden, smear score and culture positivity) and radiological parameters (chest x-ray inflammation score and cavitation).

2) Compare induced sputum MMP and pro-inflammatory cytokine concentrations in HIV-infected with -uninfected TB patients and control patients.

3) Compare peripheral blood iNKT cell frequency, as a percentage of CD3+ CD19- live lymphocytes, in HIV-infected with -uninfected TB patients and control patients.

**Longitudinal study**

*Primary objective:*

1) Characterize inflammatory pathology in HIV-infected TB patients who develop paradoxical TB-IRIS following ART initiation, in comparison to similar patients who did not develop TB-IRIS during ART initiation (non-IRIS controls).

2) Quantify MMP concentrations (induced sputum and plasma), PIIINP concentrations (plasma) and pro-inflammatory cytokine concentrations (induced sputum and plasma) at the time of developing paradoxical TB-IRIS with non-IRIS controls, in a population at high risk for TB-IRIS (HIV infected with CD4<200 cells/$mm^3$ commencing ART).

3) Quantify iNKT cell frequency as a percentage of CD3+CD19- live lymphocytes, in TB-IRIS patients in comparison to non-IRIS controls, at TB diagnosis, at ART initiation and over the first four weeks of ART.

*Secondary objectives (both studies):*

1) Correlate induced sputum MMP concentrations and iNKT cell frequency with markers of TB-related inflammatory pathology.
2) Characterize iNKT cell populations in terms of CD4 and CD8 expression, activation markers (CD95, CD161, CD40L), degranulation marker expression (CD107a) and cell anergy (PD1 expression).

The overall aim of this work is to describe patterns of differential inflammatory pathology in TB and HIV-associated TB and corresponding immunological phenotypes. With this hypothesis-driven approach, I intend to identify host factors that may improve diagnosis and provide new targets for host-directed therapy in TB and TB-HIV co-infection.

# Chapter 2 Materials and Methods

## Introduction

The TDTB2 study was an observational study comprising two parts: a cross-sectional study, to compare HIV-infected TB patients and HIV-uninfected TB patients with both respiratory symptomatic and healthy controls, and a longitudinal (retrospective cohort) study, comparing HIV-infected TB patients who developed TB-IRIS following ART initiation with HIV-infected TB patients who did not develop TB-IRIS (non-IRIS controls). The study aimed to describe clinical, microbiological and radiographic markers of inflammatory pathology in HIV-infected and HIV-uninfected TB patients, in addition to quantification of MMPs, pro-inflammatory cytokines and iNKT cells, in peripheral blood and at the site of disease (induced sputum).

## Patient recruitment

The study was approved by the University of Cape Town Human Research Ethics Committee (HREC, REF 516/2011). All participants gave informed consent prior to enrolment. Participants were recruited at Ubuntu HIV/TB clinic, Site B Khayelitsha and in the community. Clinic staff and patients were informed about the study by posters, presentations and informal discussions. Study participants were identified from patients routinely attending for assessment and/or treatment at Ubuntu clinic or at Khayelitsha Day Hospital, an outpatient facility on the same site. Additionally, participants volunteered directly from the community following community engagement exercises. Written informed consent was obtained for all participants. Participants were recruited into the

cross-sectional and / or the longitudinal study, based on study specific eligibility criteria, see Figure 2-1 and Figure 2-2. Pregnancy, age less than 18 years, confirmed multi-drug resistant tuberculosis and an inability to provide informed consent were exclusion criteria. All HIV-infected patients were ART naive at enrollment. Cross-sectional study participants who were on antituberculosis therapy were required to have had less than 3 doses prior to study samples being collected.

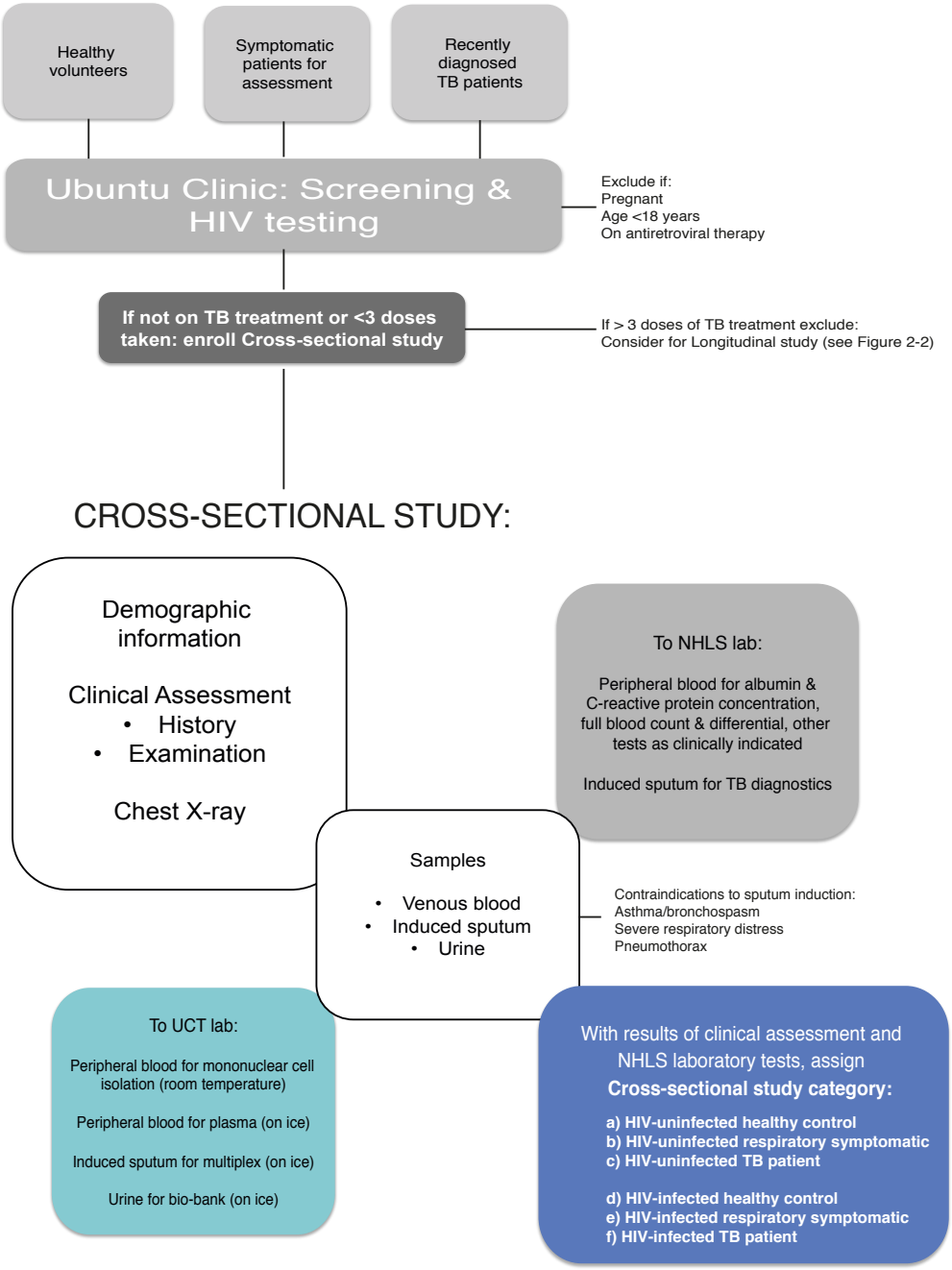



**Figure 2-1 Cross-sectional study enrolment and protocol summary**

Once eligibility was ascertained and informed consent given, history and physical examination were recorded, induced sputum and peripheral blood samples were collected and chest radiograph was performed. Following completion of enrolment and the outcome of diagnostic tests patients were categorized (groups a-f) according to HIV status, presence or absence of active TB and if not a TB patient, presence or absence of respiratory symptoms. UCT=University of Cape Town. NHLS= National Health Laboratory Service.

Cross-sectional study participants were healthy volunteers, patients with symptoms requiring assessment and recently diagnosed TB patients, starting antituberculosis therapy. Active TB was diagnosed on the basis of smear or culture positivity, or in cases of smear-negative TB, according to international guidelines (298). Once enrolled, cross-sectional study participants were provided appropriate follow up by the research team for study clinical results and were then followed up routinely by clinic staff, unless also eligible for the longitudinal study (Figure 2-2). Designation into cross-sectional study clinical categories a-f was made retrospectively, following the results of all relevant investigations (Figure 2-1 and Table 2-1). Cross-sectional study participants who had respiratory symptoms but did not meet the criteria for TB diagnosis after all investigation results were available, were designated respiratory symptomatics. Cross-sectional study participants who met eligibility criteria for the longitudinal study were co-enrolled, or if eligibility became apparent after enrolment, were invited to subsequently enroll into the longitudinal study.

## Table 2-1 Cross-sectional study case definitions

| Designation | Description | Criteria | Symptoms? | HIV status | Group |
|---|---|---|---|---|---|
| **TB patient** | TB | ○ Sputum smear positive for AFB on microscopy *or*<br>○ Gene Xpert positive for Mtb with suggestive clinical presentation *or*<br>○ Sputum culture positive for *Mycobacterium tuberculosis (Mtb)* on culture *or*<br>○ Clinical features at enrolment highly suggestive of TB with diagnostic features on chest x-ray and decision taken to start patient on antituberculosis therapy by treating clinician. | ✓ | + | f |
| | | | ✓ | - | c |
| **Control patient** | Healthy control | ○ All sputum samples obtained (minimum one induced sputum) smear negative for AFB AND if done Gene Xpert negative<br>○ All sputum cultures obtained (minimum one induced sputum) culture negative for *Mtb*<br>○ Low clinical suspicion for active TB after full clinical assessment, including review of chest x-rays where available<br>○ Not on antituberculosis therapy | **x** | + | d |
| | | | **x** | - | a |
| | Respiratory symptomatic | ○ All sputum samples obtained (minimum one induced sputum) were smear negative for AFB AND if done Gene Xpert negative<br>○ All sputum cultures obtained (minimum one induced sputum) were culture negative for *Mtb*<br>○ Low clinical suspicion for active TB after full clinical assessment, including review of chest x-rays where available<br>○ Not on antituberculosis therapy | ✓ | + | e |
| | | | ✓ | - | b |

Longitudinal study participants were ART naïve HIV-infected patients with a low CD4 count (<200 cells/mm$^3$) and a recent diagnosis of TB (Figure 2-2). Following antituberculosis therapy initiation and enrolment into the longitudinal study (study visit TB0) patients received counseling for ART initiation. ART was initiated typically two weeks after antituberculosis therapy (at study visit ARV0) and patients attended further scheduled study visits at two weeks (ARV2), four weeks (ARV4) and twelve weeks (ARV12) post-ART initiation for clinical assessment and sampling. Additionally, patients were requested to attend for assessment if any new symptoms or clinical deterioration occurred (study interim assessment). Clinical research staff telephoned participants regularly to reinforce this.

If a case of TB-IRIS was suspected study samples were collected, as at a scheduled visit, in addition to clinically indicated diagnostic tests. Patients were started on antituberculosis therapy and ART according to national guidelines (299). First-line ART at the time of the study was most often tenofovir, lamivudine, and efavirenz. Retrospective designation into one of three longitudinal study diagnostic categories (paradoxical TB-IRIS (INSHI IRIS), paradoxical TB-IRIS not meeting INSHI criteria (non-INSHI IRIS), and no paradoxical TB-IRIS (non-IRIS)) followed the results of all relevant investigations and clinical follow up, and was made on case review by a consensus panel (comprising the study clinician -NW, and two clinical specialists – GM, RJW). The clinical case definitions are provided in Table 2-2.

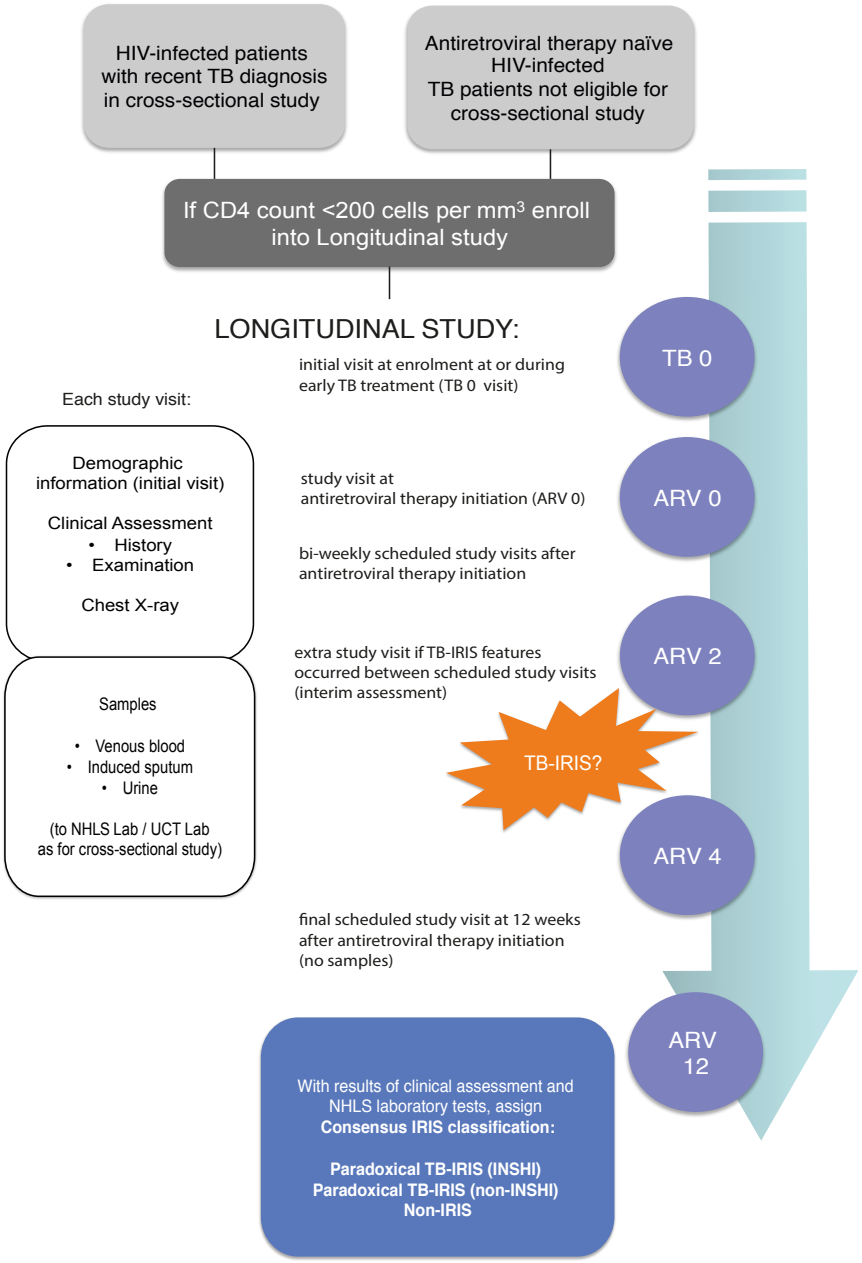



**Figure 2-2 Longitudinal study enrolment and protocol summary**

Patients recruited to the longitudinal study had a recent diagnosis of TB, were ART naïve and had baseline CD4 counts less than 200 cells/mm3. Five scheduled study visits were at TB diagnosis (TB0), ART initiation (ARV0), two weeks (ARV2), four weeks (ARV4) and twelve weeks (ARV12) post-ART initiation. Extra study visits occurred if clinical condition deteriorated suggesting possible TB-IRIS. At every visit, history and physical examination were recorded, induced sputum and peripheral blood samples were collected and chest x-ray was performed. Additional diagnostic tests were performed where clinically indicated.

## Table 2-2 Longitudinal study clinical case definitions

**Paradoxical TB-IRIS (INSHI)**

There are three components to this case definition:
(A) Antecedent requirements must be met;
(B) Clinical criteria must be fulfilled; and
(C) Alternative explanations must be excluded.

**A) Antecedent requirements**

1) **Diagnosis of tuberculosis (TB):** TB diagnosis was made prior to antiretroviral therapy (ART) initiation.
2) **Initial response to TB treatment**: The patient's condition should have stabilised or improved on appropriate TB treatment prior to ART initiation e.g. cessation of night sweats, fevers, cough, weight loss (unless starting ART within 2 weeks of starting TB treatment).

**B) Clinical Criteria**

The onset of TB-IRIS manifestations should be within three months of ART initiation.

At least 1 major criterion or 2 minor clinical criteria are required:

**Major criteria**

1) New or enlarging lymph nodes, cold abscesses or other focal tissue involvement (e.g. TB arthritis)
2) New or worsening radiological features of TB (using chest radiography, abdominal ultrasonography, computerised tomography [CT] or magnetic resonance imaging [MRI]).
3) New or worsening central nervous system TB (meningitis or focal neurological deficit [e.g. due to tuberculoma])
4) New or worsening serositis (pleural effusion, ascites or pericardial effusion)

**Minor criteria**

1) New or worsening constitutional symptoms, such as fever, night sweats or weight loss.
2) New or worsening respiratory symptoms, such as cough, dyspnoea or stridor.
3) New or worsening abdominal pain accompanied by peritonitis or hepatomegaly or splenomegaly or abdominal adenopathy on examination or ultrasound.

**C) Alternative explanations for clinical deterioration must be excluded, if possible:**

1) Failure of TB treatment due to TB drug resistance
2) Poor adherence to TB treatment
3) Another opportunistic infection or neoplasm
   (it is particularly important to exclude an alternative diagnosis in patients with smear negative pulmonary TB and extrapulmonary TB where the initial TB diagnosis has not been microbiologically confirmed).
4) Drug toxicity or reaction

**NB** It may be difficult to confirm or exclude TB drug resistance and exclude certain other infections or neoplasia in all cases with certainty. Paradoxical TB-IRIS diagnosis may be supported by resolution of clinical symptoms and signs of IRIS episode without a change to TB treatment or ART.

**Paradoxical TB-IRIS (non-INSHI)**

These cases met some but not all of the criteria required by the INSHI case definition but appeared to be typical of paradoxical TB-IRIS when reviewed retrospectively by the consensus study panel.

**Non-IRIS**

These cases did not meet the INSHI criteria for paradoxical TB-IRIS and were not considered to be typical of paradoxical TB-IRIS by the consensus study panel.

Adapted from the International network for study of HIV-associated IRIS (INSHI) guidelines (236)

### Clinical Scoring System

A chest radiograph scoring system to quantify the extent of inflammation was used, as previously described (195). Each lung field was divided into 5 areas of equal size and a score of 0-10 was assigned based on the number of areas in which inflammatory pathology was visible. Cavities were recorded as present or absent and counted. The result of sputum microscopy following auramine staining was used to derive a sputum AFB score (0-6). Scores were assigned as follows: 0=no AFB seen; 1=scanty (1-9 per 100 immersion fields) AFB seen; 2=1+ AFB seen (10-99 per 100 immersion fields); 4=2+ AFB seen (1-10 per immersion field); 6=3+ AFB seen (≥10 per immersion field).

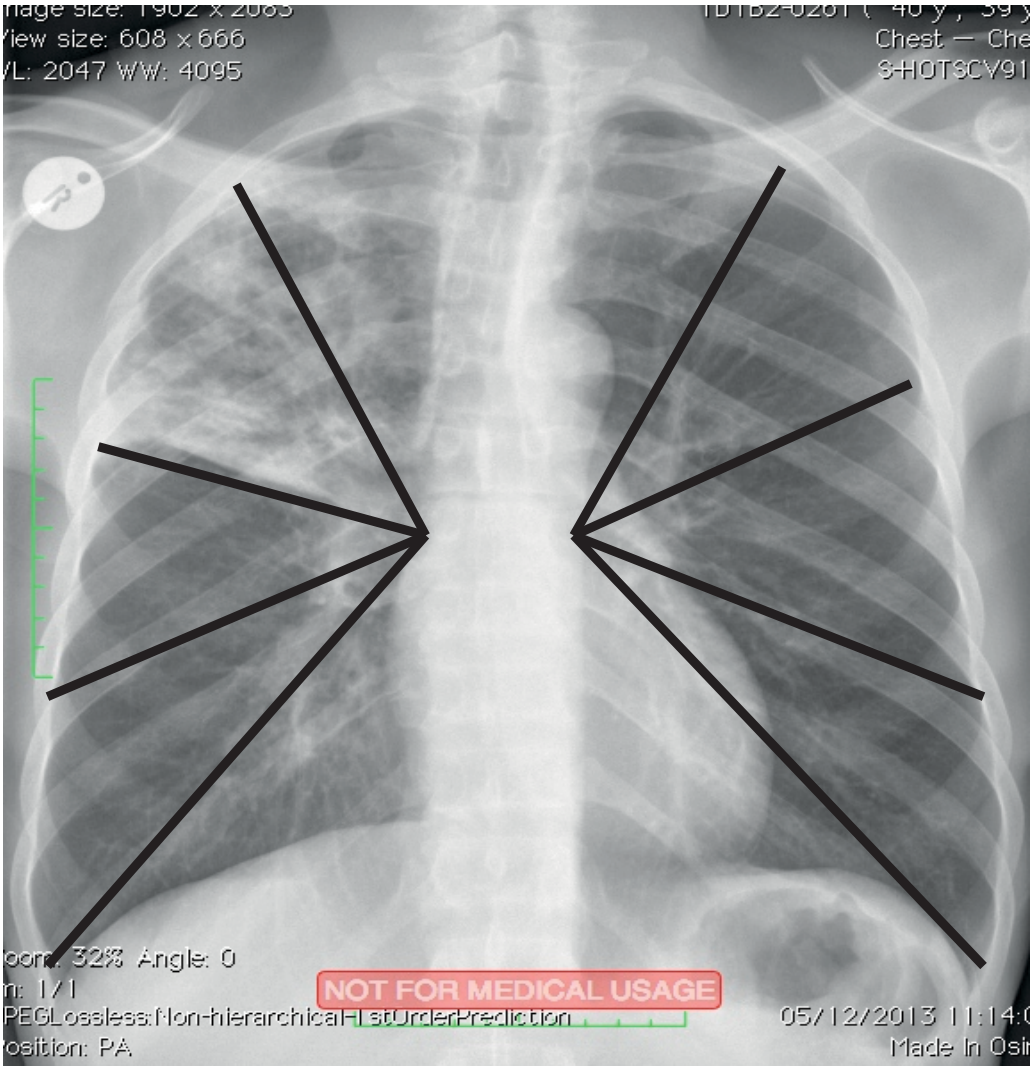



**Figure 2-3 Chest x-ray inflammation score**

Each lung field was divided into 5 areas of equal size and a score of 0-10 was assigned based on the number of areas in which inflammatory pathology was visible. This example obtained a score of 3.

## Sample collection and processing

Sputum induction was performed with 5% saline nebulized in 5-minute cycles, up to 20 minutes as tolerated. Induced sputum was not obtained if patients failed induction, refused induction or were at risk of an adverse event from induction due to respiratory distress, or history of asthma. Sputum was expectorated into two to three sterile collection containers. One or two sputum samples were sent for microbiological examination (smear microscopy and culture) by the National Health Laboratory Service (NHLS). Induced sputum for luminex analysis was transported on ice to University of Cape Town (UCT) and stored at -80$^0$C, within 2 hours of collection. Samples were thawed in batches. Mucolysis was performed by adding 0.1% dithiothreitol and agitating for 20 minutes. Samples were then centrifuged and sterile filtered through a 0.2-mm Durapore membrane (Millipore, Watford, UK) prior to aliquoting into Sarstedt containers and storing at -80$^0$C.

Venous blood was collected in sodium heparin vacutainers. Blood for luminex analysis was transported on ice to UCT and centrifuged at 3500 rpm (2800g) for 10 minutes at 4$^0$C, within two hours of collection. The supernatant was aliquoted and stored at -80$^0$C in Sarstedt containers.

Venous blood for PBMC isolation was collected in sodium heparin vacutainers, transported at room temperature to UCT, and processed within four hours of collection. PBMC were isolated by density gradient centrifugation over Ficoll and cryopreserved in heat-inactivated fetal calf serum (FCS) with 10% dimethyl sulfoxide (DMSO).

The NHLS processed blood samples for clinical laboratory analyses. Blood for full blood count and differential was collected in K2 EDTA tubes, transported at room temperature and measured using a Siemens Advia 2120I. Similarly, for HIV-infected patients, CD4 count was measured on a Beckman Coulter FC500MP. Serum was collected for albumin and C-reactive protein quantification on a Roche Modular, and for HIV-infected patients, HIV-1 viral load was measured on an Abbott M2000.

### Lipopolysaccharide testing of sodium heparin tubes

A proportion of PBMC samples were collected in sodium heparin tubes that were retrospectively identified as contaminated with lipopolysaccharide (LPS) at manufacture. Following this discovery, routine testing of each batch of sodium heparin tubes was undertaken and only used if no LPS contamination was evident. PBMC collected in LPS contaminated sodium heparin tubes were not included in the study (prospectively excluded).

### MMP, cytokine and total protein quantification

Samples were analysed on the Luminex 200 platform using MMP beads (R&D Systems, Abingdon, UK) and a 30-plex kit for cytokines, chemokines and growth factors (LHC6003M, Life Technologies, Paisley, UK), analysed as per manufacturer's instructions. Cytokines, chemokines and growth factors quantified were IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF-α, interferon [IFN]-α, IFN-γ, IL-1 receptor antagonist (IL-1RA), IL-2 receptor (IL-2R), IL-8, MIP-1α, MIP-1β, Eotaxin, MIG, MCP1, Regulated on

Activation, Normal T cell Expressed and Secreted (RANTES), IP-10, Epidermal growth factor (EGF), Hepatocyte growth factor (HGF), Vascular endothelial growth factor (VEGF), Fibroblast growth factor (FGF) basic, G-CSF and GM-CSF. Induced sputum samples were diluted 1:5 or 1:50 in assay diluent. Plasma samples were run neat. Total protein in induced sputum samples was quantified using Bradford analysis (Bio-rad, Hemel Hempstead, UK), as per manufacturer's instructions. MMP analysis was performed on all induced sputum samples. Plasma MMP analysis was undertaken on the longitudinal study samples only. Cytokine/chemokine analysis was performed on all longitudinal samples and a subset of cross-sectional study samples.

### Procollagen III N-terminal Propeptide (PIIINP) ELISA

PIIINP ELISAs (Cloud Clone Corp, USA) were performed as per manufacturers instructions. In brief, plasma samples were diluted 1:100 in phosphate-buffered saline (PBS). Positive controls for the assays were reconstituted lyophilized standard. Standard was reconstituted with standard diluent. Negative controls were standard diluent alone (blanks) provided by the assay kit. 100µl of standard/sample or blank were added to the provided ELISA plate pre-coated with PIIINP-specific antibody and incubated at 37$^{o}$C for two hours. Liquid was then removed by aspiration and 100µl Detection reagent A added to each well. The plate was covered and incubated for one hour at 37$^{o}$C. The plate was then aspirated and washed three times with wash solution. 100µl Detection reagent B was added. The plate was then covered and incubated for 30 minutes at 37$^{o}$C. Liquid was aspirated and the plate was washed a further five times. 90µl TMB substrate solution was added. The plate was covered and incubated in the dark

for 20 minutes at 37°C. 50µl Stop solution was added and the optical density measured on an ELISA plate reader at 450nm. The sensitivity of the assay was 25.6 pg/mL.

## Flow Cytometry

### Sample preparation

Flow cytometric analysis was performed using cryopreserved PBMC. Cryopreserved PBMC were rapidly thawed in warmed RPMI/10% FCS and washed. All wash steps consisted of centrifugation at 1500 rpm for 5 minutes, discarding of supernatant and disruption of the cell pellet by vortex. PBMC were counted using a Bio-rad TC20™ automated cell counter and viability was ascertained by trypan blue exclusion. For iNKT cell enumeration, one million cells per tube were transferred into two labeled fluorescence activated cell sorting (FACS) tubes per patient: one for α-galcer-loaded CD1d tetramer and one for CD1d (control) tetramer staining for each sample. Cells were washed in PBS and resuspended for viability staining with Violet LIVE/DEAD© Fixable stain kit (VIVID, Invitrogen, Paisley, UK) (1:1000 dilution, 200µl per tube) and incubated for 30 minutes at 4°C in the dark. They were then washed in PBS and resuspended in 30µl cold PBS prior to tetramer staining with α-galcer-loaded CD1d tetramer (tet+) or control CD1d tetramer (tet-cont) (Proimmune, Oxford, UK), added at 0.5ul per tube and then incubated for 30 minutes on ice. Tet+ and tet-cont-stained cells were protected from light at all times.

Following incubation, cells were washed in wash buffer (PBS, 2% HI-FCS, 0.05% $NaN_3$) and stained with the surface antibody panel for 30 minutes at 4°C (see

Table 2-3). Following surface staining, cells were washed in 2 mls PBS and resuspended in PBS, 1% Hi-FCS, 2% paraformaldehyde for 1 hour. After a further wash, cells were resuspended in 300µl wash buffer and acquired on an LSR Fortessa (BD Biosciences) within 24 hours of staining. Single fluorochrome-stained positive and negative control compensation beads were acquired for each experiment to enable fluorescence compensation. Data were analysed using Flowjo software (Tree Star, Ashland, OR).

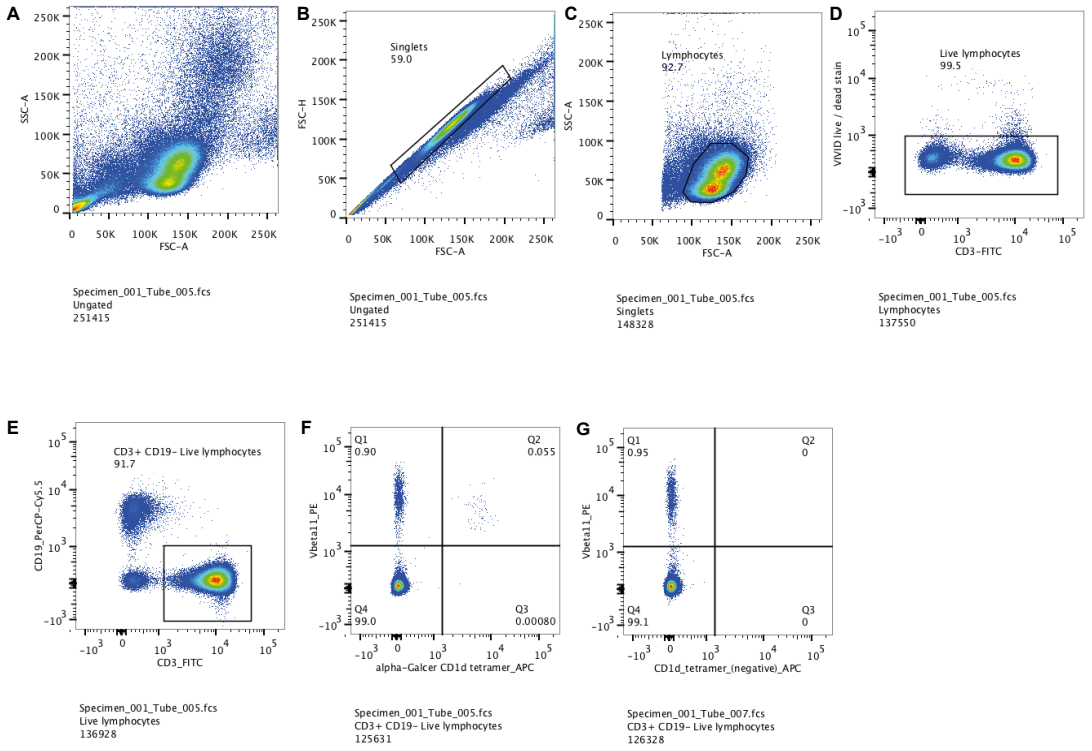
**Table 2-3 Flow cytometry surface staining panel iNKT cell identification**

| | Antibody | Function | Volume/million PBMC stained (ul) |
|---|---|---|---|
| VIVID | N/A | Viability | 200 (1:1000) |
| CD3 | FITC | Identify T cells | 1 |
| CD19 | BV510 | Gate out B cells | 3 |
| CD1d tetramer | APC | iNKT cell identification | 0.5 |
| VB11 | PE | | 5 |
| CD4 | PerCP-Cy5.5 | Assess functional maturity & Th1 (CD4+/-) /Th2 (CD4+) polarisation | 5 |
| CD8 | APC-H7 | Cytotoxicity (CD8+) | 1 |
| CD161 (NK1.1) | BV510 | Activation / maturation NK marker | 5 |
| CD107a | Alexa Fluor 700 | Degranulation | 5 |
| CD95 | BV711 | Fas- Fas ligand mediated cytotoxicity | 5 |
| PD-1 | PE-Cy7 | Induction and maintenance of iNKT cell anergy (in TB) | 5 |
| CD40L (CD154) | PE-CF594 | Activation Interaction with innate immune cells / B cells | 5 |

## Flow cytometric analysis

Cell gating strategy is shown in Figure 2-4. iNKT cell proportions were calculated as a percentage of CD3+ CD19- live lymphocytes, with subtraction of the equivalent tet-cont proportion, and reported per million CD3+CD19- live lymphocytes. Negative values were reassigned zero for analysis. Zero values

were assigned 1 if graphically represented on a log scale. Absolute iNKT cell frequency was calculated as by multiplying the iNKT cell count, as a percentage of live lymphocytes, by the total lymphocyte count per milliliter of peripheral blood, as previously reported (147). Phenotypic characteristics of iNKT cells are reported as the iNKT cell percentage expressing cell surface markers (CD4, CD8, CD107a, CD95, CD161, CD40L, PD1) and this proportion was multiplied by the total number of iNKT cells per mililiter of peripheral blood to give absolute numbers. Median fluorescence intensity (MFI) was calculated for iNKT cell populations that were positive for that surface antibody.




## Figure 2-4 Gating on iNKT cells

Flow cytometric analysis was performed on peripheral blood mononuclear cells stained with fluorescence-conjugated antibodies. A) Ungated cells visualized by forward scatter and side scatter. B) Gating on singlets. C) Identification of the lymphocyte population by forward scatter and side scatter D) Gating on VIVID (live/dead stain) negative cells. E) Gating on CD3+ CD19- cells. F) The iNKT cell population was identified as α-galcer-loaded CD1d tetramer, Vβ11 T cell receptor-positive cells (quartile, Q2). G) Sample stained with control panel using control tetramers (tet-cont, no α-galcer) demonstrating no non-specific staining in Q2. In this example, the iNKT cell frequency is 0.055% of CD3+ CD19- live lymphocytes, or 550 cells per million CD3+CD19- live lymphocytes.

The antibody panel for surface staining of PBMC was designed for iNKT cell enumeration and characterization (Table 2-3). The concentration of each antibody in the panel was determined in a series of experiments, in which antibodies were serially titrated to determine the optimal concentration (Appendix 1, panel 1). Gating of PBMC was confirmed by fluorescence-minus-one (FMO) panels in which PBMC were stained in the absence of each fluorescent-conjugated antibody, providing a negative control. This was repeated for each antibody. Experiments were designed so that all timepoints for an individual patient in the longitudinal study were acquired during a single experiment. A sample from one healthy donor was run in each experiment to identify any significant inter-experiment variability across experiments in the longitudinal study. iNKT cell frequency results from this donor were remarkably consistent (Appendix 1, panel 2). One unstained sample was also run in each experiment as an additional negative control. In a random selection of samples, iNKT cell enumeration was repeated to confirm reproducibility of results.

### Sample size calculation

In the cross-sectional study, a target sample size of 240 patients (40 patients per clinical category) was calculated, based on the standard deviation of preliminary data, which showed that 40 patients in each clinical category would give an 80% power to detect a 1.5-fold change in induced sputum MMP concentrations between HIV-infected and HIV-uninfected TB patients, with a two-sided alpha of 0.05.

There is a paucity of data on iNKT cell population size in TB-HIV co-infected patients. One study examined the difference in iNKT frequency in *Mycobacterium leprae* (*M. leprae)*-HIV co-infected patients and HIV-infected patients without evidence of *M. leprae* infection, finding reduced iNKT cell populations in *M. leprae*-HIV co-infection compared to HIV infection alone (152). Data from this study found the median iNKT cell proportion in *M. leprae*-HIV co-infected patients to be 0.022% (interquartile range (IQR) 0.007-0.051), compared to 0.072% (IQR 0.03-0.160) in HIV-infected patients without leprosy. Based on the assumption that a similar difference would exist in TB-HIV co-infected patients compared to HIV-infected patients without TB, a sample size of 32 in each group (a-f) would give a 90% power, with a two-sided alpha of 0.025, to detect a difference of 0.05% in median iNKT cell percentage between TB-HIV co-infection and HIV infection alone.

Although no preliminary data was available to allow an accurate sample size calculation for the longitudinal study, it was intended that 60-100 patients be recruited, with an expected incidence of TB-IRIS of 30-50% (28).

## Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad, UK). Normally distributed data were summarised using mean and standard deviations. Non-parametric data were summarized by median and IQR. In tables, N/A = not applicable; ND = not done; ns = not significant.

### Cross-sectional study analysis

Kruskal Wallis test was performed to compare each variable between the six

clinical categories a-f. Where Kruskal Wallis test indicated a difference with a p value <0.05, Dunn's multiple comparisons test was used in a post-test analysis, to assess the difference between individual categories. In this analysis, HIV-uninfected TB patients were compared with HIV-uninfected healthy control and HIV-uninfected respiratory symptomatics; HIV-infected TB patients were compared with HIV-infected healthy controls and HIV-infected respiratory symptomatics; HIV-infected TB patients were compared with HIV-uninfected TB patients. In the iNKT cell analysis using Dunn's multiple comparisons test, each clinical category was compared with each other category (a-f). Results of Kruskal Wallis for each comparison are reported in the Tables. Where Dunn's multiple comparison's test revealed a p value <0.05, results are indicated in the figures by horizontal bars and asterisk (see below).

Two-tailed Mann–Whitney U analysis was performed where two continuous variables were compared. A p value of <0.05 was considered to be a statistically significant difference. Correlations were assessed by Spearman's r test. Where correlations between multiple variables were assessed, a Bonferroni correction was made to account for the number of comparisons and the significance level equivalent to 0.05 was calculated.

In figures, comparisons with a p value <0.05 are shown by horizontal bars between the variables (after correction for multiple comparisons where appropriate), with * denoting p values in the range 0.01<0.05, ** 0.001<0.01, *** 0.0001<0.001 and ****<0.0001. P values >0.05 are not shown in the figures.

### Longitudinal study analysis

In the longitudinal study, some samples had to be excluded due to collection in LPS contaminated tubes. It was not possible to perform a repeated measures ANOVA due to missing data at each timepoint. Instead, a two-way analysis of variance (ANOVA) was performed for each continuous variable, categorizing samples by IRIS diagnosis: TB-IRIS or non-IRIS, and by timepoint: TB0, ARV0, ARV2, ARV4. This method calculated variance in the data by diagnosis or by timepoint and identified whether an interaction between the two variables occurred. A p value for a variance of <0.05 by timepoint, diagnosis or interaction was considered to be significant. P values <0.05 are reported in figures. Data collected at interim assessments was not included in this analysis as comparative control data was not available. However, these data were included in graphical form for illustrative purposes where informative. Chest radiograph analysis was undertaken routinely at ARV12 visit and therefore these data were included in the statistical analysis for chest- x-ray inflammation score. Where variance was attributed to diagnosis (TB-IRIS vs non-IRIS) Sidak's multiple comparison test was used to assess the difference between TB-IRIS and non-IRIS controls at each timepoint, and p values are indicated in corresponding tables using asterisks as above, * denoting p values in the range 0.01<0.05, ** 0.001<0.01, *** 0.0001<0.001 and ****<0.0001.

# Chapter 3 Cross-sectional study of TB and TB-HIV co-infection

## Introduction

In the cross-sectional study, I aimed to characterize inflammation and inflammatory mediators at TB diagnosis in HIV-infected and uninfected TB patients, compared to respiratory symptomatic and healthy controls. I compared TB patients with and without HIV-1 infection, to better understand immune response characteristics in HIV-infected and uninfected active TB, and to identify inflammatory mediators that may be amenable to host-directed therapy. By recruiting respiratory controls, in addition to healthy patients, I hoped to be able to identify clinical and immunological characteristics that are specific to active TB. My primary objectives included quantification of induced sputum MMPs, and pro-and anti-inflammatory cytokines and chemokines, as previous work had indicated their importance in the pathophysiology of TB disease and HIV infection. Additionally, in peripheral blood, I aimed to enumerate and characterize iNKT cells, cytotoxic cells capable of rapidly producing pro-inflammatory cytokines and causing mycobacterial and host cell death, thereby potentially contributing to both protection and immunopathology.

## Results

### Clinical characteristics of patient cohort

Patients were screened at Ubuntu clinic, Site B, Khayelitsha and enrolled into the study, based on inclusion and exclusion criteria as described in Chapter 2. In total, 262 patients were enrolled into the TDTB2 study. 35 HIV-infected patients with active TB had received more than 3 doses of TB treatment at time of sample

collection and were not included in the cross-sectional study. 17 enrolled patients were excluded prior to the analysis stage, leaving 210 patients remaining in the cross-sectional analysis (see Figure 3-1). Reasons for exclusion following enrolment were incomplete recruitment (8 participants enrolled who did not provide samples) and uncertain diagnosis (9 participants). A further patient inadvertently took three doses of TB treatment prior to sampling, although this was not apparent until after sample processing.

Patient demographic characteristics and reported symptoms are shown in Table 3-1. Females accounted for 29.8% (n=37) of HIV-uninfected participants compared to 53.4% (n=46) of HIV-infected participants (p=0.0009). However, females accounted for a similar proportion of TB patients and control patients (41.3% vs 40.7% respectively). There was a preponderance of current or ex-smokers in the HIV-uninfected compared to the HIV-infected participants (n=82 (66.1%) vs n=25 (29.1%), p=0.0001). However, within the HIV-infected and HIV-uninfected groups, there were similar proportions of smokers in the TB patient and control groups. Respiratory symptoms were common at TB presentation in both HIV-infected and HIV-uninfected patients. Productive cough was the most common symptom in HIV-uninfected TB patients, but was less frequent in HIV-infected TB patients (79% vs 57%, p=0.005). Night sweats (83%) were the most common presenting symptom in HIV-infected patients.

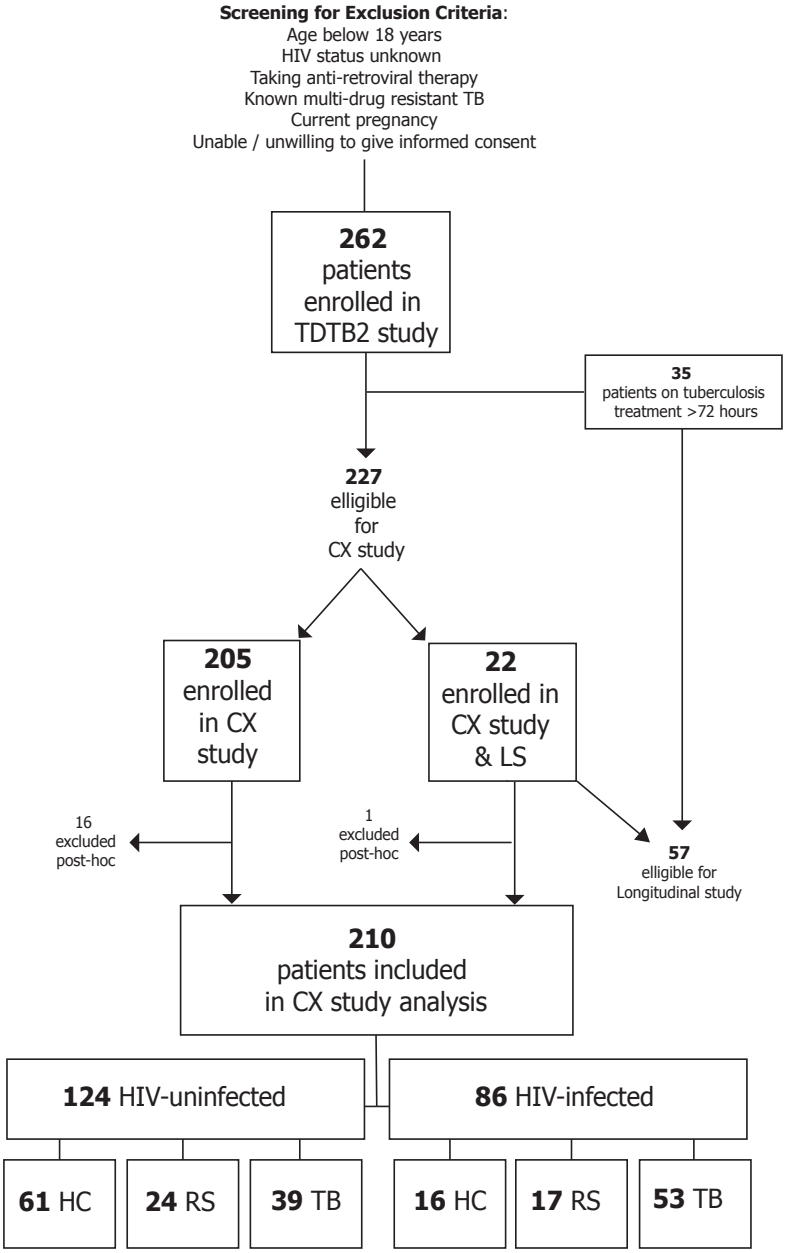



**Figure 3-1 TDTB2 Study participant enrolment**

Schematic showing Tissue Destruction in Tuberculosis 2 (TDTB2) study enrolment, indicating exclusion and inclusion into the cross-sectional (CX) study analysis. The CX study analysis included 124 HIV-uninfected patients and 86 HIV-infected patients. The frequency of patients meeting the study case definition for each of six clinical diagnostic categories is shown. HC=healthy control, RS=respiratory symptomatic, TB=patient with active TB, LS=Longitudinal study.

**Table 3-1 Demographic characteristics and symptoms of cross-sectional study participants**

| HIV status | HIV-uninfected | | | HIV-infected | | |
|---|---|---|---|---|---|---|
| Diagnostic category | HC | RS | TB | HC | RS | TB |
| Frequency, n | 61 | 24 | 39 | 16 | 17 | 53 |
| Median age, years (IQR) | 28 (23.5-33.5) | 28.5 (25.3-38) | 37 (30-42) | 32.5 (27.5-35.8) | 33 (29.5-36.5) | 32 (28.5-36) |
| Female, n (%) | 19 (31) | 5 (21) | 13 (33) | 11 (69) | 10 (59) | 25 (47) |
| Smoking history, current or ex- (%) | 43 (70) | 16 (67) | 23 (59) | 5 (31) | 6 (35) | 14 (26) |
| Productive cough, n (%) | 0 (0) | 6 (25) | 31 (79) | 0 (0) | 5 (30) | 30 (57) |
| Non-productive cough, n (%) | 0 (0) | 6 (25) | 10 (26) | 0 (0) | 8 (47) | 21 (40) |
| Haemoptysis, n (%) | 0 (0) | 0 (0) | 6 (15) | 0 (0) | 0 (0) | 8 (15) |
| Night sweats, n (%) | 1 (2) | 8 (33) | 27 (69) | 0 (0) | 8 (47) | 44 (83) |
| Weight loss, n (%) | 6 (10) | 5 (21) | 20 (51) | 3 (19) | 4 (24) | 30 (57) |
| Symptomatic, any, n (%) | 12 (20) | 24 (100) | 38 (97) | 7 (44) | 17 (100) | 53 (100) |
| Median symptom duration, days (IQR) | N/A | 14 (5-75) | 28 (14-30) | N/A | 30 (30-60) | 30 (14-30) |

Respiratory symptomatic controls were mostly patients presenting with symptoms suggestive of TB (37/41, 90%), although some of these participants presented directly from the community and revealed symptoms only on direct questioning. The median duration of symptoms in these patients was 14 (IQR 5-75) for HIV-uninfected patients and 30 (IQR 30-60) in HIV-infected patients. The cause was most often attributed to viral respiratory tract infection, although confirmatory diagnostic tests were not available. Bacterial infections, bronchiectasis and obstructive airways disease were amongst the differential diagnosis and accounted for the minority of presentations. The frequency of current or ex-smoking history was similar in the healthy control and respiratory symptomatic groups.

Clinical characteristics on examination, full blood count and biochemistry of patients in the cross-sectional study are reported in Table 3-2. 29 (55%) HIV-infected TB patients had features of extra-pulmonary TB, compared to 14 (36%) HIV-uninfected TB patients (p=0.09). HIV-uninfected and HIV-infected patients with active TB had higher temperature, heart rate, respiratory rate and C-reactive protein concentration and lower systolic blood pressure, haemoglobin (Hb) and albumin concentration than corresponding control patients (respiratory symptomatic and/or healthy controls). Statistically significant comparisons are shown in Figure 3-2. Body mass index (BMI) and diastolic blood pressure did not vary significantly between categories. Peripheral blood total white cell count and platelet count were higher in TB patients than in control patients. This trend was more marked for HIV-uninfected TB patients. HIV-infected TB patients had significantly lower total white cell and platelet counts

than HIV-uninfected TB-patients (see Figure 3-3). HIV-uninfected and HIV-infected TB patients had elevated neutrophil counts. Monocyte counts were also elevated in HIV-uninfected TB patients compared to controls. However, relative and absolute lymphocyte counts were reduced in HIV-uninfected and HIV-infected TB patients.

## Table 3-2 Clinical characteristics of cross-sectional study participants

| HIV status | HIV-uninfected | | | HIV-infected | | | |
|---|---|---|---|---|---|---|---|
| Diagnostic category | HC | RS | TB | HC | RS | TB | Kruskal Wallis |
| Median BMI | 22.9 (21.2-26.4) | 21.9 (19.9-24.2) | 21.6 (20.1-25.8) | 26.9 (22.8-32.9) | 27 (20.4-31.8) | 22.9 (20.4-25.3) | **0.03** |
| Median temperature, °C (IQR) | 36.1 (35.8-36.3) | 35.9 (35.6-36.2) | 36.2 (35.9-36.6) | 36.1 (35.8-36.5) | 35.9 (35.6-36,4) | 36.6 (36-37.5) | **<0.0001** |
| Median heart rate, beats per minute (IQR) | 72 (68-78) | 70 (66-78) | 80 (68-88) | 74 (70-82) | 70 (64-83) | 84 (76.5-97.5) | **<0.0001** |
| Median systolic blood pressure (mmHg) | 120 (119-130) | 130 (120-133) | 120 (110-120) | 120 (112-128) | 120 (111-130) | 117 (110-120) | **0.0002** |
| Median diastolic blood pressure (mmHg) | 80 (70-86.3) | 80 (70.8-85) | 80 (70-80) | 80 (70.3-83) | 80 (75.5-90) | 75 (70-80) | 0.1 |
| Median respiratory rate, breaths per minute (IQR) | 16 (16-18) | 18 (16-20) | 20 (16-24) | 18 (16-20) | 17 (16-20) | 22.5 (18-28) | **<0.0001** |
| Median O2 Saturations, % (IQR) | 99 (99-99) | 98.5 (98-99) | 98 (96-99) | 99 (98-99) | 99.5 (98.3-100) | 99 (98-99) | 0.32 |
| Median Haemoglobin concentration, g/dl (IQR) | 14.3 (13.3-15) | 14.3 (13.8-15.3) | 12.7 (12.2-13.7) | 12.6 (11.8-13.7) | 13.2 (12.7-13.9) | 10.7 (9.4-12.0) | **<0.0001** |
| Median total white cell count, $x10^9$/L (IQR) | 6.06 (4.84-7.48) | 5.81 (4.58-7.55) | 7.45 (5.84-9) | 4.95 (4.19-5.88) | 5.24 (4.28-6.28) | 5.48 (4-7.73) | **0.0003** |
| Median neutrophil count (IQR) | 3.32 (2.39-4.53) | 3.32 (2.21-4.88) | 5.11 (3.4-6.71) | 2.53 (1.9-3.59) | 2.32 (1.9-3.45) | 3.54 (2.33-5.34) | **<0.0001** |
| Median neutrophil % (IQR) | 55.2 (47.5-61.4) | 55.5 (46.4-63.6) | 65.8 (58.2-75.5) | 51.8 (46.6-59.3) | 51.4 (42.5-61.9) | 68.9 (57.5-78.1) | **<0.0001** |
| Median lymphocyte count (IQR) | 1.99 (1.61-2.4) | 1.8 (1.24-2.03) | 1.57 (1.2-2.02) | 1.53 (1.38-2.17) | 1.81 (1.25-2.5) | 1.08 (0.59-1.59) | **<0.0001** |
| Median lymphocyte % (IQR) | 32.7 (27.9-40.2) | 32.6 (24.8-42.6) | 21 (14.5-29.3) | 32.3 (24.5-39.8) | 34 (26.8-41.4) | 20.8 (13.7-29.8) | **<0.0001** |
| Median monocyte count (IQR) | 0.35 (0.26-0.43) | 0.36 (0.23-0.44) | 0.55 (0.39-0.69) | 0.37 (0.29-0.43) | 0.27 (0.22-0.42) | 0.34 (0.26-0.45) | **<0.0001** |
| Median monocyte % (IQR) | 5.2 (4.4-7.1) | 5.6 (4.5-6.5) | 6.6 (5.2-9.8) | 6.9 (6.1-9.0) | 5.9 (4.8-7.6) | 6.8 (4.55-8.4) | **0.02** |
| Median platelet count (IQR) | 309 (247-355) | 316 (262-380) | 505 (355-579) | 247 (182-327) | 325 (263-377) | 357 (249-464) | **<0.0001** |
| Median C-reactive protein (IQR)* | 1.3 (0.9-4.9) | 3.3 (0.9-8.3) | 46.9 (7.75-8.27) | 1.4 (0.9-2.55) | 2.65 (1.45-11.0) | 57.1 (18.5-85.7) | **<0.0001** |
| Median albumin concentration, g/dl (IQR) | 47 (44-49) | 47 (43.8-49.5) | 40 (37-43) | 43 (42-46) | 44 (42-45.8) | 34 (30-38) | **<0.0001** |
| Median CD4 count, cells/$mm^3$ (IQR) | N/A | N/A | N/A | 309 (194-409) | 373 (151-526) | 172 (91-351) | **0.03** |
| Median CD4 % (IQR) | N/A | N/A | N/A | 15.8 (4.63-23.4) | 18.5 (12-23.9) | 12.7 (9.5-18.9) | 0.18 |
| Median HIV viral load, copies/ml (IQR) | N/A | N/A | N/A | 39499 (5953-149691) | 25954 (9764-123420) | 174440 (59190-501603) | **0.003** |

* lower limit of assay 1.0, therefore <1.0 given value 0.9 for analytical purposes

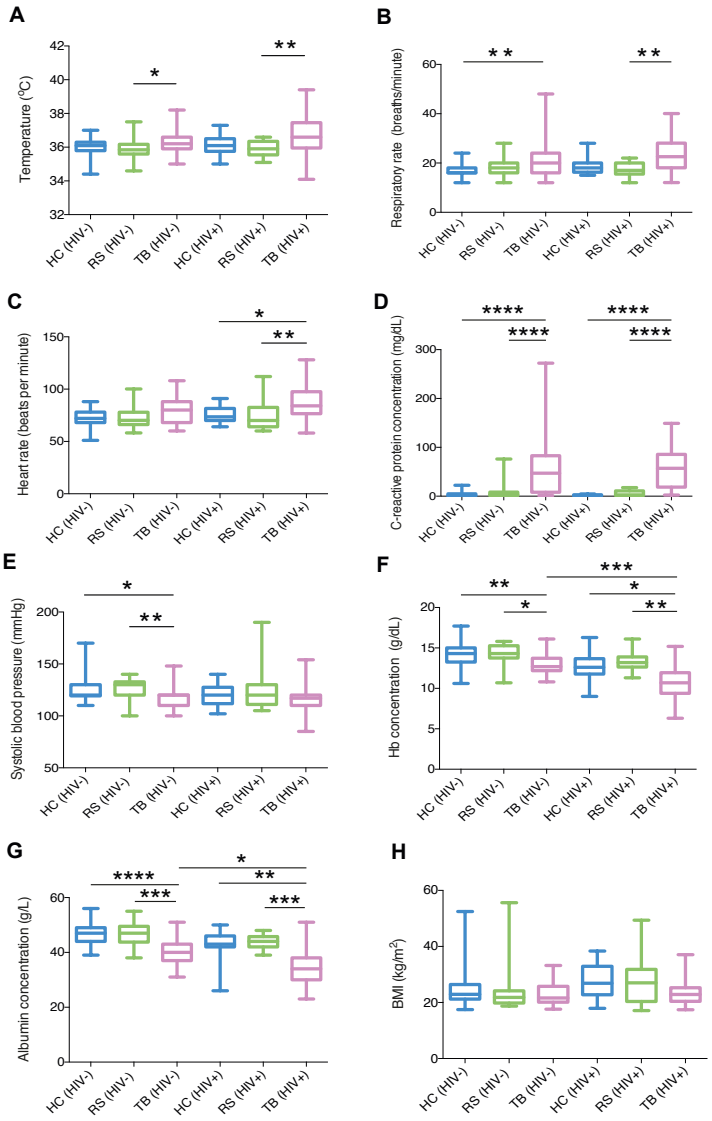



**Figure 3-2 Clinical characteristics of cross-sectional study participants**

Active tuberculosis was associated with elevated temperature (A), respiratory rate (B) heart rate (C) and C-reactive protein (D), compared to respiratory symptomatic and healthy controls. Systolic blood pressure (E), haemoglobin (Hb) (F) and albumin (G) concentration were lower in patients with active TB. These trends were evident in both HIV-infected and uninfected patients, although the magnitude of the difference was greater in HIV-infected patients. By contrast, body mass index (BMI) was similar in TB patients and control groups (H). Analysis was by Kruskal Wallis and Dunn's multiple comparison's test. Comparisons where $p<0.05$ by Dunn's tests are shown ($0.01<0.05$, ** $0.001<0.01$, *** $0.0001<0.001$ and **** $<0.0001$).

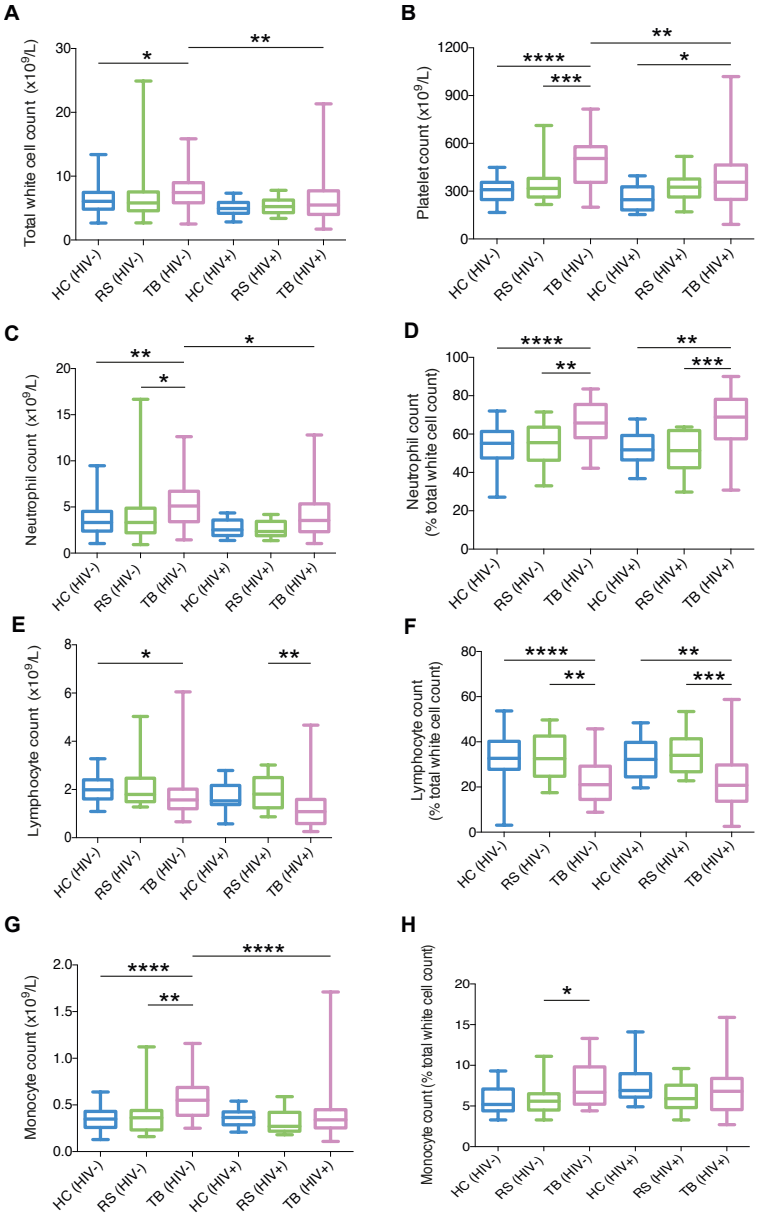



**Figure 3-3 Cross-sectional study peripheral blood white cell and platelet counts**

Peripheral blood white cell and platelet counts are shown for cross-sectional study patients. Total white cell count was elevated in HIV-uninfected TB patients but not in HIV-infected TB patients (A). Active tuberculosis was associated with elevated platelet count (B), neutrophil count (C) and neutrophil percentage (D) in HIV-infected and uninfected patients, compared to respiratory symptomatic and healthy controls. Lymphocyte count (E) and percentage (F) were lower in these patients. Total white cell count (A), neutrophil (C) and monocyte (G) counts were lower in HIV-infected TB patients compared to HIV-uninfected TB patients.

The median CD4 count of HIV-infected study participants was 213 cells/$mm^3$ (IQR 110-421). Within the HIV-infected group, whilst there was a trend towards a lower CD4 count in HIV-infected TB patients, this reached statistical significance only for the comparison between respiratory symptomatic patients and TB patients and CD4 percentages were not different between the three HIV-infected clinical groups (see Figure 3-4). Median peripheral blood HIV-1 viral loads were higher in TB patients (174440 copies/ml) than in both healthy controls (39499 copies/ml) and respiratory symptomatics (25954 copies/ml).

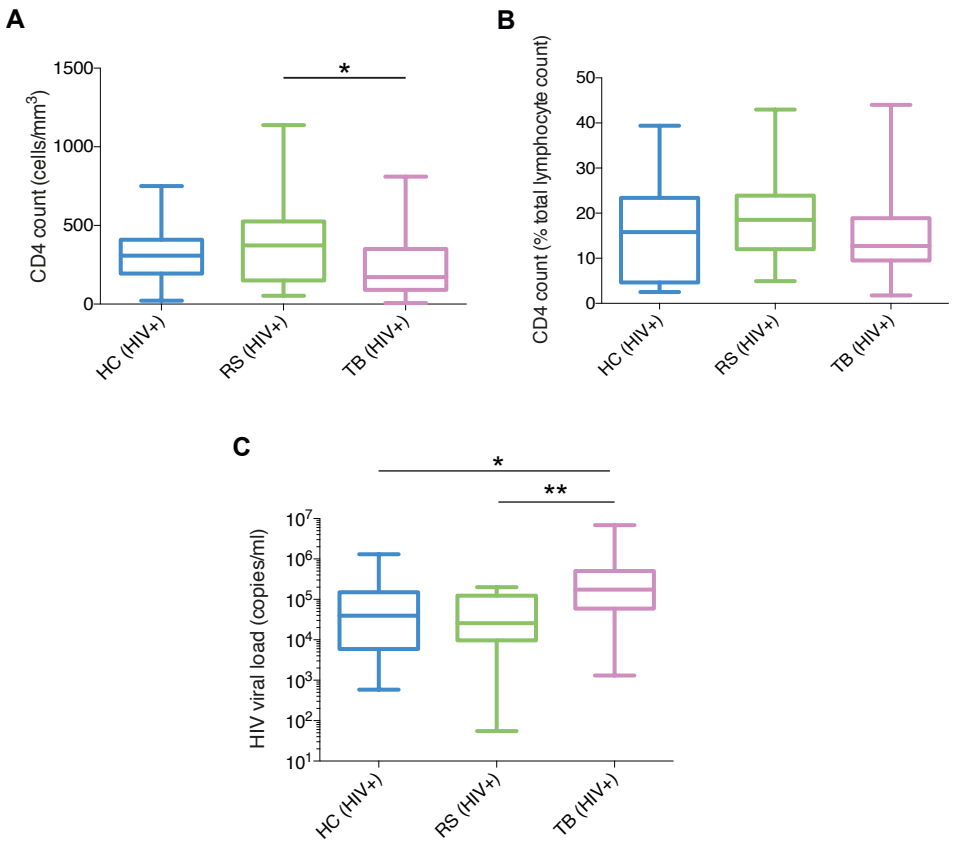



**Figure 3-4 CD4 count and HIV-1 viral load in cross-sectional study participants**

Peripheral blood absolute CD4 count was lower in HIV-infected TB patients than in HIV-infected respiratory symptomatics, although the CD4 percentage was similar. HIV-infected TB patients had an increased HIV-1 viral load than HIV-infected respiratory symptomatic and healthy controls.

HIV-infected patients had a range of comorbidities, although no concurrent major opportunistic infection (see Table 3-3). Oropharyngeal candidiasis was diagnosed in six TB patients, but none of the control patients.

**Table 3-3 Comorbidities in HIV-infected cross-sectional study participants**

| TB disease status | no TB | TB |
|---|---|---|
| Oral hairy leukoplakia, n | 0 | 1 |
| Oro-pharnygeal candidiasis, n | 0 | 6 |
| Oral ulceration, n | 1 | 3 |
| Vaginal/urethral discharge, n | 1 | 0 |
| Genital warts, n | 1 | 0 |
| Papular pruritic eruption, n | 1 | 2 |
| Molluscum contagiosum, n | 0 | 1 |
| Rash (other/unspecified), n | 2 | 2 |
| Paronychia, n | 1 | 0 |
| **Total, n** | **33** | **54** |

Variable radiographic appearance was observed in HIV-infected TB patients, with a wider range of chest x-ray inflammatory scores (0-10) recorded compared to those in HIV-uninfected TB patients (2-10) as shown in Figure 3-5A. One HIV-infected patient had no evidence of inflammation on chest x-ray, despite having sputum culture-confirmed TB. Seventeen (32%) HIV-infected patients had evidence of miliary TB on chest x-ray compared to only one (3%) HIV-uninfected TB patient (p=0.0003). Median chest x-ray inflammation score was 6 (IQR 4.5-8) and 6 (IQR 3-10) in HIV-uninfected and HIV-infected TB patients respectively. HIV-infected TB patients had significantly fewer cavities than HIV-

uninfected TB patients (median (IQR) 0 (0-1) vs 1 (0-4), p<0.0001), see Figure 3-5B.

The vast majority of healthy controls had normal chest x-rays, with a median chest x-ray inflammation score of 0 (IQR 0-0) both in HIV-uninfected and HIV-infected healthy control groups. However, chest x-ray abnormalities were common in respiratory symptomatics, present in 27/41 patients (65.9%). The median chest x-ray inflammation scores were 1 (IQR 0-2) and 1 (IQR 0-1) in HIV-uninfected and HIV-infected respiratory symptomatic control groups respectively.

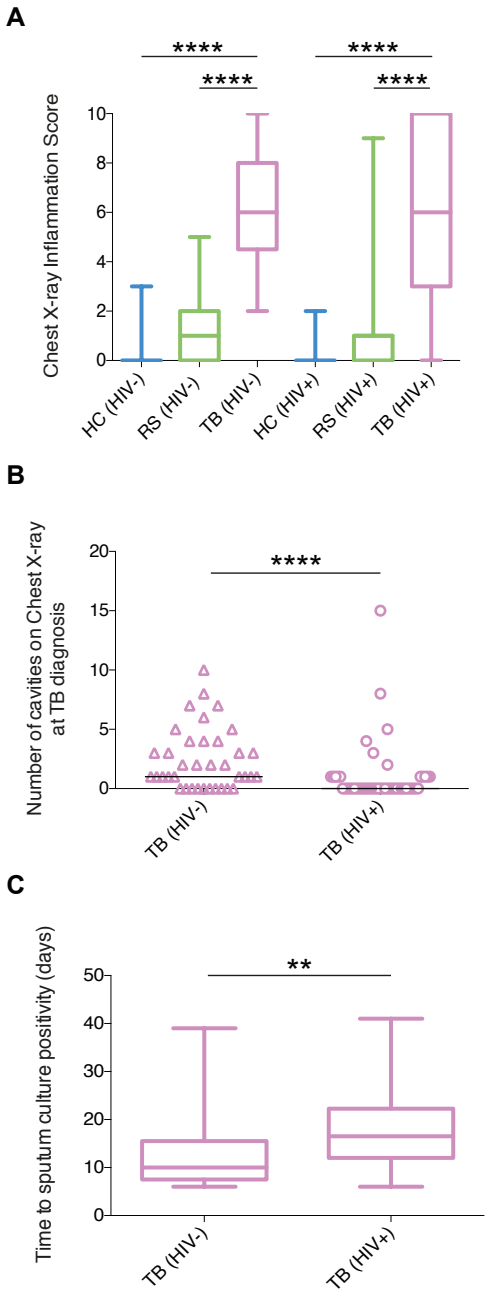



**Figure 3-5 Radiographic and microbiological characteristics of TB and HIV-associated TB**

Chest x-ray inflammation score was higher in TB patients than control patients (A). Some HIV-infected TB patients had no radiographic evidence of pulmonary inflammation. HIV-uninfected TB patients had more cavities on chest x-ray than HIV-infected TB patients, who infrequently had cavitary TB (B). Time to sputum culture positivity for M.tb. was longer in HIV-infected TB patients than HIV-uninfected TB patients (C).

Microbiological and radiological characteristics of cross-sectional study participants are reported in Table 3-4. Seven (13%) HIV-uninfected TB patients and seven (18%) HIV-infected TB patients had no microbiological confirmation of TB diagnosis (p=0.568). A greater proportion of HIV-uninfected TB patients were sputum smear-positive for AFB on auramine staining than HIV-infected TB patients (22/36 vs 17/51, p=0.016). Proportions with culture-confirmed M.tb. were similar in HIV-uninfected and HIV-infected TB patients (21/27 vs 39/51, p=1.000) but time to culture positivity was longer in HIV-associated TB (Figure 3-5C).

**Table 3-4 Microbiological and radiological characteristics of cross-sectional study participants**

| HIV status | HIV-uninfected | | | HIV-infected | | | KW/MW |
|---|---|---|---|---|---|---|---|
| Diagnostic category | HC | RS | TB | HC | RS | TB | |
| Frequency, n | 61 | 24 | 39 | 16 | 17 | 53 | |
| Sputum smear positive for AFB, n (%) | 0 (0) | 0 (0) | 22 (56) | 0 (0) | 0 (0) | 17 (32) | ND |
| Sputum smear negative for AFB, n (%) | 55 (90) | 24 (100) | 14 (36) | 16 (100) | 17 (100) | 34 (64) | ND |
| Gene Xpert positive for Mtb, n (%)* | 0 (0) | 0 (0) | 23 (59) | 0 (0) | 0 (0) | 22 (42) | ND |
| Gene Xpert negative for Mtb, n (%)* | ND | 1 (4) | 1 (3) | 1 (6) | ND | 6 (11) | ND |
| Sputum culture positive for Mtb, n (%) | 0 (0) | 0 (0) | 21 (54) | 0 (0) | 0 (0) | 39 (72) | ND |
| Sputum culture negative for Mtb, n (%) | 55 (90) | 24 (100) | 6 (15) | 16 (100) | 17 (100) | 12 (23) | ND |
| Median smear score, (IQR) | N/A | N/A | 2 (0-6) | N/A | N/A | 0 (0-2) | **0.004** |
| Median time to culture positivity, days (IQR) | N/A | N/A | 10 (7.5-15.5) | N/A | N/A | 16.5 (12-22.3) | **0.004** |
| Median chest X-ray inflammation score (IQR) | 0 (0-0) | 1 (0-2) | 6 (4.5-8) | 0 (0-0) | 1 (0-1) | 6 (3-10) | **<0.0001** |
| Cavities present on chest x-ray, n (%) | N/A | N/A | 27 (69) | N/A | N/A | 15 (28) | **<0.0001** |
| Median number of cavities on chest x-ray, n (IQR) | N/A | N/A | 1 (0-4) | N/A | N/A | 0 (0-1) | **<0.0001** |

*Not routinely performed

### Induced sputum MMP concentrations

MMP concentrations in induced sputum were analysed by Luminex array and are shown in Figure 3-6. Induced sputum MMP-1 concentrations were similar in smokers and non-smokers (median 88.8 vs 124 pg/ml, p=0.181) and in males and females (median 112 vs 100 pg/ml, p=0.87). The collagenases, MMP-1 (interstitial collagenase) and MMP-8 (neutrophil collagenase), along with MMP-3 (stromelysin-1) which activates MMP-1, were increased in TB patients compared to controls, both in HIV-uninfected and HIV-infected patients.

We have previously shown that in advanced HIV (patients with CD4 counts <200 cells/$mm^3$) sputum MMP concentrations were significantly lower than in HIV-uninfected TB patients. In this comparison between HIV-uninfected TB patients and HIV-infected TB patients with a higher median CD4 count, there was a trend towards decreased MMP concentrations in HIV-associated TB, but this did not reach statistical significance. The median MMP-1 concentration in HIV-uninfected TB patients was 2280 pg/ml (IQR 175-24284) compared to 68.1pg/ml (IQR 52.7-103) in HIV-uninfected healthy controls and 65.6 (IQR 52.7-75.1) in HIV-uninfected respiratory symptomatics. In HIV-infected patients, the median MMP-1 concentration was 413 pg/ml (IQR 163-1784) in TB, 101 (IQR 48.5-262) in respiratory symptomatics and 61.4 (IQR 39.5-140) in healthy controls. Increased concentrations of MMP-2, -7, -9 and MMP-10 were also evident in TB compared to control patients, but only in HIV-uninfected patients.

In a secondary analysis, after adjustment for total protein concentration, similar trends were evident although the difference between groups tended to be

smaller. Induced sputum MMP concentrations were significantly correlated with total protein concentration (Spearman r values: MMP-1=0.50, MMP-2=0.58, MMP-3=0.39, MMP-7=0.54, MMP-8=0.51, MMP-9=0.57, MMP-10=0.47, $p<0.0001$ in each case). Medians and IQR for raw and total protein-adjusted MMP concentrations are reported in Table 3-5.

## Table 3-5 Induced sputum MMP concentrations in cross-sectional study participants

| HIV status | HIV-uninfected | | | HIV-infected | | | |
|---|---|---|---|---|---|---|---|
| Diagnostic category | HC | RS | TB | HC | RS | TB | Kruskal Wallis |
| Induced sputum available for analysis (% patients) | 60 (98) | 24 (100) | 30 (77) | 16 (100) | 17 (100) | 46 (87) | |
| Median IS MMP-1, pg/ml (IQR) | 68.1 (52.7-103) | 65.6 (52.7-75.1) | 2280 (175-24284) | 61.4 (39.5-140) | 101 (48.5-262) | 413 (163-1784) | **<0.0001** |
| Median IS MMP-2, pg/ml (IQR) | 654 (404-1270) | 850 (279-1263) | 2357 (947-5084) | 675 (262-1137) | 972 (568-1279) | 1041 (536-2476) | **<0.0001** |
| Median IS MMP-3, pg/ml (IQR) | 102 (39.2-285) | 85.7 (44.1-194) | 3888 (245-10217) | 149 (24-242) | 147 (111-276) | 641 (331-1527) | **<0.0001** |
| Median IS MMP-7, pg/ml (IQR) | 12848 (4775-24307) | 19523 (7248-27011) | 31329 (15080-51502) | 26098 (5152-66573) | 19078 (9689-43626) | 35102 (17048-59883) | **<0.0001** |
| Median IS MMP-8, pg/ml (IQR) | 42441 (21386-103943) | 51871 (28855-243330) | 597879 (218898-1400000) | 44694 (22610-84294) | 105485 (41302-440705) | 272567 (109867-676202) | **<0.0001** |
| Median IS MMP-9, pg/ml (IQR) | 140684 (85385-256106) | 113823 (59355-254012) | 600585 (356791-976746) | 94532 (57754-202134) | 250972 (70503-479053) | 264024 (125560-453991) | **<0.0001** |
| Median IS MMP-10, pg/ml (IQR) | 884 (215-2012) | 1074 (237-1826) | 4222 (1149-15393) | 1287 (631-2554) | 1670 (889-5152) | 3423 (1515-6652) | **<0.0001** |
| Median IS MMP-1, pg/mg total protein (IQR) | 138 (80.7-263) | 95 (55.4-228) | 2402 (221-8655) | 154 (85.6-316) | 156 (86.1-259) | 956 (335-3389) | **<0.0001** |
| Median IS MMP-2, pg/mg total protein (IQR) | 1500 (847-3286) | 1430 (512-2404) | 1524 (696-3123) | 1777 (1055-2214) | 1686 (1036-2156) | 1542 (777-2952) | 0.82 |
| Median IS MMP-3, pg/mg total protein (IQR) | 203 (59.5-719) | 93.2 (47.6-366) | 1284 (244-4867) | 236 (50.9-655) | 270 (133-469) | 923 (469-4701) | **<0.0001** |
| Median IS MMP-7, pg/mg total protein (IQR) | 26015 (12274-50035) | 27989 (14766-60357) | 26921 (12588-49031) | 60138 (24045-60138) | 28055 (21297-63521) | 49780 (23664-100675) | **0.02** |
| Median IS MMP-8, pg/mg total protein (IQR) | 121332 (43811-121332) | 115318 (56266-402868) | 163998 (107865-568776) | 74328 (56093-244055) | 188496 (65953-346155) | 315338-124666-960703) | **0.001** |
| Median IS MMP-9, pg/mg total protein (IQR) | 301921 (107490-540513) | 245987 (124795-508178) | 316111 (144566-527484) | 295742 (146218-533751) | 369701 (173049-838834) | 352165 (162728-7400000) | 0.77 |
| Median IS MMP-10, pg/mg total protein (IQR) | 1554 (532-3360) | 1381 (543-3671) | 2296 (1172-4939) | 1892 (1125-6489) | 2958 (1320-5198) | 4346 (2093-7020) | **0.003** |
| Median total protein, ug/ml (IQR) | 463 (296-952) | 691 (420-1093) | 1544 (910-2274) | 578 (379-1106) | 627 (456-1279) | 764 (367-1304) | **0.0001** |

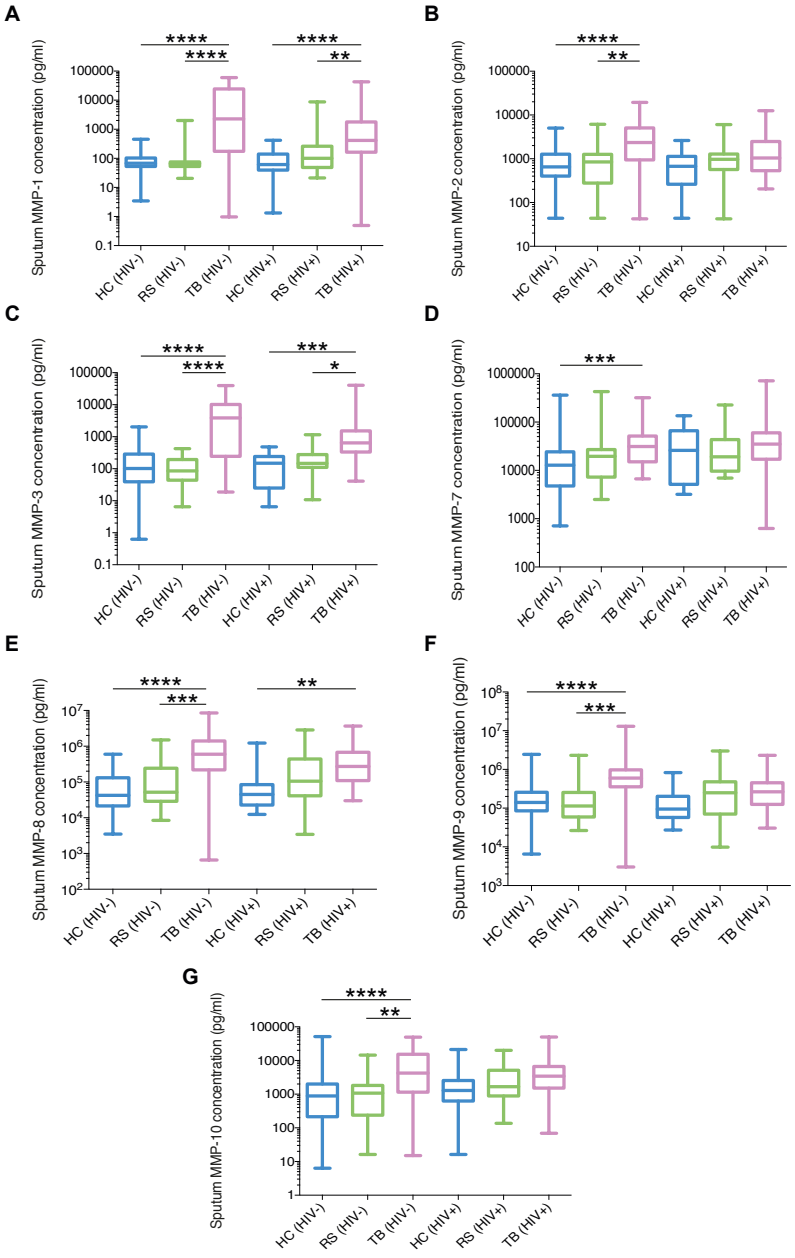



**Figure 3-6 TB and TB-HIV co-infection are associated with elevated induced sputum MMP concentrations**

The collagenases, MMP-1 (A) and MMP-8 (E), along with MMP-3 (C), were increased in TB patients compared to controls, both in HIV-uninfected and HIV-infected patients. MMP-2 (B), -7 (D) and -10 (G) were increased in HIV-uninfected TB patients, but not HIV-infected TB patients, in comparison to controls. There was a trend towards higher sputum MMP concentrations in HIV-uninfected compared to HIV-infected TB patients.

MMP-1 was significantly increased in patients with cavitation on chest x-ray compared to those without ($p=0.0007$, Figure 3-7A). The association between induced sputum MMP concentrations and chest x-ray inflammation score was evaluated by Spearman correlation. A positive correlation between chest x-ray inflammation score and MMP concentration was found for all MMP concentrations analysed (each $p<0.0001$). The strongest correlation was between MMP-1 and chest x-ray inflammation score ($r=0.59$, Figure 3-7B) followed by MMP-3 ($r=0.552$), MMP-8 ($r=0.519$), MMP-9 ($r=0.36$), MMP-2 ($r=0.322$), MMP-7 and MMP-10 (both $r=0.308$).

As HIV-infected TB patients with lower CD4 counts tend to have less marked inflammatory tissue damage on chest x-ray, I expected to find a positive correlation between induced sputum MMP and CD4 count in HIV-infected patients. However, I did not find a significant association between CD4 count and induced sputum MMP-1 concentration. As HIV-infected TB patients tend to have higher HIV-1 viral loads than HIV-infected patients without TB, I hypothesized that an effect of HIV-1 viral load on induced sputum MMP concentration could confound an association between sputum MMP concentration and CD4 count. Indeed, there was a positive correlation between plasma HIV-1 viral load and induced sputum MMP-1 ($r=0.33$, $p=0.0069$), MMP-7 ($r=0.33$, $p=0.0062$), and MMP-8 ($r=0.38$, $p=0.0031$).

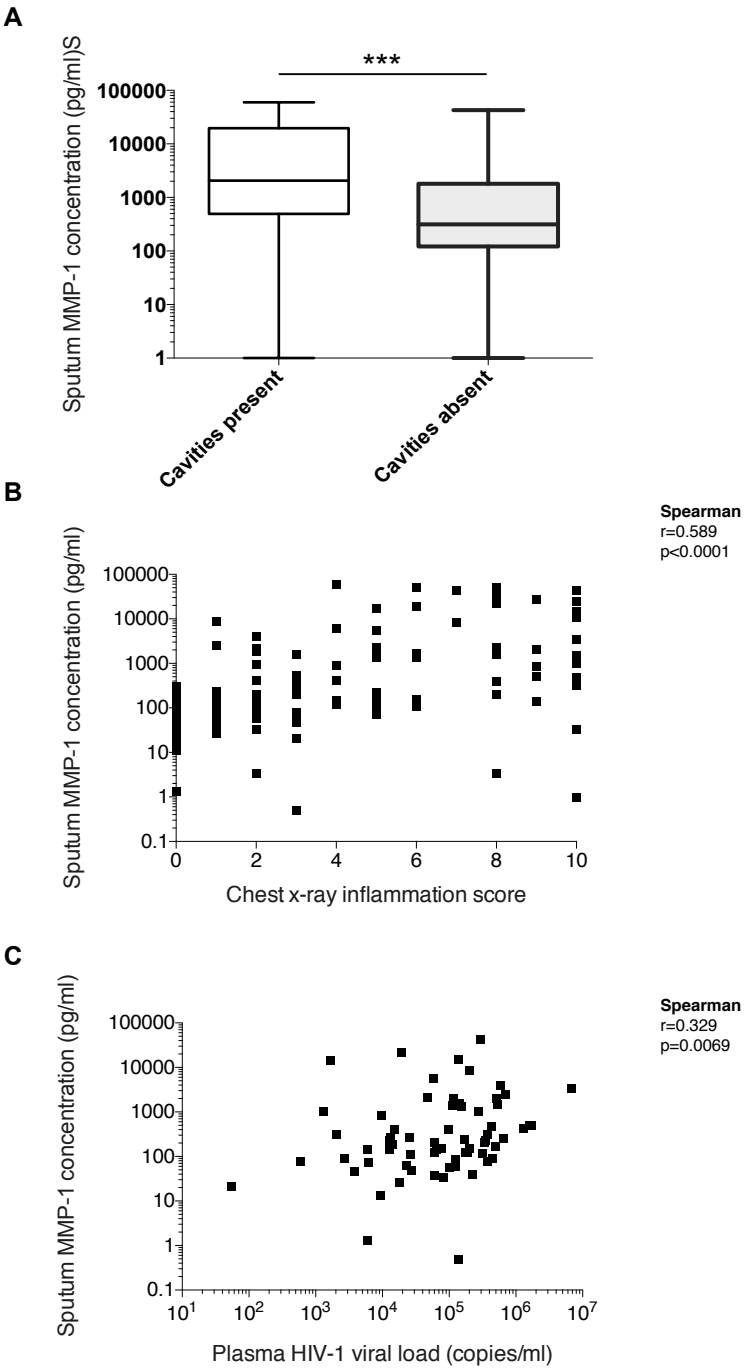



**Figure 3-7 MMP-1, Chest x-ray inflammation score and HIV-1 viral load**

Induced sputum MMP-1 was elevated in patients with cavitary TB and correlated with extent of radiographic inflammation measured by chest x-ray inflammation score. MMP-1 did not correlate with CD4 count, but did correlate positively with plasma HIV-1 viral load.

### Plasma PIIINP concentrations

We have previously demonstrated that procollagen III N-terminal propeptide (PIIINP), a matrix degradation product released from the lung during collagen turnover, was elevated in induced sputum and plasma of TB patients. In our previous study we analysed two small cohorts of patients of mixed HIV-seroprevalence, with relatively well-preserved CD4 counts, from Durban and Cape Town (204). In those cohorts, plasma PIIINP correlated with induced sputum MMP-1 concentration and chest x-ray inflammation score. In the current study, I measured PIIINP in a subgroup of 73 (39 TB, 34 control) cross-sectional study participants to develop these findings.

As in our previous analyses, I found elevated plasma PIIINP concentrations in TB patients compared to controls, median PIIINP values were 25278 pg/ml (IQR 11787-45071) and 3888 pg/ml (IQR 1278-10367) respectively ($p<0.0001$) (Figure 3-8A). HIV-uninfected and HIV-infected TB patients had higher plasma PIIINP concentrations than corresponding respiratory symptomatic and healthy controls. This difference was statistically significant for HIV-infected TB patients compared to healthy control participants on Dunn's multiple comparisons test (Figure 3-8B). There was a trend towards higher plasma PIIINP concentrations in HIV-infected TB patients (median 28458 pg/ml, IQR 14852-48886), compared to HIV-uninfected TB patients (median 11964 pg/ml, IQR 6639-28473), although this difference was not statistically significant on Dunn's multiple comparisons test (Figure 3-8B).

Plasma PIIINP concentrations significantly positively correlated with induced sputum MMP-1 ($r=0.5$, $p<0.0001$), MMP-3 ($r=0.46$, $p=0.0001$) and MMP-8 ($r=0.40$, $p=0.001$) concentrations, in addition to chest x-ray inflammation score ($r=0.43$, $p=0.0002$) and plasma HIV-1 viral load ($r=0.54$ $p=0.0019$), shown in Figure 3-8. A negative correlation ($r= -0.43$, $p=0.0057$) between peripheral blood CD4 count and plasma PIIINP concentration was observed, although this was not statistically significant after Bonferroni correction for multiple comparisons (significant difference inferred by $p<0.005$ in this analysis).

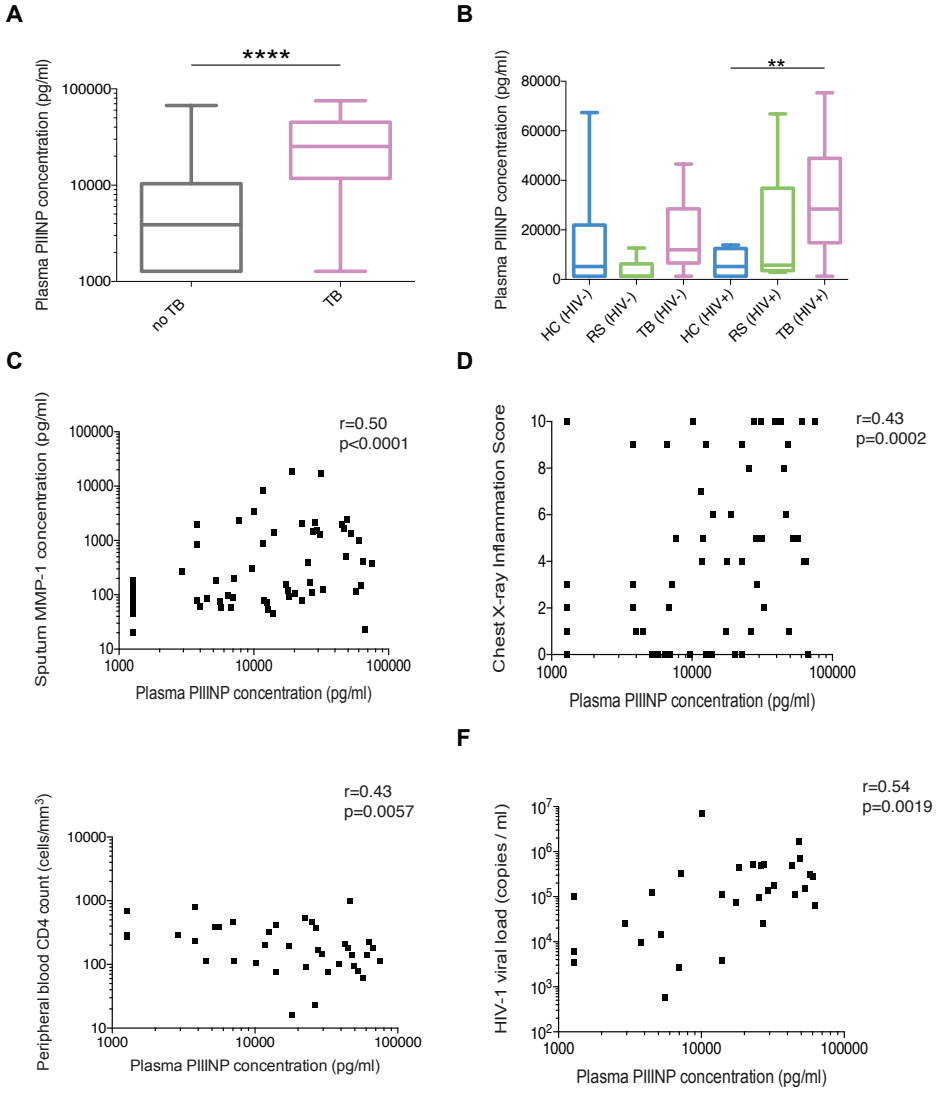



**Figure 3-8 Plasma PIIINP is elevated in TB**

Plasma PIIINP concentration was elevated in TB patients compared to healthy controls and respiratory symptomatics, in an analysis of HIV-infected and uninfected patients by Mann-Whitney U (A) and was increased in HIV-infected TB patients compared to HIV-uninfected patients, but not significantly so, in a comparison by Kruskal Wallis and Dunn's test (B). Plasma PIIINP positively correlated with induced sputum MMP-1 concentration (C), chest x-ray inflammation score (D) and plasma HIV-1 viral load (F), and negatively correlated with CD4 count (E). Plasma PIIINP also positively correlated with induced sputum MMP-3 and MMP-8 (data not shown).

### Induced sputum cytokine and chemokine concentrations

In our previously published analysis of induced sputum cytokine and chemokine concentrations, in a smaller cohort of patients, we did not find elevated cytokine and chemokine concentrations in pulmonary TB (195). Similarly, we found no difference between sputum cytokine and chemokine concentrations in HIV-uninfected and HIV-infected TB patients, after correction for total protein. In the current study, I measured 30 cytokines/chemokines in a subset of 54 cross-sectional study participants (37 TB, 17 control) and analysed both raw and protein adjusted values. There was no HIV-uninfected respiratory symptomatic control group in this analysis. Similar to our previous findings, there was no variance between groups identified by Kruskal Wallis analysis, for total protein-corrected values (data not shown). However, several pro-inflammatory cytokines and chemokines were significantly elevated in HIV-uninfected TB, compared to HIV-uninfected healthy controls in the unadjusted analysis, shown in Figure 3-9, including cytokines IL-1B, IL-8, IL-12 p40/p70, IL-4, IL-2R and TNF-α, and chemokines MIP1a (CCL3) and MIG (CXCL9). Growth factors VEGF and HGF were similarly elevated. IL-5, IL-10 and IL-17 were below the limit of detection. RANTES values were off the top of the standard curve and therefore not analysed. These data are summarised in Table 3-6. There was little difference between cytokine and chemokine concentrations in HIV-associated TB and HIV-infected control groups. However, in HIV infection, these inflammatory mediators tended to be higher in both the control groups and in TB patients than in HIV-uninfected healthy controls, but not as high as in HIV-uninfected TB patients.

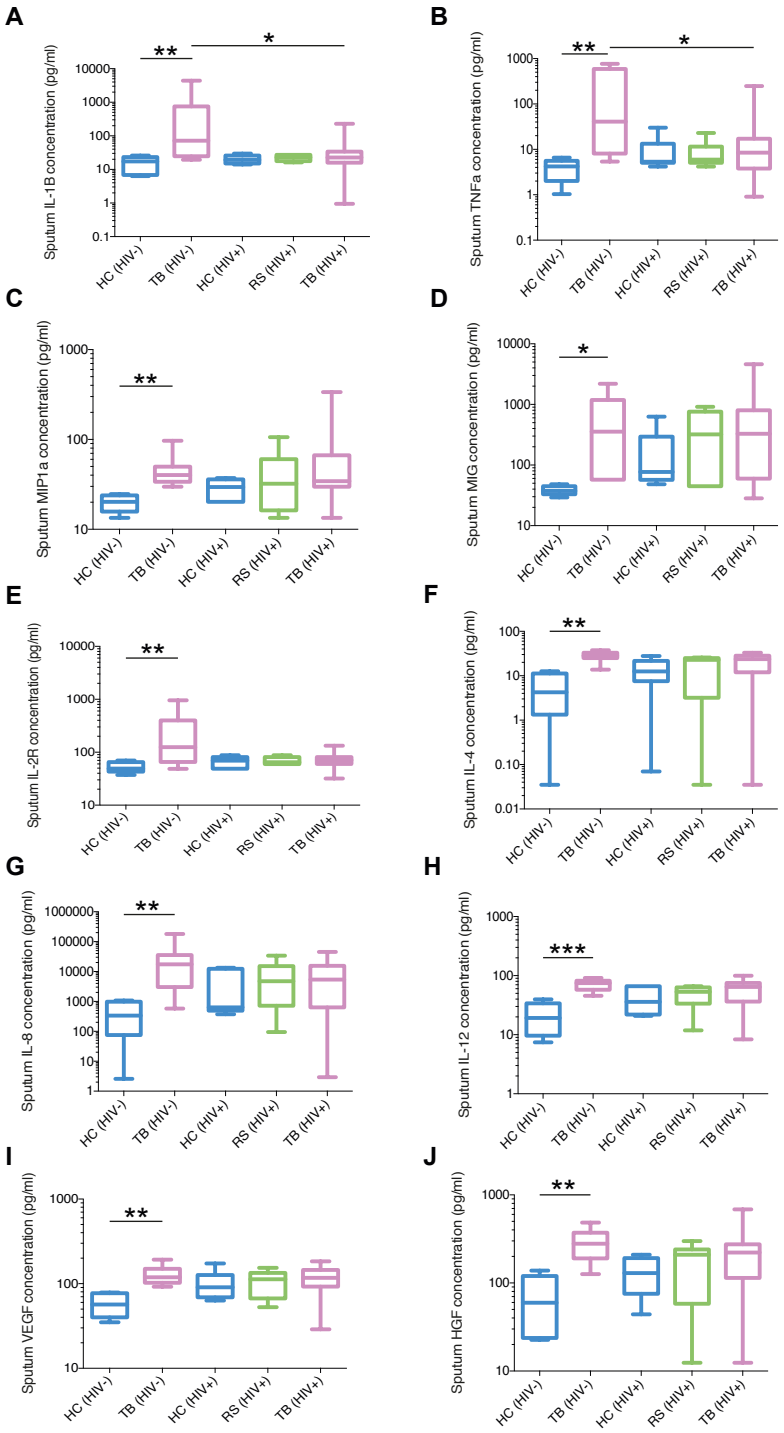



**Figure 3-9 Induced sputum cytokine and chemokine concentrations**

In HIV-uninfected TB patients, induced sputum pro-inflammatory cytokines and chemokines were elevated when compared to healthy controls. This was not the case in in HIV-infected TB patients in comparison to HIV-infected healthy controls. Values shown are not adjusted for total protein.

## Table 3-6 Induced sputum cytokine and chemokine concentrations in cross-sectional study participants

| HIV status | HIV-uninfected | | | HIV-infected | | | |
|---|---|---|---|---|---|---|---|
| Diagnostic category | HC | RS | TB | HC | RS | TB | Kruskal Wallis |
| Sample frequency, n | 5 | ND | 10 | 6 | 6 | 27 | |
| Median VEGF, pg/ml (IQR) | 56.7 (40.1-77.1) | ND | 119 (102-150) | 90.5 (69.1-126) | 113 (66.7-134) | 117 (92.3-145) | **0.035** |
| Median IL-1β , pg/ml (IQR) | 17.4 (6.86-23.4) | ND | 71.5 (24.8-754) | 19.4 (15-19.4) | 22.6 (17.4-27) | 22.7 (16-34) | **0.008** |
| Median GCSF, pg/ml (IQR) | 484 (449-714) | ND | 853 (760-891) | 585 (493-884) | 976 (522-1976) | 839 (633-1144) | 0.089 |
| Median EGF, pg/ml (IQR) | 15 (4.48-41.3) | ND | 98.4 (45.6-184) | 31.8 (11.4-128) | 95.8 (13.5-166) | 126 (27.5-266) | 0.087 |
| Median IL-10, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND |
| Median HGF, pg/ml (IQR) | 59.7 (23.7-120) | ND | 280 (190-372) | 130 (75.5-192) | 209 (58.2-240) | 222 (114-275) | **0.013** |
| Median FGF Basic, pg/ml (IQR) | 5.43 (0.97-10.8) | ND | 5.55 (0.67-14.2) | 5.76 (0.64-12.6) | 8.41 (6.65-15.1) | 7.85 (0.67-12.8) | 0.787 |
| Median IFN-α, pg/ml (IQR) | 35.5 (13.8-47.1) | ND | 72.8 (65.3-81.5) | 50.5 (40.5-79.5) | 74.6 (34.8-80) | 68.3 (48.4-84.4) | 0.073 |
| Median IL-6, pg/ml (IQR) | 22.3 (5.89-50.1) | ND | 110 (55.5-275) | 29.4 (17.9-200) | 63.4 (32.2-157) | 83.7 (41.5-179) | 0.092 |
| Median IL-12 p40/p70, pg/ml (IQR) | 19.2 (9.65-33.9) | ND | 74.7 (57.7-81.8) | 36.1 (22-66.1) | 53.1 (33.7-63.2) | 64.2 (36.4-75.2) | **0.002** |
| Median RANTES, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND |
| Median Eotaxin, pg/ml (IQR) | 0.57 (0.55-0.83) | ND | 1.24 (0.98-2.11) | 1 (0.77-3.07) | 1 (0.768-3.07) | 1.04 (0.79-2.01) | 0.093 |
| Median IL-13, pg/ml (IQR) | 16.4 (3.74-42.2) | ND | 26.9 (24.6-43.4) | 18.2 (10.8-20.4) | 18.2 (10.8-23.6) | 20 (16.4-38.5) | 0.104 |
| Median IL-15, pg/ml (IQR) | 41 (23.9-78.5) | ND | 95.6 (61-129) | 114 (53.8-171) | 159 (78.3-197) | 127 (32.7-206) | 0.253 |
| Median IL-17, pg/ml (IQR)* | ND | ND | ND | ND | ND | ND | ND |
| Median MIP-1α, pg/ml (IQR) | 20.3 (15.8-23.8) | ND | 40.1 (33.8-49.9) | 29.6 (20.3-35.9) | 32.2 (16.2-60.4) | 34.3 (29.8-66.6) | **0.014** |
| Median GM-CSF, pg/ml (IQR) | 0.38 (0.34-0.38) | ND | 0.58 (0.41-0.86) | 0.38 (0.38-0.71) | 0.38 (0.38-0.49) | 0.38 (0.38-0.64) | 0.112 |
| Median MIP-1β, pg/ml (IQR) | 12.3 (5.23-20.8) | ND | 37.3 (26.4-46.7) | 25.2 (15.3-37.2) | 33.4 (11-140) | 29.9 (14.8-91.8) | 0.104 |
| Median MCP-1, pg/ml (IQR) | 103 (14-149) | ND | 344 (306-415) | 207 (98.7-523) | 306 (82.9-462) | 366 (184-470) | 0.1 |
| Median IL-5, pg/ml (IQR)* | ND | ND | ND | ND | ND | ND | ND |
| Median IFN-y, pg/ml (IQR) | 0.87 (0.645-1.97) | ND | 3.43 (2.06-99.7) | 1.48 (1.1-3.53) | 2.67 (1.78-5.24) | 2.96 (1.68-6.74) | 0.089 |
| Median TNF-α, pg/ml (IQR) | 4.17 (2.01-5.57) | ND | 40.8 (8.08-593) | 5.36 (5.06-13.3) | 5.95 (5.06-13.3) | 8.46 (3.78-17.2) | **0.003** |
| Median IL1-RA, pg/ml (IQR) | 131285 (26020-142017) | ND | 142017 (107276-142017) | 94907 (44922-620455) | 90489 (30173-134593) | 90366 (39146-136323) | 0.495 |
| Median IL-2, pg/ml (IQR) | 5.39 (0.34-6.28) | ND | 7.25 (3.24-8.57) | 2.41 (0.36-7.82) | 3.02 (2.15-7.19) | 2.63 (0.36-5.8) | 0.239 |
| Median IL-7, pg/ml (IQR) | 6.86 (6.86-21.8) | ND | 33 (31.6-38.4) | 26.3 (6.86-46.3) | 29.2 (17.4-40.8) | 36.3 (25.8-42.9) | 0.072 |
| Median IP-10, pg/ml (IQR) | 32.3 (2.3-63.5) | ND | 28.6 (4.58-157) | 139 (3.91-299) | 298 (33.8-1988) | 244 (18.8-1221) | 0.066 |
| Median IL-2R, pg/ml (IQR) | 48.9 (42.9-64.9) | ND | 125 (65.4-399) | 69.6 (48.9-81.1) | 64.6 (59.6-81.1) | 71 (60-81) | **0.029** |
| Median MIG, pg/ml (IQR) | 37.5 (32.9-44.7) | ND | 357 (57.3-1185) | 76.8 (56.9-295) | 321 (44.8-759) | 328 (59.9-803) | **0.042** |
| Median IL-4, pg/ml (IQR) | 4.26 (1.33-11.3) | ND | 28.2 (25-33) | 12.5 (7.56-21.6) | 22.8 (3.2-25.1) | 23.8 (12-28.2) | **0.003** |
| Median IL-8, pg/ml (IQR) | 338 (75.8-980) | ND | 17411 (3054-35671) | 631 (490-12428) | 4754 (722-15295) | 5404 (638-15623) | **0.028** |

*values below standard curve

### Plasma cytokine and chemokine concentrations

Next, I measured cytokines and chemokines in plasma of all study participants. Similarly to induced sputum analysis, several pro-inflammatory mediators were identified as significantly different between the 6 clinical groups by Kruskal Wallis test, including IFN-α, IFN-γ, IL-1β IL-2, IL-6, IL-12 p40/p70 and TNF-α (Table 3-7). However, Dunn's test revealed these differences to be predominantly amongst the HIV-infected patients, with relatively increased plasma concentrations in HIV-associated TB compared to HIV-infected control patients and HIV-uninfected TB patients. Figure 3-9 and Figure 3-10 report the results for those cytokines and chemokines respectively with significant differences between groups identified by Dunn's test. Elevated plasma growth factors VEGF, EGF, HGF and FGF basic are shown in Figure 3-11. Together with the clinical findings, results of sputum MMP and cytokines/chemokine results and plasma PIIINP results, a pattern of increased inflammatory mediators and tissue damage at the site of disease in HIV-uninfected TB patients, but increased inflammation in peripheral blood in HIV-associated TB emerges.

## Table 3-7 Plasma cytokine and chemokine concentrations in cross-sectional study participants

| HIV status | HIV-uninfected | | | HIV-infected | | | |
|---|---|---|---|---|---|---|---|
| Diagnostic category | HC | RS | TB | HC | RS | TB | Kruskal Wallis |
| Sample frequency, n | 8 | 13 | 11 | 6 | 6 | 30 | |
| Median VEGF, pg/ml (IQR) | 3.84 (1.96-6.09) | 3.22 (1.77-4.3) | 5.57 (3.22-7.67) | 3.53 (2.61-5.9) | 6.51 (4.87-7.27) | 9.8 (6.66-13.4) | **<0.0001** |
| Median IL-1β , pg/ml (IQR) | 5.91 (5.3-6.36) | 5.76 (5.15-6.36) | 6.36 (5.76-6.67) | 5.76 (4.46-6.14) | 5.91 (5.46-6.51) | 6.97 (5.8-7.55) | **0.008** |
| Median GCSF, pg/ml (IQR) | 42.3 (38.7-45.8) | 31.3 (31.3-45.8) | 38.5 (31.3-54.3) | 38.5 (14-59.3) | 45.8 (26.4-59.3) | 73.2 (31.3-117) | **0.043** |
| Median EGF, pg/ml (IQR) | 11.9 (9.94-14.1) | 10.9 (9.73-12.4) | 11.2 (7.25-18.1) | 13.4 (11.0-14.9) | 13.3 (10.3-15.6) | 19.6 (15-24.4) | **<0.0001** |
| Median IL-10, pg/ml (IQR) | 10.4 (8.95-10.7) | 10.7 (9.71-14) | 11.7 (9.71-21.4) | 10.7 (10.4-12) | 12.1 (10.4-19.3) | 14.4 (12-16.2) | **0.002** |
| Median HGF, pg/ml (IQR) | 18.9 (18.9-26.5) | 18.9 (10.9-26.5) | 26.5 (18.9-40.2) | 22.7 (10.4-27.4) | 22.7 (16.8-46.7) | 52.9 (45-63.8) | **<0.0001** |
| Median FGF Basic, pg/ml (IQR) | 8.7 (8.7-12.5) | 8.7 (8.7-11.5) | 8.7 (7.91-12.8) | 10.1 (5.55-14.2) | 8.54 (1.49-16.7) | 17.6 (13.9-21) | **<0.0001** |
| Median IFN-α, pg/ml (IQR) | 21.1 (15.9-21.1) | 18.2 (16.3-21.1) | 18.2 (16.4-21.1) | 19.6 (13.5-22.3) | 20.9 (17.2-25.9) | 34.4 (25.9-42.1) | **<0.0001** |
| Median IL-6, pg/ml (IQR) | 3.25 (2.55-3.83) | 2.61 (2.61-2.86) | 4.68 (2.86-12.6) | 2.99 (2.61-3.37) | 3.38 (2.8-7.86) | 13.8 (6.7-26.6) | **<0.0001** |
| Median IL-12 p40/p70, pg/ml (IQR) | 55.8 (42.2-64.8) | 55.8 (44.9-58.7) | 51.5 (39.8-65.9) | 59.4 (46.6-80.9) | 79.3 (55.4-94.6) | 148 (102-201) | **<0.0001** |
| Median RANTES, pg/ml (IQR)* | ND | ND | ND | ND | ND | ND | ND |
| Median Eotaxin, pg/ml (IQR) | 8.34 (4.9-26.3) | 8.09 (6.97-10.5) | 19.9 (5.92-42.5) | 27 (11.5-40.9) | 7 (5.33-17.8) | 17.6 (12.4-34.1) | 0.067 |
| Median IL-13, pg/ml (IQR) | 20.1 (17.1-20.1) | 20.1 (18.1-20.1) | 20.1 (16.1-20.1) | 20.1 (16.1-20.1) | 20.1 (14.9-22.3) | 17.5 (13.1-34.1) | 0.375 |
| Median IL-15, pg/ml (IQR) | 36.6 (28.5-36.3) | 27.3 (17-29.7) | 27.3 (21.4-73.3) | 26.8 (17-36.6) | 36.6 (17-45.3) | 93.2 (66.3-129) | **<0.0001** |
| Median IL-17, pg/ml (IQR)** | ND | ND | ND | ND | ND | ND | ND |
| Median MIP-1α, pg/ml (IQR) | 24.8 (20.6-29.1) | 29.1 (25.3-29.1) | 25.3 (22.4-38.1) | 26.1 (18.8-32.5) | 30.8 (27-34.6) | 35 (27.4-47.7) | **0.027** |
| Median GM-CSF, pg/ml (IQR) | 7.73 (7.41-8.49) | 8.05 (7.41-9.15) | 8.05 (7.41-9.64) | 7.41 (7.41-8.12) | 7.73 (7.41-9.06) | 8.31 (8.05-8.87) | 0.05 |
| Median MIP-1β, pg/ml (IQR) | 12.7 (10-20) | 17.3 (16.2-21.1) | 15.8 (15.4-19.2) | 15.8 (10.8-21.9) | 25.8 (21.1.-32.5) | 25 (17.1-29.3) | **0.004** |
| Median MCP-1, pg/ml (IQR) | 109 (85.2-144) | 115 (97.9-156) | 127 (89.1-158) | 158 (64.4-176) | 114 (106-215) | 237 (155-338) | **<0.0001** |
| Median IL-5, pg/ml (IQR) | 2.1 (1.88-2.66) | 2.32 (2.32-2.32) | 2.32 (2.32-2.77) | 2.32 (2.27-2.32) | 2.55 (2.32-3.45) | 2.82 (2.49-3.73) | **0.0006** |
| Median IFN-γ, pg/ml (IQR) | 8.16 (5.12-9) | 7.27 (5.82-8.77) | 8.77 (6.39-14.1) | 6.98 (4.15-7.72) | 7.13 (4.98-8.24) | 18.9 (13.4-39.3) | **<0.0001** |
| Median TNF-α, pg/ml (IQR) | 5.26 (5.26-6.18) | 5.26 (4.66-5.26) | 5.26 (4.05-5.87) | 5.88 (4.96-6.49) | 4.66 (4.05-5.57) | 6.52 (6.49-6.9) | **0.0001** |
| Median IL1-RA, pg/ml (IQR) | 68.4 (56.7-141) | 65 (54.1-80.8) | 81.4 (70.7-107) | 82.4 (71.1-142) | 82.9 (57.3-187) | 123 (82.4-213) | **0.003** |
| Median IL-2, pg/ml (IQR) | 6.41 (5.6-6.52) | 6.07 (5.6-6.3) | 6.07 (5.6-6.96) | 6.07 (5.12-6.52) | 6.18 (5.36-6.74) | 7.42 (6.43-8.14) | **0.0004** |
| Median IL-7, pg/ml (IQR) | 4.73 (1.2-4.73) | 1.2 (1.2-6.5) | 1.2 (1.2-11.2) | 4.73 (1.2-8.26) | 8.26 (6.13-10.3) | 8.71 (5.74-16.6) | **<0.0001** |
| Median IP-10, pg/ml (IQR) | 7.85 (6.21-9.28) | 10.2 (7.92-10.8) | 21 (8.08-64.6) | 15.2 (12.2-17.7) | 16.7 (13.6-28.9) | 109 (57.5-168) | **<0.0001** |
| Median IL-2R, pg/ml (IQR) | 171 (143-249) | 191 (154-252) | 377 (149-252) | 234 (163-353) | 355 (223-1257) | 923 (518-1939) | **<0.0001** |
| Median MIG, pg/ml (IQR) | 14.3 (12.2-20.8) | 12.2 (7.59-16.4) | 61.5 (20.4-161) | 36.8 (22.3-68.4) | 42 (35.1-193) | 281 (186-438) | **<0.0001** |
| Median IL-4, pg/ml (IQR) | 21.1 (20.3-21.9) | 21.9 (20.3-21.9) | 20.3 (18.6-21.9) | 21.1 (19.9-22.3) | 21.1 (18.6-23.5) | 23 (21.9-25.1) | **0.001** |
| Median IL-8, pg/ml (IQR)** | ND | ND | ND | ND | ND | ND | ND |

*values above standard curve
**values below standard curve

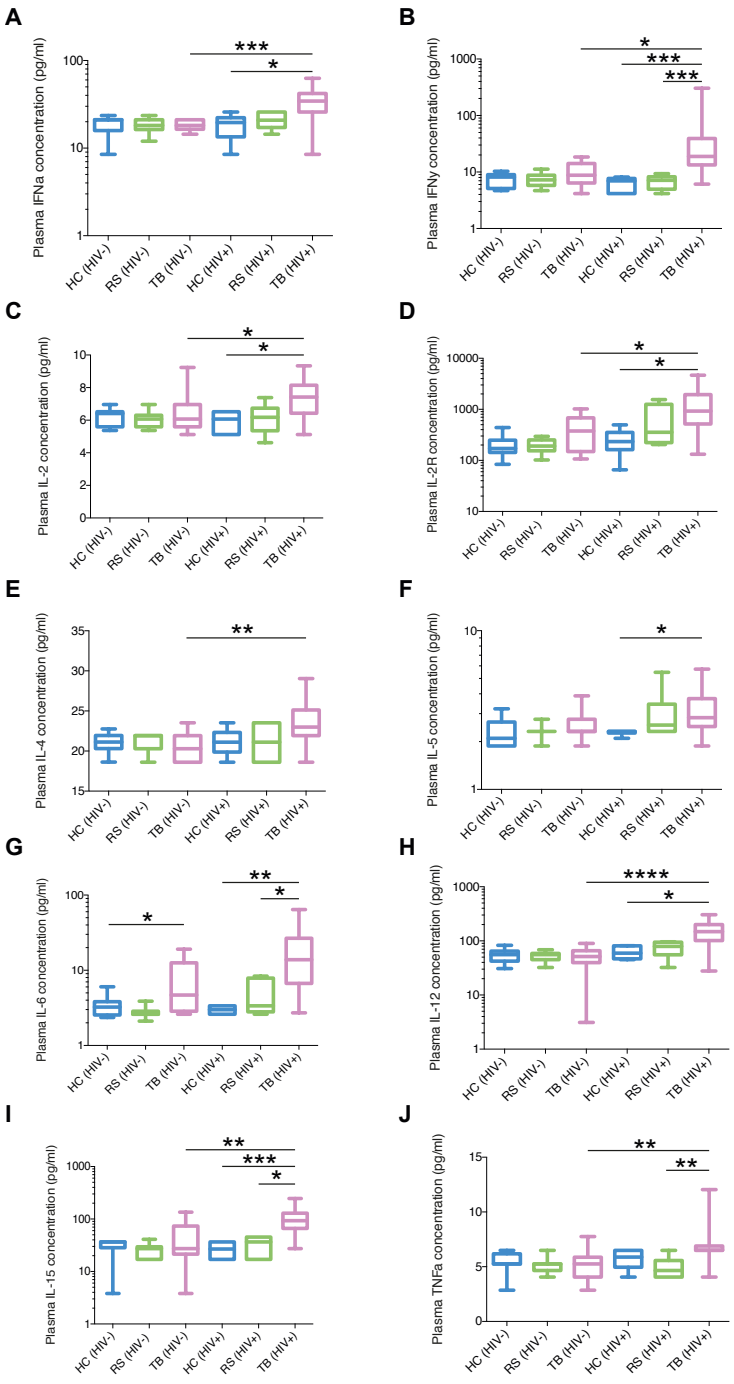



**Figure 3-10 Elevated plasma cytokine concentrations in TB-HIV co-infection**

Pro-inflammatory cytokines were elevated in plasma in TB-HIV co-infection compared to HIV-infected controls but not in HIV-uninfected TB patients compared to HIV-uninfected controls.

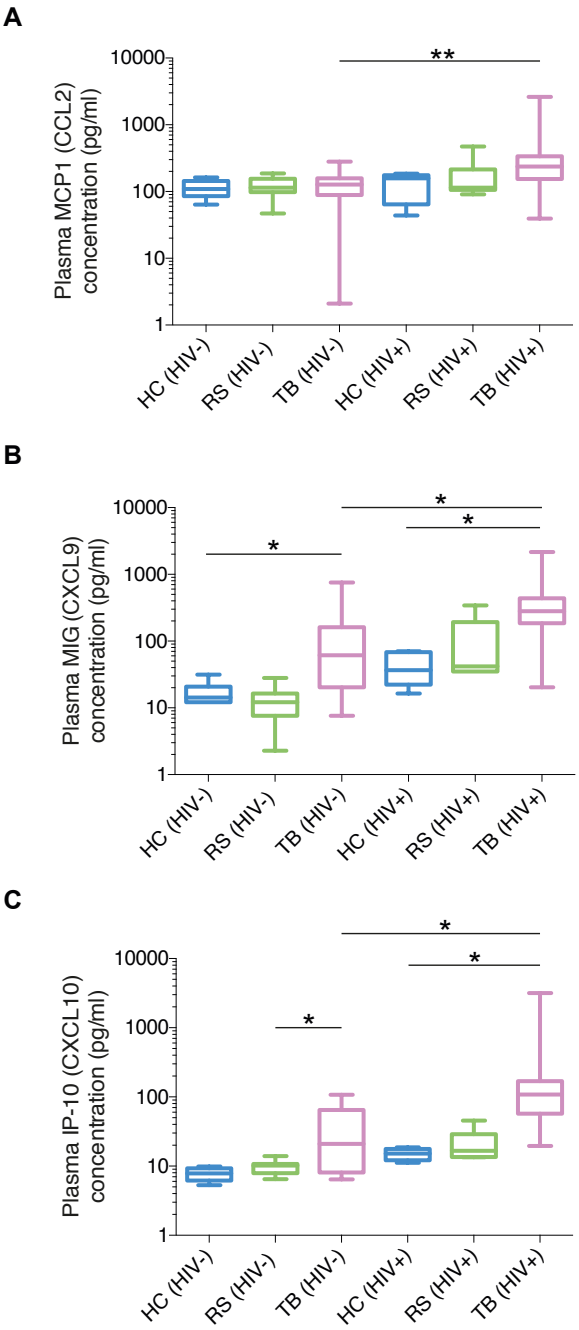



**Figure 3-11 Elevated plasma chemokine concentrations in TB-HIV co-infection**

Pro-inflammatory chemokines MCP1 (CCL2), MIG (CXCL9) and IP-10 (CXCL10) were elevated in plasma in HIV-infected TB patients, in comparison to HIV-uninfected patients with TB.

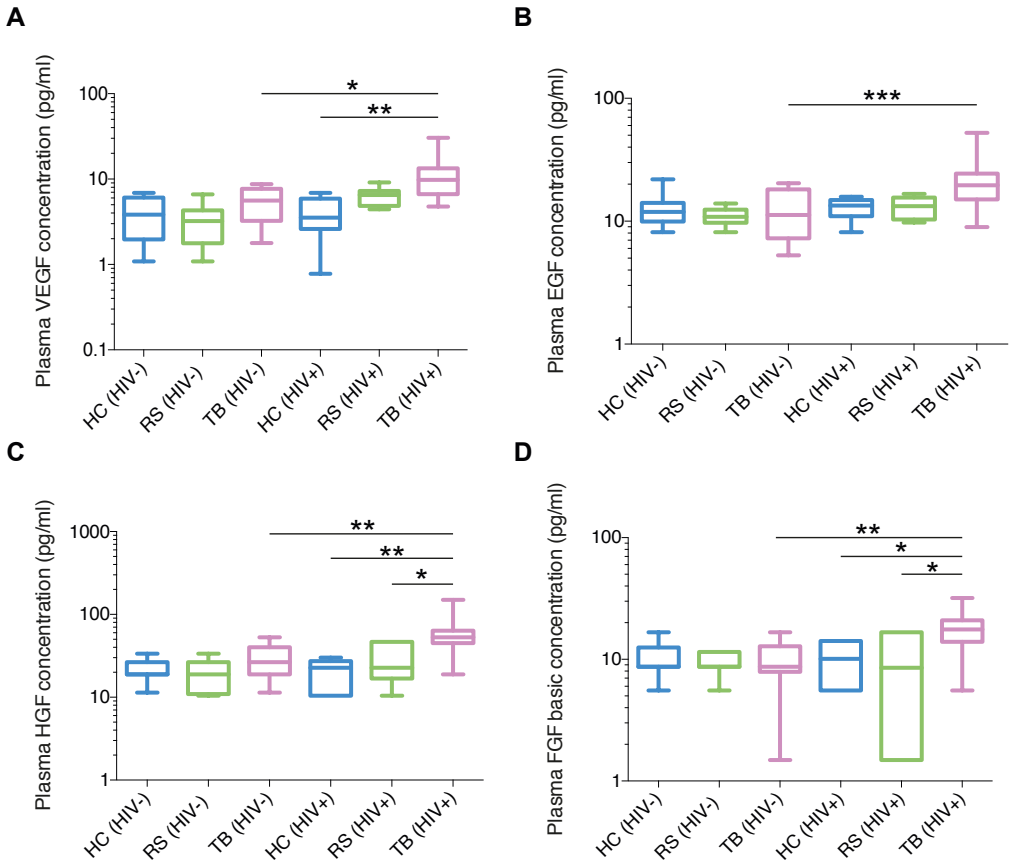



**Figure 3-12 Elevated plasma growth factor concentrations in TB-HIV co-infection**

Growth factors VEGF, EGF, HGF and FGF were elevated in plasma in HIV-infected TB patients in comparison to HIV-uninfected TB patients and HIV-infected controls.

### INKT cell frequency in HIV infection and TB

Previous studies have demonstrated reduced peripheral blood iNKT cell frequency associated with untreated pulmonary TB and untreated HIV infection (146, 147, 149-151). Treatment of either infection has been associated with an increase in iNKT cell frequency, suggesting that iNKT cell reductions are a consequence of infection, rather than a predisposition (146, 151). No previous studies have examined iNKT cell frequency in TB-HIV co-infection, although one former study has shown that co-infection with HIV and *M. leprae* was associated with more profound iNKT cell reduction than with either infection alone (152). I aimed to enumerate peripheral blood iNKT cells in HIV infection, active TB and TB-HIV co-infection, compared to control patients, by flow cytometry using α-galcer-loaded CD1d tetramers, a highly sensitive and specific method for iNKT cell identification. I quantified iNKT frequency both as a proportion of CD3+ CD19- live lymphocytes (reported as cells per million CD3+ CD19- live lymphocytes), and as an absolute frequency of iNKT cells per mililiter of peripheral blood.

In this study population, HIV-1-infected patients had significantly lower iNKT proportions and absolute numbers than HIV-uninfected patients (Figure 3-13, A and B). The median iNKT cell frequency per million CD3+ CD19- live lymphocytes was 1400 (IQR 630-2525) in HIV-uninfected patients, compared to 335 (IQR 92.8-1625) in HIV-infected patients ($p=0.0001$). Absolute values were also reduced, with a median of 166922 (IQR 82489-362495) cells per ml in HIV-uninfected patients, compared to 32048 (IQR 9200-180097) cells per ml in HIV-infected patients ($p<0.0001$). Similarly, patients with active TB also had

significantly lower iNKT cell proportions and absolute numbers than patients without active TB, although the magnitude of the difference was less (Figure 3-13, C and D). The median frequency of iNKT cells per million CD3+ CD19- live lymphocytes in patients without TB was 1200 (IQR 340-2300), compared to 550 (140-1800) in TB patients (p=0.032) and the median absolute frequencies of iNKT cells per milliliter were 156339 (IQR 42060-323038) and 35269 (IQR 12704-157514) respectively (p=0.0018).

In a comparison between TB patients, respiratory symptomatics and healthy controls, I observed significant differences by Kruskal Wallis, with a greater effect of TB in the HIV-uninfected patients compared to the HIV-infected patients. In both HIV-uninfected and HIV-infected patients respiratory symptomatics had higher median absolute and proportional iNKT cell frequencies than TB patients, although this was not statistically significant by Dunn's test (Figure 3-14). Median iNKT frequencies for the six clinical categories with IQR are shown in Table 3-8.

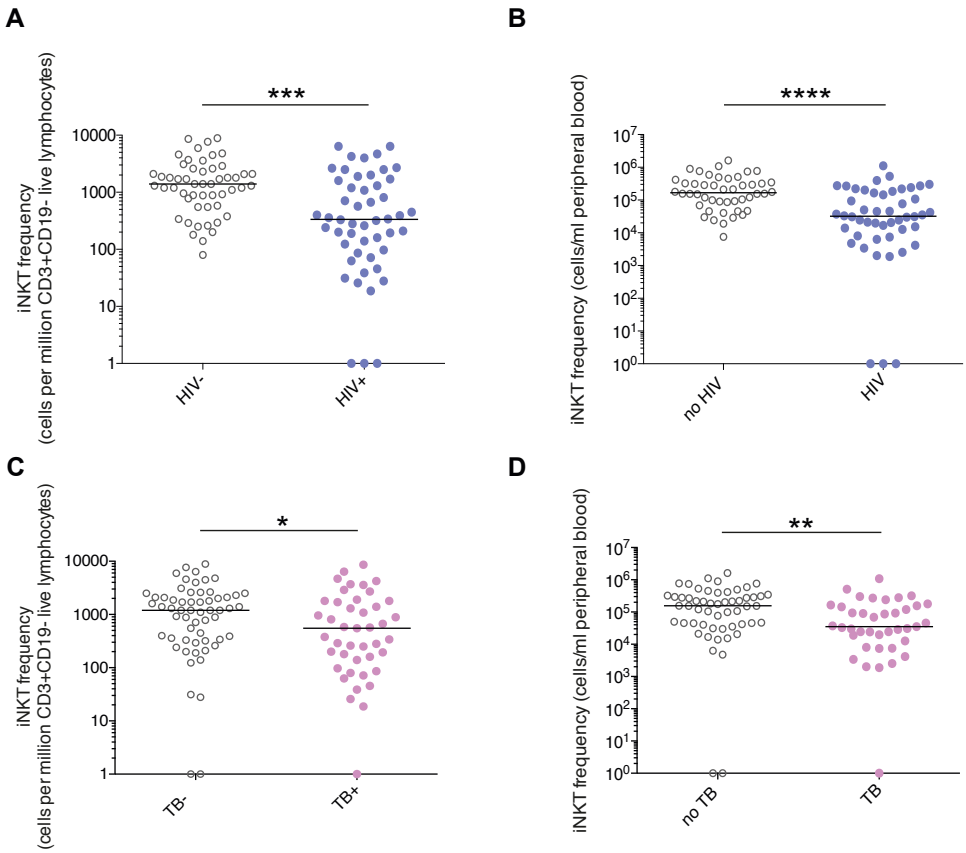



**Figure 3-13 HIV infection and TB are associated with reduced iNKT cell frequency**

INKT cell frequency was enumerated by flow cytometry using α-galactosylceramide-loaded CD1d tetramers (tet+). Control tetramer (tet-cont) non-specific staining was subtracted from tet+ frequencies. Where results were negative they were assigned 1 for representation on a log scale. Both HIV infection (A and B) and TB disease (C and D) were associated with reduced iNKT cell frequency, enumerated either as a proportion of CD3+CD19- live lymphocytes (A and C) or as cell number per milliliter of peripheral blood (B and D). Statistical analysis was by Mann-Whitney U test (*0.01<0.05, ** 0.001<0.01, *** 0.0001<0.001 and ****<0.0001).

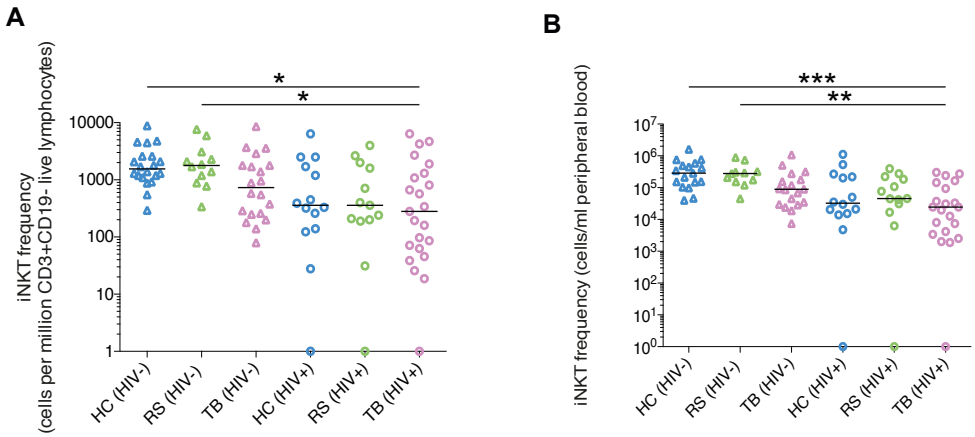



**Figure 3-14 INKT cell frequency in HIV-infected and -uninfected TB patients, respiratory symptomatics and healthy controls**

Reduced iNKT frequency in HIV-infection and TB disease is shown comparing the six study groups. The dominant effect is from HIV infection. An additive reduction in TB-HIV co-infection is not statistically significant. Analysis was by Kruskal Wallis and then Dunn's multiple comparisons test (*p<-0.05-0.01, **p<0.01-0.001, ***p<0.001-0.0001, ****p<0.0001).

**Table 3-8 INKT cell frequency in HIV-infected and -uninfected TB and control participants**

| HIV status | HIV-uninfected | | | HIV-infected | | | |
|---|---|---|---|---|---|---|---|
| Diagnostic category | HC | RS | TB | HC | RS | TB | Kruskal Wallis |
| Sample frequency, n | 20 | 12 | 20 | 14 | 13 | 23 | |
| Median iNKT cells per million CD3+CD19- live lymphocytes (IQR) | 1550 (1125-2600) | 1785 (968-2900) | 735 (253-1800) | 360 (136-1900) | 360 (195-1800) | 280 (62.7-1300) | **0.002** |
| Median total iNKT count/ml (IQR) | 286896 (127611-546992) | 278360 (155377-323038) | 88580 (29600-263771) | 32451 (14921-231208) | 45298 (24039-202817) | 24439 (3789-119449) | **<0.0001** |

### INKT cell phenotype in HIV infection and TB

INKT cells are thought to bridge the gap between the innate and acquired immune system due to their potential to produce abundant cytokines rapidly in response to stimulation and their cytotoxic capability. I aimed to explore phenotypic characteristics of iNKT cells in HIV infection and TB by studying cell surface expression of CD4 and CD8, PD1, CD40L, CD95, CD161 and CD107a. iNKT cells can express either CD4 or CD8, and the presence of both or neither marker and has been associated with Th cytokine polarization and activation status. For example, CD4 expression is associated with Th2 polarisation and CD8 expression has been associated with activation and increased cytotoxic potential (144, 300). CD4 expression was prominent in the NKT cell populations in HIV-uninfected healthy controls (median 38.7%, IQR 27.2-57.5), respiratory symptomatics (median 48.8%, IQR 35.9-80.2) and TB patients (median 38.9%, IQR 16.3-67.1), whereas CD8 expression was much less frequent (Figure 3-14). CD4+ CD8- cells and CD4- CD8- (double negative) cells were the predominant subsets in HIV-uninfected patients (Figure 3-15).

As HIV binds to CD4 and may directly infect iNKT cells, I expected the CD4+ iNKT subsets to be lower in HIV infection and I found that this was the case, more so in HIV-associated TB than in HIV-infected healthy controls and HIV-infected respiratory symptomatics, although this comparison was not statistically significant (Figure 3-15).

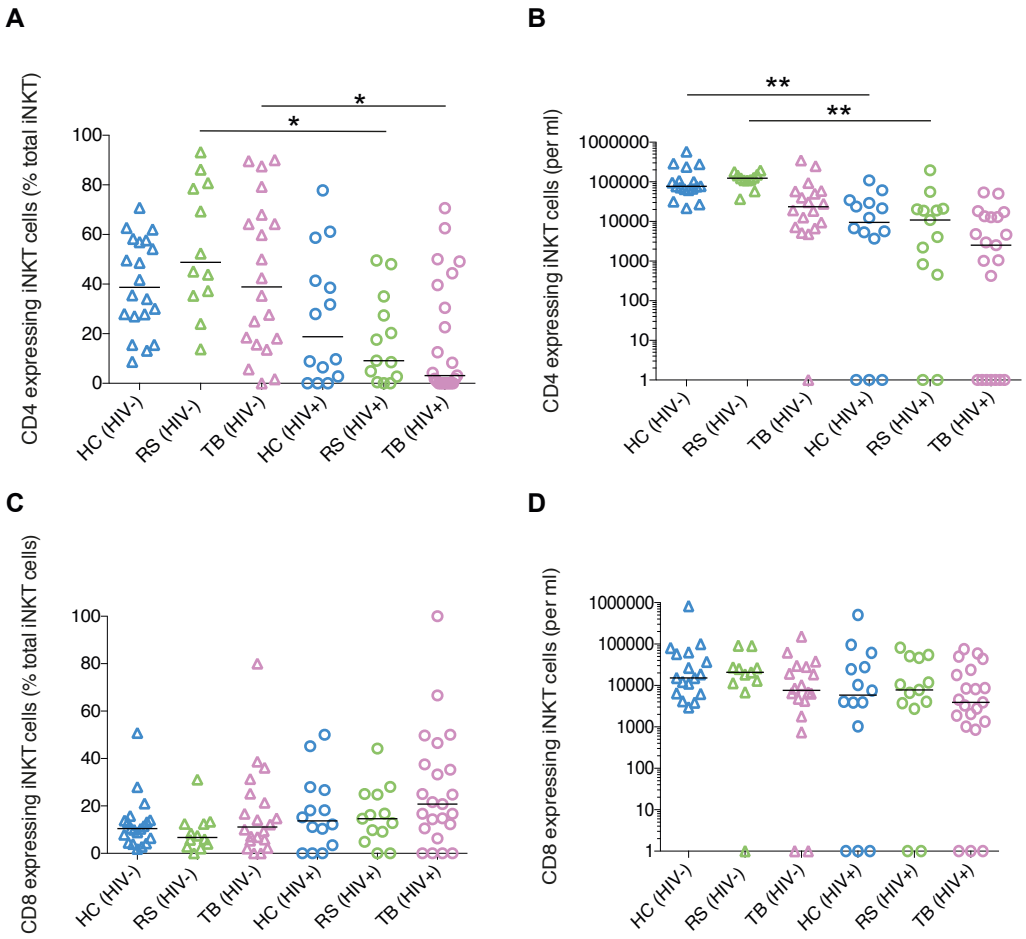



**Figure 3-15 CD4 and CD8 iNKT cell expression in HIV infection and TB**

Proportions of iNKT cells expressing CD4 and CD8 were quantified (A and C) and absolute values were obtained by multiplication of the proportional value by the absolute iNKT cell number (B and D). CD4-expressing iNKT cells were reduced in HIV infection, markedly so in HIV-associated TB. Reductions in absolute number of CD8 expressing cells were less marked and proportionally this population was slightly increased in HIV-associated TB. Significant differences identified on Dunn's test following Kruskal Wallis analysis are shown. Additionally, significant differences not shown were observed in panel (A) between HC (HIV-) and TB (HIV+), and RS (HIV-) and TB (HIV+), and in panel (B) between RS (HIV-) and HC (HIV+), HC (HIV-) and RS (HIV+), HC (HIV-) and TB (HIV+), RS (HIV-) and TB (HIV+). HC=healthy control; RS=respiratory symptomatic; TB=TB patient.

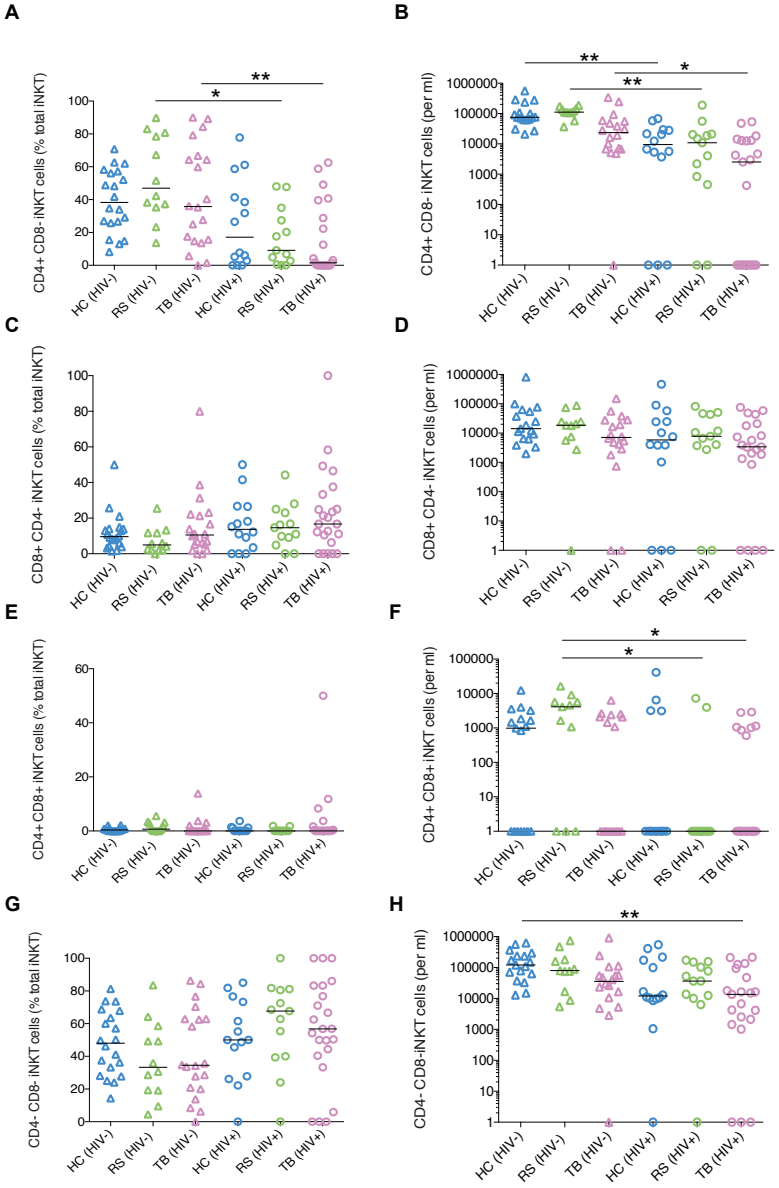



**Figure 3-16 CD4 and CD8 iNKT cell subsets in HIV infection and TB**

The CD4+CD8- iNKT cell subset was the most significantly reduced, of CD4/CD8 subsets, in HIV-infected patients compared to HIV-uninfected controls. Significant differences identified on Dunn's test following Kruskal Wallis analysis are shown. Additionally, significant differences not shown were observed in panel (A) and (B) between HC (HIV-) and TB (HIV+), and RS (HIV-) and TB (HIV+).

Cell surface marker expression of CD95 and CD161, CD107a, PD1 and CD40 ligand are reported in Table 3-9. There was a trend towards higher proportions of iNKT cells expressing of activation receptors CD95 and CD161 in TB patients than in healthy controls and higher CD95 and CD161 MFI values in TB than in healthy controls in both HIV-infected and HIV-uninfected TB patients, but absolute values were slightly lower than in controls (Figure 3-16). CD107a and CD40L expression were low with no significant difference between the clinical groups on Kruskal Wallis analysis. Higher expression of PD1, an inhibitory receptor that together with its ligand have been implicated in iNKT cell anergy, have been previously observed on peripheral blood iNKT cells from pulmonary TB and extra-pulmonary TB patients compared to healthy and latently infected control patients. In my current analysis I found that PD1 expression was universally high on iNKT cells in controls and TB patients, with a trend towards higher proportions (but not absolute numbers) of iNKT cells expressing PD1 in respiratory symptomatics and in TB patients, both in HIV-uninfected and HIV-infected patients, along with higher (although not significantly so) PD1 MFIs (Figure 3-17).

## Table 3-9 INKT cell phenotype and surface marker expression

| HIV status | HIV-uninfected | | | HIV-infected | | | |
|---|---|---|---|---|---|---|---|
| Diagnostic category | HC | RS | TB | HC | RS | TB | Kruskal Wallis |
| PD1 expressing iNKT (% iNKT) | 77.1 (72.8-92) | 84.6 (63.8-87.2) | 85.6 (72.8-90.5) | 80.9 (61.4-92.6) | 85.5 (72.6-95.3) | 86.9 (50-100) | 0.928 |
| CD40L expressing iNKT (% iNKT) | 0 (0-0.143) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.79) | 0 (0.0) | 0.232 |
| CD107a expressing iNKT (% iNKT) | 0 (0-0.555) | 0 (0-1.88) | (0-0.89) | 0 (0-0.188) | 0 (0-0.445) | 1.4 (0-9.61) | 0.999 |
| CD95 expressing iNKT (% iNKT) | 77.3 (71.5-87.3) | 89.1 (82.2-92.2) | 91 (74.2-100) | 85.6 (78.2-95.9) | 80 (73.8-84.4) | 87.8 (74.5-99.6) | **0.047** |
| CD161 expressing iNKT (%iNKT) | 60.1 (21.7-79.4) | 73.4 (29.1-87.2) | 65.5 (42.9-90.8) | 75.1 (51.5-83.6) | 74.5 (44.9-89.2) | 85.7 (41.7-94.5) | 0.356 |
| PD1 expressing iNKT (per ml) | 105333 (39974-214530) | 184579 (16470-241083) | 26052 (12637-127213) | 19177 (5757-190248) | 39232 (14155-89175) | 19680 (2078-96534) | **0.025** |
| CD40L expressing iNKT (per ml) | 0 (0-728) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-542) | 0.308 |
| CD107a expressing iNKT (per ml) | 0 (0-1166) | 0 (0-5625) | 0 (0-1258) | 0 (0-732) | 0 (0-824) | 853 (0-2073) | 0.443 |
| CD95 expressing iNKT (per ml) | 161965 (99226-476298) | 266947 (111250-295580) | 78178 (24093-178740) | 28999 (12050-207777) | 37889 (19324-169324) | 19014 (2758-110199) | **<0.0001** |
| CD161 expressing iNKT (per ml) | 105333 (39974-214530) | 184579 (16470-241083) | 26052 (12637-127213) | 19177 (5757-190248) | 39232 (14155-89175) | 19680 (2078-96534) | **0.025** |
| PD1 expressing iNKT MFI | 945 (714-1450) | 1148 (930-1598) | 1304 (701-1789) | 1193 (838-1461) | 944 (731-1318) | 1699 (847-317) | 0.227 |
| CD40L expressing iNKT MFI | 2400 (1842-11887) | 3766 (3490-4041) | 2495 (2064-3176) | ND | ND | 3406 (1942-6377) | ND |
| CD107a expressing iNKT MFI | 4308 (2092-6211) | 2978 (1533-31803) | 7974 (1849-8562) | 3180 (1776-3682) | 10821 (2549-17045) | 5862 (2461-17045) | 0.524 |
| CD95 expressing iNKT MFI | 4021 (3341-5230) | 4940 (4172-6141) | 4951 (4034-6591) | 4986 (4087-5897) | 3739 (3289-5826) | 5393 (3988-7111) | 0.088 |
| CD161 expressing iNKT MFI | 2159 (1804-2694) | 2382 (1788-2770) | 2849 (1983-3413) | 2739 (2215-3421) | 2640 (2420-3329) | 3151 (2485-3731) | **0.004** |

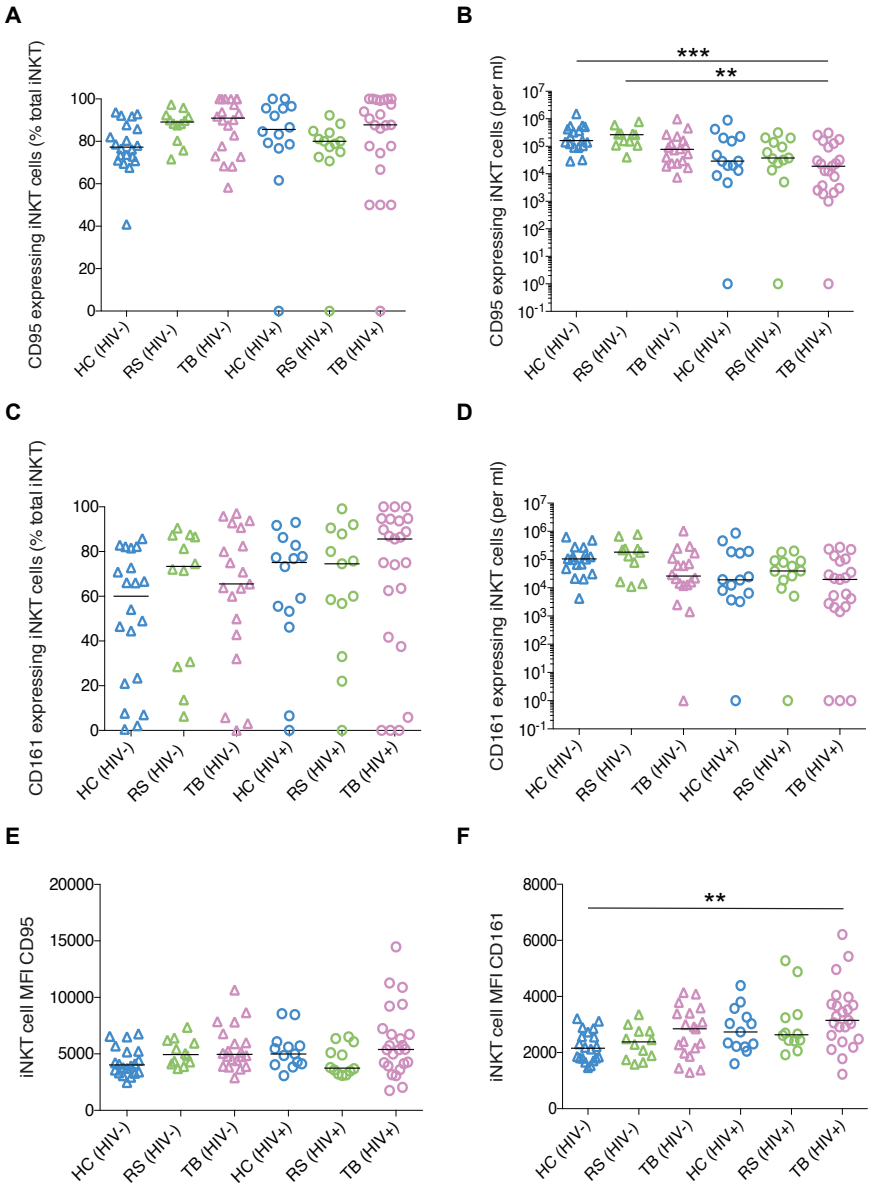



**Figure 3-17 INKT cell expression of activation receptors CD95 and CD161**

There was a trend towards higher proportions of iNKT cells expressing of activating receptors CD95 (A and B) and CD161 (C and D) in TB patients than in healthy controls and higher CD95 (E) and CD161 (F) MFI values in TB than in healthy controls in both HIV-infected and HIV-uninfected TB patients, but absolute values were slightly lower than in controls.

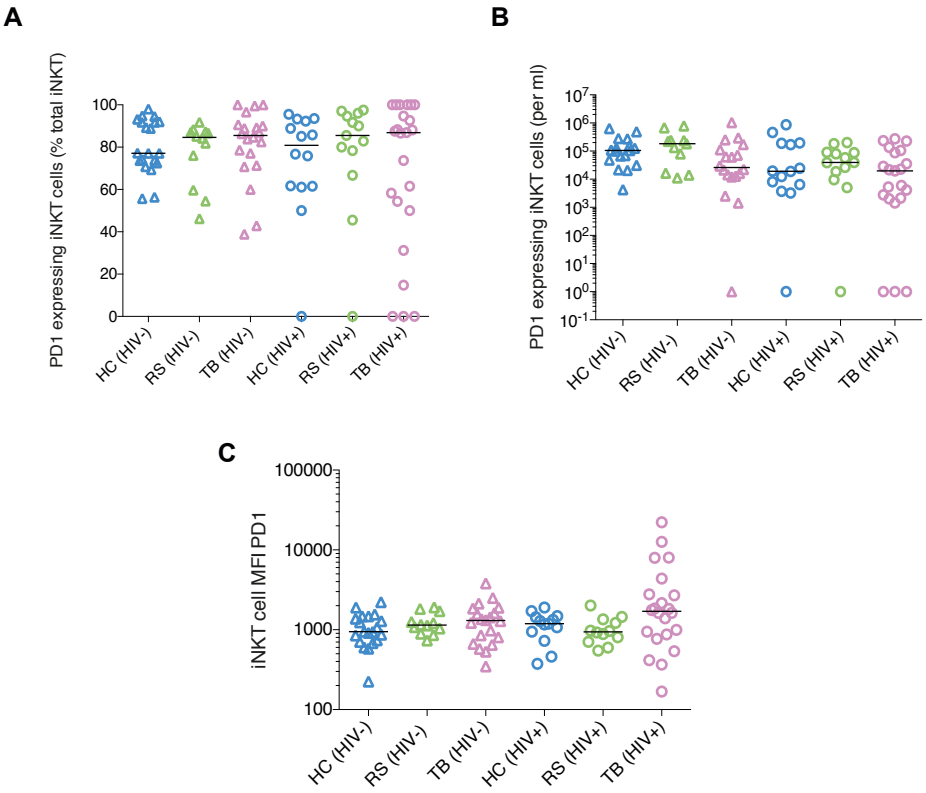



**Figure 3-18 INKT cell PD-1 expression**

PD1 expression was high on iNKT cells in controls and TB patients, with a trend towards higher proportions (A) but not absolute numbers (B) of iNKT cells expressing PD1 in respiratory symptomatics and in TB patients, both in HIV-uninfected and HIV-infected patients, along with a trend towards higher PD1 MFI.

### *iNKT cell frequency and inflammatory markers*

In order to explore a relationship between iNKT cell activity and immune status, I assessed a correlation between peripheral blood CD4 count and percentage, HIV-1 viral load and plasma PIIINP, key cytokines and chemokines in plasma and sputum MMPs (Table 3-10). Peripheral blood CD4 count and percentage, HIV-1 viral load did not correlate with iNKT frequency. There was a negative correlation between iNKT cell frequency and C-reactive protein, PIIINP and a number of cytokines (including IL-1β, TNF-α, IL-12 p40/p70). However, after adjustment for multiple comparisons, these did not reach significance (indicated by $p<0.002$).

To investigate this further, I examined a correlation between CD4+ iNKT cells and these analytes. CD4+ iNKT cells strongly correlated with total CD4 percentage and negatively correlated with HIV-1 viral load. In this comparison several pro-(IFN-α, IFN-γ, IL-6, IL-12 p40/p70, IL-15, MCP1, MIG, IP-10) and anti-inflammatory (IL-1RA, IL-2R) mediators significantly inversely correlated with CD4+ iNKT cell proportions. There was also a trend towards an inverse correlation with C-reactive protein, PIIINP, IL-1β, IL-4, IL-5 and IL-8. Some similarities were found in correlations between these parameters and total peripheral blood CD4 count, but these did not reach significance. In contrast, no correlation was observed between iNKT cell frequency and induced sputum MMP concentrations.

## Table 3-10 Correlation between iNKT cell frequency, HIV status and inflammatory mediators

| Sample | Analyte | iNKT count | | CD4+ iNKT % | | Peripheral blood CD4 % | |
|---|---|---|---|---|---|---|---|
| | | r | p value | r | p value | r | p value |
| Blood | HIV-1 viral load | -0.014 | 0.930 | -0.571 | **0.0001** | -0.546 | **< 0.0001** |
| | CD4 count | 0.003 | 0.982 | -0.179 | 0.463 | 0.070 | 0.738 |
| | CD4% | 0.067 | 0.786 | 0.523 | **< 0.0001** | 1.000 | N/A |
| | C-reactive protein | -0.267 | 0.015 | -0.180 | 0.103 | -0.308 | 0.006 |
| | PIIINP | -0.326 | 0.011 | -0.370 | 0.004 | -0.350 | 0.031 |
| | IL-1B | -0.274 | 0.036 | -0.303 | 0.020 | -0.028 | 0.868 |
| | IL-10 | -0.094 | 0.478 | -0.309 | 0.017 | -0.532 | **0.001** |
| | IFN-α | -0.243 | 0.064 | -0.460 | **0.0002** | -0.392 | 0.014 |
| | IL-6 | -0.247 | 0.059 | -0.445 | **0.0004** | -0.346 | 0.031 |
| | IL-12 | -0.340 | 0.009 | -0.506 | **< 0.0001** | -0.422 | 0.008 |
| | GM-CSF | -0.025 | 0.851 | -0.066 | 0.623 | -0.246 | 0.132 |
| | MCP1 | -0.171 | 0.200 | -0.464 | **0.0002** | -0.467 | 0.003 |
| | IFN-y | -0.088 | 0.506 | -0.338 | 0.009 | -0.274 | 0.092 |
| | TNF-α | -0.337 | 0.009 | -0.284 | 0.030 | -0.235 | 0.149 |
| | IL1-RA | -0.031 | 0.818 | -0.418 | **0.001** | -0.141 | 0.391 |
| | IL-2 | -0.241 | 0.066 | -0.277 | 0.034 | -0.301 | 0.063 |
| | IP-10 | -0.293 | 0.024 | -0.523 | **< 0.0001** | -0.385 | 0.016 |
| | IL-2R | -0.198 | 0.132 | -0.420 | **0.001** | -0.332 | 0.039 |
| | MIG | -0.294 | 0.024 | -0.572 | **< 0.0001** | -0.483 | 0.002 |
| | IL-4 | -0.266 | 0.042 | -0.355 | 0.006 | -0.432 | 0.006 |
| | IL-5 | -0.234 | 0.075 | -0.351 | 0.006 | -0.348 | 0.030 |
| | IL15 | -0.205 | 0.122 | -0.462 | **0.0003** | -0.455 | 0.004 |
| | IL-8 | 0.119 | 0.381 | -0.360 | 0.007 | -0.095 | 0.613 |
| Sputum | MMP-1 | 0.010 | 0.926 | -0.136 | 0.202 | -0.133 | 0.243 |
| | MMP-2 | 0.038 | 0.724 | -0.058 | 0.590 | 0.105 | 0.358 |
| | MMP-3 | 0.024 | 0.823 | -0.181 | 0.088 | -0.128 | 0.262 |
| | MMP-7 | -0.115 | 0.283 | -0.162 | 0.128 | -0.176 | 0.121 |
| | MMP-8 | 0.028 | 0.792 | -0.206 | 0.052 | -0.156 | 0.170 |
| | MMP-9 | 0.052 | 0.626 | -0.166 | 0.119 | 0.111 | 0.329 |
| | MMP-10 | 0.000 | 0.998 | -0.180 | 0.089 | -0.150 | 0.188 |

## Discussion

### Summary of main findings of cross-sectional study

In the cross-sectional study, I aimed to characterize clinical and immunological markers of inflammation and tissue damage at TB diagnosis, in HIV-infected and -uninfected TB patients, respiratory symptomatic and healthy controls. Clinical presentation differed between HIV-infected and -uninfected TB patients. Night sweats were the predominant symptom in HIV-associated TB, whereas in HIV-uninfected TB patients the most common presentation was productive cough. Extra-pulmonary features of TB were more common at presentation in HIV-infected TB patients. These patients had lower Hb and albumin concentrations, than HIV-uninfected TB patients and HIV-infected controls, suggesting possibly either disseminated TB, or more advanced HIV-infection. HIV-1 viral loads were higher in TB-HIV co-infection than in HIV-infected healthy controls. However, CD4 counts were similar. This is consistent with the hypothesis that TB infection amplifies HIV replication, potentially by driving greater NF-κβ activation (74). HIV-infected TB patients had lower total white cell counts, neutrophil counts and monocyte counts than HIV-uninfected TB patients, although proportionally neutrophils were elevated compared to control patients both in HIV-infected and -uninfected TB patients, and were the dominant leucocyte population.

TB in HIV-infected patients was characterized radiographically by fewer cavities and more commonly a miliary appearance, compared to TB in HIV-uninfected patients. Typically, HIV-infected TB patients were smear-negative for AFB on microscopy and time to sputum culture positivity was longer than in HIV-

uninfected patients. Together, this is consistent with the reported observations of reduced pulmonary tissue destruction and reduced M.tb. bacterial load in sputum, in advanced HIV-induced immunosuppression (301, 302).

TB was associated with increased MMPs in induced sputum, particularly MMP-1, -3 and -8, which were increased in comparison to healthy controls and respiratory symptomatics, and were increased in HIV-infected TB patients compared to HIV-uninfected healthy controls. In addition, these MMPs strongly correlated with chest x-ray inflammation score. Induced sputum MMP-1, -3 and -8 also correlated with plasma PIIINP, a peripheral marker of matrix turnover, which was elevated in TB patients compared to controls. In contrast to induced sputum MMP results, plasma PIIINP was more prominently elevated in HIV-infected patients in comparison to controls, than in HIV-uninfected patients compared to controls.

Elevated sputum cytokines in HIV-uninfected TB patients, including IL-1β and TNF-α, suggest a local, antigen-driven, pro-inflammatory innate immune response, that may drive or be driven by MMP production (185). The absence of these cytokines in induced sputum of HIV-infected TB patients suggests either the absence of such immune responses in the lung, or that these immune responses are not in communication with airways. This may occur if there is less matrix destruction in the lung in HIV-associated TB. The elevation of plasma cytokines and chemokines in HIV-associated TB, in comparison to HIV-uninfected TB, does not support down-regulation of immune responses, but rather implies that the lung is not the focal point of inflammation. The immune

response may be systemic, not focal, due to disseminated rather than localized antigen. This fits with the clinical picture of less frequent smear positivity and fewer cavities, but more extra-pulmonary and disseminated TB in HIV-infected TB patients. Elevated PIIINP in plasma suggests that there is systemic inflammation and matrix turnover in HIV-associated TB, whilst in TB in HIV-uninfected patients, matrix turnover is localized to the pulmonary compartment.

Finally, I have demonstrated reduced iNKT cell populations in TB and HIV infected patients and in TB-HIV co-infection, compared to control patients. This reduction is most marked in HIV infection, where there is a pronounced reduction in iNKT cell proportions and a highly significant absolute reduction in iNKT cells per milliliter of peripheral blood. This reduction most substantially affected the CD4+ iNKT cell population, which is particularly depleted in TB-HIV co-infection. This suggests a direct effect of HIV virus on CD4+ iNKT cells, which may be productively infected and destroyed by HIV (149). Increased CD95+ and CD161+ iNKT cells in TB-HIV co-infection, suggest increased activation of these cells, which may increase their susceptibility to HIV-1 infection (149).

### Relationship to other studies

My study confirms previous reports that the presentation of active TB in HIV-infected patients is atypical and is dominated by smear-negative and extra-pulmonary disease, with a preponderance of atypical chest x-ray features. Chronic cough, a cardinal symptom of TB in HIV-uninfected patients, is a less frequent symptom in HIV infection (303). Clinical screening tools for TB, such as the WHO algorithm, that rely on chronic cough and smear positivity are less

sensitive in HIV-infected patients. Additionally, a considerable burden of asymptomatic TB has been identified in HIV-infected patients in high TB burden settings on active case finding, although the clinical significance of this is unclear (25, 26).

In one report, from Zimbabwe, 26% HIV-infected patients with TB had no radiographic abnormalities on chest x-ray (301). This was higher than the proportion of patients in my study, although the Zimbabwean cohort may have had more advanced immunosuppression. In the Zimbabwean study, in TB-HIV co-infection, reduced proportions of patients had cavitation and an increased number had miliary TB, compared to HIV-uninfected TB patients. However, the proportion of patients classified as having a moderate or severe degree of lung pathology on chest radiograph was similar (301).

Challenges in microbiological diagnosis in HIV-infected TB are not due to absence of the organism. A post-mortem study of 94 TB-HIV co-infected patients with advanced HIV revealed that HIV-associated TB was predominantly multibacillary, with highly dense AFB, but non-reactive, with granular necrosis, ill-formed or absent granulomas and scanty or absent giant and epithelioid cells (46). Rather, diagnostic difficulty in advanced HIV is mainly attributable to our reliance on detection of AFB in the airways, which occurs as a consequence of cavitary matrix-degrading immunopathology in TB, which is less common in advanced HIV.

This study supports my previous work showing that MMPs are upregulated in sputum of both HIV-infected and uninfected patients. In my previous study, I investigated patients who had CD4 counts less than 200 cells/mm$^3$ and found lower sputum MMP concentrations than in HIV-uninfected TB patients. In my current study, there was a trend towards lower sputum MMPs in HIV-infected patients but this was not significant, and no inverse correlation was found between CD4 count and MMP-1. My current study included patients with higher CD4 counts than in the previous study. TB-HIV co-infected patients with relatively preserved CD4 counts presented with a wide spectrum of inflammatory pathology, suggesting that HIV infection alone does not inhibit matrix degradation in TB. HIV-1 viral load correlated positively with sputum MMP-1, -7 and -8 and was higher in TB-HIV co-infected patients than HIV-infected controls.

The relationship between immune status and MMP upregulation is complex. It is unclear whether HIV has an effect on MMP production that is independent of TB disease status, although *in vivo* work supports this theory (61). Here, I show that sputum MMPs correlate with chest x-ray inflammation score and associate with cavitation, similarly to our previous study. The median CD4 count in TB-HIV co-infected patients in the current study was relatively preserved, at 172 cells/mm$^3$. CD4 count may drop acutely with active TB and these patients may have had higher CD4 counts prior to TB diagnosis, so low CD4 count at TB diagnosis may not be a reliable indicator of severe chronic immune suppression.

The finding of increased neutrophil proportions in TB and TB-HIV co-infection adds to the body of literature suggesting that neutrophils play a role in TB immunopathology. Neutrophil activity in TB has been associated both with increased severity of disease and with protection (72, 73). Neutrophils may play a protective role early in infection and a pathological role as disease progresses (304). In the UK, a study of 1236 TB patients in comparison to 214 contacts demonstrated neutrophilia to be associated with active TB at diagnosis. In that study a neutrophil count greater or equal to 7.5 cells $x10^9$/L was associated with a three-fold risk of death from TB (304). In a previous study of HIV-associated TB, neutrophils were elevated in sputum and were increased with patients with higher sputum mycobacterial load (305). Neutrophils are a source of MMP-8 (neutrophil collagenase). A recent study examining the role of neutrophil-derived MMP-8 in TB found MMP-8 and MMP-9 co-localising with neutrophils in lung biopsies from TB patients. In the same study, MMP-8 in induced sputum in TB patients correlated with neutrophil markers and with clinical and radiological disease severity in pulmonary TB patients (306). I did not measure sputum neutrophil counts in my study. However, together these results suggest that increased neutrophils are a source of elevated MMP-8 in TB and TB-HIV co-infection.

MDPs have not been extensively studied in TB. In the only prior analysis, we reported elevated induced sputum and plasma PIIINP concentrations in TB patients compared to controls in two relatively small cohorts. In a comparison including patients of mixed HIV serostatus, plasma PIIINP was 3-fold higher in TB patients than controls. In the current analysis, plasma PIIINP concentrations

were 2.3-fold higher in HIV-infected TB patients than HIV-uninfected TB patients. Elevated plasma PIIINP has been demonstrated in inflammatory (Acute Respiratory Distress Syndrome, ARDS) granulomatous (sarcoid) and fibrotic (fibrosing alveolitis) lung conditions and is modulated was modulated by corticosteroid therapy in the treatment of ARDS (201-203). Plasma concentrations of PIIINP in TB patients in this study were of similar magnitude to those detected in patients requiring ventilation for ARDS (201). Elevated plasma PIIINP in HIV-infected TB patients, in the absence of extensive lung destruction, suggests that there is a global systemic immune response causing matrix destruction.

Similarly to previous studies, my study demonstrates profound depletion of iNKT cells in HIV, particularly affecting the CD4+ CD8- subset (145). This subset has been shown to be the most susceptible to infection and depletion by HIV-1 virus (149). My findings support previous studies of human TB that have shown decreased iNKT cell proportions in HIV-uninfected patients with pulmonary TB and extra-pulmonary TB (145-147, 149). In one study, increased PD1 expression after cell culture with α-galcer supported a role for functional anergy of iNKT cells in TB (147). In my analysis, PD-1 expression and MFI was slightly increased in TB patients compared to controls, although this did not reach significance. In the same study, iNKT cell frequencies inversely correlated with C-reactive protein. This was similar to the finding of an inverse correlation between C-reactive protein and iNKT cell count in my study. Although no prior studies have examined iNKT cells in TB-HIV co-infection, a previous study found a synergistic effect of HIV and *M. leprae* on iNKT reduction and I have shown a similar trend,

although the dominant effect in TB-HIV co-infection appears to be due to HIV (152). This is supported by the inverse correlation between iNKT cell frequency and HIV-1 viral load.

### Strengths and Limitations

A strength of the study is the inclusion of both healthy and respiratory symptomatic controls. My results suggest that abnormalities identified in TB patients were specific to TB rather than cause by an a non-specific infective or inflammatory process. Respiratory symptomatic patients were similar to TB patients in terms of presenting symptoms and were largely patients attending clinic for TB screening. However, the respiratory symptomatic control group was more similar to healthy controls in clinical examination findings, peripheral blood inflammatory markers and suggesting that inflammation was relatively mild compared to in TB patients. In future studies, it will be important to include a control group of patients with non-TB severe respiratory illness, e.g. patients with acute bacterial pneumonia, to control for severity of inflammation.

A limitation of the study is that the HIV-infected and uninfected groups were not matched for sex and smoking status, and a preponderance of females in the HIV-infected groups and smokers in the HIV-uninfected groups may have affected results. In previous studies, both gender and smoking status have been linked to differential MMP activity (307-309). However, I think this is unlikely to have significantly affected my results. MMP-1 concentrations in sputum did not significantly differ between men and women and between smokers and non-smokers. The relationship between smoking status, HIV and MMPs is complex. In

one study, an effect of smoking on MMP gene expression occurred predominantly in HIV-infected rather than -uninfected patients (309). This is an area for further work and in future analyses, stratification by smoking status and by sex, will help me to identify or eliminate an effect of smoking on MMP concentrations in sputum and blood.

The use of sputum induction was highly successful to obtain sputum samples and the quality and abundance of these samples enabled microbiological confirmation of TB on a high proportion of TB patients, in addition to sampling the site of disease for immunological analysis. However, induced sputum may not always completely reflect the immunological environment at the site of disease, especially in patients with extra-pulmonary TB. MMPs may be rapidly inactivated by the TIMPs. I did not measure TIMPS in this analysis. Several prior analyses have demonstrated that TIMPS are not upregulated in TB infection, leading to a matrix degrading phenotype. However, quantification of TIMPS in HIV-associated TB is an area for future work.

The study was one of the largest of its kind, but even so the sample size was limited, particularly for the iNKT analysis, due to LPS contamination (at manufacture) of sodium heparin tubes used to collect blood. The study was not powered well for subgroup analyses requiring adjustment for multiple comparisons. Whilst the null hypothesis cannot be rejected on the basis of results that do not meet the threshold of significance, the alternative hypothesis cannot be rejected on this basis either, where the sample size is small.

## Future work

The results so far showing elevated matrix turnover in TB and TB-HIV co-infection and correlations between matrix turnover and TB immunopathology strongly support an interventional study of MMP inhibition in humans as an adjunct to TB treatment. Attempts to reduce the duration of TB therapy with antimycobacterial agents have not been successful (310, 311). However, cellular and animal studies support the addition of MMP inhibitors (195, 306). The potential benefits of MMP inhibition in TB include reduction in long-term respiratory morbidity and reduced transmissibility. Candidate MMP inhibitors include doxycycline and Ro32-3555. I have previously shown that doxycycline, which directly inhibits MMP-1 upregulation in TB, also has antimycobaterial activity (195). Many other agents, including vitamin D and other antibiotics, modulate MMP activity and could potentially be used for this purpose (312, 313).

Diagnosis of TB can be extremely challenging in patients with advanced HIV, due to factors demonstrated in this study such as low smear positivity and minimal chest x-ray findings. Whilst evidence of TB at the site of primary infection in the lung may be scarce, plasma PIIINP is significantly elevated in TB-HIV co-infection and correlates with markers of immunopathology. This suggests that plasma PIIINP could be useful in TB diagnosis.

Subgroup analyses of patients by high and low CD4 count and by chest x-ray inflammation score may allow dissection of the relationship between HIV-infection and MMPs. Additionally, as I have measured a large number of variables, I plan to perform principal component analysis to reduce the

dimensionality of the data and to identify the principal components along which variation of the data is maximal. In principal component analysis, samples are plotted along the axis of greatest variance (principal components)(314). This may help me to generate hypotheses of causality and identify variables that could facilitate TB diagnosis.

## Conclusions

This study builds on my previous work, demonstrating a consistent pattern of MMP upregulation in TB and HIV-associated TB. Additionally, it supports previous studies showing reduced iNKT cell populations in TB and HIV infection and provides the first report of reduced iNKT cells in TB-HIV co-infection. Causal relationships cannot be inferred by an observational study. However, a longitudinal analysis may provide stronger evidence for causality than a cross-sectional analysis and this will be the subject of my next chapter.

# Chapter 4 Longitudinal study of TB-IRIS and non-IRIS patients

## Introduction

In the longitudinal study, I aimed to characterize inflammation and inflammatory mediators at the time of TB diagnosis (TB0), at ART initiation (ARV0) and subsequently over the first twelve weeks of ART. By comparing patients who developed symptoms of TB-IRIS during this period with patients who did not develop TB-IRIS (non-IRIS controls), I hoped to identify inflammatory mediators associated with immunopathology. The longitudinal study design enabled me to describe the immunological profile at the time of IRIS onset and to identify possible effectors of tissue damage. In addition, I could analyse features prior to the onset of TB-IRIS symptoms that might indicate factors that predispose to the development of paradoxical TB-IRIS. I measured secreted MMPs and pro-and anti-inflammatory cytokines and chemokines, in induced sputum and plasma. I also enumerated and characterized iNKT cells in peripheral blood, cytotoxic cells which are a potential source of pro-inflammatory cytokines and a cause of cell death.

## Results

### Clinical characteristics

In total, 57 patients were enrolled in the cross-sectional study, summarised in Figure 4-1. Prior to analysis, 10 patients were excluded, including one HIV-infected patient with an undetectable viral load on repeated samples, who was likely to be an elite controller and therefore immunologically different. A second patient who had a hepatotoxic reaction to antituberculosis therapy, and was not

commenced on ART until five months after antituberculosis therapy initiation, was excluded. These two patients were followed up to three months post-ART initiation and neither developed TB-IRIS. Eight patients were excluded due to loss to follow up or ineligibility and were not followed up to study completion. Of the 49 patients followed up to study completion, 29 developed TB-IRIS, according to the review panel consensus, giving a study TB-IRIS incidence of 59.2%. INSHI criteria were met in 25 of these cases. Four cases were designated likely TB-IRIS but did not meet INSHI criteria. Diagnostic criteria for the TB-IRIS patients are reported in Appendix 3.

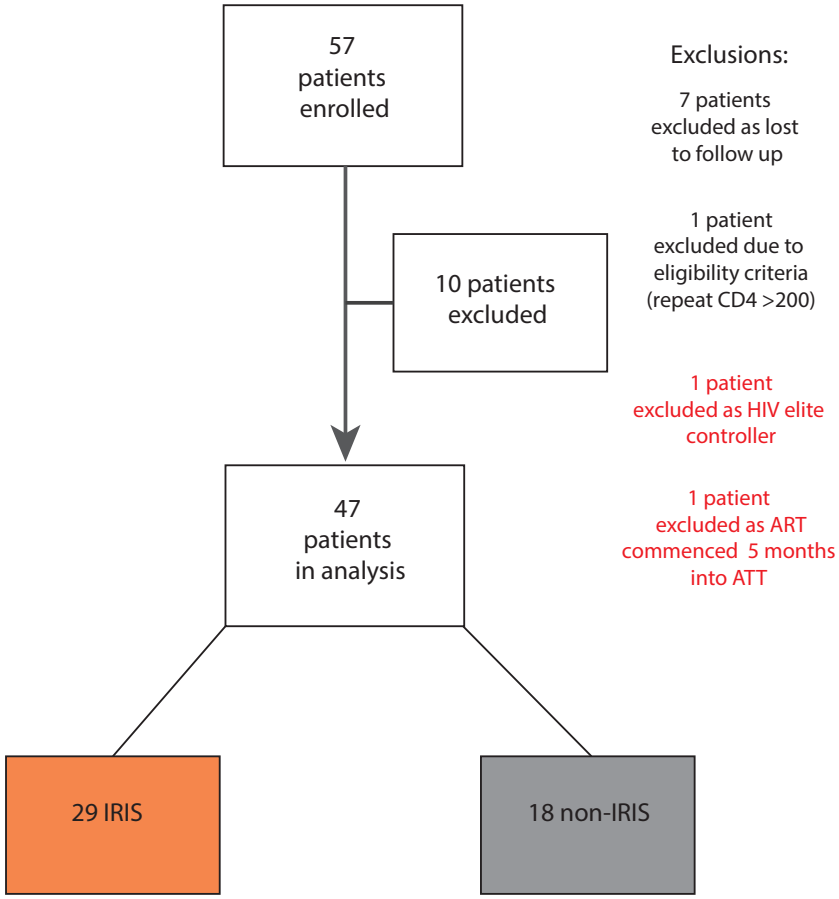



**Figure 4-1 Patient recruitment to TDTB2 Study longitudinal cohort**

Recruitment and exclusion of participants to the study cohort is summarised. The study cohort comprised 47 patients of which 29 developed paradoxical TB-IRIS, and 18 patients, who did not develop paradoxical TB-IRIS, who were designated non-IRIS controls.

The subsequent analysis describes the results for 47 patients, summarised in Figure 4-1, comprising 29 TB-IRIS patients and 18 non-IRIS controls. The majority (33/47) had resided in Khayelitsha for more than ten years. Four patients had a history of prior TB (all TB-IRIS patients). A history of BCG vaccination was reported (or evident due to BCG scar) in 17 TB-IRIS patients and 9 non-IRIS controls (p=0.763). Additional demographic information is provided in Table 4-1.

**Table 4-1 Demographic and clinical characteristics of longitudinal study participants**

| | Diagnostic category | | |
|---|---|---|---|
| | non-IRIS | IRIS | p value |
| Frequency, n | 18 | 29 | N/A |
| Median age, years (IQR) | 36 (30.8-43) | 35 (29.5-42) | 0.867 |
| Female, n (%) | 10 (56) | 14 (48) | 0.766 |
| Smoking history, current or ex-, n (%) | 4 (22) | 9 (31) | 0.739 |
| BCG vaccination, n (%) | 9 (50) | 17 (58.6) | 0.763 |
| Median days of TB treatment to ART initiation (IQR) | 21.5 (13.8-39.8) | 15 (14-28) | 0.127 |

Study visits and sample yields are shown in Figure 4-2. 24 (51%) patients were seen at or shortly after TB diagnosis (TB0). The remaining patients were recruited at ART initiation (ARV0). Induced sputum samples were not obtained at every visit. Induced sputum was not attempted on patients who were in respiratory distress, those who had wheeze on auscultation, had a possible pneumothorax or were hospitalized. Very rarely was a patient unable to produce sputum on induction. Patients missed scheduled visits mainly due to hospitalization or travel to visit family outside the Western Cape (common over the Christmas holiday period). Whenever possible, patients were visited during hospital admission and study blood samples were collected. Symptoms reported at TB diagnosis and during scheduled follow up visits are reported in Table 4-2.

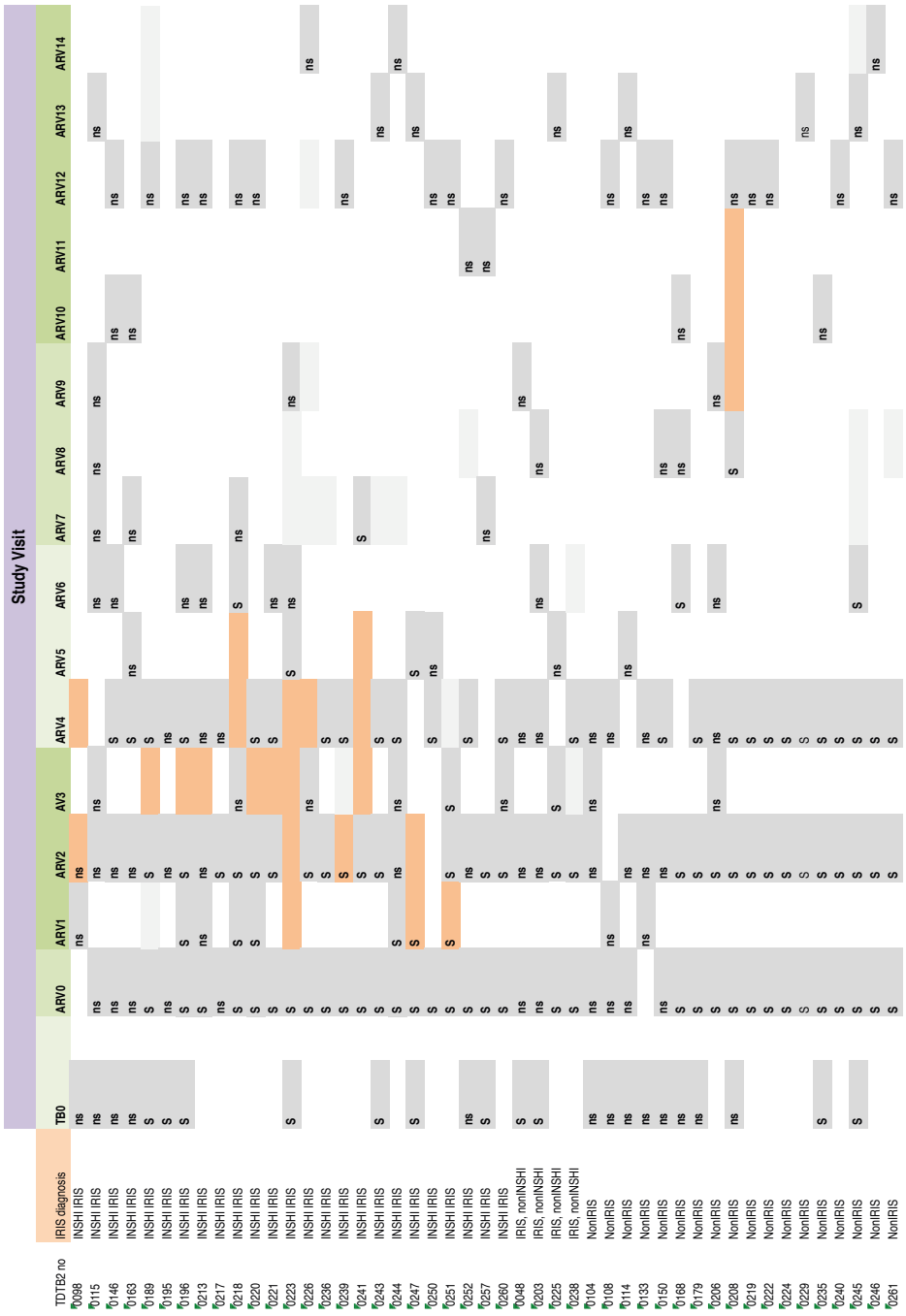



**Figure 4-2 Study visit log**

Study visits shaded dark grey were either scheduled (TB0, ARV0, ARV2, ARV4, ARV12) or unscheduled (ARV1, ARV3, ARV5-11, ARV13, ARV14). Unscheduled visits occurred if patients became unwell or if patients missed appointments and attended more than 3 days either side of their scheduled appointment. Periods of hospital admission are recorded in orange. Blood sample collection and availability for analysis is indicated (ns=no sample available; S=sample availability). Sputum sampling is not shown. Light grey boxes indicate visits for clinical review.

The median time to TB-IRIS symptom onset was six days (IQR 3.5-9.5, range 1-23) post-ART initiation and patients presented at a median of 14 days (IQR 9-15, range 4-29). Clinical features of TB-IRIS presentations are reported in Table 4-3 (and Appendix 3). The predominant features were constitutional (n=29, 100%) and pulmonary (n=27, 93.1%). At ARV2, 10 TB-IRIS patients had fever, compared to six TB-IRIS patients at ARV0 and one non-IRIS control at ARV2. Weight loss was a prominent symptom in TB-IRIS patients and was more commonly reported in TB-IRIS patients than non-IRIS controls at TB diagnosis (p=0.02). Hospital admission was required in 13 TB-IRIS (44.8%) patients compared to one (5.6%) non-IRIS control. There were no deaths in study patients during follow up.

## Table 4-2 Symptom profile of longitudinal study cohort

| Symptom | Diagnostic category and sample timepoint | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | | ARV0 | | | ARV2 | | | ARV4 | | |
| | non-IRIS | IRIS | p value | non-IRIS | IRIS | p value | non-IRIS | IRIS | p value | non-IRIS | IRIS | p value |
| Frequency, n | 10 | 14 | N/A | 17 | 28 | N/A | 17 | 26 | | 16 | 20 | |
| Fever, n (%) | 5 (50) | 6 (42.9) | 1 | 3 (17.6) | 6 (21.4) | 1 | 1 (5.9) | 10 (38.5) | **0.03** | 0 (0) | 6 (30) | **0.024** |
| Productive cough, n (%) | 4 (40) | 7 (50) | 0.697 | 3 (17.6) | 6 (21.4) | 1 | 2 (11.8) | 8 (30.8) | 0.269 | 2 (12.5) | 5 (25) | 0.426 |
| Non-productive cough, n (%) | 6 (60) | 5 (35.7) | 0.408 | 8 (47.1) | 6 (21.4) | 0.757 | 8 (47.1) | 15 (57.7) | 0.545 | 6 (37.5) | 6 (30) | 0.73 |
| Haemoptysis, n (%) | 0 (0) | 0 (0) | N/A | 0 (0) | 1 (3.6) | 1 | 0 (0) | 1 (3.9) | 1 | 0 (0) | 0 (0) | 1 |
| Night sweats, n (%) | 8 (80) | 13 (92.9) | 0.55 | 7 (41.2) | 7 (25) | 0.326 | 6 (35.3) | 4 (15.4) | 0.158 | 4 (25) | 6 (30) | 1 |
| Weight Loss, n (%) | 6 (60) | 13 (92.9) | **0.02** | 9 (52.9) | 13 (46.4) | 0.763 | 5 (29.4) | 16 (61.5) | 0.062 | 3 (18.8) | 8 (40) | 0.277 |
| Shortness of breath, n (%) | 3 (30) | 8 (57.1) | 0.24 | 5 (29.4) | 6 (21.4) | 0.722 | 3 (17.6) | 11 (42.3) | 0.112 | 3 (18.8) | 6 (30) | 0.7 |
| Pleuritic chest pain, n (%) | 5 (50) | 7 (50) | 1 | 2 (11.8) | 6 (21.4) | 0.69 | 1 (5.9) | 7 (26.9) | 0.119 | 1 (6.25) | 2 (10) | 1 |
| Symptomatic, any, n (%) | 10 (100) | 14 (100) | N/A | 16 (94.1) | 26 (93.9) | 1 | 14 (82.4) | 26 (100) | 0.055 | 15 (93.8) | 16 (80) | 0.355 |

**Table 4-3 Clinical features of TB-IRIS presentation**

| Presentation | n | % | Examples |
|---|---|---|---|
| Pulmonary | 27 | 93.1 | Shortness of breath, pleural effusion, worsening radiological features |
| Constitutional | 29 | 100 | Fever, night sweats, weight loss |
| Gastrointestinal | 19 | 65.5 | Abdominal pain, intra-abdominal lymphadenopathy, hepatomegaly |
| Neurological | 10 | 34.5 | Headache, seizure, meningitis |
| Cardiac | 1 | 3.4 | Myocarditis, pericardial effusion |
| Nodal | 8 | 27.6 | Tender, fluctuant lymphadenopathy |
| Dermatological | 4 | 13.8 | Rash |
| Other | 11 | 3.4 | Inflammatory arthritis |

Corticosteroid therapy was administered to 18 (62.1%) TB-IRIS patients during the follow-up period. In one patient, corticosteroids were commenced prior to ART initiation for CNS tuberculosis. Corticosteroid therapy was prescribed for TB-IRIS in the remaining 17 patients. Four (22.2%) non-IRIS patients received prednisone therapy. In one case, this was for suspected TB-IRIS that was not confirmed on consensus case review. In one case it was for HIV-related facial nerve palsy, present at TB diagnosis, in another for TB pericarditis at TB diagnosis and in one case the indication was not clearly documented.

Clinical variables on examination are reported in Table 4-4. TB-IRIS patients had higher heart rates ($p=0.0002$) and respiratory rates ($p<0.0001$), than non-IRIS controls (Figure 4-3A and 4-3B). This trend was present at TB diagnosis, and for the first four weeks following ART initiation. Median respiratory rate at TB0 was 20 breaths per minute (IQR 18-22.6) in non-IRIS controls compared to 27 (IQR 20-28) in TB-IRIS patients. Recorded temperature was slightly higher in TB-IRIS patients, but the difference was less marked than for heart rate and respiratory rate (Figure 4-3C). Median BMIs were similar between the two groups (see Figure 4-3D).

## Table 4-4 Clinical features on examination of longitudinal study cohort

| Clinical variable | Diagnostic category and sample timepoint | | | | | | | | | | | | Two-way ANOVA | | | Sidak's multiple comparisons test (non-IRIS vs IRIS) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | | ARV0 | | | ARV2 | | | ARV4 | | | | | | | | | |
| | non-IRIS | IRIS | p value | non-IRIS | IRIS | p value | non-IRIS | IRIS | p value | non-IRIS | IRIS | p value | Interaction | Timepoint | Diagnosis | TB0 | ARV0 | ARV2 | ARV4 |
| Median BMI | 23.1 (20.7-24.4) | 21.3 (20.5-23.6) | 0.474 | 20.2 (17.9-23.1) | 20.7 (19.2-24.3) | 0.573 | 21.7 (18.3-25.6) | 19.4 (18.7-23.1) | 0.784 | 21.4 (18.5-25.1) | 20 (19.3-23.5) | 0.972 | 0.868 | 0.502 | 0.649 | | | | |
| Mean temperature, oC (SD) | 36.5 (36-37.1) | 36.6 (35.3-37) | 0.875 | 35.8 (35.4-36.8) | 36.2 (35.7-36.4) | 0.568 | 36.1 (35.5-36.4) | 36.4 (35.9-37) | 0.076 | 36 (35.6-36.4) | 36.2 (35.7-36.7) | 0.226 | 0.414 | 0.347 | 0.196 | | | | |
| Median heart rate, beats per minute (IQR) | 79.5 (75.8-92.5) | 94 (86-110) | **0.025** | 88 (77-111) | 100 (92-112) | 0.179 | 97.5 (89-117) | 112 (97.5-120) | 0.074 | 90.5 (80-104) | 96.5 (89.5-108) | 0.127 | 0.706 | **0.005** | **0.0002** | ns | ns | ns | ns |
| Median systolic blood pressure (mmHg) | 120 (116-123) | 110 (108-125) | 0.213 | 110 (100-114) | 119 (107-131) | **0.014** | 116 (109-125) | 110 (101-127) | 0.423 | 120 (111-125) | 113 (100-132) | 0.486 | **0.031** | 0.636 | 0.852 | | | | |
| Median diastolic blood pressure (mmHg) | 76.5 (68.8-88.5) | 78 (68.8-81.3) | 0.851 | 70 (62.5-81) | 77 (70-87) | **0.049** | 74 (70-82.8) | 70 (69-80) | 0.404 | 74 (69-80.8) | 73 (62-82) | 0.915 | 0.257 | 0.809 | 0.57 | | | | |
| Median respiratory rate, breaths per minute (IQR) | 20 (18-22.5) | 27 (20-28) | **0.025** | 20 (18-22) | 22 (20-24) | 0.11 | 20 (16-24.8) | 24 (20.5-26.8) | **0.043** | 18 (17.3-22) | 20 (18-24) | 0.137 | 0.804 | 0.249 | **<0.0001** | ns | ns | ns | ns |
| Median O2 Saturations, % (IQR) | 98.5 (98-99) | 99 (93.5-100) | 0.905 | 99 (97-99.5) | 99 (98-100) | 0.656 | 99 (98.5-99) | 99 (99-99.3) | 0.591 | 99 (99-99.3) | 99 (97.5-100) | 0.715 | 0.652 | 0.415 | 0.402 | | | | |
| Median Haemoglobin concentration, g/dl (IQR) | 11.4 (8.98-12.6) | 8.9 (7.95-10.9) | 0.119 | 11 (8.95-13.1) | 10.5 (9.7-11.2) | 0.295 | 11.2 (9.83-12.6) | 9.6 (8.68-11.2) | 0.089 | 11 (9.5-13.3) | 10.3 (8.9-12.1) | 0.113 | 0.795 | 0.141 | **0.008** | ns | ns | ns | ns |
| Median total white cell count, x109/L (IQR) | 4.15 (3.61-6.2) | 4.3 (3.05-5.02) | >0.999 | 5.6 (2.85-6.35) | 3.8 (2.8-5.2) | 0.201 | 6 (3.73-7.68) | 6.15 (5.35-8.38) | 0.397 | 5.8 (3.9-8.4) | 6.5 (4.4-7.7) | 0.939 | 0.515 | **0.0007** | 0.885 | | | | |
| Median neutrophil count (IQR) | 2.56 (1.82-3.57) | 2.79 (2.07-3.86) | 0.473 | 2.9 (1.5-4.15) | 2.45 (1.58-4) | 0.735 | 3.35 (2-5.48) | 5 (3.2-7.08) | **0.023** | 3.3 (1.5-6.6) | 4.45 (3.13-5.38) | 0.48 | 0.392 | **0.002** | 0.075 | | | | |
| Median neutrophil % (IQR) | 61.6 (47.1-72.9) | 69.7 (67.6-83) | **0.025** | 54.8 (44.5-71.3) | 68.7 (58.4-73.3) | 0.12 | 61.8 (46.9-70.4) | 78 (71.3-85.4) | **0.0009** | 59.8 (40.6-74.9) | 71.5 (64.5-77.8) | 0.056 | 0.435 | 0.143 | **<0.0001** | ns | ns | ** | ** |
| Median lymphocyte count (IQR) | 1.08 (0.78-1.44) | 0.59 (0.485-0.885) | **0.012** | 1.1 (0.85-1.55) | 0.7 (0.5-1.3) | **0.035** | 1.2 (0.75-1.5) | 0.7 (0.45-1.0) | **0.008** | 1.4 (0.8-1.65) | 0.75 (0.58-1.3) | 0.106 | <0.0001 | <0.0001 | 0.799 | | | | |
| Median lymphocyte % (IQR) | 25.2 (17.6-36.7) | 17.2 (10.6-20.7) | **0.012** | 28.6 (7.2-39.8) | 18.5 (15.1-27.9) | 0.123 | 20.8 (14.3-38.3) | 11.1 (8.08-13.4) | **0.0007** | 21.2 (10.9-31.8) | 16.7 (12.4-18.6) | 0.329 | 0.135 | **0.005** | **0.001** | ns | ns | ** | ns |
| Median monocyte count (IQR) | 0.25 (0.175-0.39) | 0.32 (0.263-0.415) | 0.347 | 0.2 (0.2-0.3) | 0.3 (0.25-0.4) | 0.154 | 0.3 (0.2-0.4) | 0.4 (0.3-0.6) | **0.038** | 0.3 (0.1-0.4) | 0.4 (0.3-0.5) | 0.193 | 0.114 | 0.337 | 0.206 | | | | |
| Median monocyte % (IQR) | 7.05 (6.15-8.4) | 6.8 (4.52-8.4) | 0.684 | 5.4 (3.5-6.1) | 7.7 (6.3-8.4) | **0.006** | 5.3 (4-6.5) | 5.3 (4-7.7) | 0.618 | 4.1 (3.5-5.25) | 6.1 (5-7.1) | 0.022 | 0.414 | 0.504 | **0.018** | ns | * | ns | ns |
| Median platelet count (IQR) | 275 (245-463) | 363 (263-469) | 0.455 | 283 (212-484) | 328 (227-455) | 0.571 | 326 (282-626) | 423 (283-615) | 0.551 | 403 (321-504) | 501 (351-555) | 0.425 | 0.984 | 0.158 | 0.578 | | | | |
| Median C-reactive protein (IQR)* | 17.1 (10.7-73.6) | 72.9 (17.9-89.4) | **0.094** | 5.4 (2-35.7 | 14.8 (5.9-35.5) | 0.071 | 33.1 (7.15-48.2) | 80.1 (39.9-127) | **0.001** | 17.4 (4.38-56.8) | 50.7 (30.5-63.4) | **0.032** | 0.773 | **<0.0001** | **<0.0001** | ns | ns | ** | * |
| Median albumin concentration, g/dl (IQR) | 33.5 (32.5-40) | 30 (27.8-32.3) | **0.014** | 35 (32-40.5) | 35 (32-38) | 0.636 | 35 (31-39) | 32 (29-35.8) | 0.161 | 36.5 (31.5-41) | 34 (31-37) | 0.151 | 0.578 | 0.518 | **0.002** | ns | ns | ns | ns |
| Median CD4 count, cells/mm3 (IQR) | 145 (79.5-182) | 95 (63.5-142) | 0.355 | 123 (66.5-215) | 101 (69-160) | 0.77 | 171 (101-352) | 160 (102-218) | 0.434 | 176 (94.5-337) | 206 (122-354) | 0.976 | 0.987 | **0.004** | 0.953 | | | | |
| Median CD4 % (IQR) | 10.2 (8.65-16.1) | 11.9 (9.76-14.5) | 0.584 | 9.99 (6.34-13.1) | 11 (9.18-16.5) | 0.268 | 13.1 (10.2-18.1) | 17.4 (11.3-22.1) | 0.176 | 16.4 (9.38-20) | 21.5 (11.5-28) | 0.069 | 0.822 | **<0.0001** | **0.04** | ns | ns | ns | ns |
| Median HIV viral load, copies/ml (IQR) | 140960 (94095-522253) | 504590 (274751-1430000) | **0.046** | 352397 (140351-857711) | 589221 (169612-1100000) | 0.297 | 1133 (469-1964) | 2174 (734-3118) | **0.045** | 474 (226-749) | 883 (365-2043) | 0.162 | 0.55 | **<0.0001** | **0.032** | ns | ns | ns | ns |

Laboratory parameters of Hb (Figure 4-4A) and albumin concentrations (Figure 4-4B) were lower in TB-IRIS patients than non-IRIS controls, from TB diagnosis for the first four weeks of ART ($p=0.008$, $p=0.002$). Hb concentration was consistently below the normal range in TB-IRIS, with lowest values at TB0. C-reactive protein concentrations were higher in TB-IRIS patients ($p<0.0001$) and varied significantly according to timepoint ($p<0.0001$). Typically, in TB-IRIS patients, C-reactive protein concentration was elevated at TB0, reduced in the first weeks of antituberculosis therapy but then sharply rose after ART initiation. In TB-IRIS patients sampled at ARV1, median C-reactive protein concentration was 142mg/dL (range 44.9-293) compared to 80 (range 2.1-14.8) in patients at ARV0 (Figure 4-5B).

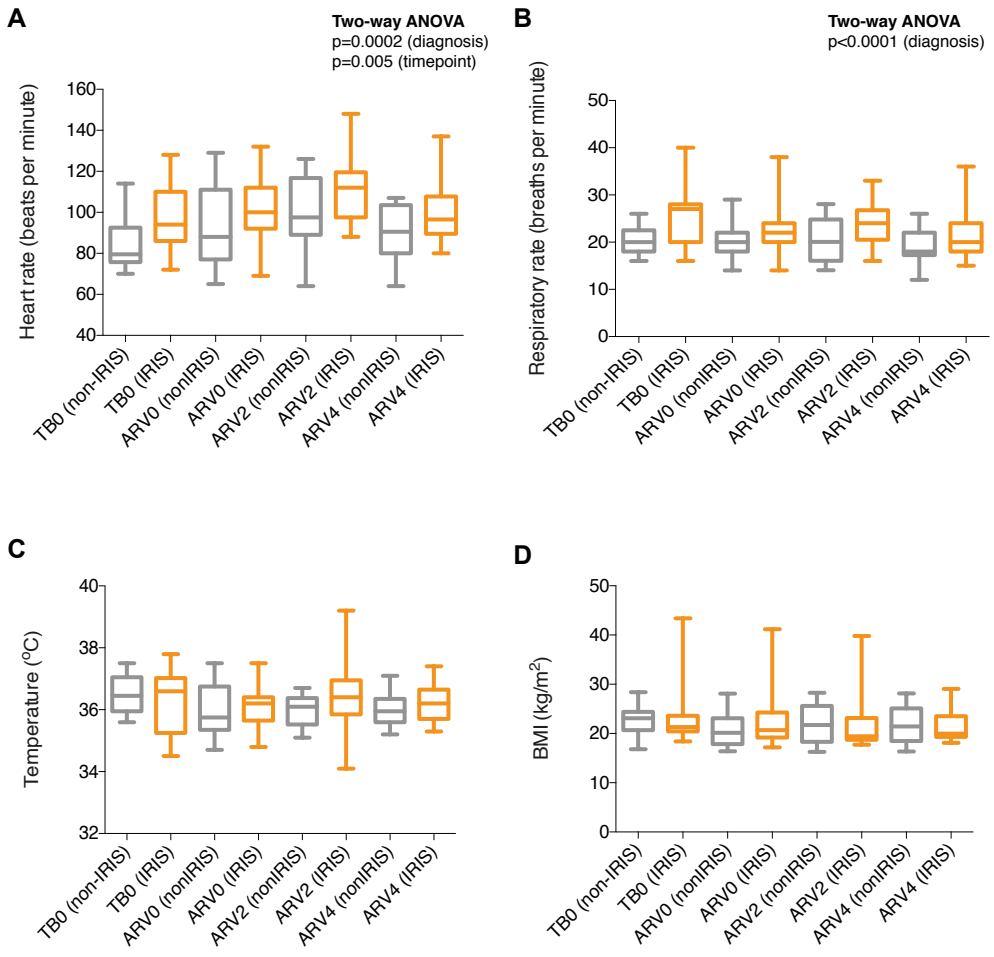



**Figure 4-3 Clinical characteristics on examination of patients in longitudinal study cohort**

TB-IRIS patients had higher heart rate (A) and respiratory rate (B) than non-IRIS controls. Median temperature was elevated in TB-IRIS patients, although the difference was not statistically significant (C). BMI, by contrast, was similar between TB-IRIS and non-IRIS controls throughout study follow up (D). Statistical analysis was by two-way ANOVA to detect variability according to TB-IRIS or non-IRIS designation (diagnosis) or according to study visit (timepoint). Significant variance (indicated by $p<0.05$) is reported for diagnosis or timepoint. Sidak's post-test comparison (corrected for multiple comparisons) was performed for variables with significant differences on ANOVA, to compare TB-IRIS and non-IRIS controls at each study visit, but no statistically significant differences were found.

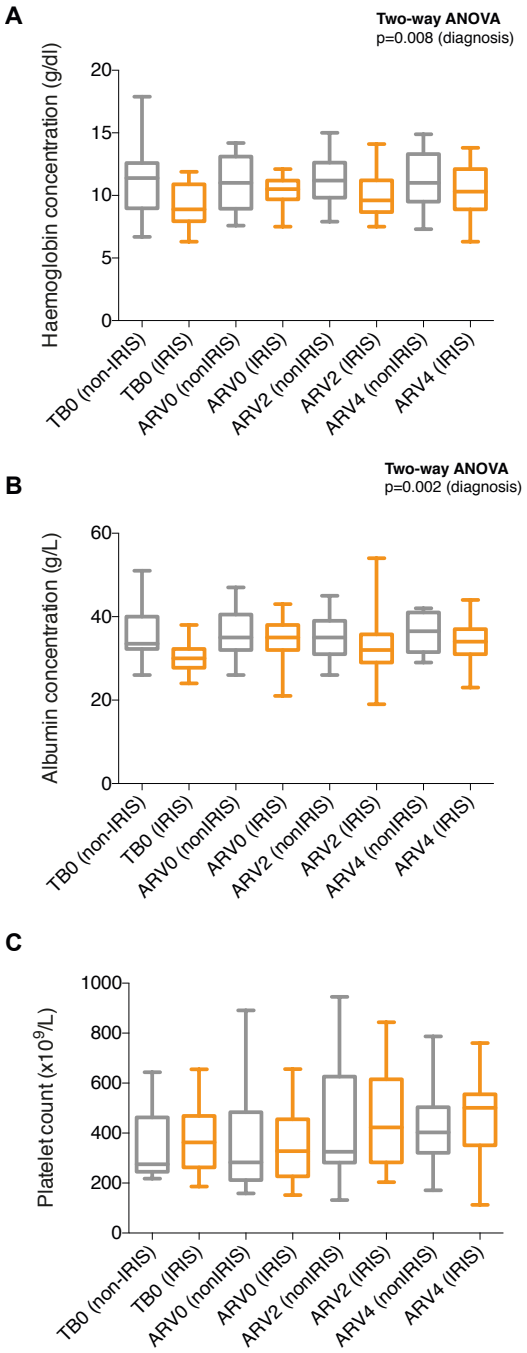



**Figure 4-4 Clinical laboratory parameters in TB-IRIS patients compared to non-IRIS controls.**

Peripheral blood haemoglobin concentration (A) and albumin concentration (B) were lower in TB-IRIS patients compared to non-IRIS controls, suggesting more severe illness. Platelet count by contrast was similar in both patient groups (C).

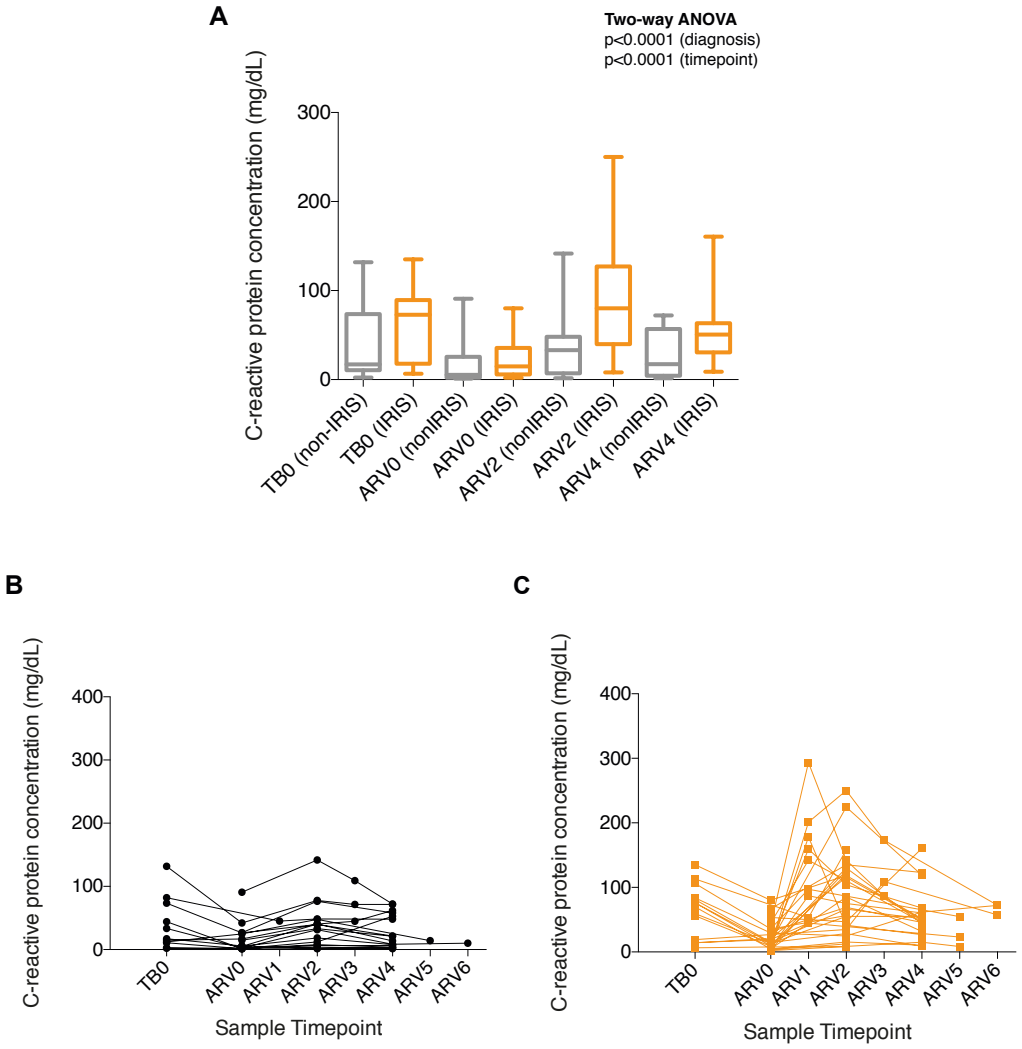



**Figure 4-5 C-reactive protein in TB-IRIS patients and non-IRIS controls**

TB-IRIS was associated with elevated peripheral blood C-reactive protein concentrations compared to non-IRIS controls (A). Typically, in TB-IRIS patients, C-reactive protein levels rose sharply after ART initiation to peak at one (ARV1) or two (ARV2) weeks post-ART initiation (C), the usual time of TB-IRIS symptom onset. This pattern was not evident in non-IRIS controls, where elevations in C-reactive protein during ART were of lesser magnitude (B). Mean difference between TB-IRIS patients and non-IRIS controls was significant at ARV2 and ARV4 on Sidak's test.

Peripheral blood leukocyte counts are shown in Figure 4-6. In both TB-IRIS patients and non-IRIS controls, total white cell count increased from TB0 to ARV2. TB-IRIS was characterized by higher neutrophil percentages ($p<0.001$, Figure 4-6B) and lower lymphocyte percentages ($p=0.001$, Figure 4-6C). At ARV2, typically the time of TB-IRIS presentation, neutrophils constituted a median 78% (IQR 71.3-85.4) of the total white cell population, compared to lymphocytes 11.1% (IQR 8.08-13.4).

CD4 counts rose during the first four weeks of ART (Figure 4-7). CD4 percentages were higher in TB-IRIS compared to non-IRIS controls ($p=0.04$), increasing from a median of 10.2% (IQR 8.65-16.1) at TB0 in non-IRIS controls to 16.4% (IQR 9.38-20) at ARV4, and from a median of 11.9% (IQR 9.76-14.5) at TB0 in TB-IRIS to 21.5% (IQR 11.5-28) at ARV4, although absolute CD4 numbers were similar in the two groups.

HIV viral load was higher in TB-IRIS patients than non-IRIS controls ($p=0.032$, Figure 4-8). In both groups, there was a sharp decrease in HIV-1 viral load evident even after just one week of ART in TB-IRIS patients at ARV1 (Figure 4-8B). An increase in HIV-1 viral load in one non-IRIS control at ARV4 indicated inadequate ART adherence (Figure 4-8C). Results of all clinical laboratory parameters are summarised in Table 4-4.

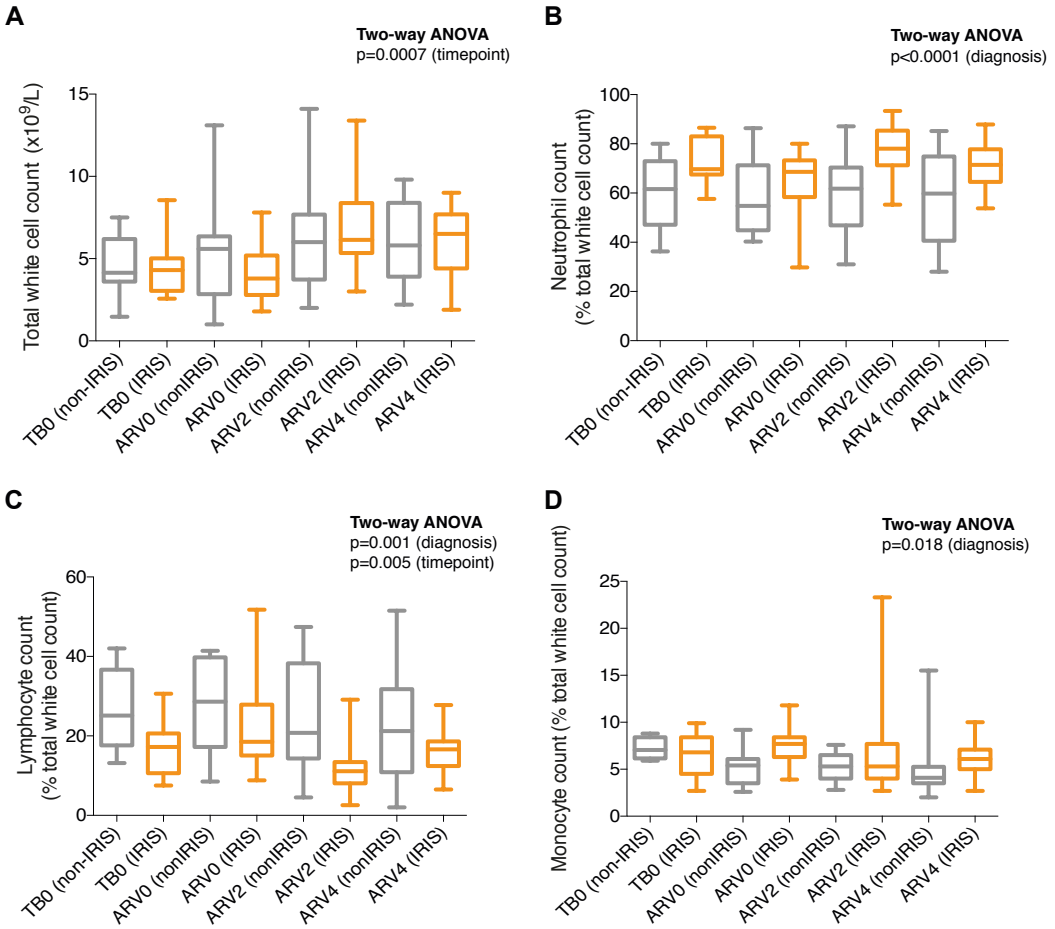



**Figure 4-6 Peripheral blood leucocyte counts in TB-IRIS and non-IRIS controls**

Peripheral blood total white cell counts increased following ART initiation, similarly in TB-IRIS patients and non-IRIS controls. Neutrophils were the predominant leucocyte in peripheral blood in TB-IRIS and non-IRIS control patients. TB-IRIS diagnosis was associated with increased neutrophil percentages and decreased lymphocyte percentages at all scheduled study visits, from TB diagnosis (TB0) to week 4 (ARV4) following ART initiation. This difference was significant on Sidak's test at ARV2 for both cell populations, and at ARV4 for neutrophil percentage. TB-IRIS was associated with an increase in monocyte proportions at ARV0 and ARV4.

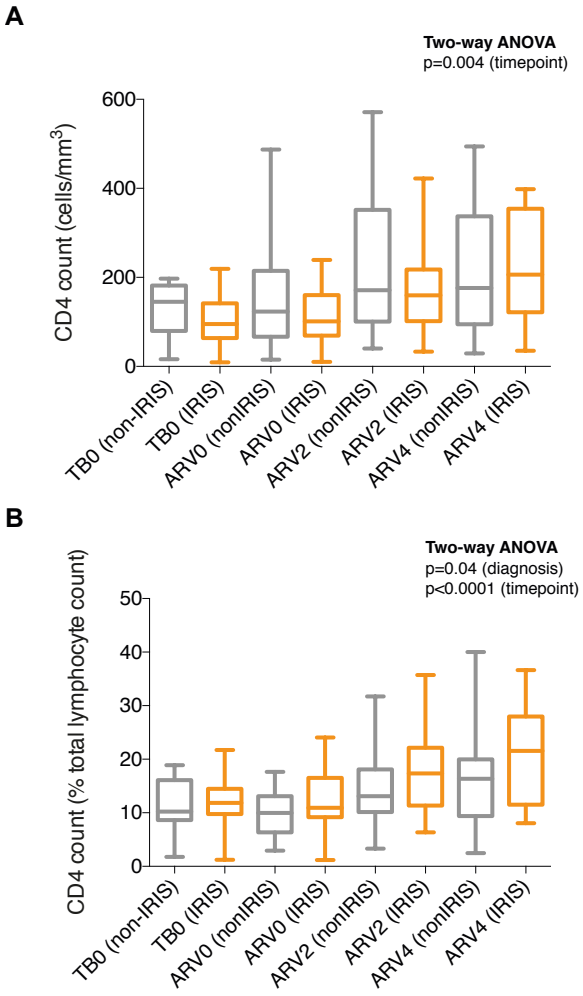



**Figure 4-7 Inflammatory phenotype at TB diagnosis in TB-IRIS patients**

Absolute and proportional peripheral blood CD4 count increased following ART initiation in both TB-IRIS patients and non-IRIS controls. TB-IRIS was associated with higher CD4 percentages, evident at all timepoints assessed (TB0, ARV0, ARV2 and ARV4).

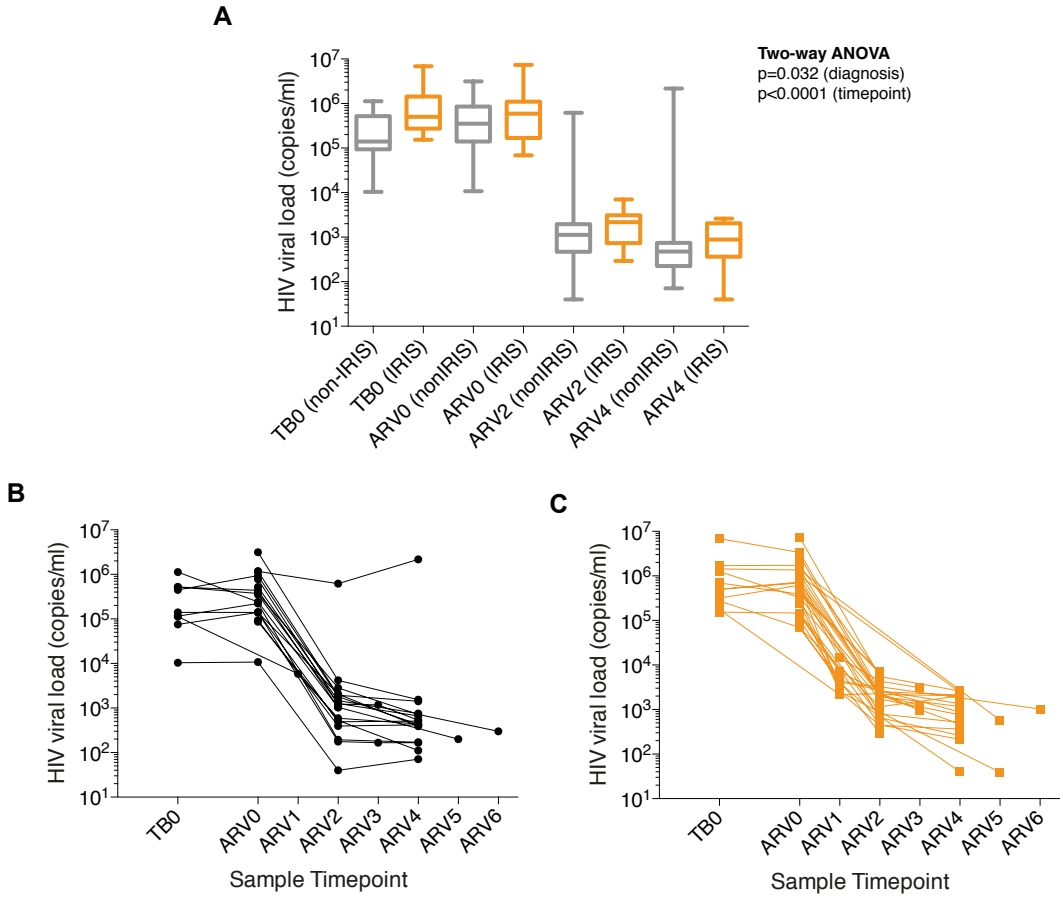



**Figure 4-8 HIV-1 viral load in TB-IRIS patients and controls**

Peripheral blood HIV-1 viral loads fell dramatically following ART initiation in longitudinal study participants. At ARV2, median values had reduced by almost three logs compared to ARV0, in both TB-IRIS and control patients (A). TB-IRIS was associated with increased HIV-1 viral loads, compared to non-IRIS controls at the time of TB diagnosis and ART initiation (C). However, reductions in HIV-1 viral load were similar in magnitude in TB-IRIS patients and non-IRIS controls and were most pronounced in the first two weeks of ART (B, C). One non-IRIS patient had an increased HIV-1 viral load at ARV4 compared to ARV0, which indicated poor adherence to ART (B).

Comparisons between TB-IRIS patients and non-IRIS controls at TB0 were limited, due to enrolment of only half the study cohort at this first timepoint. In Figure 4-9, results for key clinical variables (heart rate, respiratory rate, C-reactive protein, albumin concentration and HIV viral-load) at TB0 for TB-IRIS patients and non-IRIS controls were compared with the corresponding results for the larger population of ART naïve HIV-infected patients recruited in the cross-sectional study, who were assessed at TB diagnosis but not differentiated according to TB-IRIS status. Compared to the population of newly diagnosed TB patients, TB-IRIS were representative of those with a more severe inflammatory phenotype at TB diagnosis, compared to non-IRIS controls, with abnormalities suggestive of a more severe systemic inflammatory response (higher heart rate and respiratory rate, Figure 4-9A and B), acute phase response (higher C-reactive protein and lower albumin, Figure 4-9C and D) and a higher HIV-1 viral load (Figure 4-9E).

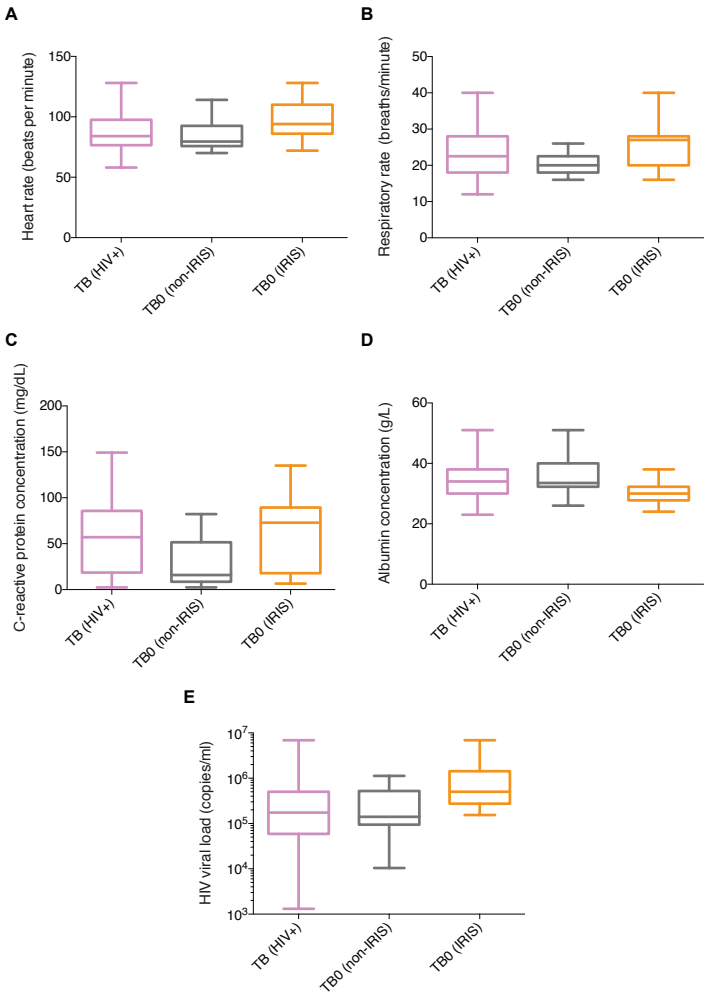



**Figure 4-9 Inflammatory phenotype at TB diagnosis in TB-IRIS patients**

Clinical parameters measured in TB-IRIS patients and non-IRIS controls at TB diagnosis (TB0) were compared to those measured in TB-HIV co-infected patients recruited into the cross-sectional study (TB(HIV+)) at TB diagnosis. TB-IRIS patients were typical of those cross-sectional study patients with a more pro-inflammatory phenotype, suggestive of a systemic inflammatory response, with elevated heart rates (A), respiratory rates (B) and C-reactive protein concentrations (D), and lower albumin concentrations relative to non-IRIS controls. HIV-1 viral load measurements in TB-IRIS patients were representative of values in the upper two quartiles of the cross-sectional study population. Comparisons shown were not significantly different by Mann-Whitney U analysis.

### Radiological characteristics

There was a great variety of inflammatory disease present on chest x-ray at TB diagnosis and throughout study follow up. Representative chest x-rays are shown in Appendix 2. Twenty one (72.4%) TB-IRIS patients had new or worsening radiographic features during a TB-IRIS episode. Chest x-rays were scored 0-10 based on number of zones with inflammatory abnormalities evident. Higher scores indicated more widespread inflammation, whereas lower scores indicated more focal inflammation. The results of chest x-ray inflammation score, summarised in Figure 4-10, demonstrate a wide range of scores in both TB-IRIS and non-IRIS patients during study follow up.

TB-IRIS was associated with higher chest x-ray scores in comparison to non-IRIS controls, indicating a greater extent of inflammation. At TB0 the median score was 9 (IQR 2-10) in TB-IRIS, compared to 6 (IQR 4-9) in non-IRIS controls. In TB-IRIS, the peak in median chest x-ray inflammation score in those patients who presented as TB-IRIS cases at ARV1, suggests that these patients presenting with early TB-IRIS had widespread inflammation on chest x-ray at this time, rather than focal disease restricted to one or two zones (e.g. lung apices). The score does not indicate whether this inflammation was destructive (e.g. cavitation) or granulomatous (e.g. in miliary TB). However, a high score of 9 or 10 was frequently due to miliary TB affecting the majority of lung parenchyma. Indeed, ten (34.5%) TB-IRIS patients, compared to three (16.4%) non-IRIS controls had evidence of miliary TB on baseline chest radiograph and therefore high scores at baseline in TB-IRIS patients are reflective of this pathology.

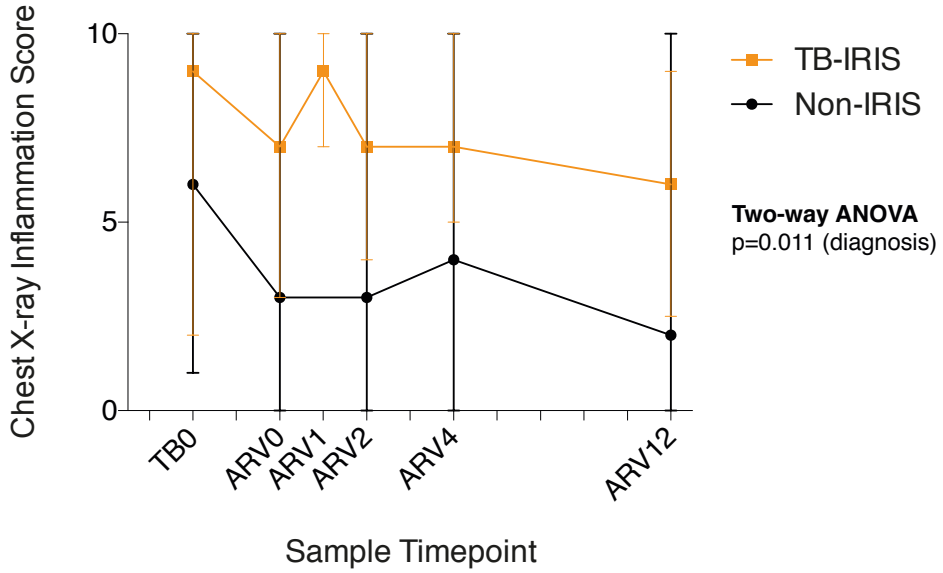



**Figure 4-10 Chest x-ray inflammation score in TB-IRIS patients and non-IRIS controls**

Chest radiographs were scored (0-10) based on extent of radiographic inflammation. Median score and IQR is shown for TB-IRIS patients (orange) and control patients (black). TB-IRIS patients had higher scores demonstrating that these patients had more widespread inflammation.

I had not expected patients with low CD4 counts (<200 cells/mm$^3$) enrolled in the cross-sectional study to present with cavitary TB at TB diagnosis, as this is uncommon in advanced immunosuppression. However, 11 patients (3 (16.7%) non-IRIS, 8 (27.6%) TB-IRIS) patients had cavities on ARV0 study chest x-ray. Proportions of patients with cavities are reported in Table 4-5. Three TB-IRIS patients without cavities on baseline chest x-ray developed cavities during TB-IRIS. An example is shown in Figure 4-11.

## Table 4-5 Radiographic features of TB-IRIS patients and non-IRIS controls

| Chest x-ray appearance | Diagnostic category and sample timepoint | | | | | | | | | | Two-way ANOVA | | | Sidaks multiple comparisons test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB diagnosis | | ARV0 | | ARV2 | | ARV4 | | ARV12 | | Interaction | Timepoint | Diagnosis | |
| | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | | | | |
| Cavities visible on Chest x-ray, n (%) | 2 (11.1) | 5 (17.2) | 3 (16.7) | 8 (27.6) | 4 (22.2) | 10 (34.5) | 4 (22.2) | 2 (6.9) | 3 (16.7) | 6 (20.7) | ND | | | ND |
| No cavities visible, n (%) | 7 (38.9) | 10 (34.5) | 12 (66.7) | 17 (58.6) | 6 (33.3) | 9 (31) | 5 (27.8) | 9 (31) | 8 (44.4) | 7 (24,1) | | | | |
| Data not available, n (%) | 9 (50) | 14 (48.3) | 3 (16.7) | 4 (13.8) | 8 (44.4) | 10 (34.5) | 9 (50) | 18 (62.1) | 7 (38.9) | 11 (37.9) | | | | |
| Median Chest X-ray inflammation score (IQR) | 6 (4-9) | 9 (2-10) | 3 (1-7) | 7 (3-10) | 4 (0-10) | 7 (4-10) | 4 (2.5-10) | 7 (5-10) | 2 (0-4) | 6 (2.5-9) | 0.687 | 0.071 | **0.011** | ns |

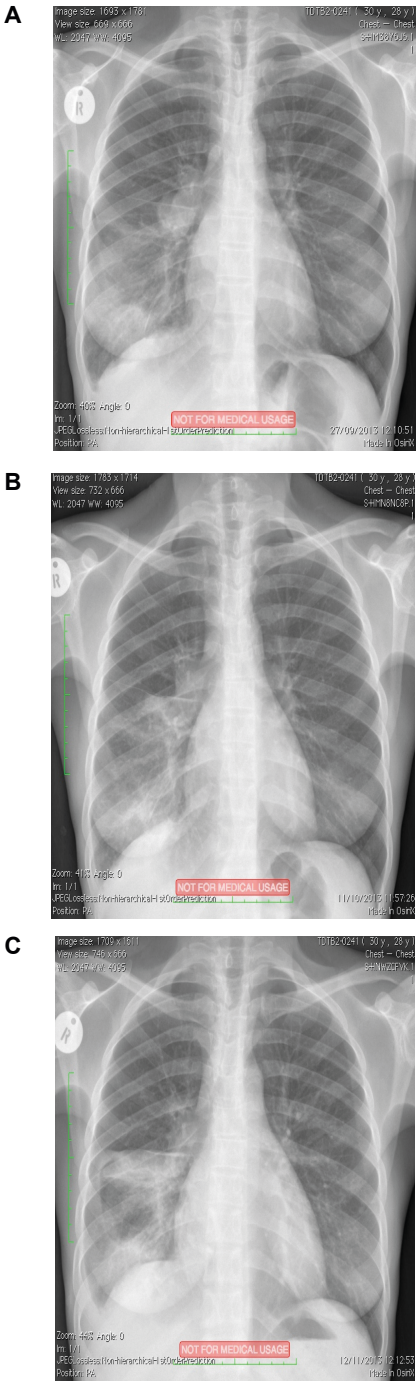



**Figure 4-11Radiographic progression and cavitation in a patient with TB-IRIS**

This chest x-ray series shows radiographic progression in a patient with right lower lobe consolidation and hilar adenopathy on chest x-ray at ART initiation (ARV0). After fourteen days of ART, worsening infiltrates and small cavities were present (B) which had extended at six weeks post-ART initiation (C).

### Microbiological characteristics

A higher proportion of TB-IRIS patients were smear and culture positive for M.tb. than non-IRIS groups at TB diagnosis, although this difference was not statistically significant (Table 4-6 and Figure 4-12). Smear and culture positivity may reflect increased pulmonary mycobacterial load. However, this may be confounded by the greater frequency of TB-IRIS patients with cavitation, introducing ascertainment bias, as cavitation facilitates release of mycobacteria into the airway, to be detected in sputum. No patients converted from smear-negative to smear-positive during study follow up, although one patient converted from culture negative to culture positive at TB-IRIS onset. Sputum smear scores increased in one TB-IRIS patient and one non-IRIS control during study follow up. In those patients who were culture positive for M.tb., time to sputum culture positivity increased over the study period in both TB-IRIS patients and non-IRIS controls and was not different between study groups (Figure 4-12).

**Table 4-6 Microbiological characteristics of TB-IRIS patients and non-IRIS controls**

| Microbiological variable | Diagnostic category and sample timepoint | | | | | | | | | Two-way ANOVA | | | Sidak's test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | At TB diagnosis | | | ARV0 | | ARV2 | | ARV4 | | | | | |
| | non-IRIS | IRIS | p value | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | Interaction | Timepoint | Diagnosis | |
| Sputum smear positive for AFB, n (%) | 5 (27.8) | 14 (45.3) | 0.235 | 3 (16.7) | 4 (13.8) | 1 (5.56) | 4 (13.8) | 1 (5.56) | 1 (3.44) | | | | |
| Sputum smear negative for AFB, n (%) | 12 (66.7) | 15 (51.7) | | 12 (66.7) | 19 (66.5) | 7 (38.9) | 12 (41.4) | 11 (61.1) | 13 (44.8) | | | | |
| Gene Xpert positive for Mtb, n (%)* | 8 (44.4) | 18 (62.1) | 1 | ND | ND | ND | ND | ND | ND | | | | |
| Gene Xpert negative for Mtb, n (%)* | 3 (16.7) | 6 (20.7) | | ND | ND | ND | ND | ND | ND | | | | |
| Sputum culture positive for Mtb, n (%) | 12 (66.7) | 21 (72.4) | 0.722 | 6 (33.3) | 7 (24.1) | 5 (27.8) | 4 (41.4) | 6 (33.3) | 15 (51.7) | | | | |
| Sputum culture negative for Mtb, n (%) | 3 (16.7) | 8 (27.5) | | 9 (50) | 14 (48.3) | 5 (27.8) | 10 (34.5) | 2 (11.1) | 2 (6.9) | | | | |
| Sputum culture not done/not known, n (%) | 3 (16.7) | 0 (0) | N/A | 3 (16.7) | 8 (27.6) | 8 (44.4) | 15 (51.7) | 10 (55.6) | 12 (41.4) | | | | |
| Median smear score, (IQR) | 0 (0-1) | 0.5 (0-4) | 0.18 | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.5) | 0 (0-0) | 0 (0-0) | | | | |
| Median time to culture positivity, days (IQR) | 15 (12-28.5) | 12 (9-20.3) | 0.252 | 21 (14.8-29.3) | 19 (18-23) | 24 (21.5-34) | 24 (22-32) | 34 (31-37) | 30.5 (29-32) | 0.814 | **0.001** | 0.369 | ns |

*Not routinely performed

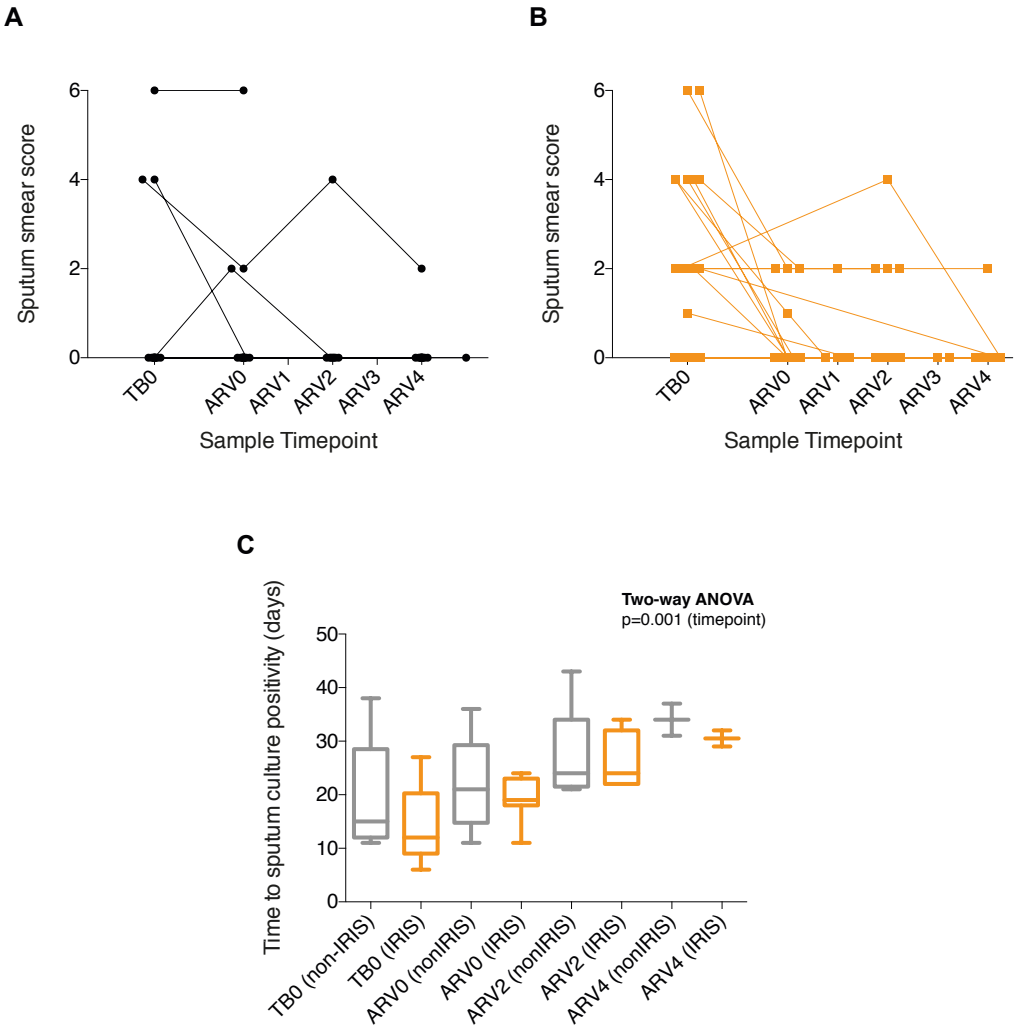



**Figure 4-12 Microbiological characteristics of TB-IRIS patients and non-IRIS controls**

Sputum scores for acid-fast bacilli were calculated from auramine microscopy results: negative=0, scanty=1, 1+=2, 2+=4, 4+=6. Sequential results for individual patients on serial induced sputum samples are shown for non-IRIS (A) and TB-IRIS patients (B). The majority of non-IRIS patients were smear-negative and remained so throughout study follow up. TB-IRIS patients were more frequently smear-positive at TB diagnosis and throughout the study follow up period, although in the majority the sputum smear score reduced. In patients who were culture positive for *M tuberculosis*, time to culture positivity increased throughout the study follow up period, in both TB-IRIS and non-IRIS control patients (C).

### Induced sputum MMP concentrations

To investigate the potential mediators of immunopathology in TB-IRIS, induced sputum MMP concentrations were measured by luminex and are reported in Table 4-7, Figure 4-13 and Figure 4-14. Samples were available from between 44.8% and 92.9% of enrolled participants at each timepoint (Table 4-7). Similar sample yields were obtained from TB-IRIS patients and non-IRIS controls, with the exception of ARV4, when a lower proportion of induced sputum samples were available from TB-IRIS patients than control patients (44.8% vs 77.8% respectively). Analysis was performed on results without adjustment for total protein content in sputum. MMP-12 and MMP-13 were not assessed as concentrations were below the level of detection of the assay.

There was a trend towards increased MMP-1, 2, -3, -7 and -10 in TB-IRIS patients compared to controls at ARV0, ARV2 and ARV4 (Figure 4-13). The difference was most evident for MMP-7 ($p=0.014$) and MMP-10 ($p=0.026$), where the difference was most marked at ARV4. In non-IRIS controls, a consistent pattern of reduced median MMP concentrations at ARV0 compared to TB0 was observed for all MMPs analysed. This was not the case for TB-IRIS patients with increased MMP-2, -7 and -10 at ARV0 compared to TB0 and more consistently elevated concentrations between TB0 and ARV4 than in control patients. Median MMP-8 and MMP-9 concentrations increased between ARV0 and ARV2 in both TB-IRIS patients and non-IRIS controls (Figure 4-14).

## Table 4-7 Induced sputum MMP concentrations in TB-IRIS patients and non-IRIS controls

| Induced sputum MMP concentration | Diagnostic category and sample timepoint | | | | | | | | Two-way ANOVA | | | Sidaks test (non-IRIS vs TB-IRIS) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | ARV0 | | ARV2 | | ARV4 | | | | | | | | |
| | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | Interaction | Timepoint | Diagnosis | TB0 | ARV0 | ARV2 | ARV4 |
| Sample frequency n, (% enrolled participants) | 8 (80) | 13 (92.9) | 14 (77.8) | 23 (79.3) | 11 (61.1) | 17 (58.6) | 14 (77.8) | 13 (44.8) | | | | | | | |
| Median MMP-1, pg/ml (IQR) | 1491 (123-2625) | 503 (218-1456) | 127 (59.2-403) | 391 (160-953) | 354 (142-1118) | 575 (130-2915) | 87.4 (16.1-754) | 255 (89.5-1409) | 0.727 | 0.109 | 0.191 | | | | |
| Median MMP-2, pg/ml (IQR) | 1146 (650-2335) | 597 (409-898) | 936 (533-1764) | 1296 (589-2683) | 1181 (443-1396) | 1163 (693-1690) | 598 (330-954) | 794 (573-1004) | 0.246 | **0.031** | 0.615 | | | | |
| Median MMP-3, pg/ml (IQR) | 1199 (178-2529) | 632 (332-830) | 176 (80.5-445) | 174 (116-582) | 456 (66.1-791) | 510 (146-2646) | 137 (25-732) | 208 (99.2-1437) | 0.751 | 0.066 | 0.486 | | | | |
| Median MMP-7, pg/ml (IQR) | 34732 (26035-107564) | 38808 (17487-58786) | 31824 (14470-50602) | 65463 (29760-119562) | 33880 (18379-55301) | 60731 (28786-103122) | 14217 (8096-45956) | 34192 (15912-115429) | 0.341 | 0.374 | **0.014** | ns | ns | ns | ns |
| Median MMP-8, pg/ml (IQR) | 314589 (100810-1100000) | 598292 (159413-999657) | 84673 (35752-719541) | 119014 (76539-415186) | 229007 (39419-597879) | 383157 (136111-929025) | 190006 (19449-605951) | 105939 (70850-701067) | 0.769 | 0.039 | 0.143 | | | | |
| Median MMP-9, pg/ml (IQR) | 245854 (81844-425451) | 264024 (152684-366811) | 109865 (38895-294402) | 205443 (72409-300945) | 379195 (130253-443602) | 343397 (182106-656466) | 175029 (86195-512131) | 223230 (101471-519814) | 0.942 | **0.016** | 0.319 | | | | |
| Median MMP-10, pg/ml (IQR) | 3718 (2339-9056) | 4421 (1621-6711) | 2361 (872-5125) | 5969 (1750-10882) | 2283 (1062-8365) | 4175 (1711-7259) | 983 (464-5057) | 4569 (903-6525) | 0.452 | 0.25 | **0.026** | ns | ns | ns | ns |

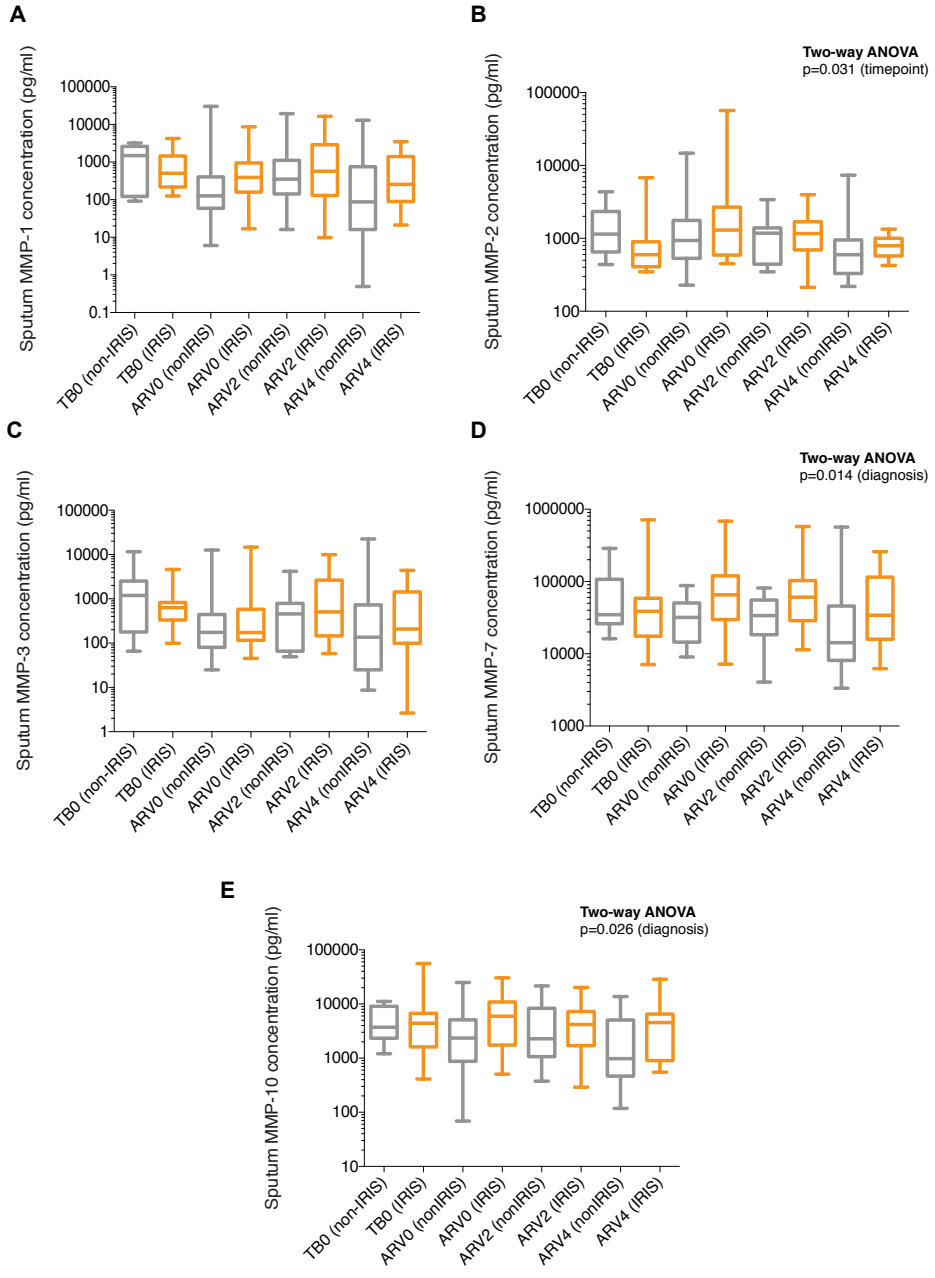



**Figure 4-13 Elevated MMP concentrations in induced sputum of TB-IRIS patients**

TB-IRIS was associated with elevated induced sputum MMP -1 -2, -3, -7 and -10. MMP -7 and MMP-10 were significantly increased compared to non-IRIS controls on two-way ANOVA analysis. In non-IRIS control patients, median MMP concentrations tended to fall following TB treatment initiation and were reduced in comparison to TB0 at ARV4. However in TB-IRIS patients, MMP concentrations at ARV4 tended to be similar to those at TB0.

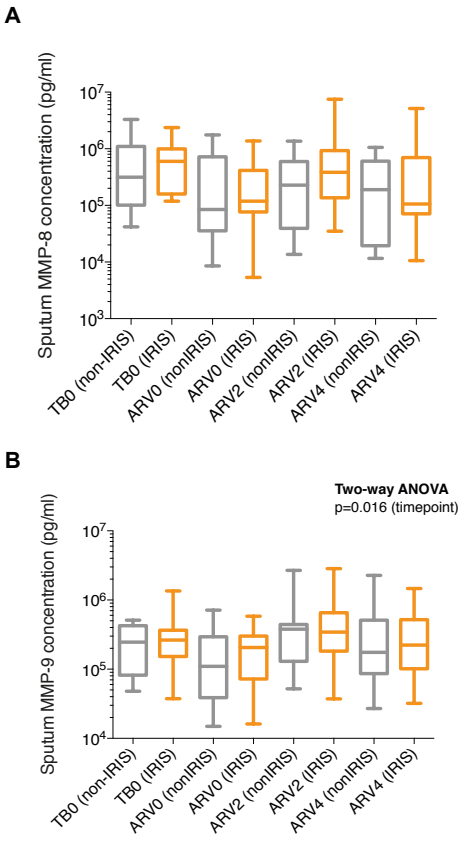



**Figure 4-14 Induced sputum MMP-8 and MMP-9 in TB-IRIS**

In TB-IRIS patients and non-IRIS controls, induced sputum MMP-8 and -9 increased between ARV0 and ARV2. However, although there was a trend to increase in both MMP-8 and -9 in patients who developed TB-IRIS, there was no significant difference between TB-IRIS and non-IRIS controls.

### Plasma MMP concentrations

Samples were available from the majority of enrolled participants at each timepoint (Table 4-8). Plasma MMP-1, MMP-3 and MMP-8 were significantly increased in TB-IRIS compared to non-IRIS controls (p=0.003, 0.0002, <0.0001 respectively). At ARV2 the difference between TB-IRIS and non-IRIS controls was significant on Sidak's multiple comparisons test. MMP-8 was also significantly increased at ARV4 in TB-IRIS compared to non-IRIS controls. This pattern was evident in MMP-7 and MMP-10, although comparisons did not reach significance (Figure 4-15). Elevated median concentrations of MMP-1, -3, -7, and -8 were present at all study timepoints analysed, including TB0 and ARV0, prior to TB-IRIS onset. MMP-2 and MMP-9 were similar between TB-IRIS and controls, although MMP-9 did increase over time (<0.0001, Figure 4-16). Whilst these findings are similar to MMP results in sputum, the elevated MMP concentrations at TB0 were not apparent in sputum. Additionally, MMP-2 was elevated in sputum but not plasma and MMP-8 was elevated in plasma and not sputum.

## Table 4-8 Plasma MMP and PIIINP concentrations in TB-IRIS and non-IRIS control patients

| Plasma MMP and PIIINP concentrations | Diagnostic category and sample timepoint | | | | | | | | Two-way ANOVA | | | Sidaks multiple comparisons test | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | ARV0 | | ARV2 | | ARV4 | | | | | | | | |
| | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | Interaction | Timepoint | Diagnosis | TB0 | ARV0 | ARV2 | ARV4 |
| Sample frequency, n (% enrolled participants) | 10 (100) | 14 (100) | 17 (94) | 29 (96.6) | 17 (94) | 25 (86.2) | 16 (88.9) | 18 (62.1) | | | | | | | |
| Median MMP-1, pg/ml (IQR) | 2971 (1806-3489) | 4073 (2026-5148) | 2123 (1152-3268) | 3485 (1714-5721) | 2194 (1099-5349) | 4820 (2348-7115) | 3166 (1446-4583) | 3918 (2155-6524) | 0.756 | 0.5 | **0.003** | ns | ns | * | ns |
| Median MMP-2, pg/ml (IQR) | 151140 (134705-195738) | 192297 (126289-243711) | 219671 (156073-286765) | 207052 (162984-291484) | 211157 (148907-268753) | 197745 (161620-265572) | 209497 (179101-290903) | 207084 (170303-282518) | 1 | 0.078 | 0.658 | | | | |
| Median MMP-3, pg/ml (IQR) | 11146 (8003-15467) | 23039 (14192-32693) | 13689 (8455-17022) | 17081 (11483-23055) | 12025 (7373-19487) | 17867 (12351-28800) | 15846 (10129-19166) | 17833 (12218-23420) | 0.67 | 0.912 | **0.0002** | ns | ns | * | ns |
| Median MMP-7, pg/ml (IQR) | 3956 (3082-6329) | 5327 (2359-9348) | 3995 (2767-7853) | 5163 (3719-7469) | 4115 (3170-9818) | 6802 (4989-11350) | 4099 (2971-8055) | 5420 (2938-11848) | 0.919 | 0.438 | 0.141 | | | | |
| Median MMP-8, pg/ml (IQR) | 526 (326-2306) | 4582 (2617-9090) | 378 (353-1162) | 946 (35.3-2617) | 1095 (297-3929) | 6104 (3702-12617) | 1209 (137-5664) | 3787 (1852-5862) | 0.145 | **<0.0001** | **<0.0001** | ns | ns | ** | * |
| Median MMP-9, pg/ml (IQR) | 8316 (4515-11231) | 10051 (739-13214) | 10203 (6601-16015) | 8185 (6673-11185) | 19182 (10673-35031) | 17781 (10612-33208) | 18168 (11974-36697) | 15864 (11679-29911) | 0.551 | **<0.0001** | 0.746 | | | | |
| Median MMP-10, pg/ml (IQR) | 1248 (1045-2821) | 2153 (1770-2727) | 1631 (1227-2530) | 1967 (1386-2327) | 1735 (1007-2374) | 1801 (1247-2602) | 1937 (1513-2745) | 1487 (1224-2280) | 0.378 | 0.974 | 0.116 | | | | |
| Median PIIINP concentration, pg/ml (IQR) | 21651 (17757-33196) | 43600 (30021-63913) | 27333 (14057-33154) | 25121 (18434-39468) | 27093 (16616-41442) | 39478 (26296-56996) | 22424 (16892-31546) | 46763 (29104-70641) | 0.342 | 0.143 | **<0.0001** | ns | ns | * | ** |

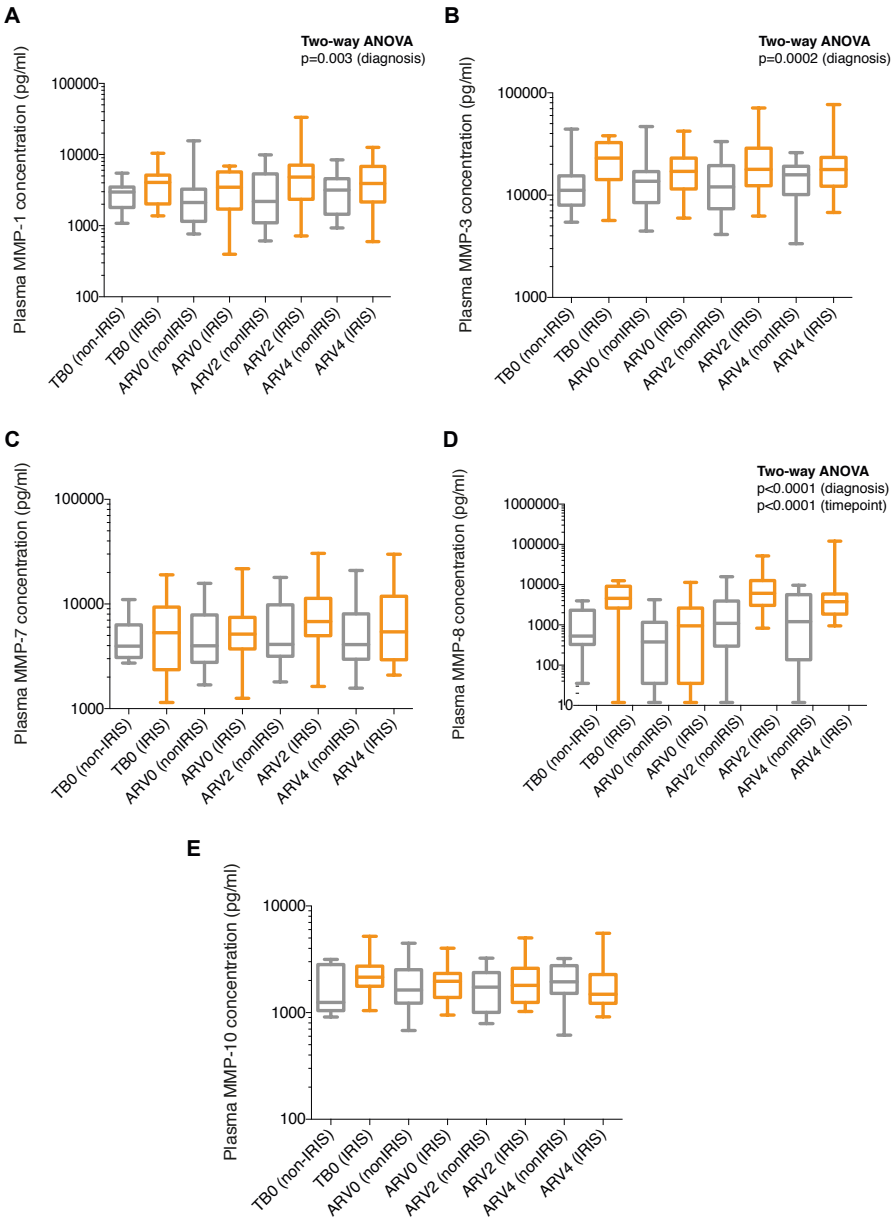



**Figure 4-15 Elevated plasma MMP-1, -3, -7, -8 and -10 in TB-IRIS**

Plasma concentrations of MMP-1, -3, -7, -8 and -10 were elevated in TB-IRIS patients compared to non-IRIS control patients. This was evident at TB0, prior to ART initiation. Median concentrations were higher in TB-IRIS patients compared to control patients at all timepoints with the exception of MMP-10 at ARV4. The difference between patient groups was significant on Sidak's multiple comparisons test for MMP-1, -3, and -8 at ARV2 and for MMP-8 at ARV4.

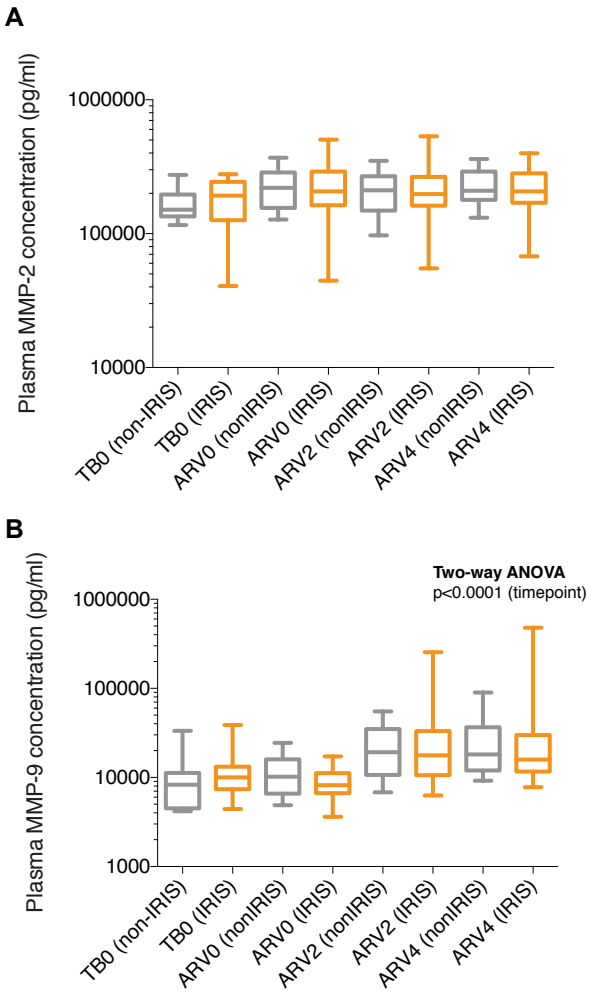



**Figure 4-16 Plasma MMP-2 and MMP-9 in TB-IRIS and non-IRIS control patients.**

The gelatinases, MMP-2 and MMP-9, increased during the first four weeks of antiretroviral therapy in both TB-IRIS patients and non-IRIS controls, but were not significantly different between the two groups of patients.

### Plasma PIIINP concentrations

PIIINP is released during type III collagen turnover and therefore may represent a peripheral marker of immunopathology. Plasma PIIINP was elevated in TB-IRIS compared to control patients ($p<0.0001$) with higher median values at TB0, ARV2 and ARV4, in comparison to non-IRIS controls (Figure 4-17). At TB0, in TB-IRIS patients, the median plasma PIIINP was over double the value in non-IRIS controls, 43600 pg/ml (IQR 30021-63913) compared to 21651 pg/ml (IQR 17757-33196) respectively. This comparison did not reach significance on Sidak's multiple comparison test. However, at timepoints ARV2 and ARV4, the difference in median values had reached significance.

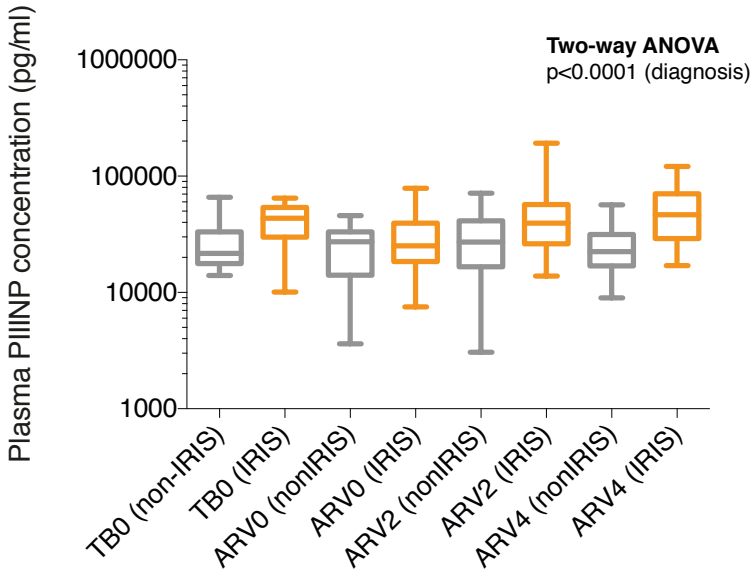



**Figure 4-17 Elevated plasma PIIINP in TB-IRIS patients**

Plasma PIIINP was measured by ELISA on plasma samples from TB-IRIS patients and non-IRIS controls. TB-IRIS was associated with elevated PIIINP compared to non-IRIS patients. This comparison was significant on Sidak's multiple comparisons test at ARV2 and ARV4, although the trend was also evident at TB diagnosis (TB0).

### Induced sputum cytokine and chemokine concentrations

Induced sputum concentrations of 30 key cytokines and chemokines were assessed by Luminex to investigate immunological mediators of pathology. Sample availability was as for MMP analysis (Table 4-8). Seven analytes were detected at levels below the standard curve and therefore not quantified (IL-2, IL-4, IL-5, IL-7, IL-10, IL-17, Eotaxin) and one analyte was so abundant that it was above the standard curve (RANTES) and therefore also not analysed. TB-IRIS diagnosis was associated with increased pro-inflammatory mediators associated with an innate immune response, IL-8 ($p=0.016$), G-CSF ($p=0.047$), IL-6 ($p=0.005$) compared to non-IRIS controls, as shown in Figure 4-18. However, at TB0 concentrations of these three analytes were similar in the two groups. IL-7 was also increased in TB-IRIS compared to controls $p=0.007$ (Figure 4-19). Other cytokines/chemokines were not different in TB-IRIS and non-IRIS controls on two-way ANOVA analysis and tended to be present at low concentrations, with the exception of IL-1RA, MCP1, IP-10 and MIG, which were abundant in both patient groups. Table 4-9 reports median and IQR values for all cytokines and chemokines assessed in induced sputum.

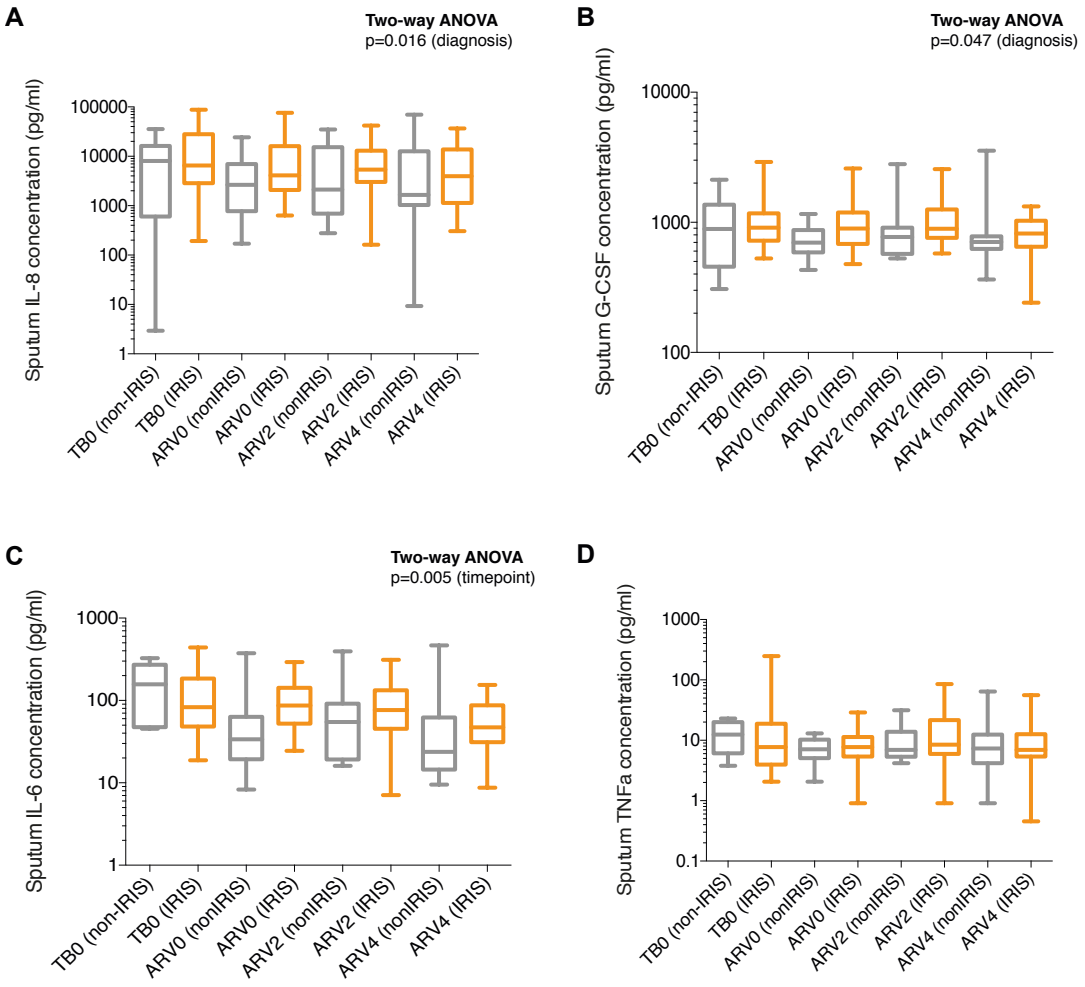



**Figure 4-18 Elevated innate pro-inflammatory cytokines and chemokines in induced sputum in TB-IRIS patients compared to non-IRIS controls**

TB-IRIS diagnosis was associated with increased innate pro-inflammatory mediators, IL-8 (A), G-CSF (B), and IL-6 (C) compared to non-IRIS controls at ARV0, ARV2, and ARV4. At TB0, concentrations of these three analytes were not increased. By contrast, concentrations of TNF-α (D), and IL-1β (not shown) were relatively low and were similar in TB-IRIS and non-IRIS control patients (D).

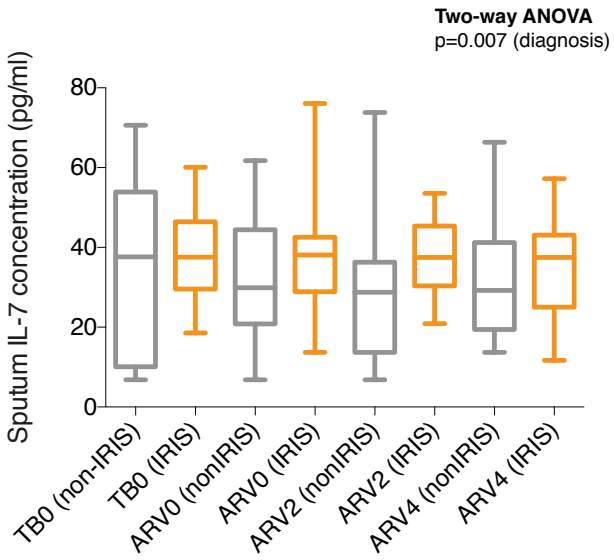



**Figure 4-19 Elevated induced sputum IL-7 in TB-IRIS patients**

Induced sputum IL-7 was elevated in TB-IRIS patients compared to non-IRIS controls, most prominently at two weeks post-ART initiation.

## Table 4-9 Induced sputum cytokines and chemokines in TB-IRIS and non-IRIS patients

| Induced sputum cytokine/chemokine concentration | Diagnostic category and sample timepoint | | | | | | | | Two-way ANOVA | | | Sidaks test (non-IRIS vs TB-IRIS) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | ARV0 | | ARV2 | | ARV4 | | | | | | | | |
| | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | Interaction | Timepoint | Diagnosis | TB0 | ARV0 | ARV2 | ARV4 |
| Median VEGF, pg/ml (IQR) | 126 (53.3-166) | 130 (98.9-145) | 101 (90.4-122) | 116 (95.9-134) | 112 (86.2-161) | 121 (98.3-149) | 94.4 (76.9-138) | 105 (83.8-153) | 0.874 | 0.492 | 271 | | | | |
| Median IL-1β , pg/ml (IQR) | 25 (16.2-30) | 23.8 (21.4-45.9) | 16.3 (10.8-30.1) | 20.3 (16-25.1) | 20.9 (7.69-29.6) | 22.7 (19.8-33.5) | 20.6 (13.7-33.6) | 22.7 (15.2-27.2) | 0.679 | 0.142 | 0.458 | | | | |
| Median GCSF, pg/ml (IQR) | 889 (456-1365) | 912 (722-1172) | 699 (588-873) | 896 (683-1191) | 772 (573-911) | 893 (759-1255) | 708 (624-780) | 820 (650-1029) | 0.75 | 0.507 | **0.047** | ns | ns | ns | ns |
| Median EGF, pg/ml (IQR) | 107 (20.1-573) | 140 (71.9-297) | 91.4 (68.5-242) | 186 (68.5-242) | 70.7 (36.9-206) | 191 (41.6-335) | 113 (29.2-243) | 75 (22.5-277) | 0.881 | 0.774 | 0.427 | | | | |
| Median IL-10, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median HGF, pg/ml (IQR) | 200 (50.4-308) | 222 (162-261) | 162 (108-233) | 190 (148-275) | 182 (85.9-420) | 272 (166-388) | 151 (126-293) | 178 (133-302) | 0.933 | 0.64 | 0.102 | | | | |
| Median FGF Basic, pg/ml (IQR) | 7.57 (0.648-9.68) | 12 (0.67-16) | 5.99 (1.12-12.8) | 12 (4.75-14.2) | 9.47 (4.75-12.4) | 7.98 (5.22-14.7) | 6.08 (0.67-10.6) | 13.3 (4.22-16.2) | 0.775 | 0.536 | 0.147 | | | | |
| Median IFN-α, pg/ml (IQR) | 75.9 (22.4-90.6) | 68.4 (63.3-78.4) | 69.2 (47.4-74.9) | 71.8 (54.1-79.6) | 61.3 (44.7-76.3) | 71 (61.1-78.9) | 54.8 (51.7-75.9) | 60.9 (48.4-75.1) | 0.661 | 0.408 | 0.075 | | | | |
| Median IL-6, pg/ml (IQR) | 158 (47.5-271) | 82.8 (48.3-184) | 33.8 (19.3-63.4) | 87 (52.5-143) | 54.7 (19.2-91.3) | 76.4 (45.3-133) | 23.8 (14.5-62.2) | 47.2 (31.2-87.6) | 0.241 | **0.005** | 0.053 | | | | |
| Median IL-12 p40/p70, pg/ml (IQR) | 61.8 (37.3-82.8) | 72.5 (47.2-78.6) | 55.2 (34.1-67.6) | 59.3 (48.9-73.4) | 53.1 (31-83.5) | 69.8 (54.8-78.3) | 47 (32.9-62.7) | 58.7 (39.7-73.4) | 0.991 | 0.459 | 0.193 | | | | |
| Median RANTES, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median Eotaxin, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IL-13, pg/ml (IQR) | 21.6 (17.3-26.7) | 26.9 (14.3-46) | 16.4 (6.57-26.3) | 26.9 (20-38.5) | 20 (12.2-46) | 38.5 (23.1-46) | 18.2 (12.2-35.1) | 26.9 (11.4-46) | 0.712 | 0.509 | 0.068 | | | | |
| Median IL-15, pg/ml (IQR) | 155 (32.7-298) | 184 (54.6-281) | 131 (66.8-265) | 154 (94.8-243) | 61.6 (47.7-105) | 136 (70.5-167) | 94.5 (44.1-160) | 122 (82-202) | 0.839 | 0.215 | 0.164 | | | | |
| Median IL-17, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median MIP-1α, pg/ml (IQR) | 47.7 (20.6-22.6) | 43.7 (34.3-96.2) | 35.5 (27.9-44.1) | 40.5 (31.8-48.6) | 32.2 (27.7-67.4) | 36.4 (31.8-45.7) | 28.6 (25.4-41.7) | 39.3 (31.0-42) | 0.941 | 0.09 | 0.808 | | | | |
| Median GM-CSF, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median MIP-1β, pg/ml (IQR) | 60.9 (12.3-687) | 47.7 (31.2-178) | 23.6 (16-52.2) | 29.4 (20.3-101) | 20.3 (20.1-55.6) | 33.4 (21.9-69.8) | 28.1 (16-43.2) | 40.9 (20.1-47.7) | 0.815 | 0.101 | 0.793 | | | | |
| Median MCP-1, pg/ml (IQR) | 410 (54.4-734) | 374 (293-472) | 247 (197-363) | 297 (211-575) | 224 (167-379) | 328 (228-442) | 279 (167-432) | 288 (187-387) | 0.94 | 0.745 | 0.09 | | | | |
| Median IL-5, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IFN-γ, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median TNF-α, pg/ml (IQR) | 12.4 (6.1-20) | 7.71 (3.98-18.8) | 7.13 (5.06-10.3) | 7.72 (5.4-11.4) | 6.95 (5.36-13.9) | 8.46 (5.97-21.6) | 7.34 (4.17-12.4) | 6.95 (5.4-12.6) | 0.951 | 0.402 | 0.883 | | | | |
| Median IL1-RA, pg/ml (IQR) | 107358 (42730-242355) | 84195 (33823-139360) | 90773 (25511-160657) | 42889 (15707-142017) | 97273 (26888-172861) | 88139 (41060-150202) | 71934 (31360-151641) | 79393 (43082-131312) | 0.498 | 0.785 | 0.57 | | | | |
| Median IL-2, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IL-7, pg/ml (IQR) | 37.6 (10.1-53.9) | 37.6 (29.6-46.5) | 29.9 (20.99-44.4) | 38.1 (28.9-42.6) | 28.8 (13.7-36.3) | 37.5 (30.4-45.4) | 29.2 (19.4-41.2) | 37.5 (25-43.1) | 0.605 | 0.938 | **0.007** | ns | ns | ns | ns |
| Median IP-10, pg/ml (IQR) | 638 (52.4-8658) | 528 (53.9-6785) | 261 (73.4-2716) | 471 (103-1548) | 137 (6.17-232) | 240 (112-391) | 145 (9.12-510) | 210 (91.6-895) | 0.663 | 0.114 | 0.184 | | | | |
| Median IL-2R, pg/ml (IQR) | 71.9 (42.7-77.8) | 78.9 (64.9-102) | 70.3 (57.4-83.3) | 69.6 (48.6-81) | 71 (48.6-97.3) | 71 (60.3-81) | 67.1 (59.6-79.4) | 69.6 (48.6-76) | 0.513 | 0.692 | 0.899 | | | | |
| Median MIG, pg/ml (IQR) | 708 (40-1852) | 497 (159-946) | 362 (86.2-1007) | 237 (65.5-603) | 94.9 (57.3-850) | 284 (72.3-1431) | 136 (54.3-794) | 356 (81.1-1238) | 0.744 | 0.864 | 0.445 | | | | |
| Median IL-4, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IL-8, pg/ml (IQR) | 8072 (607-16228) | 6515 (2859-28148) | 2656 (778-2955) | 4121 (2081-16010) | 2115 (689-15274) | 5392 (3038-12963) | 1657 (1030-12665) | 3981 (1143-13725) | 0.884 | 0.844 | **0.016** | ns | ns | ns | ns |

ND=not done (off standard curve), ns=not significant

### Plasma cytokine and chemokine concentrations

Table 4-10 reports median and IQR values for all cytokines and chemokines assessed in plasma. RANTES was not analysed as values were above the standard curve. Several analytes were not analysed as they were below the level of detection (G-CSF, EGF, FGF basic, IL-4, IL-5, IL-7, IL-8, IL-10, IL-13, IL-17, GM-CSF, MIP-1α). I observed evidence of increased innate and Th1 pro-inflammatory cytokines and chemokines in TB-IRIS compared to control patients (Figure 4-20 and Figure 4-21). Cytokines IL-6 ($p<0.0001$), IFN-γ ($p<0.001$), chemokine receptor IL-2R ($p<0.0001$) and chemokines MIG (CXCL9, $p<0.0001$) and IP-10 (CXCL10, $p=0.0001$) were elevated at all timepoints in TB-IRIS compared to non-IRIS controls. Plasma IFN-γ and IP-10 were significantly elevated in TB-IRIS patients compared to non-IRIS controls at TB0 on Sidak's multiple comparison test, with median values 25 pg/ml (IQR 17.9-48.9) vs 15 (IQR 10.6-20.9) and 131 (103-218) vs 62.6 pg/ml (IQR 29.9-115) respectively. Anti-inflammatory mediator, IL-1RA was elevated in TB-IRIS compared to non-IRIS controls ($p=0.001$, Figure 4-22). Additionally, growth factors VEGF and HGF not previously evaluated in TB-IRIS patients, were elevated in TB-IRIS compared to non-IRIS controls ($p<0.0001$ and $p=0.001$ respectively, Figure 4-23).

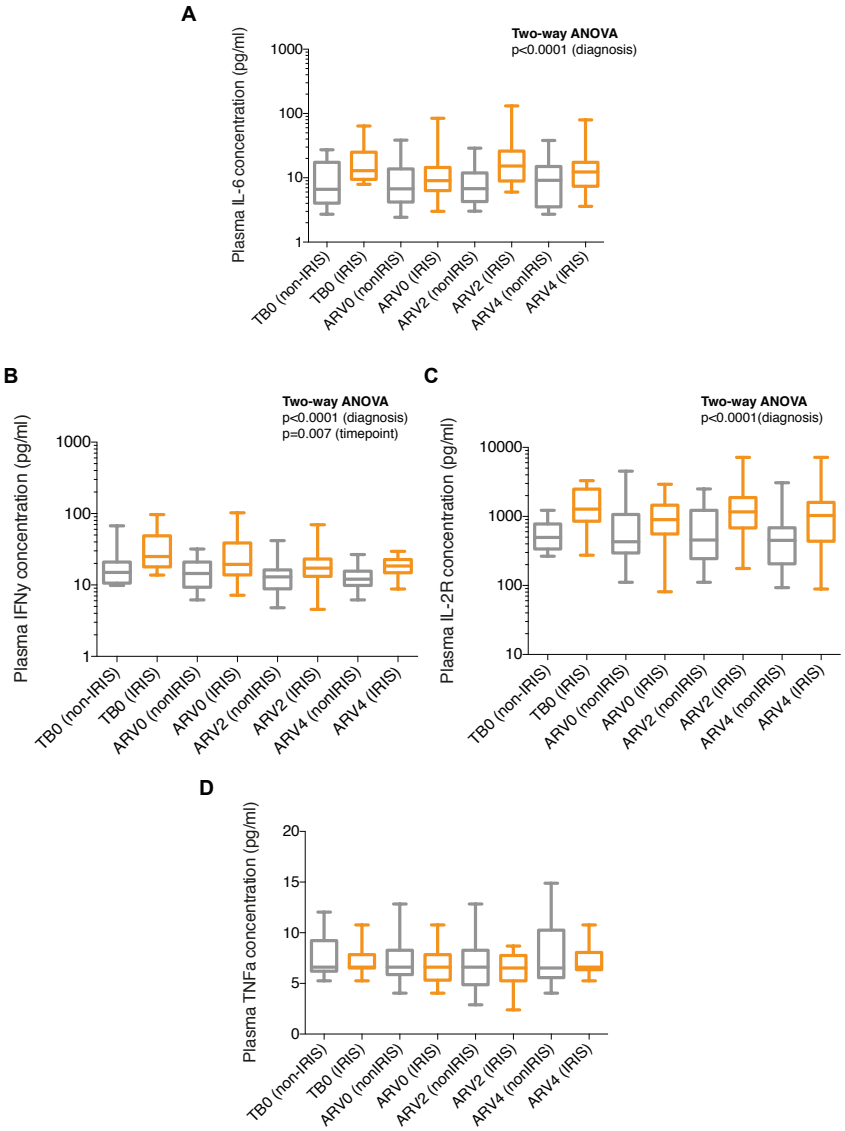



**Figure 4-20 Pro-inflammatory cytokines in plasma in TB-IRIS and non-IRIS control patients**

Pro-inflammatory mediators in plasma, IL-6 (A), IFN-γ (B) and IL-2R (C), were elevated at all timepoints assessed in TB-IRIS patients compared to non-IRIS controls. TNF-α (D) by contrast was not elevated.

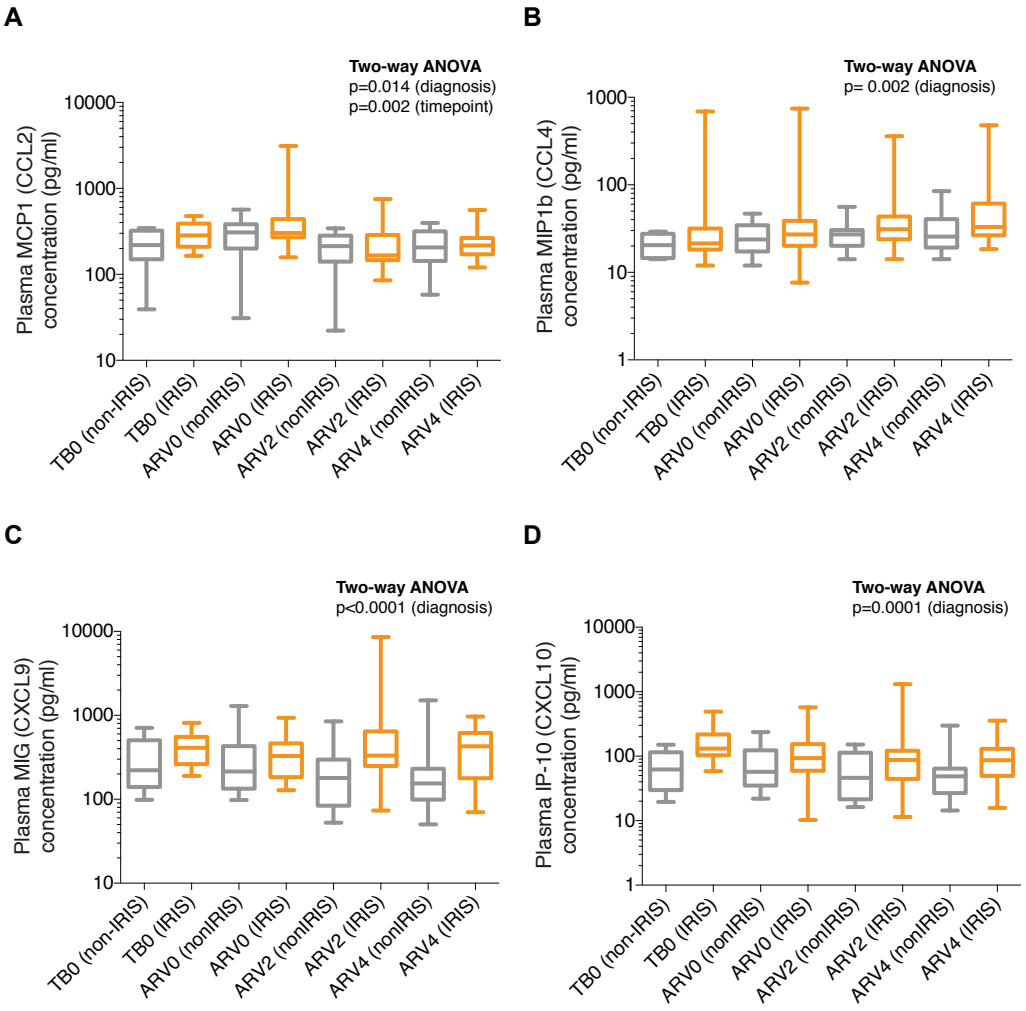



**Figure 4-21 Elevated plasma chemokines in TB-IRIS compared to non-IRIS control patients**

Plasma MCP1 (CCL2), MIP-1β (CCL4), MIG (CXCL9) and IP-10 were elevated in peripheral blood in TB-IRIS patients compared to non-IRIS control patients.

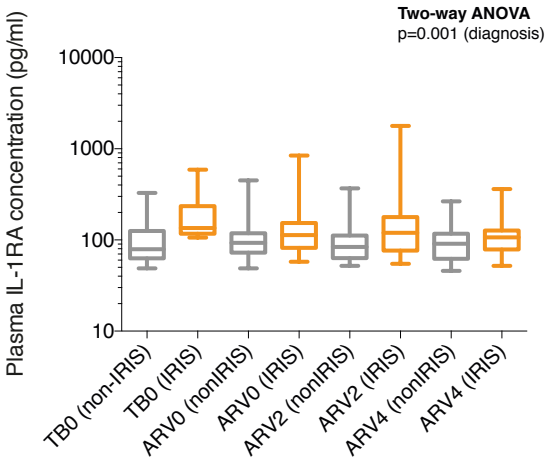



**Figure 4-22 Plasma IL-1RA in TB-IRIS and non-IRIS control patients**

Ant-inflammatory mediator, IL-1RA was elevated in TB-IRIS compared to control patients.

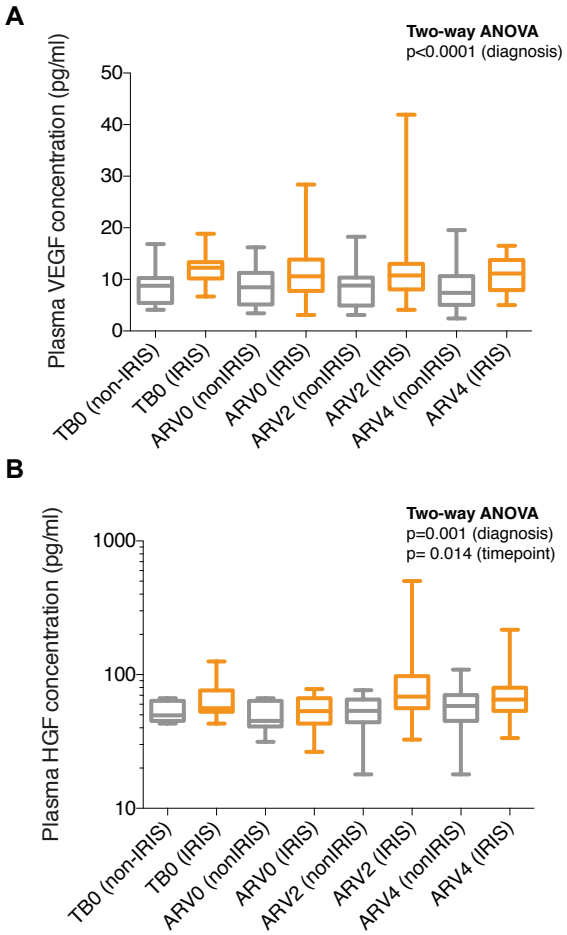



**Figure 4-23 Elevated plasma growth factors in TB-IRIS patients**

Plasma growth factors, VEGF and HGF were elevated in TB-IRIS compared to control patients. HGF also increased with increasing time from TB diagnosis.

## Table 4-10 Plasma cytokines and chemokines in TB-IRIS and non-IRIS control patients

| Plasma cytokine/chemokine concentration | Diagnostic category and sample timepoint | | | | | | | | ANOVA | | | Sidaks test (non-IRIS vs TB-IRIS) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | ARV0 | | ARV2 | | ARV4 | | | | | | | | |
| | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | Interaction | Timepoint | Diagnosis | TB0 | ARV0 | ARV2 | ARV4 |
| Median VEGF, pg/ml (IQR) | 8.78 (5.44-10.3) | 12.3 (10.2-13.4) | 8.46 (5.14-11.3) | 10.6 (7.78-13.9) | 8.8 (4.95-10.3) | 10.8 (8.08-13) | 7.42 (5.05-10.6) | 11.2 (7.94-13.8) | 0.946 | 0.83 | **<0.0001** | ns | ns | * | ns |
| Median IL-1β , pg/ml (IQR) | 6.23 (5.79-7.81) | 7.23 (6.44-8.48) | 5.8 (5.75-8) | 7.23 (5.8-7.58) | 6.36 (5.29-7.39) | 7.23 (6.65-8.59) | 6.65 (6.43-8.31) | 6.36 (5.75-8) | 0.264 | 0.881 | 0.935 | | | | |
| Median GCSF, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median EGF, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IL-10, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median HGF, pg/ml (IQR) | 49.6 (45-63.8) | 56.2 (52.7-76.4) | 45 (41-63.8) | 53.3 (43-66.6) | 53.7 (44-65.2) | 68.7 (56.1-97.6) | 58.4 (45-70.3) | 65.2 (53.7-80) | 0.264 | **0.014** | **0.001** | ns | ns | ** | ns |
| Median FGF Basic, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IFN-α, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IL-6, pg/ml (IQR) | 6.65 (4.03-17.4) | 13 (9.49-25.1) | 6.75 (4.22-13.7) | 9.04 (6.33-14.5) | 6.75 (4.26-11.8) | 15.2 (8.92-26.1) | 9.17 (3.54-15) | 12.4 (7.38-17.5) | 0.362 | 0.281 | **<0.0001** | ns | ns | ** | ns |
| Median IL-12 p40/p70, pg/ml (IQR) | 153 (135-204) | 161 (147-212) | 155 (131-197) | 149 (104-202) | 146 (116-189) | 163 (135-210) | 138 (119-172) | 165 (116-207) | 0.434 | 0.657 | 0.715 | | | | |
| Median RANTES, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median Eotaxin, pg/ml (IQR) | 15.8 (12.9-36.1) | 19.7 (14.6-37.9) | 24.6 (15.3-54.8) | 21 (14.7-48.6) | 19 (14.5-47.6) | 14.7 (10.1-41.1) | 20.4 (15.2-39.9) | 20.2 (13.9-37.5) | 0.803 | 0.234 | 0.683 | | | | |
| Median IL-13, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IL-15, pg/ml (IQR) | 78.8 (54-103) | 98.8 (78.8-132) | 67.6 (54-105) | 84.8 (67.6-127) | 56.5 (38.5-88.1) | 84.8 (58-118) | 60.7 (53.7-84.8) | 86.2 (64.8-134) | 0.811 | 0.565 | 0.076 | | | | |
| Median IL-17, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median MIP-1α, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median GM-CSF, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median MIP-1β, pg/ml (IQR) | 20.5 (14.6-27.7) | 21.4 (18.3-31.7) | 23.8 (17.3-34.6) | 27.1 (20.1-38.9) | 27.1 (20.1-30.4) | 31.2 (23.9-43.5) | 25.6 (19.2-40.5) | 33.1 (26.6-61) | 0.95 | 0.313 | **0.002** | ns | ns | ns | ns |
| Median MCP-1, pg/ml (IQR) | 219 (150-323) | 284 (209-391) | 308 (200-384) | 302 (270-440) | 213 (141-282) | 167 (147-288) | 206 (144-317) | 217 (171-265) | 0.705 | **0.002** | **0.014** | ns | ns | ns | ns |
| Median IL-5, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IFN-γ, pg/ml (IQR) | 15 (10.6-20.9) | 25 (17.9-48.9) | 14.5 (9.32-20.9) | 19.4 (13.8-38.8) | 13 (8.76-16.2) | 17.2 (13.2-23.1) | 12.1 (9.83-15.7) | 18.5 (14.9-22.7) | 0.853 | **0.007** | **<0.0001** | * | * | ns | ns |
| Median TNF-α, pg/ml (IQR) | 6.61 (6.2-9.22) | 6.61 (6.52-7.84) | 6.61 (5.88-8.27) | 6.61 (5.33-8.27) | 6.61 (4.88-8.27) | 6.52 (5.25-7.75) | 6.52 (5.57-10.3) | 6.61 (6.36-8.06) | 0.996 | 0.104 | 0.438 | | | | |
| Median IL1-RA, pg/ml (IQR) | 79.4 (63.1-125) | 135 (117-236) | 93.1 (72.5-118) | 113 (82-154) | 83.7 (63.1-112) | 120 (76.5-179) | 90.9 (62.1-117) | 107 (79-127) | 0.425 | 0.396 | **0.001** | ns | ns | ns | ns |
| Median IL-2, pg/ml (IQR) | 7.54 (7.29-8.14) | 7.66 (6.95-8.39) | 6.91 (6.14-8.06) | 7.19 (6.91-8.06) | 7.29 (6.18-8.39) | 7.39 (6.77-8.23) | 7.42 (6.77-8.21) | 7.66 (7.29-8.06) | 0.782 | 0.539 | 0.772 | | | | |
| Median IL-7, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IP-10, pg/ml (IQR) | 62.6 (29.9-115) | 131 (103-218) | 57.4 (35.2-12.4) | 93.9 (59.5-154) | 46.3 (21.5-114) | 87.6 (44.5-122) | 48.8 (26.9-64.1) | 86.5 (49.5-130) | 0.61 | 0.206 | **0.0001** | * | ns | ns | ns |
| Median IL-2R, pg/ml (IQR) | 498 (338-778) | 1274 (854-2486) | 430 (297-1068) | 901 (559-1462) | 457 (245-1226) | 1164 (683-1875) | 450 (206-685) | 1034 (438-1599) | 0.651 | 0.641 | **<0.0001** | ns | ns | * | ns |
| Median MIG, pg/ml (IQR) | 222 (140-504) | 411 (263-555) | 215 (134-431) | 327 (184-465) | 180 (84-298) | 331 (248-645) | 155 | 427 (179-620) | 0.282 | 0.517 | **<0.0001** | ns | ns | ** | * |
| Median IL-4, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |
| Median IL-8, pg/ml (IQR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | | | |

ND=not done, above or below the standard curve
ns=not significant

### The effect of corticosteroid therapy on inflammatory mediators

Corticosteroid therapy was administered to 18 (62.1%) of TB-IRIS patients during follow up. Corticosteroid therapy has broad immunomodulatory effects and has been demonstrated as of therapeutic benefit in paradoxical TB-IRIS, reducing symptom severity and hospital admissions (265). In most cases of TB-IRIS, prednisone therapy was commenced between ARV2 and ARV4 visits, by which time the diagnosis of IRIS was considered most likely and key differential diagnoses (such as lobar pneumonia or bacterial sepsis) had been excluded. At ARV4, 13 TB-IRIS patients were receiving corticosteroid therapy, whilst 15 TB-IRIS patients were not. In one TB-IRIS case, timing of prednisone initiation was not recorded.

To evaluate the immunomodulatory effect of corticosteroid therapy in key analytes, I selected analytes that were relatively increased in TB-IRIS patients, compared to non-IRIS control patients across the timepoints assessed, but relatively reduced by ARV4 compared to earlier timepoints, in sputum (MMP-1, MMP-2, MMP-3 and MMP-7) and in plasma (MMP-1, -3, -7, -8, IL-6, IFN-γ, IL-2R), in addition to plasma PIIINP. The results are reported in Table 4-11 and Figure 4-24). In induced sputum, corticosteroid therapy appeared to be associated with reduced MMP-1, -2 -3, -7 concentrations. Median MMP-1 concentration was 6-fold lower in corticosteroid treated patients compared to patients not on corticosteroid treatment at ARV4 (157 pg/ml, IQR 39.7-512 vs 1005 pg/ml, IQR 792-1269 respectively), median MMP-3 was 7.5-fold lower (802 pg/ml, IQR 144-2362 vs 106 pg/ml, IQR 93.5-288 respectively) and median MMP-7 concentration was 3.5-fold lower, although these differences did not reach

statistical significance on Mann-Whitney U test, likely due to the small sample size. Median MMP-2 was 1.5-fold lower in corticosteroid therapy treated patients vs patients not on corticosteroid treatment at ARV4 (802 pg/ml, IQR 144-2362 vs 106 pg/ml, IQR 93.5-288, p=0.035). In plasma, MMP-1 was slightly lower in corticosteroid therapy treated patients, although this difference was not marked. The other plasma analytes assessed did not appear to differ according to corticosteroid treatment group.

**Table 4-11 Induced sputum and plasma MMP and cytokine concentrations at ARV4 in corticosteroid-treated and -untreated patients**

| | ARV4 | TB-IRIS | | Mann-Whitney U |
|---|---|---|---|---|
| | | No corticosteroid | Corticosteroid treated | p value |
| | n (% enrolled participants) | 15 (51.7) | 13 (44.8) | |
| Induced sputum | Samples available for analysis, n (%) | 6 (40) | 7 (53.8) | ND |
| | Median MMP-1, pg/ml (IQR) | 1005 (225-3369) | 157 (39.7-512) | 0.07 |
| | Median MMP-2, pg/ml (IQR) | 1004 (792-1268) | 641 (572-794) | 0.035 |
| | Median MMP-3, pg/ml (IQR) | 802 (144-2362) | 106 (93.5-288) | 0.192 |
| | Median MMP-7, pg/ml (IQR) | 115429 (22029-209410) | 32167 (15701-49151) | 0.181 |
| Plasma | Samples available for analysis, n (%) | 9 (60) | 9 (69.2) | ND |
| | Median MMP-1, pg/ml (IQR) | 5890 (1992-8519) | 3510 (2165-6315) | 0.931 |
| | Median MMP-3, pg/ml (IQR) | 18727 (10541-21224) | 16938 (13558-35876) | 0.136 |
| | Median MMP-7, pg/ml (IQR) | 5307 (2920-22373) | 5532 (3434-9789) | 0.863 |
| | Median MMP-8, pg/ml (IQR) | 2427 (1300-6133) | 4118 (2346-10566) | 0.297 |
| | Median PIIINP concentration, pg/ml (IQR) | 44860 (27604-62510) | 46763 (27222-97271) | 0.963 |
| | Median IL-6, pg/ml (IQR) | 10.9 (7.17-15.4) | 13.8 (7.9-19.8) | 0.34 |
| | Median IFN-y, pg/ml (IQR) | 17.8 (14.6-20.6) | 19.4 (13.8-26.7) | 0.449 |
| | Median IL-2R, pg/ml (IQR) | 663 (391-2295) | 1264 (696-1579) | 0.546 |

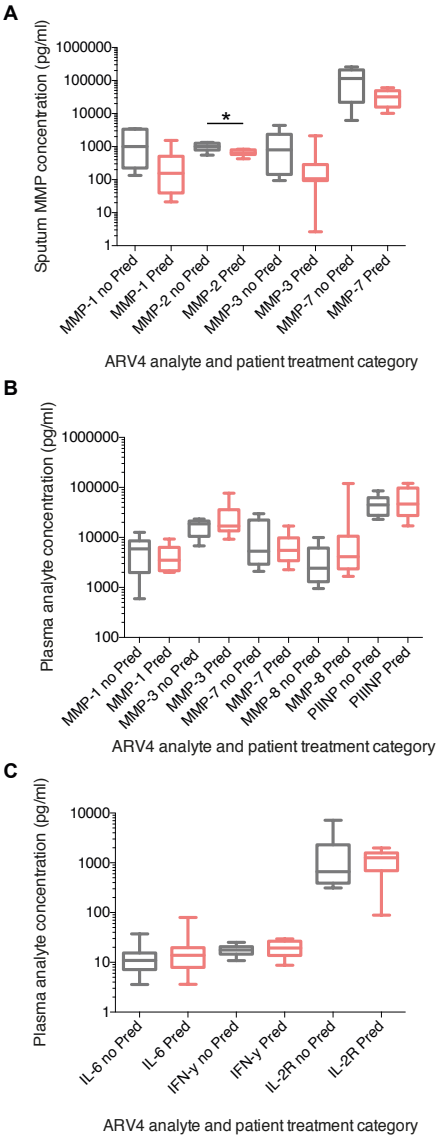



**Figure 4-24 Corticosteroid therapy and inflammatory mediators in TB-IRIS patients**

The effect of corticosteroid therapy at ARV4 in TB-IRIS patients on sputum MMP concentration (A), plasma MMP concentration (B) and plasma IL-6, IFN-γ and IL-2R concentration (C) was assessed by comparing concentrations in patients on prednisone therapy at ARV4 with those who were not. Sputum MMPs appeared to be reduced in patients taking prednisone, whereas plasma analytes did not appear to be affected.

### INKT cell frequency

After exclusion of 41 samples affected by LPS contamination of sodium heparin tubes, 100 PBMC samples were available for iNKT cell enumeration. Median iNKT cell frequency was increased in TB-IRIS compared to non-IRIS controls (p=0.003), see Figure 4-25. Although underpowered for a multiple comparisons, the trend towards increased iNKT cell frequency was evident at ART initiation, when median iNKT cell frequency was over 6-fold higher in TB-IRIS patients compared to non-IRIS controls (median 600 (IQR 78.6-464.5) vs 90 (IQR24.5-619) cells per million CD3+CD19- live lymphocytes). At ARV2, the most usual time for TB-IRIS presentation, median iNKT cell frequency per million CD3+CD19- live lymphocytes was almost ten-fold higher in TB-IRIS patients compared to non-IRIS controls (median 992 cells, IQR 166-5682 vs 100 cells, IQR 23.6-440 respectively, Table 4-12).

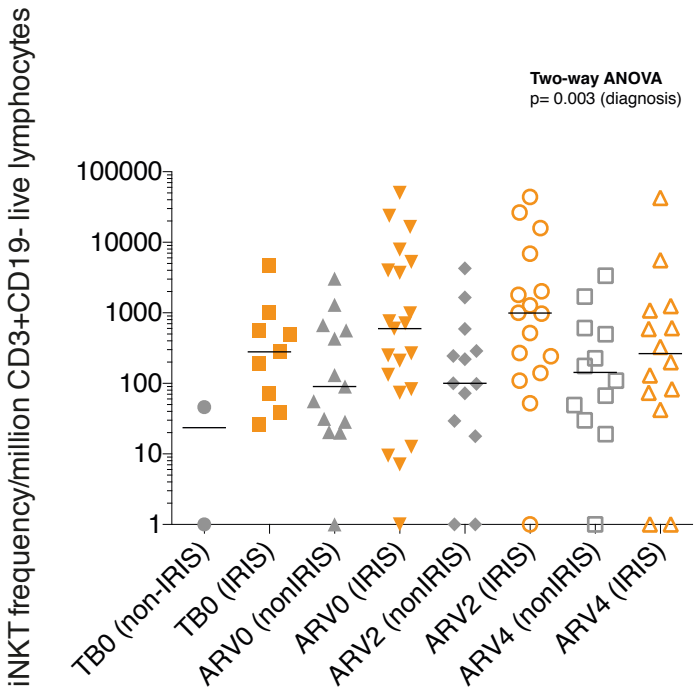



**Figure 4-25 INKT cell frequency in TB-IRIS patients and non-IRIS controls**

TB-IRIS was associated with increased iNKT cell frequency, measured as cells per million CD3+CD19- live lymphocytes, after control tetramer subtraction, compared to non-IRIS controls. This was most marked at ARV2, the most common time of TB-IRIS presentation.

## Table 4-12 INKT cell frequency in TB-IRIS patients and non-IRIS controls

| Invariant Natural Killer T cell frequency | Diagnostic category and sample timepoint | | | | | | | | ANOVA | | | Sidaks multiple comparisons test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | ARV0 | | ARV2 | | ARV4 | | | | | |
| | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | non-IRIS | IRIS | Interaction | Timepoint | Diagnosis | |
| Sample freqency, n | 2 | 9 | 13 | 21 | 13 | 16 | 12 | 14 | | | | |
| Median iNKT per million CD3+CD19- live lymphocytes (IQR) | 23 (0-46) | 280 (55.2-785) | 90 (24.5-619) | 600 (78.6-4645) | 100 (23.6-440) | 992 (166-5682) | 143 (34.8-582) | 265 (66.1-1123) | 0.458 | 0.401 | **0.003** | ns |
| Median total iNKT cells / ml peripheral blood (IQR) | 2465 (0-4930) | 12704 (1265-56850) | 5537 (1589-51032) | 30561 (3566-413956) | 7337 (1886-57678) | 39298 (9000-171767) | 11675 (6250-30274) | 22227 (4658-60347) | 0.993 | 0.695 | 0.059 | ns |
| Median iNKT per million CD3+ CD19- live lymphocytes - no control subtraction (IQR) | 156 (61.4-250) | 280 (55.2-1000) | 220 (46.8-705) | 770 (112-4700) | 260 (99.5-480) | 1100 (238-6575) | 220 (49.1-590) | 310 (114-1375) | 0.739 | 0.217 | **0.012** | ns |

In comparison to the cross-sectional study, considerable non-specific staining of cells with control tetramer occurred in the longitudinal study. In order to assess the effect of control staining on the association between iNKT cell frequency and TB-IRIS, I enumerated iNKT cell frequency without subtraction of control tetramer frequency (see Table 4-12). The observation of increased iNKT cell frequency in TB-IRIS remained and was more pronounced without control subtraction (p=0.012). As TB-IRIS patients have lower lymphocyte percentages than non-IRIS controls, I also calculated the absolute iNKT frequency per milliliter of peripheral blood by multiplying the iNKT cell percentage of lymphocytes by the total lymphocyte count per milliliter of peripheral blood. Despite reduced lymphocyte counts in TB-IRIS, absolute iNKT cell counts remained higher in TB-IRIS patients than non-IRIS controls, (p=0.059, Figure 4-26) although the difference was less marked than in the analysis by proportion.

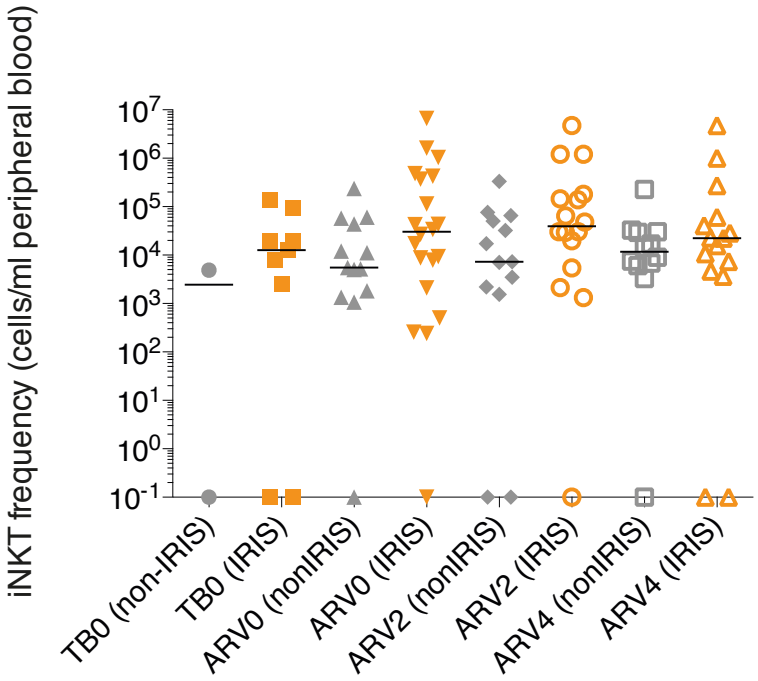



**Figure 4-26 INKT cell frequency per milliliter of peripheral blood in TB-IRIS and non-IRIS controls**

INKT cell frequency results are shown per milliliter of peripheral blood, demonstrating increased iNKT cell frequency in TB-IRIS (p=0.059).

Differential expression of CD4 and CD8 is associated with iNKT cell phenotype. CD4+CD8- and double negative (DN) cells are the most frequent cell type in the absence of disease (144). The CD4+ iNKT cell subset has been found to inversely correlate with total iNKT cell count in HIV-uninfected patients, whereas the DN subset positively correlates (144). CD4 expression is associated with an immunomodulatory phenotype with higher IL-4 production whilst IFN-γ production has been found to be similar in subsets. Double negative cells are thought to have a more pro-inflammatory phenotype. There was a trend towards lower frequencies of CD4-expressing cells in TB-IRIS but there were low frequencies of this subset in both TB-IRIS and non-IRIS controls (Figure 4-27). CD4+CD8- and CD4+CD8+ subsets were infrequent, whereas CD8+CD4- and DN cells were more frequent in both TB-IRIS and controls (Figure 4-28). There was a trend towards higher DN iNKT cells in TB-IRIS patients at TB0 and ARV0 but numbers of samples for this comparison were relatively small. As surface marker proportions were skewed even after log transformation, comparisons between TB-IRIS patients and non-IRIS controls for this analysis were assessed by Mann-Whitney U at each timepoint. P values from these comparisons were all >0.05. Results are given in Table 4-13.

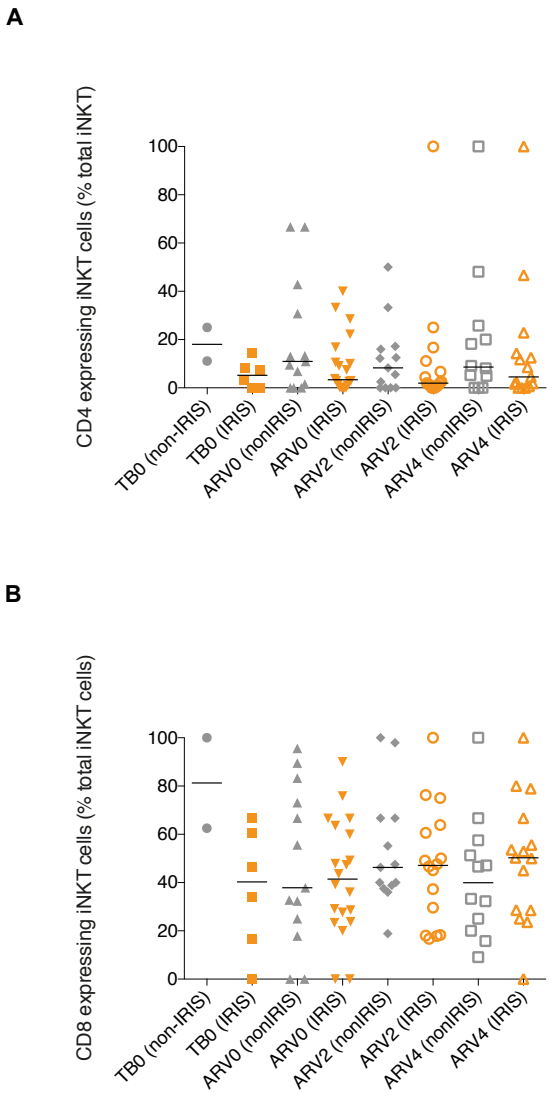



**Figure 4-27 INKT cell CD4 and CD8 expression**

CD4 and CD8 expression was measured as a percentage of the total iNKT cell count in TB-IRIS and non-IRIS control patients. A trend towards reduced CD4+ iNKT cell percentages in TB-IRIS is shown. Statistical analysis was performed using Mann-Whitney U at each timepoint and no significant differences were found.

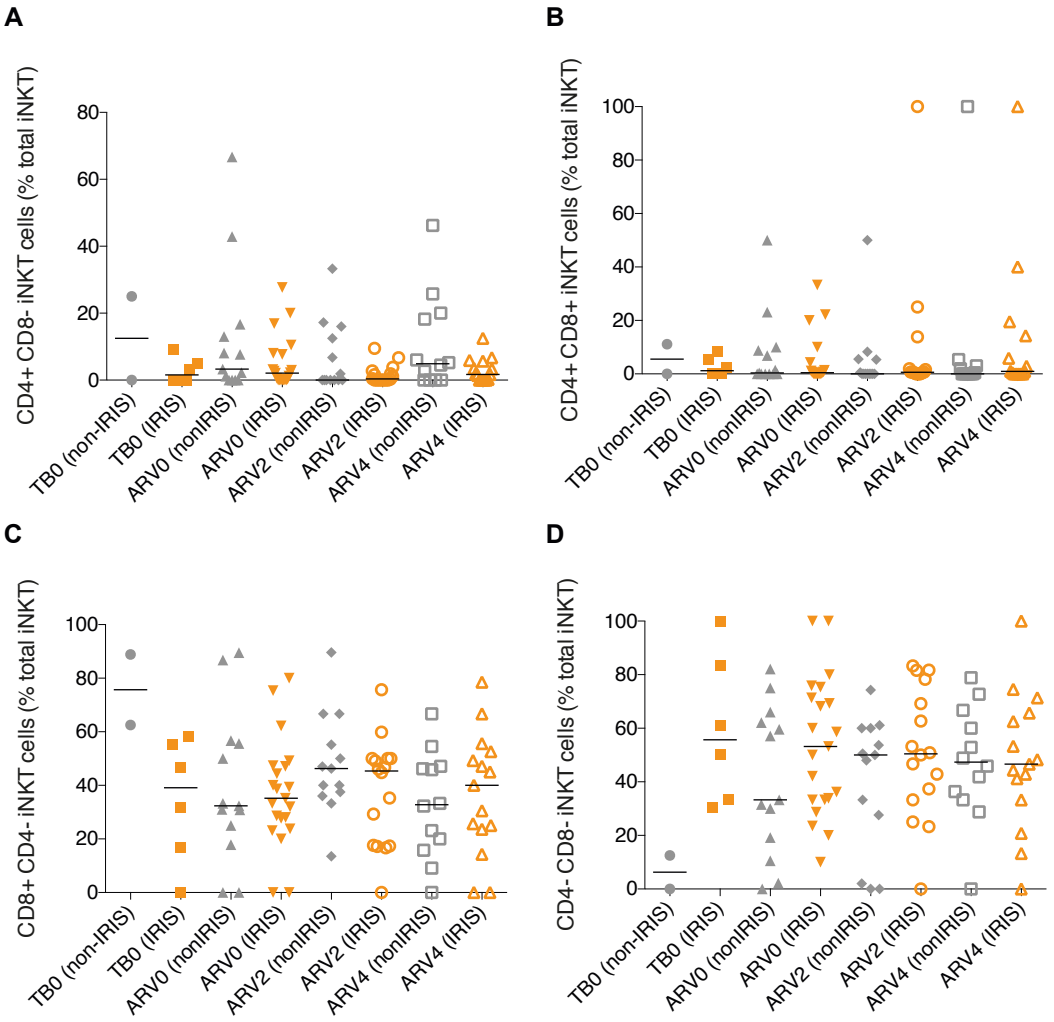



**Figure 4-28 CD4 and CD8 iNKT cell subsets in TB-IRIS and non-IRIS control patients**

CD4+CD8- iNKT cells and CD4+CD8+ (double positive, DP) iNKT cells were low in proportion, in contrast to CD8+CD4- and CD4-CD8- (double negative, DN) iNKT cells. Significant differences between TB-IRIS patients and non-IRIS controls were not observed (analysis by Mann-Whitney U). At early timepoints (TB0 and ARV0), there was a trend towards higher DN cells in TB-IRIS patients.

## Table 4-13 INKT cell CD4 and CD8 expression

| iNKT cell surface marker | Diagnostic category and timepoint | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | MWU analysis | ARV0 | | MWU analysis | ARV2 | | MWU analysis | ARV4 | | MWU analysis |
| | non-IRIS | IRIS | | non-IRIS | IRIS | | non-IRIS | IRIS | | non-IRIS | IRIS | |
| CD4 expressing iNKT (% iNKT) | 18.1 (11.1-25) | 5.24 (0-9.87) | 0.107 | 11 (0.78-36.8) | 3.4 (0.48-15.3) | 0.275 | 8.33 (0-16.6) | 1.96 (0.78-10) | 0.465 | 8.6 (1.24-24.4) | 4.54 (0.7-14.3) | 0.537 |
| CD8 expressing iNKT (% iNKT) | 81.3 (62.5-100) | 40.3 (12.5-62.1) | 0.143 | 37.9 (21.5-78.2) | 41.1 (24.8-62.7) | 0.669 | 46.3 (38.2-66.7) | 47.1 (21.1-63.1) | 0.627 | 40 (21.3-56) | 50.3 (28.6-66.7) | 0.42 |
| CD4+ CD8- iNKT (% iNKT) | 12.5 (0-25) | 1.58 (0-5.96) | 0.714 | 3.33 (0-14.9) | 2.13 (0.7.85) | 0.417 | 0 (0-14.3) | 0.4 (0-2.57) | 0.621 | 4.89 (0-19.6) | 1.67 (0-5.71) | 0.247 |
| CD8+ CD4- iNKT (% iNKT) | 75.7 (62.5-88.9) | 39.1 (12.5-56.1) | 0.071 | 32.4 (21.5-56.2) | 35.2 (25.8-47.3) | 0.923 | 50 (14.8-60) | 50.5 (34.3-76) | 0.334 | 47.4 (34.1-65) | 46.6 (33.3-65.7) | 0.605 |
| Double positive (CD4+CD8+) iNKT (% iNKT) | 5.55 (0-11.1) | 1.22 (0-6.03) | 0.714 | 0.39 (0-9.4) | 0.46 (0-2.83) | 0.749 | 0 (0-5.49) | 0.656 (0.073-1.87) | 0.29 | 0 (0-2.75) | 0.95 (0-14.3) | 0.337 |
| Double negative (CD4-CD8-) iNKT (% iNKT) | 6.25 (0-12.5) | 55.7 (32.6-87.5) | 0.071 | 33.3 (14.9-64.1) | 53.2 (33.3-73.4) | 0.19 | 50 (14.8-60) | 50.5 (34.3-76) | 0.368 | 47.4 (34.1-65) | 46.6 (33.3-65.7) | 1 |

CD107a, a marker of degranulation, has been observed in increased frequency in NK cells from TB-IRIS patients (123). PD1, is a marker of iNKT cell anergy. CD40L has a role in dendritic cell priming (148, 315, 316). Expression of these surface markers was assessed for further phenotypic characterization of iNKT cells in the longitudinal study. Results are shown in Figure 4-29 and Table 4-14. Expression of these markers was similar in TB-IRIS patients and non-IRIS controls, with high expression of PD-1 and low expression of CD40L. CD107a expression tended to be low but was high in some patients. Expression of activation markers CD161 and CD95 was also similar in the two groups. MFIs of these markers were assessed but were not found to differ between groups.

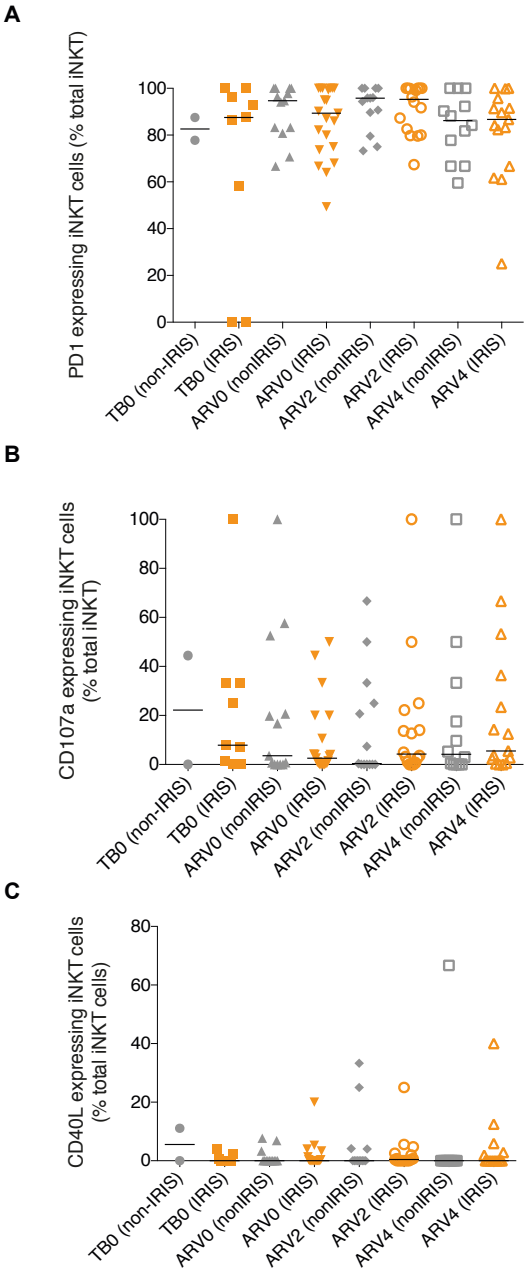



**Figure 4-29 INKT cell surface expression of PD1, CD107a and CD40L in TB-IRIS and non-IRIS control patients**

Programmed death 1 (PD-1), CD107a and CD40 ligand (CD40L) expression on iNKT cells was measured as a percentage of total iNKT cells. PD-1 expression was high in both TB-IRIS patients and controls (A). CD107a (B) and CD40L (C) expression were low, although considerable heterogeneity in CD107a expression was observed.

**Table 4-14 INKT cell surface marker expression in TB-IRIS patients and non-IRIS controls**

| iNKT cell surface marker | Diagnostic category and timepoint | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TB0 | | MWU analysis | ARV0 | | MWU analysis | ARV2 | | MWU analysis | ARV4 | | MWU analysis |
| | non-IRIS | IRIS | | non-IRIS | IRIS | | non-IRIS | IRIS | | non-IRIS | IRIS | |
| PD1 expressing iNKT (% iNKT) | 82.7 (77.8-87.5) | 87.5 (29.2-98.1) | 0.782 | 94.7 (82.1-100) | 89.4 (74.4-100) | 0.552 | 95.8 (84.6-100) | 95.3 (80.7-100) | 0.913 | 86.2 (69.5-98.1) | 86.5 (66.7-95.7) | 0.904 |
| CD40L expressing iNKT (% iNKT) | 5.55 (0-11.1) | 0 (0-1.62) | 0.618 | 0 (0-1.67) | 0 (0-0.89) | 0.431 | 0 (0-4.09) | 0.44 (0-2.4) | 0.483 | 0 (0-0) | 0 (0-2.86) | 0.145 |
| CD107a expressing iNKT (% iNKT) | 22.2 (0-44.4) | 7.89 (0.785-33.3) | 1 | 3.57 (0-36.7) | 2.57 (0.19-15.3) | 0.615 | 0.39 (0-29.2) | 4.27 (0.813-20.2) | 0.546 | 4.15 (0-29.4) | 5.56 (0.65-36.4) | 0.491 |
| CD95 expressing iNKT (% iNKT) | 100 (100-100) | 99.6 (89.7-100) | 0.464 | 96.3 (81.6-100) | 99.3 (94.6-100) | 0.19 | 96 (88.2-100) | 98.5 (94.3-100) | 0.557 | 95.7 (91-100) | 94.4 (86.7-99.1) | 0.386 |
| CD161 expressing iNKT (%iNKT) | 23.6 (22.2-25) | 67.7 (28.6-95.4) | 0.286 | 71.4 (18.7-81.8) | 80.5 (47.1-92.8) | 0.126 | 75 (18.3-82.6) | 86.6 (52.8-95.3) | 0.089 | 71.1 (59.3-82.3) | 73.5 (33.3-91.5) | 0.71 |

Correlations were assessed between iNKT cell proportions and 13 key peripheral blood parameters that were identified prospectively as of interest for this analysis: CD4 count and proportions, HIV-1 viral load, C-reactive protein concentration, plasma MMP-1, -3 and -8, plasma PIIINP, IL-1β, IL-6, IL-12 p40/p70, TNF-α and IFN-γ. Results are reported in Table 4-15 and Figure 4-30. Plasma MMP-8 was the most significantly correlated with iNKT cell count (Spearman r=0.327, p=0.007). Bonferroni correction for multiple comparisons indicated that significance could be inferred by a p value <0.004 in this analysis. Lesser correlations were observed with C-reactive protein (r=0.27, p=0.03) and CD4 count (r=0.26, p=0.041), IL-12 p40/p70 (r=0.21, p=0.091) and IL-1β (r=0.208, p=0.096).

**Table 4-15 Correlation between iNKT cell proportions and peripheral blood immune parameters**

| iNKT cell correlation | Spearman r | 95% CI | p value |
|---|---|---|---|
| CD4 count (cells/mm3) | 0.255 | 0.004 to 0.475 | **0.041** |
| CD4 (% total white cell count) | 0.193 | 0.061- to 0.424 | 0.123 |
| HIV viral load, copies/ml | 0.111 | -0.1482 to 0.356 | 0.387 |
| C-reactive protein concentration (mg/dL) | 0.27 | 0.020 to 0.487 | **0.03** |
| Plasma MMP-1 concentration (pg/ml) | 0.071 | -0.181 to 0.314 | 0.573 |
| Plasma MMP-3 concentration (pg/ml) | -0.037 | -0.283 to 0.214 | 0.77 |
| Plasma MMP-8 concentration (pg/ml) | 0.327 | 0.085 to 0.532 | **0.007** |
| Plasma IL-1B concentration (pg/ml) | 0.208 | -0.045 to 0.436 | 0.096 |
| Plasma IL-6 concentration (pg/ml) | 0.103 | -0.15 to 0.343 | 0.41 |
| Plasma IL-12 concentration (pg/ml) | 0.21 | -0.041 to 0.436 | 0.091 |
| Plasma IFN-y concentration (pg/ml) | 0.095 | -0.158 to 0.336 | 0.447 |
| Plasma TNFa concentration (pg/ml) | 0.029 | -0.221 to 0.276 | 0.815 |
| Plasma PIIINP concentration (pg/ml) | 0.092 | -0.173 to 0.345 | 0.485 |

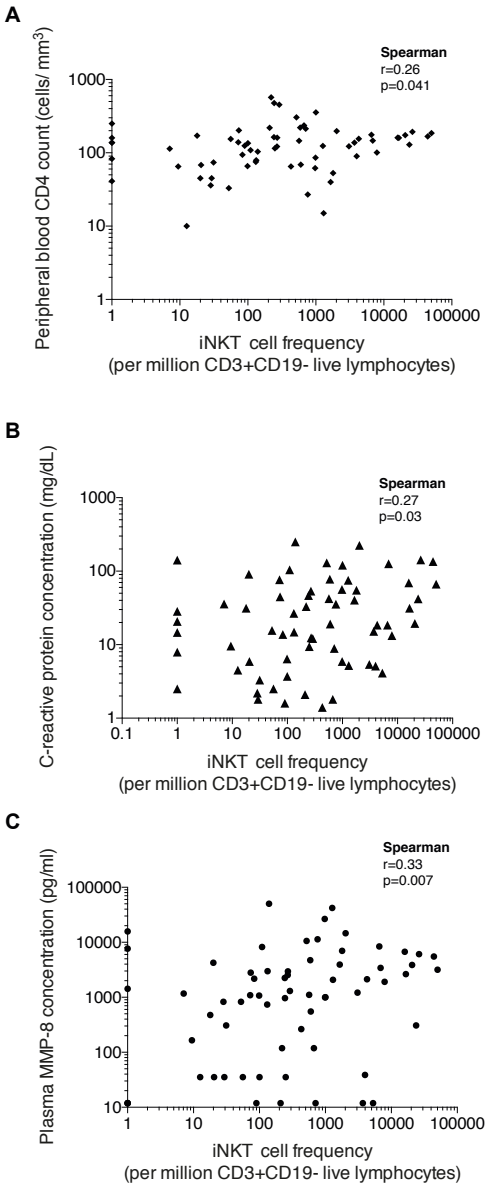



**Figure 4-30 Relationship between iNKT cell frequency and peripheral blood CD4 count, C-reactive protein and plasma MMP-8 concentration**

A weak correlation was found between peripheral blood CD4 count and iNKT cell frequency (A), but not CD4 percentage (not shown). C-reactive protein (B) and plasma MMP-8 (C) did correlate with iNKT cell frequency, but this did not reach significance after Bonferroni correction for multiple comparisons.

## Discussion

### Summary of main findings of longitudinal study

In the longitudinal study, I aimed to characterize inflammation and inflammatory mediators at TB diagnosis (TB0), at ART initiation (ARV0) and subsequently over the first twelve weeks of ART. By comparing patients who developed symptoms of TB-IRIS with patients who did not develop TB-IRIS, I hoped to identify inflammatory mediators responsible for immunopathology. I found an extremely high incidence of TB-IRIS, occurring in 59% of longitudinal study patients. Morbidity was high, with 45% requiring hospital admission and over half requiring corticosteroid therapy. TB-IRIS was associated with a systemic inflammatory response, elevation in heart rate, respiratory rate and C-reactive protein, a fall in Hb and albumin concentration and a peripheral blood neutrophilia and lymphopenia.

TB-IRIS tended to occur in patients who had a higher heart rate, respiratory rate and C-reactive protein and higher HIV-1 viral load at TB diagnosis, suggesting an association with more severe illness at presentation, which may reflect increased and/or disseminated M.tb. antigen. Whilst an elevated HIV-1 viral load was associated with TB-IRIS diagnosis, HIV virus itself was unlikely to be directly responsible for the immunopathology in TB-IRIS, as a dramatic fall in HIV-1 viral load occurred within one week of ART. However, it is possible that falling HIV-1 viral load had an immunomodulatory effect that was permissive of increased inflammation during TB-IRIS.

Worsening radiological features were common at TB-IRIS diagnosis. TB-IRIS patients tended to have higher chest x-ray inflammation scores than non-IRIS patients. There was a trend towards increased frequency of sputum smear and culture-positivity in TB-IRIS patients, but time to culture positivity was similar.

TB-IRIS was associated with evidence of increased matrix breakdown. In TB-IRIS patients, sputum MMPs were elevated compared to non-IRIS controls, particularly MMP-7 and -10, which were significantly increased. Plasma MMPs were also increased in TB-IRIS patients. A trend was evident from TB diagnosis for MMP-1, -2 and -8, which were significantly increased in TB-IRIS compared to non-IRIS at two weeks post-ART initiation, the most common time of TB-IRIS presentation. Plasma PIINP was similarly increased in TB-IRIS. In plasma, I did not find a difference in MMP-7 in TB-IRIS and control groups, demonstrating a compartment effect on MMP upregulation.

In sputum, elevated pro-inflammatory cytokines IL-6, IL8 and G-CSF at ARV2 suggested an exaggerated innate immune response in TB-IRIS patients. This was supported by findings in plasma where pro-inflammatory cytokines, chemokines and the chemokine receptor IL-2R were significantly elevated. There was also evidence of an increased, possibly compensatory, anti-inflammatory response. Corticosteroid therapy was associated with reduced sputum MMPs but did not appear to have an effect on plasma MMPs or cytokines.

iNKT cell proportions were elevated in TB-IRIS compared to non-IRIS controls, at ART initiation (ARV0) and at two weeks post-ART initiation (ARV2). Relatively increased proportions of the double negative (CD4- CD8-) iNKT cell subset in TB-IRIS patients at TB diagnosis and at ART initiation may indicate that these cells have a pro-inflammatory phenotype in TB-IRIS. There was no clear difference in markers of activation or degranulation capacity.

### Relationship to previously published work

As previous studies have reported reduced inflammatory pathology in advanced TB-HIV co-infection, I had not expected to find evidence of severely destructive pulmonary pathology at TB diagnosis in longitudinal study patients with relatively low CD4 counts. However, there was a great variety of chest radiograph appearance at TB diagnosis. Some patients had little evidence of inflammatory pathology on chest radiograph. However, some patients presented with severe pulmonary damage, including cavitary TB. It is possible that the CD4 count fell with active TB infection, and therefore was not reflective of chronic advanced immunosuppression. Alternatively, it suggests there is heterogeneity in destructive immunopathology in TB, even in advanced HIV.

In the cross-sectional study, I reported higher neutrophil proportions in TB patients compared to controls. Here, I have shown that neutrophil proportions are significantly higher in TB-IRIS patients prior to and at the time of TB-IRIS, compared to those in non-IRIS control patients. Neutrophils may contribute to innate immunity in TB but they have also been associated with poor outcomes (72, 304). Increased neutrophil proportions in TB-IRIS support the hypothesis

that TB-IRIS patients have increased M.tb. antigen, as an association between neutrophil count and sputum mycobacterial load in TB has previously been shown (305). A previous study of TB meningitis IRIS has shown higher CSF neutrophil counts in TB-IRIS patients at TB meningitis diagnosis and further expansions in neutrophil counts at the time of TB-IRIS diagnosis (254). This study found an association between CSF culture-positivity and TB-IRIS diagnosis, supporting the hypothesis that M.tb. antigen load is increased in TB-IRIS patients. Neutrophils upregulate MMP-8 in TB and drive tissue destruction (306). They are likely to be a source of increased plasma MMP-8 detected in TB-IRIS.

The finding of increased plasma MMP-1, -3, and -8 in TB-IRIS is consistent with our previous report of MMP upregulation in PBMC from TB-IRIS patients at the time of TB-IRIS, in response to restimulation with M.tb. In that study, MMP-7 was found to be increased in serum and was corticosteroid modulated. In my current study, I found significantly increased MMP-7 in sputum in TB-IRIS, which was reduced in corticosteroid treated patients. MMP-7 was not significantly increased in plasma in TB-IRIS patients in the current study, but there was a trend towards increased levels at all timepoints. The finding that MMPs appear to be immunomodulated by corticosteroid therapy, supports previous reports of corticosteroid modulation of MMP activity in TB (317). MMP inhibition may be one mechanism by which corticosteroids improve outcomes in TB-IRIS (265).

In addition to enzymatic activity causing matrix breakdown, MMPs may drive inflammatory cell recruitment in TB-IRIS (318). MMP-7 mediated elastin

degradation, causes the release of elastin-derived peptides that are chemotactic (187, 319, 320). There is now considerable evidence of exaggerated pro-inflammatory innate immune responses during TB-IRIS, supported by my findings (258, 273, 291, 321). However, the cellular source of increased pro-inflammatory mediators, including IL-6 and IFN-γ, is not known (291). iNKT cells can produce abundant pro-inflammatory cytokines on stimulation without priming, including IL-6 and IL-1β and IFN-y. However, in my study I did not find a correlation between iNKT cell count and these cytokines.

Previous longitudinal studies have found that iNKT cell frequencies in humans are remarkably stable in under normal physiological conditions (144). iNKT cells comprise a relatively small proportion of the total lymphocyte count, but a considerable variety in the proportions has been demonstrated in healthy subjects (144). However in TB, longitudinal studies have shown iNKT cell frequencies to increase in six months with antituberculosis therapy, to levels comparable to TB contacts (146). Similarly, a two-fold increase in iNKT cell frequency has been observed in patients taking ART by three months (151). In my study, total iNKT cell frequency, whether measured as a proportion of T lymphocytes or as an absolute frequency per milliliter of peripheral blood, did not vary significantly over time. However, these timepoints were relatively early in treatment in comparison to the previous studies that identified increases with ART and antituberculosis therapy.

It is possible that having a low iNKT cell frequency causes a predisposition to TB. However, it seems more likely that peripheral blood iNKT cells decrease in

response to active TB, due to migration to the lung or other site of inflammation. The recruitment of iNKT cells to sites of inflammation may be controlled by chemokines released from macrophages or dendritic cells at the site of an infection (322). Human iNKT cells express receptors for extra-lymphoid tissue and inflammation-related chemokines, unlike conventional T cells (322). However, it is not clear whether iNKT cell recruitment is protective or pathological in TB. Whilst iNKT cells may not be essential for protection against TB, they have an important regulatory function (131, 323). Such a regulatory function has been demonstrated in a number of other diseases including cystic fibrosis, where iNKT cells are thought to suppress autoimmunity (324). Additionally, iNKT cells have been shown to induce neutrophil accumulation in a mouse model of hepatitis, through release of IL-4 (325).

### Strengths and Limitations

My study was limited by the relatively small sample size in the control group, due to a higher proportion of patients developing TB-IRIS than expected, and the LPS contamination of blood collection tubes, causing samples from some timepoints to be discarded. Additionally, the difficulty collecting induced sputum samples from patients with severe illness due to TB-IRIS may have introduced ascertainment bias. This is likely to lead to an underestimation of effect rather than an overestimation.

I expected TB-IRIS patients to present with features of IRIS at two weeks post-ART initiation. The study was designed so that the control group would also be sampled at that time. ARV2 was the most common time of IRIS presentation.

However, a number of TB-IRIS patients presented after one week of ART. I do not have sufficient control samples for a meaningful comparison at this timepoint.

Another limitation of the study is that TB-IRIS patients could have been mis-classified as non-IRIS cases and vice versa. The diagnosis of TB-IRIS is clinical and follows consensus guidelines. In this study I minimized the risk of mis-classification by extensive diagnostic testing for differential diagnosis and thorough case review by three experienced clinicians. Several patients in the control group developed new symptoms during study follow up, but on consensus panel review an alternative diagnosis was considered more likely. Future subgroup analyses of these symptomatic patients to will be of interest. This study limitation highlights the need for improved diagnostic tests for TB-IRIS, especially to improve study of interventions.

A strength of the iNKT cell analysis is the robust method of iNKT quantification (326). PBMC yields were low in this group of TB-HIV co-infected patients with advanced immunosuppression. Non-specific tetramer binding is a recognized limitation of tetramer-based assays. In this study, I controlled for non-specific binding by setting up a CD1d tetramer without α-galcer (tet-cont), for every sample analysed. I then subtracted control tetramer cell frequencies from α-galcer-loaded tetramer (tet+) frequencies to give the iNKT cell frequency. Tet-cont staining was variable and in some patients was similar to tet+ staining, resulting in zero or negative values for iNKT cell frequencies. This was robust for iNKT quantification. The same pattern of increased iNKT cell frequency in TB-IRIS patients was also seen when the uncorrected data was analysed.

## Future work

Future work will focus on three areas:

1) Further analysis of current dataset to confirm findings and generate causal hypotheses.

I plan to perform repeated measures ANOVA on samples available from individuals at several timepoints to confirm my current findings. I will also calculate fold changes in MMP and PIIINP concentrations at timepoints relative to the time of IRIS onset. I plan to use principal component analysis to investigate causal relationships between markers of inflammatory pathology. Principle component analysis offers the benefit of improving power to find significant associations between multiple variables in relatively small sample sizes. I would also like to repeat iNKT cell enumeration on samples from TB patients who participated in the TDTB2 study following TB treatment completion, to evaluate whether iNKT cell frequencies have increased in the absence of active TB.

2) INKT cell interrogation in laboratory studies to explore a mechanistic link between NKT cell activity and TB tissue damage.

In a new flow cytometric panel, using cryopreserved iNKT cells from study participants, I plan to perform intracellular staining for key cytokines and cytotoxic mediators. I would particularly like to study Granzyme B, Perforin and IL-4, which will help to characterize iNKT cells by cytotoxic and regulatory functions (144, 296).

I also plan to investigate a mechanistic link between NKT cell proliferation and tissue damage in a cell culture system. Firstly, I plan to analyse cytokine expression by purified NKT cells from TB-IRIS and non-IRIS control patients by RT-PCR. Next, I will stimulate M.tb.-infected monocyte-derived macrophages with recombinant iNKT cell-expressed cytokines and analyse MMP gene expression and secretion to determine whether iNKT cells amplify M.tb.-induced MMP secretion. Together, these experiments will dissect iNKT-macrophage-MMP networks amplifying TB-driven immunopathology.

3) Human intervention studies to explore therapeutic benefits of MMP inhibition in TB and TB-IRIS.

Evidence for MMP-driven inflammatory pathology in TB is sufficient to warrant clinical intervention studies of MMP-inhibition in humans. This will be the subject of my discussion in Chapter 5.

## Conclusions

In a longitudinal study of TB-HIV co-infected patients at high risk of TB-IRIS, I have demonstrated that elevated MMPs in sputum and plasma associate with TB-IRIS diagnosis. The matrix degradation product, PIIINP was also increased in plasma in TB-IRIS patients, compared to non-IRIS controls, indicating increased matrix breakdown. Inhibition of MMP activity may have a role in prevention and treatment of TB-IRIS. Additionally, peripheral blood iNKT cell proportions were increased in TB-IRIS patients compared to non-IRIS controls. Further work is required to elucidate the importance of iNKT cells in TB-IRIS pathology.

# Chapter 5 Discussion

The overall aim of my work was to describe patterns of differential inflammatory pathology in TB and HIV-associated TB and corresponding immunological phenotypes to identify host factors that may improve diagnosis and provide new targets for host-directed therapy in TB and TB-HIV co-infection.

## The need for new diagnostics for TB-HIV co-infection and TB-IRIS

Diagnosis of TB can be extremely challenging in patients with advanced HIV, due to factors demonstrated in this study, such as lack of productive cough, low smear positivity and minimal chest x-ray findings (171, 327). The need for new diagnostics is highlighted by studies that show a substantial proportion of patients die of undiagnosed TB, or early during therapy, possibly as consequence of late diagnosis (20, 327, 328). Sputum is hard to obtain from hospitalized patients. In these patients, the differential diagnosis of TB in a HIV-infected person is wide, and similarly at TB-IRIS presentation, there are multiple other diagnoses that require consideration, and may delay instigation of corticosteroid therapy (236, 262). Highest TB-HIV incidence areas are often poorly resourced, with inferior health care access. These are areas where sputum diagnostics are extremely challenging and the technology for cell culture required for IGRA is not available (327).

## The rationale MMP/MDP-based diagnostics

Whilst evidence of TB at the site of primary infection in the lung may be scarce, plasma PIIINP is significantly elevated in TB-HIV co-infection and correlates with markers of immunopathology. This suggests that plasma PIIINP could be useful

in TB diagnostics, as matrix degradation, whilst not specific to TB amongst respiratory illness, may be relatively specific to TB amongst respiratory infections. Another potential role for plasma PIIINP monitoring in TB is in intervention studies, when it could be used as a peripheral marker of inflammatory pathology (201). It may be more objective than chest x-ray inflammation scores and is more convenient than sputum-based assays. PIIINP could be combined with a panel of MMPs and clinical variables for greater specificity. There is the potential for quantification using a lateral flow device given that, in contrast to IGRA, no incubation step is required.

### The need for host-directed therapy for TB inflammatory pathology

Attempts to reduce the duration of TB therapy with antimycobacterial agents have so far failed (310, 311). One reason for this is that M.tb. is contained in relatively immunopriviledged sites, such as cavities, where high concentrations of drug cannot be obtained (329). Drug delivery into the cell is also a challenge. The mycobacterial cell wall is remarkably resistant to penetration by antibiotics, that may otherwise effectively inhibit essential mycobacterial enzymes (330). Although promising in pre-clinical and phase 1 studies, efficacy studies of T cell vaccines, enhancing cell-mediated immune responses to TB to prevent active TB, have been disappointing. It is becoming apparent that elements of the immune response to TB favour bacterial persistence (43). Too much immunity may be as bad as too little. Inhibiting specific aspects of TB immunity, rather than boosting them, may prove a more rewarding approach (331).

## The rationale for MMP inhibition in TB and TB-IRIS

My results demonstrating elevated matrix turnover in TB and TB-HIV co-infection and correlations between matrix turnover and TB immunopathology strongly support an interventional study of MMP inhibition in humans as an adjunct to TB treatment. Cellular and animal studies support the addition of MMP inhibitors (195, 306). MMP inhibition has been demonstrated to be of benefit in other conditions, where pathology is attributable to matrix remodeling, including cardiac disease and gingivitis (332).

The potential benefits of MMP inhibition in TB include reduction in long-term respiratory morbidity and reduced transmissibility. Candidate MMP inhibitors include doxycycline and Ro32-3555 (329). I have shown that doxycycline, which directly inhibits MMP-1 upregulation in TB, also has antimycobaterial activity (195). Many other agents, including vitamin D and other antibiotics, have modulatory effects on MMP activity and could potentially be used for this purpose (312, 313).

There is a strong economic argument for re-purposing drugs currently licensed for other indications, as manufacturing and development costs will be lower (333). A sense of urgency exists considering that more patients died in the last year from TB than in the worst Ebola virus outbreak on record (334). If drugs of potential benefit are known to be affordable and safe, there is an imperative to put them to the test in clinical trials.

### INKT cells and immunomodulatory therapy

My study is the first report of iNKT cell frequency and phenotype in TB-HIV co-infection and there is much further study required to elucidate a mechanism by which iNKT cells contribute to TB immunity or pathology. Vaccination boosting strategies are effective against mycobacterial infections in mice (143). Administration of α-galcer has been shown to improve responses to TB treatment in mice (142). However, findings in mice will not always concur with human studies and more human studies are required. Therapeutic administration of α-galcer to boost anti-cancer immunity, the subject of some exploratory clinical studies, has been relatively well-tolerated and associated with some favourable responses (316).

### Future work

I plan to put in an intermediate fellowship application to study the therapeutic effects of MMP inhibition in TB. This will be an exploratory study, including both HIV-infected patients and –uninfected patients. Outcome measures will include time to sputum smear conversion, time to culture conversion, radiographic scores and lung function at 12 months and incidence of TB-IRIS in the TB-HIV subgroup and PIIINP concentrations. Ideally, this study will include a subset of patients with MDR-TB, as this group of patients is desperately in need of treatment-shortening interventions and improved outcomes. Additionally, I intend to collaborate on studies of MDPs in TB diagnosis, using proteomics. Whilst plasma PIIINP is unlikely to be sufficiently specific for alone, it could be

combined with a panel of MMPs/ MDPs for improved diagnosis of TB and TB-IRIS.

## Conclusions

Active TB in HIV-infected and -uninfected patients is associated with inflammatory pathology that causes considerable morbidity. In cross-sectional and longitudinal analyses of TB-HIV co-infected patients and controls, I have demonstrated an important role for MMPs in inflammatory pathology at TB diagnosis and during TB-IRIS. This and previous work strongly support human intervention studies targeting MMP activity. The role of iNKT cells in TB-HIV immunopathology is unclear. This work, the first study of iNKT cells in TB-HIV co-infection, supports previous laboratory and human studies in TB and HIV, suggesting iNKT cells are important. However, it is unclear whether iNKT cells play a protective or pathological role and further study is required.

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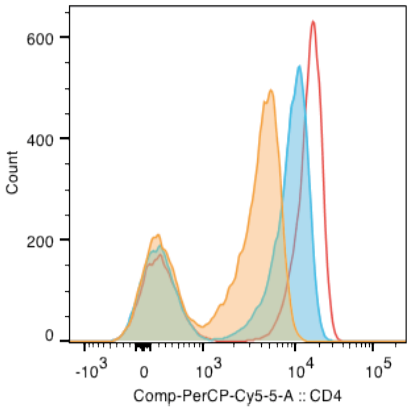
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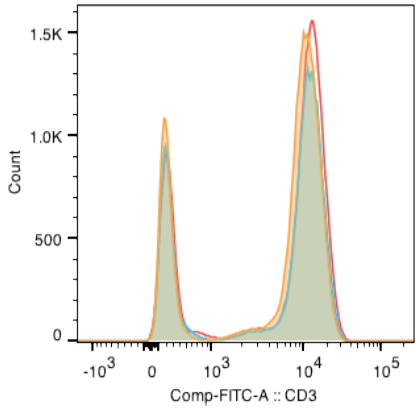
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# Appendix 1 Supplementary Methods

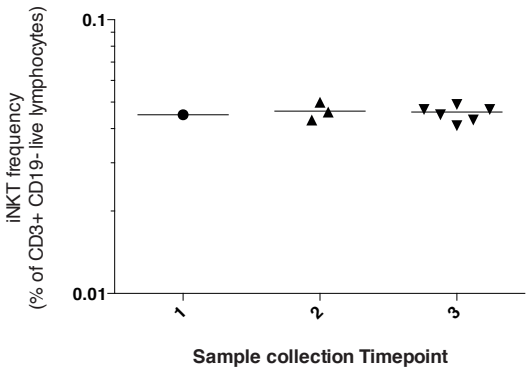



| | Sample Name | Subset Name | Count |
|---|---|---|---|
| | Specimen_001_Tube_003.fcs | CD3+ lymphocytes | 22380 |
| | Specimen_001_Tube_002.fcs | CD3+ lymphocytes | 20900 |
| | Specimen_001_Tube_001.fcs | CD3+ lymphocytes | 19031 |



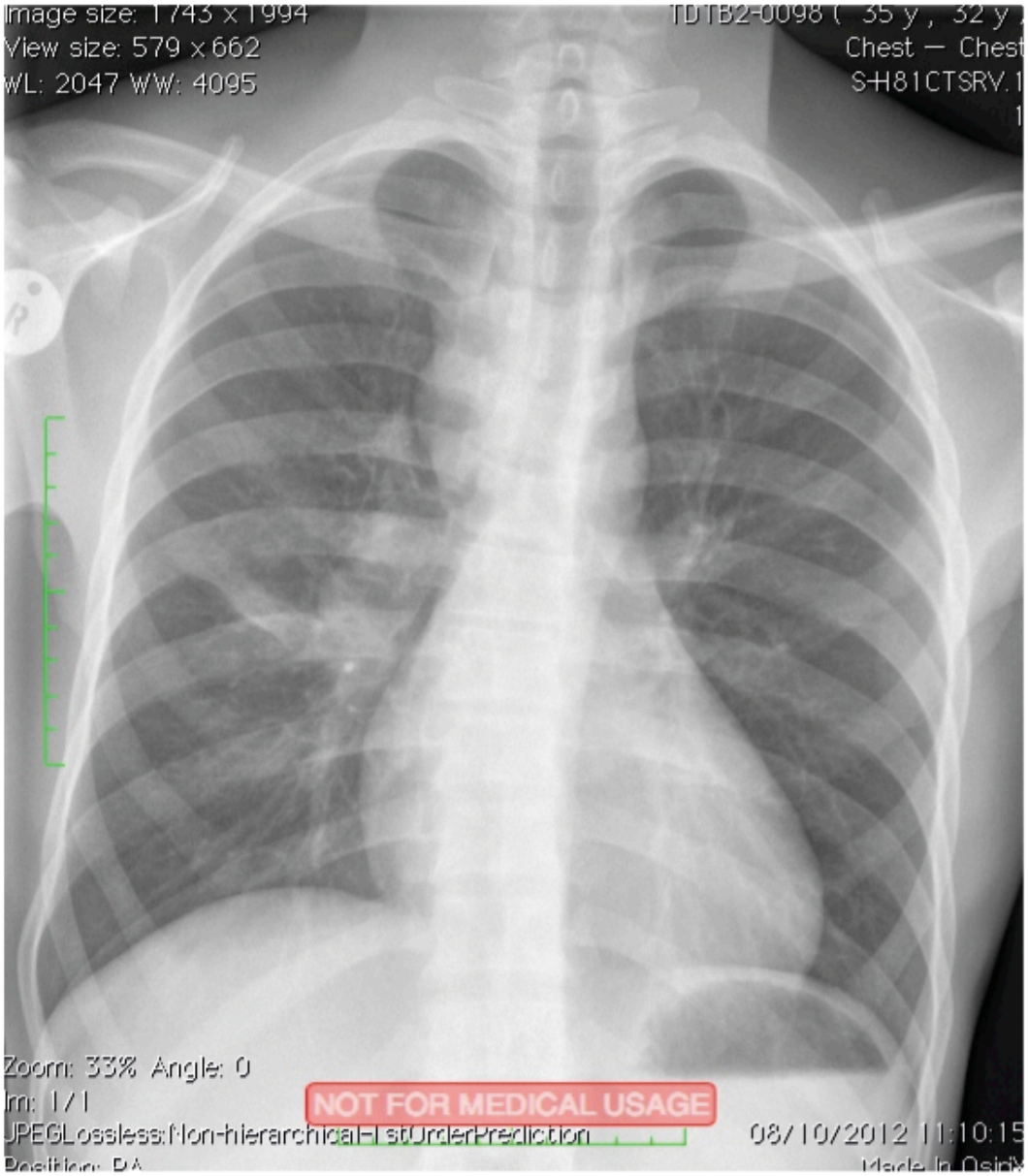

| | Sample Name | Subset Name | Count |
|---|---|---|---|
| | Specimen_001_Tube_003.fcs | Live lymphocytes | 53688 |
| | Specimen_001_Tube_002.fcs | Live lymphocytes | 46570 |
| | Specimen_001_Tube_001.fcs | Live lymphocytes | 52479 |

Panel 1: Fluorescent antibody titration. An example is shown of results for titration of CD4 Per-CP-Cy5.5 (upper figure) and CD3 FITC (lower figure). 5µl (tube 1), 3µl (tube 2) and 1µl (tube 3) fluorescent-conjugated antibody were used to stain peripheral blood mononuclear cells. Optimal separation of positive and negative cell populations required 5µl CD4 Per-CP-Cy5.5. CD3 FITC performed similarly in all three conditions and therefore the lowest concentration of antibody was selected.

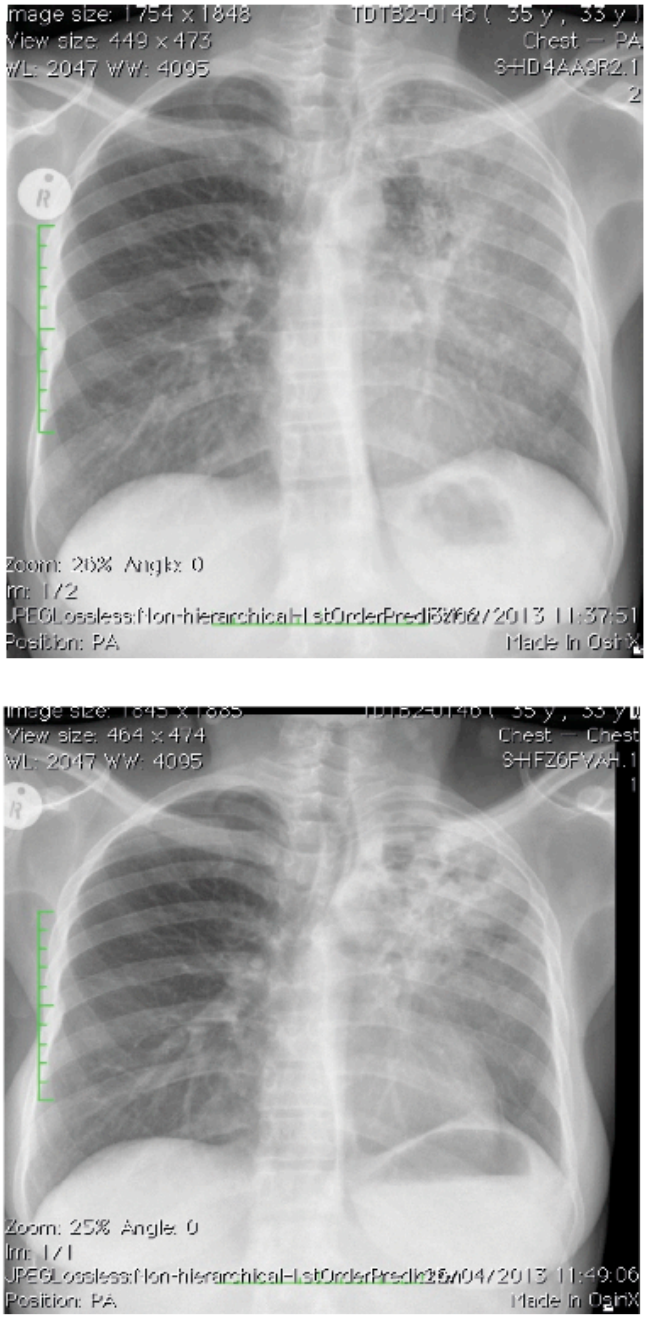



Panel 2: Replicate iNKT cell frequency results for a single healthy donor. One healthy donor provided peripheral blood mononuclear cells (PBMC) on three separate occasions for optimization and control analyses. Three timepoints labeled 1, 2 and 3 represent single episodes of peripheral venous blood sampling separated by a period of several months. INKT frequency was quantified in experiments over subsequent months to give frequency data points. Timepoint 2 includes a comparison of results using fresh and cryopreserved PBMC. Results at timepoint 3 were for cryopreserved PBMC quantified as controls during experiments in the TDTB2 longitudinal study analysis.

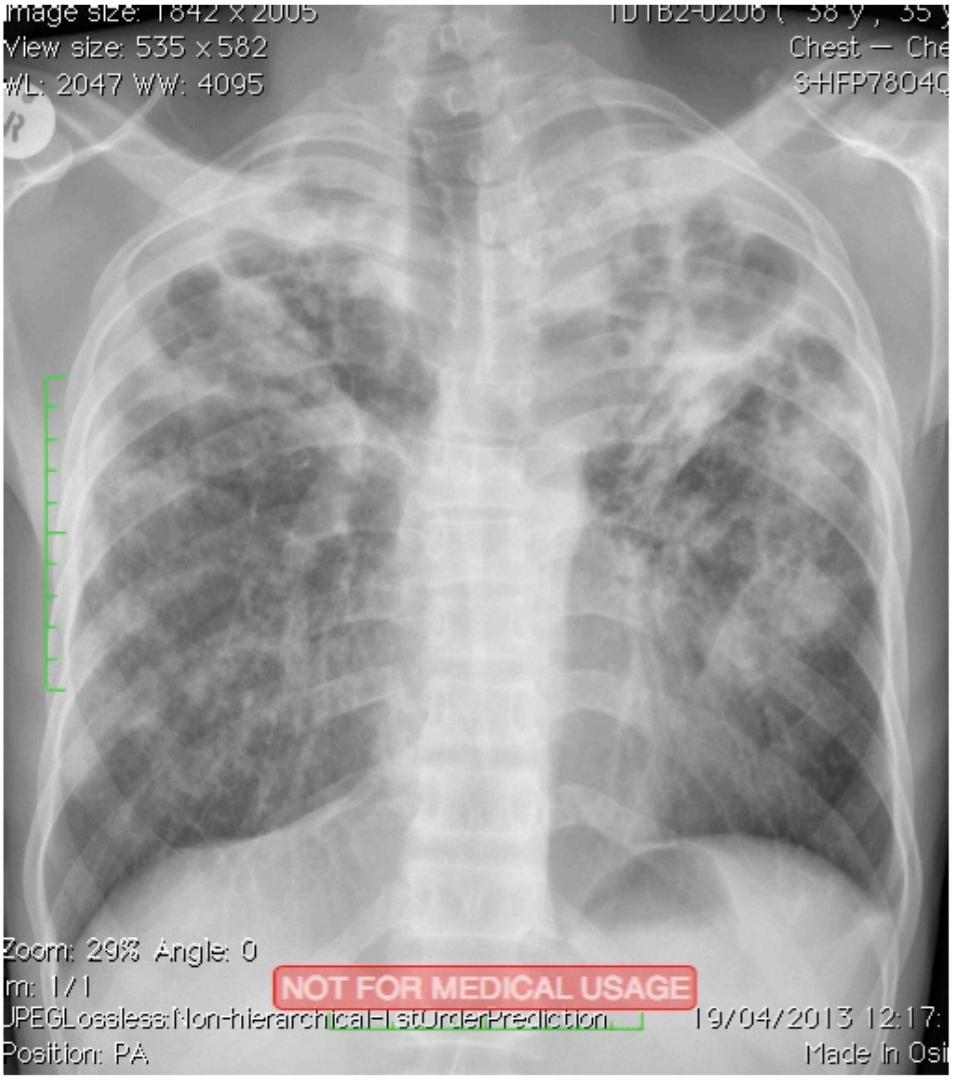
## Appendix 2 Chest radiographs in TB-HIV co-infection




Panel 1: Chest radiograph showing hilar adenopathy but little parenchymal abnormality, in advanced TB-HIV co-infection. Pre-ART CD4 nadir was 89 cell/mm$^3$.




Panel 2: Diffuse pathology in the left lung field at TB diagnosis (upper image) with focal cavitary consolidation developing in the left upper lobe during TB-IRIS (lower image). Pre-ART CD4 nadir was 11 cells/mm$^3$.




Panel 3: Severe cavitary disease at baseline in a patient with a pre-ART CD4 nadir of 45 cells/mm$^3$. Such destructive pulmonary pathology at TB diagnosis is not commonly reported in patients with low CD4 counts at TB diagnosis.

# Appendix 3 Diagnostic criteria for TB-IRIS patients

| TDTB2 | Patient Study Number | 98 | 115 | 146 | 163 | 189 | 195 | 196 | 213 | 217 | 218 | 220 | 221 | 223 | 226 | 236 | 239 | 241 | 243 | 244 | 247 | 250 | 251 | 252 | 257 | 260 | 48 | 203 | 225 | 238 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| INSHI CRITERIA | A Evidence of initial response TB Rx | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| | B Onset <2/12 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Major | New / enlarging LNs could abscesses or other focal involvement | Yes | No | No | No | Yes | No | No | Yes | No | No | No | Yes | No | Yes | No | No | No | No | No | Yes | No | No | No | No | Yes | No | No | No | No |
| | New / worsening radiological features of TB | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | No | No | No | No | Yes | No | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | No | No | No |
| | New / worsening CNS TB | Yes | No | No | No | Yes | Yes | No | Yes | Yes | No | No | No | No | Yes | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No |
| | New / worsening serositis (e.g. pleural effusion, ascites, pericardial effusion) | No | No | No | Yes | No | Yes | Yes | No | No | No | No | Yes | No | No | No | No | Yes | No | No | No | No | Yes | No | No | No | No | No | No | No |
| Minor | New / worsening constitutional symptoms | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | Yes |
| | New / worsening respiratory symptoms | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | No | No | No |
| | New / worsening abdominal pain (with features of peritonitis / lymphadenitis or organomegaly) | Yes | No | No | No | No | No | No | Yes | No | Yes | Yes | Yes | No | No | No | No | No | Yes | No | Yes | No | No | No | No | No | No | No | No | No |
| Exclusions | C Failure of TB treatment due to TB drug resistance | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No |
| | C Poor Adherence | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No |
| Comorbities | Notes: Other opportunistic infection | Possible bacterial pneumonia | HSV recurrence | Possible concurrent drug toxicity with hepatic involvement | Possible EFZ toxicity causing neuropsychiatric presentation | Hypo-natraemia | Possible hepatotoxicity | Possible cerebral toxoplasmosis (treated) | Viral LRTI | Klebsiella bacteraemia | Possible EFZ neurotoxicity | Possible PZA arthropathy | Ascariasis | EFZ dizziness | Possible viral LRTI | Oral candidiasis | Genital ulceration | |
| | Notes: Drug toxicity or reaction | No | No | No | No | No | No | No | No | No | No (switched to NVP) | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | Possible PZA arthropathy | No |
| Classification Consensus TB-IRIS classification (INSHI/non-INSHI) | | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | INSHI | non-INSHI | non-INSHI | non-INSHI | non-INSHI |

INSHI=International network for study of HIV-associated Immune Reconsitution Inflammatory Syndrome, TB=tuberculosis
LNs=lymph nodes, LRTI=lower respiratory tract infection, EFZ=efavirenz, NFV=nevirapine, HSV=herpes simplex virus

# Appendix 4 Publications, presentations and awards

## Published original research articles relating to this work

**Cytotoxic mediators in paradoxical HIV-associated tuberculosis immune reconstitution inflammatory syndrome**

Wilkinson KA*, Walker NF*, Meintjes G, Deffur A, Nicol MP, Skolimowska KH, Matthews K, Tadokera R, Seldon R, Maartens G, Rangaka MX, Besra GS, Wilkinson RJ. *J Immunol.* 2015;194(4):1748-54.

**Membrane type-1 matrix metalloproteinase regulates monocyte migration and collagen destruction in tuberculosis**

T Sathyamoorthy, Tezera LB, Walker NF, Saraiva L, Mauri FA, Wilkinson RJ, Friedland JS, Elkington PE. *J Immunol* 2015;195(3):882-91

**HIV-1 Infection of Macrophages Dysregulates Innate Immune Responses to Mycobacterium tuberculosis by Inhibition of Interleukin-10.**

Tomlinson GS, Bell LC, Walker NF, Tsang J, Brown JS, Breen R, Lipman M, Katz DR, Miller RF, Chain BM, Elkington PT, Noursadeghi M.

*J Infect Dis*. 2014; 209(7):1055-65

**Matrix metalloproteinases and tissue damage in HIV-tuberculosis immune reconstitution inflammatory syndrome.**

Tadokera R, Meintjes GA, Wilkinson KA, Skolimowska KH, Walker NF, Friedland JS, Maartens G, Elkington PT, Wilkinson RJ.

*Eur J Immunol*. 2014; 44(1):127-36

**Procollagen III N-terminal Propeptide and Desmosine are Released by Matrix Destruction in Pulmonary Tuberculosis.**

Seddon J*****, Kasprowicz V*, Walker NF* Yuen HM, Sunpath H, Tezera L, Meintjes G, Wilkinson RJ, Bishai WR, Friedland JS, Elkington PT

*J Infect Dis.* 2013; 208(10):1571-9

**Doxycycline and HIV infection suppress tuberculosis-induced matrix metalloproteinases.**

Walker NF, Clark SO, Oni T, Andreu N, Tezera L, Singh S, Saraiva L, Pedersen B, Kelly DL, Tree JA, D'Armiento JM, Meintjes G, Mauri FA, Williams A, Wilkinson RJ, Friedland JS, Elkington PT. *Am J Respir Crit Care Med.* 2012; 185(9):989-97

**MMP-1 drives immunopathology in human tuberculosis and transgenic mice**

Elkington P, Shiomi T, Breen R, Nuttall RK, Ugarte-Gil CA, Walker NF, Saraiva L, Pedersen B, Mauri F, Lipman M, Edwards DR, Robertson BD, D'Armiento J, Friedland JS. *J Clin Invest.* 2011; 121(5):1827-33

* Authors contributed equally

## Published review articles relating to this work

**HIV-associated immune reconstitution inflammatory syndrome.**

Walker NF, Scriven J, Meintjes GA, Wilkinson RJ.

*HIV AIDS (Auckl).* 2015;7:49-64.

**HIV-1 and the immune response to TB**

Walker NF, Meintjes G, Wilkinson RJ.

*Future Virol.* 2013; 8(1)**:** 57–80

## Presentations relating to this work

**Invariant natural killer T cells in HIV-associated paradoxical tuberculosis immune reconstitution inflammatory syndrome** Walker NF, Elkington PT, Meintjes G, Goliath R, Peyper J, Friedland JS, Wilkinson RJ Wilkinson KA. FAIS, December 1-4, 2014, Nairobi, Kenya (Oral).

**Cytotoxic cells in Paradoxical HIV-associated tuberculosis Immune reconstitution inflammatory syndrome (TB-IRIS)** Walker NF, Meintjes G, Skolimowska KH, Matthews K, Rebecca Tadokera R, Nicol MP, Rangaka MX, Besra GS, Wilkinson RJ, Wilkinson KA Keystone Symposia: Host Response in Tuberculosis (X7) March 13 - 18, 2013, Whistler Conference Centre, Whistler, British Columbia (Poster)

**Matrix metalloproteinases drive immunopathology in TB-HIV co-infection**

European Society of Clinical Microbiology and Infectious Diseases ECCMID 2012 Birmingham, UK (Oral)

**Matrix metalloproteinases drive immunopathology in TB-HIV coinfection.** Royal Society of Tropical Medicine and Hygiene Research in Progress Meeting. 14th December 2011. Awarded 1st Prize. (Oral)

## Awards relating to this work

**CROI Young investigator Scholarship Award**

Conference on Retroviruses and Opportunistic Infections, December 2015.

I was unfortunately unable to attend the conference due to travel restrictions imposed following work on the Ebola outbreak response.

**Federation of African Immunological Societies (FAIS) Scholarship**

December 2014

**Ruth Bowden Scholarship, British Federation of Women Graduates**

July 2013

**Royal Society of Tropical Medicine and Hygiene Research in Progress Prize**

December 2011

**Wellcome Trust Clinical Tropical Medicine Research Training fellowship**

October 2011

## Appendix 5 Copyright permissions

**Re: HIV-associated immune reconstitution inflammatory syndrome.**

Walker NF, Scriven J, Meintjes GA, Wilkinson RJ *HIV AIDS (Auckl).* 2015;7:49-64.

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Walker NF, Meintjes G, Wilkinson RJ. *Future Virol.* 2013; 8(1): 57–80

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