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CHEMOKINE PRODUCTION IN HIV-1 INFECTION AND PULMONARY TUBERCULOSIS

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University of the Witwatersrand, Johannesburg

for the Degree of Master of Science in Medicine

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ABSTRACT

Introduction Circulating levels, and the *ex vivo* production, of the chemokines CCL3, CCL4, CCL5, CXCL8 and CXCL12 (known to play an important role in the pathogenesis of either human immunodeficiency virus type 1 (HIV-1) or tuberculosis (TB)) were examined in the context of both single infections with HIV-1 or *Mycobacterium tuberculosis* (*Mtb*) and coinfection with both organisms. We hypothesised that *CCL3L1* gene copy number (known to affect CCL3 production, associated with susceptibility to and disease progression of HIV-1) would be associated with mother-to-child transmission (MTCT) of HIV-1, and that the IL8-251T→A single nucleotide polymorphism (SNP) (associated with enhanced CXCL8 production and susceptibility to TB in African Americans) would be highly represented in the South African Black population.

Methods Samples used included (i) plasma, DNA samples and cell culture supernatants from control, HIV-1, TB and HIV-1/TB groups, (ii) DNA samples from mothers and their infants (grouped as HIV-1 exposed-uninfected, infected *in utero*, or infected intrapartum), and (iii) DNA samples from a population-based study cohort. Chemokines were quantified by enzyme-linked immunosorbent assay (ELISA), *CCL3L1* gene copy numbers were determined by real-time polymerase chain reaction (PCR), and a real-time PCR method was developed for identification of the IL8-251T→A SNP. DNA sequencing was used for confirmation.

Results We found reduced *ex vivo* chemokine production in response to phytohaemagglutinin (PHA) together with increased plasma levels of chemokines in HIV-1 and TB patients. In contrast to that seen in Caucasians

(median *CCL3L1* copy number of 2), in Black individuals (median *CCL3L1* copy number of 5) circulating levels of CCL3 did not correlate with *CCL3L1* gene copy number; in addition, a high proportion of Black individuals were found to have *CCL3L1* copy numbers below their population-specific median. Using MTCT as a model for studying HIV-1 transmission, infants who became infected with HIV-1 had significantly reduced *CCL3L1* gene copy numbers. IL8-251A allele frequencies were found to be 0.41 for Caucasian groups, and 0.85 for Black groups; due to study limitations, the possible association of IL8-251T→A with TB susceptibility could not be addressed.

Discussion The increased plasma levels of chemokines seen in HIV-1 and TB, likely due to chronic immune activation *in vivo*, may result in T cell anergy which in turn might be the cause of reduced PHA-stimulated *ex vivo* chemokine production. Our results suggest that Black South Africans may be at particularly high risk for acquiring HIV-1 (at least with respect to *CCL3L1* gene copy number), and further imply the presence of other genetic polymorphisms which may influence plasma CCL3 levels. In addition, the high IL8-251A allele frequency (if indeed associated with TB in South African populations) in Black individuals suggests a greater risk for infection with *Mtb*. It will be important, in larger studies, to gain a more in-depth understanding of the relationships between host genotype and chemokine production phenotype, and to relate these measures to infection outcomes.

Conclusions Together, these results highlight the importance of gaining an understanding of the effects of host genotype on the development of innate and acquired immunity to HIV-1 and TB, which will be key in the design of efficient therapies and prevention strategies.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

Samantha Louise Donninger

_____day of _____ , 2008

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CHAPTER 1

INTRODUCTION

1.1 The twin epidemics of HIV-1 and TB

1.1.1 The HIV-1 pandemic

Human Immunodeficiency Virus or HIV (a lentivirus of the retrovirus family; Figure 1), in particular HIV-1, and Acquired Immune Deficiency Syndrome or AIDS (refer to Appendix A for a full list of abbreviations) are growing problems world-wide, with sub-Saharan Africa the region by far the hardest hit. In 2007, according to UNAIDS (http://www.unaids.org/en/), 68% of adults and nearly 90% of children infected with HIV world-wide were living in sub-Saharan Africa, 32% in southern Africa alone. In addition, sub-Saharan Africa accounted for 76% of all deaths due to AIDS in 2007 (Figure 2).

The primary risk factors for HIV-1 infection are unprotected sexual intercourse, sharing of syringes, and receiving contaminated blood products. Infants born to infected mothers comprise another group at high risk.

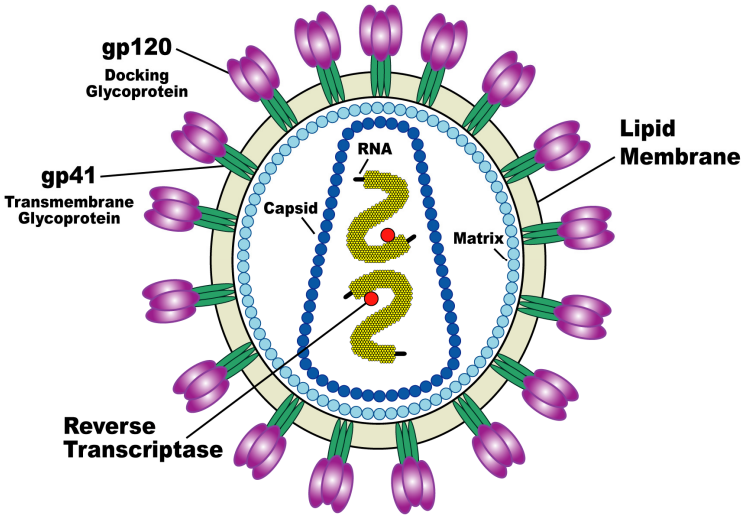


Figure 1
Organisation of the HIV-1 virion.
(from http://en.wikipedia.org/wiki/Hiv)

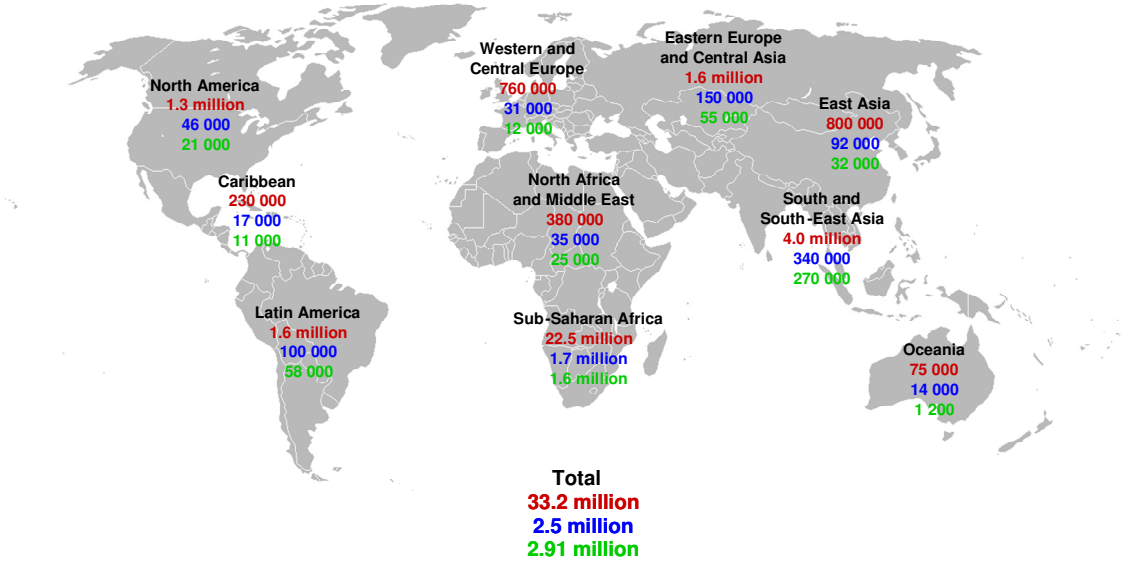


Figure 2
Global HIV-1 estimates for adults and children, 2007.
Estimates for people living with HIV-1 are shown in red, new HIV-1 infections in blue, and deaths due to AIDS in green.
(adapted from UNAIDS;
http://data.unaids.org/pub/EPISlides/2007/2007_epiupdate_en.pdf)

1.1.1.1 Mechanisms of infection by HIV-1

When HIV-1 binds to its receptor CD4, the envelope glycoprotein gp120 undergoes conformational changes. This results in the exposure of previously buried gp120 domains which then interact with one of the HIV-1 coreceptors CCR5 and CXCR4, which stabilises virus binding (Amara *et al*., 1997), leading to membrane fusion mediated by gp41. Inside the cell, viral RNA is reverse transcribed within the cytoplasm, whereupon the preintegration complex moves to the nucleus. Within the nucleus, viral cDNA is integrated as provirus into the host genome. New virions bud from the surface of infected cells *in vivo* a minimum of 1.2 days on average after infection (Perelson *et al*., 1996) (Figure 3).

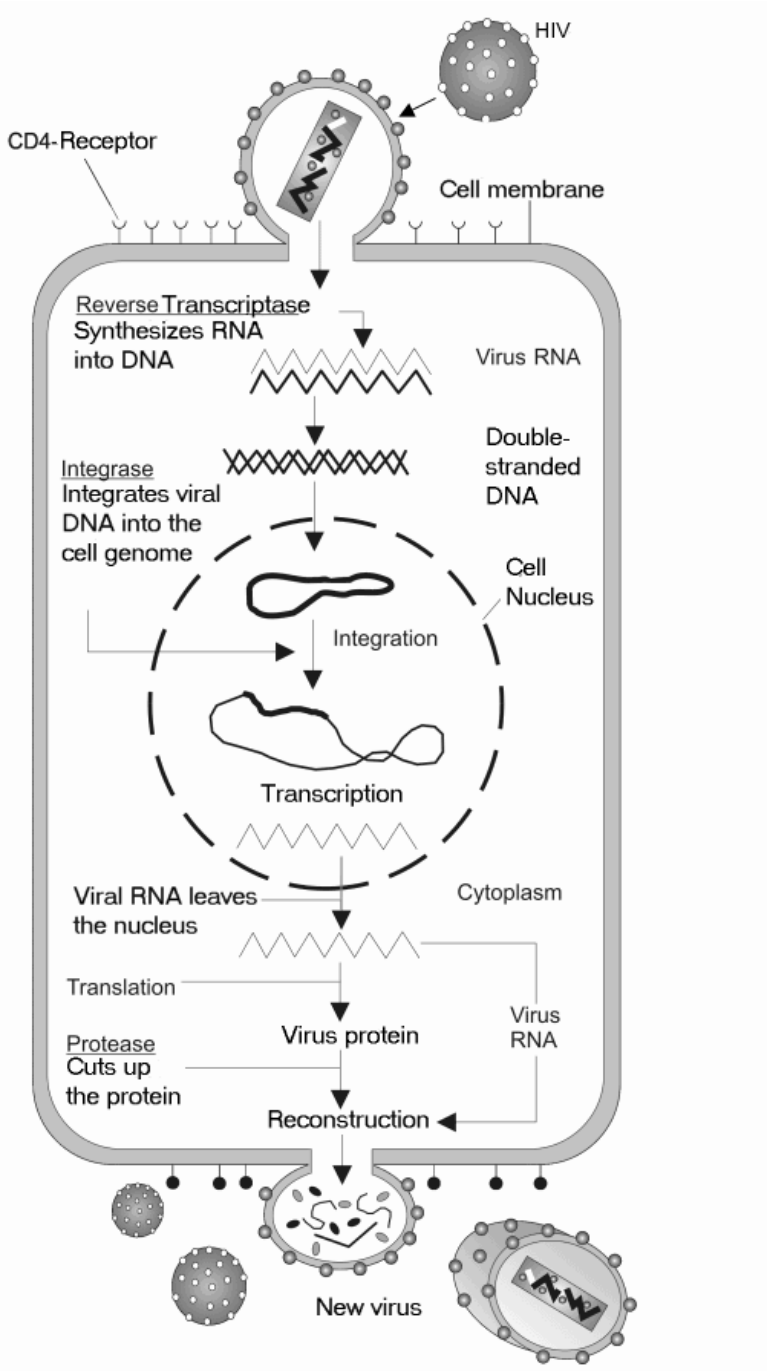


Figure 3
HIV-1 lifecycle.
(from http://en.wikipedia.org/ wiki/Hiv)

1.1.2 The TB epidemic

Tuberculosis or TB, caused by the *Mycobacterium tuberculosis* (*Mtb*) bacillus (Figure 4) has been declared "a global health emergency" by the World Health Organisation (WHO) – around 2 billion people worldwide (a third of the world's population) are infected, with 8–10 million people becoming infected each year (Figure 5). There are 2 million deaths annually as a result of TB, although around 90% of people infected with *Mtb* never develop the active disease, in the absence of HIV-1. In a pattern similar to that seen for HIV-1, the major burden of TB falls on sub-Saharan Africa (Figure 6).

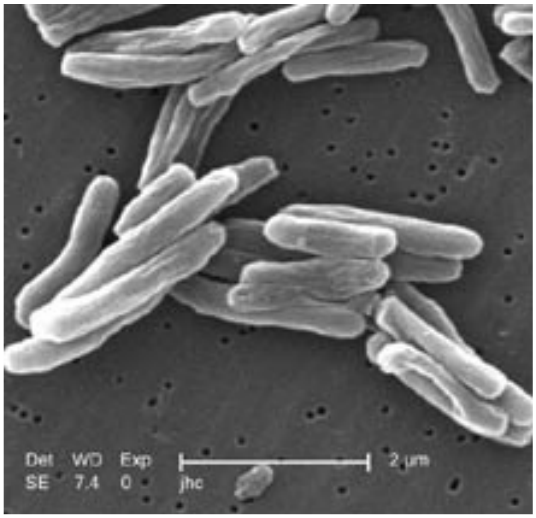


Figure 4
Scanning electron micrograph of *Mycobacterium tuberculosis*.
(from http://en.wikipedia.org/ wiki/Tuberculosis)

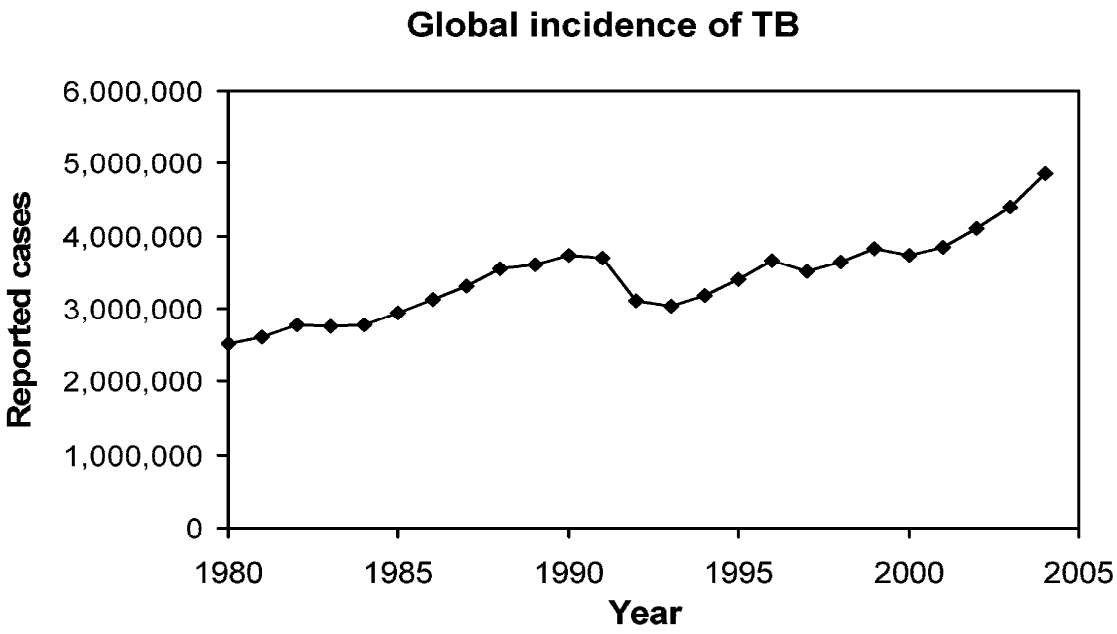


Figure 5
Annual number of new reported TB cases.
(from http://en.wikipedia.org/wiki/Tuberculosis)

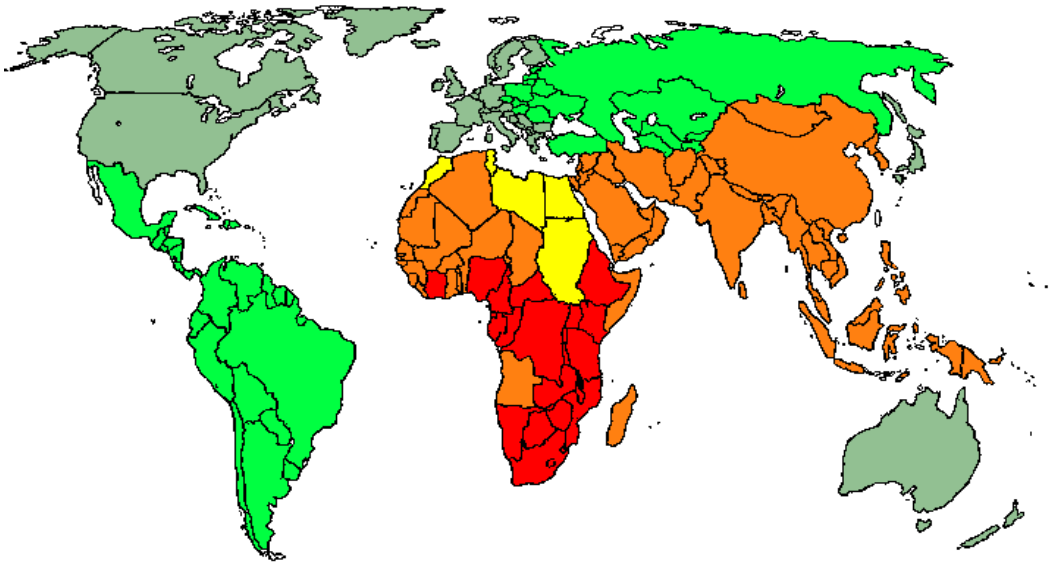


Figure 6
World TB incidence (2006).
Cases per 100,000; red >300, orange 200-300; yellow 100-200; dark green 50-100 and light green <50.
(from http://en.wikipedia.org/wiki/Tuberculosis)

The TB epidemic has become of even greater concern with the emergence first of multi-drug resistant strains of *Mtb* (MDR-TB), and, more recently, extreme (or extensive) drug resistance (XDR-TB) (http://www.who.int/tb/en/). MDR-TB is defined as having resistance to at least isoniazid and rifampicin, while XDR-TB is resistant to rifampicin and isoniazid, as well as to any fluoroquinolone and at least one of three second-line injectable drugs (kanamycin, amikacin and capreomycin); this makes XDR-TB almost impossible to treat. In KwaZulu Natal in August 2006, of 53 patients diagnosed with XDR-TB, 52 died on average within 25 days (http://www.who.int/tb/xdr/xdrmap_20june_en.pdf). Both MDR-TB and XDR-TB are a world-wide problem – WHO figures show that of 17 690 TB isolates tested between 2000 and 2004 in 49 countries, 20% were MDR-TB and 2% XDR-TB (http://www.who.int/tb/xdr/xdrmap_20june_en.pdf).

Furthermore, cases of TB resistant to all known drugs have recently been described in Italy, with worrying implications (Migliori *et al.*, 2007).

1.1.2.1 Mechanisms of infection by TB

During initial TB infection, *Mtb* bacilli are taken up by alveolar macrophages (and possibly dendritic cells (DCs)) in the lung alveoli. The bacteria are not destroyed, due to the presence of thick waxy cell walls, but persist within the host cells and are transported to the draining lymph nodes. There, a lesion develops with the attraction of monocytes; large numbers of $CD4^+$ T cells accumulate. A granuloma is formed through the induction of antigen-specific T cells; this eventually becomes surrounded by fibrosis, which can persist for many years, for as long as immunocompetence persists. Interferon gamma

(IFN-γ) and tumour necrosis factor alpha (TNF-α) are important cytokines in this containment of infection (Kaufmann & McMichael, 2005).

1.1.3 Opportunistic infections in HIV

Infections associated with HIV-1 are the main causes of admission to hospitals and death in many African countries; most are caused by preventable and treatable infections such as *Mtb*, *Salmonella*, other enteric pathogens, bacterial pneumonia and malaria (Corbett *et al*., 2002).

HIV-1 is known to interact with other diseases – as individuals become immunosuppressed, so opportunistic infections flourish. In turn, HIV-1 infectivity and clinical course can be altered by other infections (Figure 7) (Corbett *et al*., 2002).

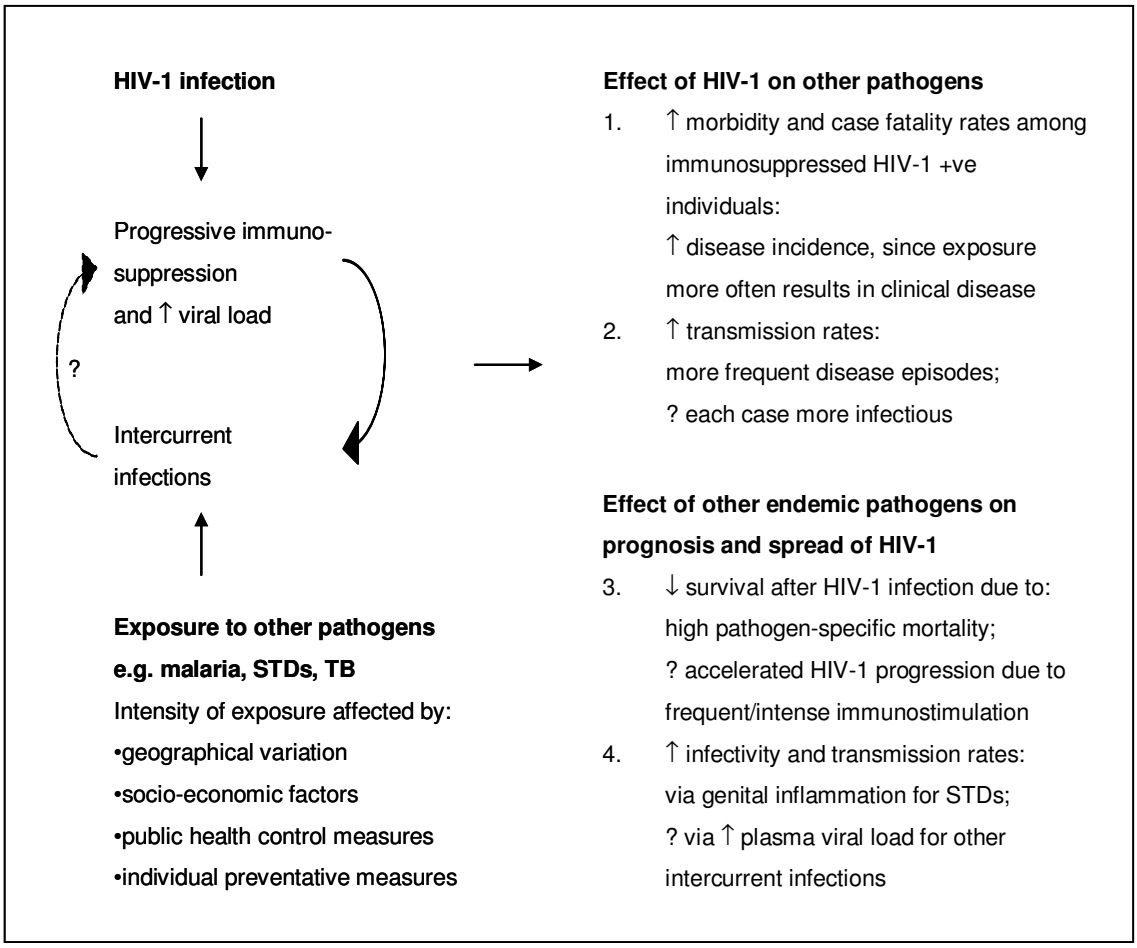


Figure 7
Potential interactions between HIV-1 and other infectious diseases.
(reproduced from (Corbett *et al.*, 2002))
STD: sexually-transmitted disease

1.1.3.1 HIV-1 and TB coinfection

TB is one of the most frequent serious HIV-1-associated infections, occurring as an early complication in 50-67% of the almost 40 million people world-wide living with HIV-1/AIDS (Ellner, 1990). The majority of HIV-1/TB coinfected people live in sub-Saharan Africa. Without proper treatment, approximately 90% of those living with HIV-1 die within months of contracting TB; TB kills up to a half of all AIDS patients world-wide. In comparison to the 10% of HIV-1/TB

coinfected individuals who develop active TB within a year, individuals infected with TB alone have a 10% lifelong risk of developing active TB (Kaufmann & McMichael, 2005).

HIV-1 infection increases the risk of both reactivation of latent TB (Selwyn *et al.*, 1989), reinfection with *Mtb* (Small *et al.*, 1993), and rapidly progressive newly acquired TB (Narain *et al.*, 1992). In fact, in a large cohort of South African miners, the risk of TB has been shown to double within a year of HIV-1 infection (Sonnenberg *et al.*, 2005). In turn, *Mtb* accelerates the progress of AIDS.

Between 1990 and 2005, countries with a high adult HIV-1 prevalence (>4%) saw a far larger increase in TB incidence than countries with a lower HIV-1 prevalence (Nunn *et al.*, 2005). This suggests that TB incidence is directly related to HIV-1 prevalence, as has been demonstrated for sub-Saharan African countries (Figure 8).

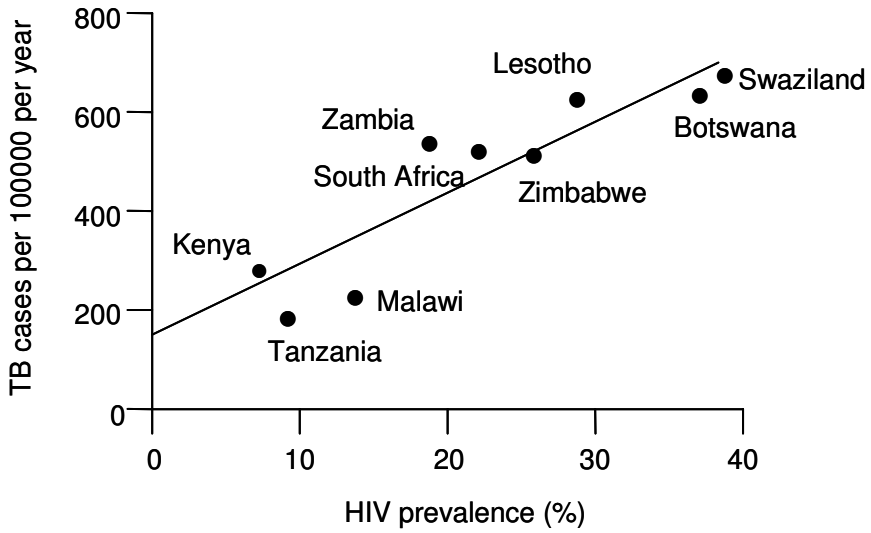


Figure 8
Relationship between incidence of infectious TB and adult HIV-1 prevalence (2002).
(reproduced from (Reid *et al.*, 2006))

TB in the context of HIV-1 infection presents the following problems (according to the WHO; http://www.who.int/tb/hiv/faq/en/):

- "TB is harder to diagnose in HIV-positive people.
- TB progresses faster in HIV-infected people.
- TB in HIV-positive people is almost certain to be fatal if undiagnosed or left untreated.
- TB occurs earlier in the course of HIV-1 infection than many other opportunistic infections."

TB is the most common cause of death in HIV-1 positive Africans; at least 30% of HIV-1 positive Africans are coinfected with *Mtb* (Corbett *et al.*, 2002, Corbett *et al.*, 2003). Incidence of TB is similar among patients infected with HIV-1 or

HIV-2 (adjusted for CD4 count), and mortality rates are similar in both groups (van der Sande *et al*., 2004). In addition, HIV-1 coinfection with TB leads to increased risk of acquiring new secondary infections compared with HIV-1 alone (Whalen *et al*., 1995).

HIV-1 infection is facilitated in TB patients due to an increased expression of CXCR4 and CCR5 (Rosas-Taraco *et al*., 2006). In addition, it has been shown in clinical (Martin *et al*., 1995, Wallis *et al*., 1993, Whalen *et al*., 1995) and *in vitro* (Shattock *et al*., 1994, Zhang *et al*., 1995) studies that infection with *Mtb* promotes the progression of HIV-1 disease. Mycobacterial products are also known to induce cytokines such as TNF-α (Wallis *et al*., 1993), which may promote HIV-1 expression in latently infected cells (Folks *et al*., 1987).

It has been shown, however, using combined data from several study cohorts in countries where TB treatment is widely available, that the progression of HIV-1 is not necessarily inherently faster in individuals who develop TB when compared with other individuals with AIDS, of the same duration of HIV-1 infection (Del Amo *et al*., 2003).

Even though $CD4^+$ T cell counts improve after HAART, *Mtb*-specific $CD4^+$ T cells have a reduced capacity for IFN-γ secretion, as in cases of primary HIV-1 infection (Sutherland *et al*., 2006). *Mtb* infection has also been shown to modify T cell phenotype in HIV-1 infection, with markers such as CD3, CD45RA, CD45RO, and CD27 showing less decrease in coinfected patients, together with an increase in CD95 (Bernal-Fernandez *et al*., 2006).

In macaque monkeys infected with SIV, infection with BCG leads to the development of a tuberculosis-like disease within 4 months (Shen *et al*., 2002). In SIV-BCG coinfected macaques with clinically latent disease, BCG can be reactivated by bacterial superantigen challenge (independent of an increase in SIV viral load) (Shen *et al*., 2004). This suggests that reactivation of latent TB in HIV-infected individuals may be partly due to further superinfection. The role of translocated microbial products (from the gastrointestinal tract) in the chronic activation of the immune system seen in HIV-1 infection is well-documented (Brenchley *et al*., 2006); this may have implications in the reactivation of latent TB.

1.2 Immune abnormalities in HIV-1 and TB infection

1.2.1 Basic immunology

1.2.1.1 Innate immunity

In mammals, the immune system comprises two arms, innate and acquired (or adaptive) immunity. The innate immune system is the first line of defence against invading pathogens, governed primarily by constitutively expressed, germ-line encoded pattern recognition receptors (PRRs) on cells including phagocytes (such as macrophages and DCs) that recognise molecules characteristic of bacteria and viruses (known as pathogen-associated molecular patterns (PAMPs)). Innate immune activation is non-specific, and targets neutrophils, monocytes/macrophages, DCs, natural killer cells (NKs) and

complement. The best characterised of these receptors are the Toll-like receptors (TLRs); the role of cytoplasmic PRRs is less well understood but is critical to the immune response (Akira *et al*., 2006).

On ligand binding by TLRs, signalling cascades lead to the selective transcriptional activation of genes involved in host defence, including proinflammatory cytokines and type 1 interferons (Akira *et al*., 2006). Macrophage killing of *Mtb* is evaded via the inhibition of IFN-γ-mediated signalling and hence the innate arm of the immune system (Akira *et al*., 2006).

Innate immunity is critical in host defence in infants, since neonatal adaptive immune responses are naïve (Marodi, 2006).

Adaptive immune responses are simultaneously initiated through the activation and maturation of DCs, which are professional antigen-presenting cells (Kabelitz & Medzhitov, 2007), thereby providing a link between the innate and adaptive immune responses.

1.2.1.2 T-helper 1 and T-helper 2 cells

T-helper (Th) cells can be divided into two subsets, Th1 and Th2. Th1 responses are characterised by IFN-γ and IL-2 production, and are associated with cell-mediated immunity; Th2 responses are characterised by production of IL-4, IL-5 and IL-13, and are associated with humoral immunity (Elliott *et al*., 2004). It has been proposed that a Th1 response, leading to macrophage activation, is required for protective immunity to TB, while a Th2 response,

which is expected to suppress type 1 responses, would be associated with susceptibility to TB (Elliott *et al.*, 2004). It has been postulated that a switch from Th1 to Th2 might be associated with the pathogenesis of HIV-1 (Clerici & Shearer, 1993), although this remains controversial. While a study of healthy adolescents (both HIV-1 positive and HIV-1 negative) failed to detect shifts from Th1 to Th2 cytokine production early in disease (Becker, 2004), a phenotypic shift from Th1 to Th2 has been demonstrated at the single-cell level during the course of HIV-1 infection (Klein *et al.*, 1997), and in HAART patients (with more advanced stages of disease), viremia and the presence of opportunistic infections were correlated with levels of cytokines characteristic of a Th2 response in serum, and production by $CD4^+$/$CD8^+$ T cells *ex vivo* (Sindhu *et al.*, 2006)

1.2.2 The effect of HIV-1 on the immune system

It was previously believed that HIV-1 caused depletion of $CD4^+$ T cells slowly and progressively, eventually leading to AIDS. However, it is now apparent that HIV-1 infection has an initial phase of extensive destruction of memory $CD4^+$ T cells, particularly within the gut mucosa (Brenchley *et al.*, 2006), followed by a phase of chronic immune activation and a gradual loss of remaining $CD4^+$ T cells, in the slow and progressive stage of the disease (Mattapallil & Roederer, 2006). This loss of $CD4^+$ T cells is correlated with markers of immune activation (Hazenberg *et al.*, 2003).

In HIV-1 positive individuals, depletion of $CD4^+$ T cells is associated with a decrease in production of type 1 and type 2 cytokines in response to mycobacterial antigens (Elliott *et al*., 2004).

Immune abnormalities in HIV-1 infected patients include defects in the microbicidal responses of phagocytic cells, which could contribute to impaired defence against opportunistic pathogens (Meddows-Taylor *et al*., 2001a). Neutrophils from HIV-1 infected adults also show functional defects including phagocytosis (Lazzarin *et al*., 1986, Shalekoff *et al*., 1998), chemotaxis (Ellis *et al*., 1988, Meddows-Taylor *et al*., 1998, Valone *et al*., 1984), oxidative burst (Chen *et al*., 1993, Pitrak *et al*., 1993, Shalekoff *et al*., 1998), bacterial killing (Ellis *et al*., 1988, Murphy *et al*., 1988) and degranulation (Baley & Schacter, 1985).

Individuals coinfected with HIV-1 and *Mtb* show a greater impairment of polymorphonuclear leukocyte (PMNL) function to those infected with HIV-1 alone (Baley & Schacter, 1985, Tiemessen *et al*., 2000b), consistent with the increased susceptibility to secondary infections found in HIV-1/TB coinfection compared to HIV-1 alone (Barin *et al*., 2006).

Bentwich *et al.* (1998) suggest that the pathogenesis of AIDS, particularly in Africa, is at least partly due to chronic immune activation caused by other chronic infections, thereby increasing susceptibility to HIV-1 infection (Bentwich *et al*., 1998). The same authors also propose that disease progression is influenced by the persistent stimulation of the immune system by HIV-1

infection itself, causing an “ageing-like process” in the functioning of the immune system. During HIV-1 infection, the reduction in blood $CD4^+$ T cell counts has been ascribed to both their redistribution to the lymphoid tissue, and to the attenuation of proliferation (Grundstrom & Andersson, 2006). T cell activation, depending on the type of stimulus, leads to “full activation” (characterised by proliferation and often cell death), or to “partial activation” (resulting in selective expression of cellular functions and anergy to full activation). Both forms of activation are induced in HIV-1 infection, explaining the functional defects in peripheral T cells in HIV-1 infection (Gougeon *et al.*, 1996).

1.2.3 The effect of *Mtb* on the immune system

In patients with active pulmonary TB, the immune activation of peripheral monocytes enhances susceptibility to HIV-1 infection *in vitro*; *Mtb* and its antigens can also upregulate *in vitro* HIV-1 replication (Wolday *et al.*, 2005). In *Mtb* infection (both *in vitro* and *in vivo*), human monocyte-derived macrophages, alveolar macrophages and $CD4^+$ T cells show increased expression of CCR5 and/or CXCR4, and their ligands (Wolday *et al.*, 2005). In the population groups we are studying, CXCR4 expression is reduced in patients with TB, HIV-1, and dual TB/HIV-1 infections (with TB exerting the greater effect), while CCR5 expression is increased (Shalekoff *et al.*, 2001).

In HIV-1 with acute phase TB coinfection, plasma HIV-1 viremia is increased (at CD4 levels >500/µl) (Toossi *et al*., 2001) and mycobacteremia is frequently observed (Archibald *et al*., 1998). In studies of African HIV-1/TB coinfected patients receiving anti-TB treatment, $CD4^+$ T cell count does not increase, and plasma viral load remains high (Wolday *et al*., 2005). In contrast, a study in Western patients showed TB treatment associated with a reduction in HIV-1 viral load (Goletti *et al*., 1996), highlighting the importance of the choice of study cohort to exclude confounding factors, including genetic background.

1.3 Chemokines and their receptors – a general overview

1.3.1 Characteristics of chemokines

Chemokines are chemotactic cytokines, which have the following characteristics:

- They are small secreted protein molecules (8-17kDa) with potent chemotactic activity.
- They are involved in organ development, angiogenesis, angiostasis, homeostatic leukocyte recirculation and immune regulation (Chensue, 2001).
- They mediate inflammatory processes including activation and chemotaxis of neutrophils, monocytes and lymphocytes to sites of infection (Miller & Krangel, 1992).

Cytokines themselves have the following characteristics (Alfano & Poli, 2002):

- They act locally, although an overproduction can lead to systemic effects e.g. sepsis.
- More than one can induce the same effect (redundancy).
- The same cytokine can be produced by different cell types (pleiotropy).
- A single cytokine/chemokine can interact with more than one receptor.

The first chemokine to be cloned and characterised was interleukin-8 (IL-8/CXCL8) (Matsushima *et al.*, 1988); since then approximately 50 human chemokines have been identified using expressed sequence tag (EST) libraries (Chensue, 2001).

Cytopathology of infected cells is probably the initial source of chemokines, which recruit and activate inflammatory leukocytes. This activation leads to the induction of cytokines (e.g. IFN-γ and TNF-α), which in turn amplify chemokine synthesis. The nett result is the destruction of infected cells, frequently associated with bystander damage.

1.3.2 Classification of chemokines

The chemokine superfamily is subclassified based on the arrangement of cysteine residues in the N-terminal region of the mature protein: C, CC (β-chemokines), CXC and CXXXC (or CX_3C) (Murphy *et al.*, 2000), where C represents the N-terminal cysteine residues, and X the number of intervening

amino acids (Figure 9) (refer to Appendix B for a Table of chemokine nomenclature).

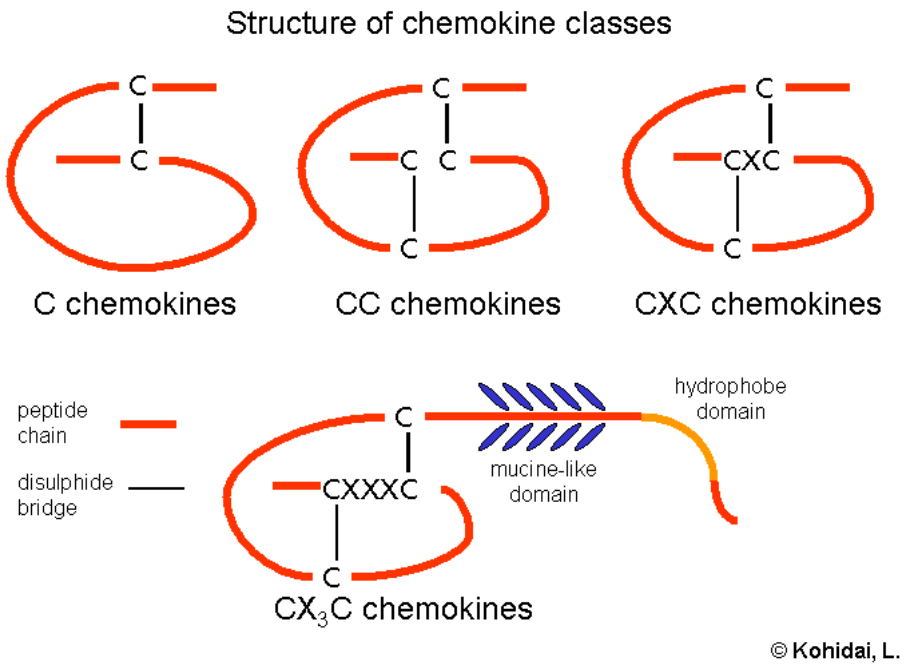


Figure 9
Chemokine structure.
(from http://en.wikipedia.org/wiki/Chemokine)

The CXC subfamily (which acts predominantly on neutrophils) is further classified, based on the presence or absence of a Glutamic acid-Leucine-Arginine (ELR) triplet amino acid motif preceding the first of the N-terminal cysteine amino acids, into ELR and non-ELR types (angiogenic and angiostatic, respectively (Keane *et al.*, 1998)). CXC ELR+ chemokines (encoded on chromosome 4 in a multigene array) attract neutrophils to sites of inflammation, and induce the respiratory burst and granule exocytosis; production is

stimulated by, for example, IL-1, TNF-α or bacterial lipopolysaccharide (LPS) (Charo & Ransohoff, 2006).

1.3.3 Characteristics of chemokine receptors

Chemokine receptors are members of the guanosine nucleotide-protein-coupled receptors (GPCR) superfamily; they are 320-380 amino acids in length, with significant sequence homology. Chemokine receptors have seven transmembrane hydrophobic domains with three intracellular and three extracellular hydrophilic loops; the extracellular amino-terminal region is involved in chemokine binding; the intracellular carboxy-terminal region is involved in G-protein linking and regulation (Chensue, 2001).

Chemokine (and chemokine receptor) genes are, for the most part, clustered within four distinct regions of the genome (Clark & Dean, 2004):

CC	17q11-21
CXC	4q12-21
CCR and CXCR	3p21-24
CXCR	2q21-35

1.4 Chemokines and chemokine receptors important in HIV-1 and TB infection

1.4.1 General background

HIV-1 expression in latently infected cells can be upregulated by pro-inflammatory cytokines (including IL-1 (Poli *et al*., 1994), IL-6 (Poli *et al*., 1990, Poli *et al*., 1994), TNF-α and TNF-β (Folks *et al*., 1989, Matsuyama *et al*., 1989), and GM-CSF (Folks *et al*., 1987)); immunosuppressive cytokines (IL-4 (Schuitemaker *et al*., 1992) and TGF-β (Poli *et al*., 1991)) inhibit HIV-1 expression (Table 1) (refer to Appendix C for a Table of cytokines involved in HIV infection).

Table 1
Cytokine effects on driving HIV-1 latency and replication.
(reproduced from (Alfano & Poli, 2002))

Latency	LIF, IFN-α/β, IL-1, IL-13, IL-16, RANTES, MIP-1α/β, MDC
Replication	TNF-α/β, CD30L, M-CSF, GM-CSF, IL-1α/β, IL-3, IL-6, IL-7, IL-8, MCP-1
Latency/replication (depending on other factors)	TGF-β, IFN-γ, IL-2, IL-4, IL-10, IL-12, IL-15

In TB, HIV-1, and TB/HIV-1 coinfection, Th1 responses to both TB and HIV-1 specific antigens are generated, but still disease progresses, leading to the theory that a “subversive Th2 component” undermines the Th1 response and

therefore causes pathogenesis (Dheda *et al.*, 2005). Th2 cytokines seem to play a role in HIV-1/TB coinfection, but not in HIV-1 alone (Dheda *et al.*, 2005).

CC chemokines (or β-chemokines) are best studied for their central role in HIV-1 infection, as CCL3, CCL4 and CCL5 are the natural ligands for the HIV-1 coreceptor CCR5. *In vitro*, CC chemokines can block replication of R5 HIV-1 strains (Cocchi *et al.*, 1995). They are also involved in the regulation of cell-mediated immunity (Matsukawa *et al.*, 2000).

Pulmonary TB, with or without concomitant HIV-1 infection, is associated with higher levels of CXCL12 (Shalekoff & Tiemessen, 2003b). IL-7 levels, on the other hand, are increased in HIV-1 infection, with or without concomitant pulmonary TB (Shalekoff & Tiemessen, 2003b).

1.4.2 CCL3 and other CC chemokines

CCL3 (previously known as macrophage inflammatory protein (MIP)-1α) is a CC chemokine produced during inflammation as part of the innate immune response. CCL3 (with CCL4) is produced by epithelial cells, lymphocytes and platelets, and is a chemoattractant for monocytes, NK cells, eosinophils and DCs (Lillard *et al.*, 2003). In studies on mice, the development of humoral and cellular mucosal and systemic immunity is enhanced by CCL3 and CCL4; CCL3 drives both Th1 and Th2 responses, while CCL4 is limited to Th2 (Lillard *et al.*, 2003).

Through interaction with its receptors (CCR1 and CCR5), CCL3 helps mediate the local accumulation of leukocytes. Deletion of the *CCL3* gene in mice (present in single allelic form) has no apparent effect on development, but has a profound effect on immunological responses in a variety of disease models (Cook *et al*., 1995).

1.4.2.1 CC chemokines and HIV-1

During HIV-1 infection, intracellular levels of the CC chemokines CCL3 and CCL4 tend to be increased in lymphocytes, with a corresponding decrease in their secretion into lymphocyte culture supernatants (Jennes *et al*., 2004). In contrast, in HIV-1 patients CCL5 tends to be increased both intracellularly and in culture supernatants (Jennes *et al*., 2004). After more than 3 years of HAART, intracellular levels of CCL4 and CCL5, and CCL3 and CCL4 in supernatant, reach normal levels, coincident with higher $CD4^+$ T cell counts (Jennes *et al*., 2004).

In the population groups under investigation in this study, stimulated cultures of cord-blood mononuclear cells (CBMCs) from infants born to HIV-1 positive mothers show an increase in CCL3 and CCL4 production compared to those born to HIV-1 negative mothers; a deficiency in this stimulated CCL3 production (seen in both infants and mothers) is associated with susceptibility to intrapartum mother-to-child transmission (MTCT) of HIV-1 infection (Meddows-Taylor *et al*., 2006). This suggests an important role for this particular chemokine in protection from infection.

Disease progression in HIV-1 infection is less rapid in individuals showing high levels of CC chemokine production by phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) (Ullum *et al*., 1998), while $CD8^+$ T cells from asymptomatic HIV-1 infected individuals produce higher levels of CCL3 and CCL4 upon PHA stimulation, in comparison with those from AIDS patients (Cocchi *et al*., 2000). In HIV-1 uninfected haemophiliacs repeatedly exposed to HIV-1-contaminated clotting Factor VIII, PHA-activated PBMCs show an overproduction of CC chemokines (Zagury *et al*., 1998). In HIV-1 infected long-term non-progressors (LTNPs), enhanced production of CC chemokines, as a result of vigorous HIV-1-specific $CD4^+$ T cell proliferative responses, is associated with control of viremia (Rosenberg *et al*., 1997).

In addition, CC chemokines have been associated with protective immunity in animal models. In rhesus macaques vaccinated against SIV, the production of CC chemokines (specifically CCL4 and CCL5) by $CD8^+$ T cells is associated with protective immunity (Lehner *et al*., 1996), while CC chemokine production correlates with protection against infection by SIV in both naïve and vaccinated macaques (Ahmed *et al*., 2005).

It has been shown, however, that expression of CCL3, CCL4 and CCL5 is very high in the peripheral lymph nodes of patients during early acute HIV-1 infection (Nilsson *et al*., 2007). This suggests that during the dissemination of HIV-1 infection, viral replication may not necessarily be suppressed by CC chemokines, which highlights the complex interplay of innate immunity.

1.4.3 CCR5 and CXCR4: HIV-1 entry coreceptors

Along with CD4, the chemokine receptors CCR5 and CXCR4 are coreceptors for HIV-1 entry into $CD4^+$ T cells. CXCR4 is utilized by T cell tropic or X4 HIV-1 strains (previously known as syncitia-inducing, or SI), CCR5 by macrophage-tropic or R5 strains (previously known as non-syncitia-inducing, or NSI). HIV-1 strains utilizing both receptors are termed dual-tropic, or R5X4. R5 strains are considered to be primarily responsible for disease transmission, as they are generally the virus strains recovered following seroconversion, while X4 strains are found preferentially during late-stage disease (Amara *et al.*, 1997). However, exceptions can occur, as demonstrated by the fact that individuals homozygous for mutations resulting in a complete lack of cell-surface CCR5 can still be infected (although rarely) with X4 or R5X4 dual-tropic HIV-1 strains (Gray *et al.*, 2006).

The specific ligands for CCR5 are MIP-1α/CCL3, MIP-1β/CCL4 and RANTES/CCL5 (Cocchi *et al.*, 1995); additional agonists for CCR5 are CCL7 and CCL14 (high affinity) and CCL13 (weak affinity), while CCL8 is a natural antagonist (Amara *et al.*, 1997). To date, the only ligand known for CXCR4 is SDF-1α/CXCL12 (Amara *et al.*, 1997).

CCL3, CCL4 and CCL5 are all inhibitors of HIV-1 binding – in fact, clinical trials are currently underway to investigate the use of chemokine receptor-directed agents as HIV-entry blockers (Mills & DeMartino, 2004). Maraviroc is the first CCR5 antagonist to receive FDA approval, granted in August 2007, and

European Commission approval, granted in September 2007. The inhibitory effects of chemokines are only partly due to steric hindrance through the blocking of their receptor CCR5; also important is the desensitisation and internalisation of CCR5 through ligand binding. The most significant factor might be the complex interaction of immune system components in cytokine-induced immune cell recruitment and regulation, including (but not limited to) the development of Th1 and Th2 responses. A further, recently-described effect of CCL3 binding to CCR5 on $CD4^+$ T cells and DCs is to increase the *de novo* synthesis of APOBEC3G (Pido-Lopez *et al.*, 2007), which is a potent post-entry inhibitor of HIV-1 infectivity.

In most infected individuals in Europe/USA, CD4 depletion is associated with a mutational shift in HIV-1 *env* coreceptor preference from CCR5 to CXCR4 ("R5 to X4 tropism") (O'Brien & Nelson, 2004). The emergence of X4 and/or R5X4 viruses is associated with the acceleration of $CD4^+$ T cell depletion and disease progression (Kaleebu *et al.*, 2007, Philpott, 2003).

In *Mtb* infection of macrophages, surface expression of CXCR4 is raised, and the chemokines CCL4, CCL5 and CX3CL1 are upregulated. This would be permissive for replication of X4 viruses, while inhibiting R5 viruses, providing a further possible mechanism for the acceleration of AIDS by TB coinfection (Hoshino *et al.*, 2004).

CCR5 is more abundant on the surface of Th1 cells; CXCR4 is upregulated by IL-4, so linked to Th2 differentiation (Alfano & Poli, 2001). This implies that

CCR5, linked with the Th1 type immune response, may be associated with control of TB infection; CXCR4, linked with the Th2 type of immune response, may be associated with susceptibility to TB, while at the same time contributing to the switch from R5 to X4 strains of HIV-1 and hence progression to AIDS.

Contrasting results show that there is a significant reduction in the proportion of leukocytes expressing CXCR4 in patients infected with HIV-1 and/or TB, with TB having the most dramatic effect (Shalekoff *et al*., 2001). Correspondingly, there is an increase in the proportion of CCR5-expressing leukocytes; this could have the direct effect of increasing viral entry of R5 HIV-1 strains (Shalekoff *et al*., 2001).

1.4.3.1 Polymorphisms in coreceptors influence susceptibility to HIV-1

A 32-bp deletion within the *CCR5* gene results in a truncated protein which is not expressed at the cell surface, but is retained within the endoplasmic reticulum and rapidly degraded; individuals homozygous for this mutation are highly resistant to HIV-1 infection (Liu *et al*., 1996, Samson *et al*., 1996). Two mechanisms seem to be involved in this resistance: the loss of surface CCR5 expression, and the scavenging of CXCR4 (Agrawal *et al*., 2004). Individuals heterozygous for CCR5 Δ32/wt, when compared to CCR5 wt/wt individuals, show a lower cell-surface expression of CCR5 (and lower levels of circulating CXCL12), with a concomitant increase in CXCR4 expression (Shalekoff & Tiemessen, 2003a). There are other *CCR5* mutations that affect the coding sequence and the promoter, most of which are relatively rare, and some of

which can affect ligand binding and HIV-1 disease progression (reviewed in (Amara *et al*., 1997)).

1.4.4 CXCL8

CXCL8 (previously known as IL-8) is an 8-kDa polypeptide; it is a CXC chemokine, in the ELR^+ group, important in the inflammatory process as part of the innate immune response. It is a potent chemoattractant for the recruitment of leukocytes to inflammatory sites (Pace *et al*., 1999); however, in recruiting neutrophils, it can cause significant bystander damage (Chensue, 2001). CXCL8 also activates monocytes (Charo & Ransohoff, 2006) and neutrophils, the response of which includes respiratory burst induction and chemotaxis (Matsushima & Oppenheim, 1989), lysosomal enzyme release and enhanced killing of microorganisms (including *Mtb*) (Liles & Van Voorhis, 1995). CXCL8 production is stimulated by a wide variety of microorganisms, including bacteria, mycoplasma, mycobacteria, protozoa, helminths and viruses (Chensue, 2001). It is produced predominantly by monocytes/macrophages (Yoshimura *et al*., 1987), but also by stimulated human blood mononuclear leukocytes (Yoshimura *et al*., 1987), T lymphocytes, endothelial cells, keratinocytes, fibroblasts (Liles & Van Voorhis, 1995), and polymorphonuclear neutrophils (Cassatella *et al*., 1992).

1.4.4.1 CXCL8 and TB

The role of CXCL8 in TB has become of great interest, as elevated levels of CXCL8 are found in tuberculous pleural exudate, bronchoalveolar lavage fluid and cerebrospinal fluid (Mastroianni *et al.*, 1994). High levels of CXCL8 are expressed in tuberculous granulomas heavily infiltrated by neutrophils, and plasma CXCL8 concentrations are higher in fatal cases of TB compared to survivors (Friedland *et al.*, 1995). CXCL8 is up-regulated in macrophages as part of the macrophage activation program in response to *Mtb* infection, and sputum levels are reduced in parallel to *Mtb* in response to anti-TB treatments (Ribeiro-Rodrigues *et al.*, 2002). CXCL8 has been shown to reduce the survival of *Mtb* within macrophages, while inhibition of CXCL8 is associated with proliferation of intracellular *Mtb* (O'Kane *et al.*, 2007). In rabbits, anti-CXCL8 antibodies inhibit granuloma formation, suggesting that CXCL8 may be essential for host defence to *Mtb* (Larsen *et al.*, 1995). Resting PBMC production of CXCL8 is lowered in extrapulmonary TB (in HIV-negative patients) compared to controls (Sterling *et al.*, 2001). In addition, fibroblasts in culture are strongly stimulated to produce CXCL8 in response to conditioned medium from *Mtb*-infected monocytes (O'Kane *et al.*, 2007).

Furthermore, latent *Mtb* infection can be distinguished from active disease by gene expression of CXCL8 in activated PBMCs (raised in latent vs. active TB), together with forkhead box P3 (FOXP3) (raised in active TB only) and IL-12β (raised in latent vs. active TB) (Wu *et al.*, 2007).

1.4.4.2 CXCL8 and HIV-1 or HIV-1/TB

CXCL8 levels are upregulated in both serum (Matsumoto *et al*., 1993) and plasma (Thea *et al*., 1996) of HIV-1 infected adults, although not in HIV-1 infected children (Meddows-Taylor *et al*., 2001b). Circulating levels of CXCL8 are also significantly raised in HIV-1 patients with pulmonary TB, but not in patients with TB alone (Meddows-Taylor *et al*., 1999a). CXCL8 suppresses HIV-1 replication in naturally-infected $CD4^+$ T cells (Mackewicz *et al*., 1994) and in promonocytic U1 cells as a model of HIV-1 latency (Tiemessen *et al*., 2000a). However, CXCL8 can either decrease or increase HIV-1 replication on induction by proinflammatory cytokines, depending on the nature of the cytokine and CXCL8 concentration (Tiemessen *et al*., 2000a). HIV-1 replication in macrophages and T lymphocytes is stimulated by CXCL8, while levels of CXCL8 in lymphoid tissue are increased in AIDS patients (Lane *et al*., 2001). U1 cells (with latent HIV-1 infection) show an increase in spontaneous secretion of CXCL8, but a reduced ability to secrete CXCL8 in response to cytokines (Tiemessen & Martin, 2000).

1.4.4.3 CXCR1 and CXCR2

There are two types of CXCL8 receptor, CXCR1 and CXCR2. These are found on all PMNLs, monocytes, and 5-25% of total lymphocytes, with CXCR2 dominant on monocytes and lymphocytes (Chuntharapai *et al*., 1994). Of the CXCR positive lymphocytes, 7-42% are $CD8^+$ T cells, and 39-76% are $CD56^+$ NK cells (Chuntharapai *et al*., 1994). Both receptors mediate chemotaxis, granule enzyme release, and cytosolic Ca^{2+} changes, although only CXCR1

triggers the respiratory burst (Jones *et al*., 1996). Surface expression of both receptors is down-regulated on PMNLs from HIV-1 infected patients, and in patients coinfected with HIV-1 and *Mtb* (Meddows-Taylor *et al*., 1998), relating to impaired CXCL8-induced degranulation (Meddows-Taylor *et al*., 1999b). These decreases are independent of the patients immunologic status (Meddows-Taylor *et al*., 1998). Children infected vertically with HIV-1 have significantly altered expression of CXCR2, but not CXCR1, compared to HIV-1 uninfected children; CXCR2 is significantly reduced in HIV-1 infected children with severe disease (Meddows-Taylor *et al*., 2001a). In HIV-1 exposed-uninfected infants, CXCR1 and CXCR2 expression decreases with age from 12 months, as does the proportion of PMNLs expressing the receptors (Meddows-Taylor & Tiemessen, 2004).

CXCL8 is known to regulate its own receptor expression – receptor expression is down-regulated in response to CXCL8, but the down-regulated receptor can be rapidly recycled to the neutrophil cell surface (Samanta *et al*., 1990). This suggests that the down-regulation of CXCL8 receptors seen in HIV-1, and in coinfection with HIV-1 and TB, may be modulated by the up-regulation of CXCL8 itself, or alternatively by altered recycling of the receptors (Meddows-Taylor *et al*., 1998).

Dysregulation of CXCL8 (and its receptors) in HIV-1 infection could alter cell trafficking, which could significantly alter the role of neutrophils in bacterial coinfections, and in HIV-1 dissemination by altered trafficking of T lymphocytes (Tiemessen & Martin, 2000).

Chemokine receptors can also cross-regulate the functions of each other (Ali *et al*., 1999, Richardson *et al*., 2000). CXCL8 activation of CXCR1 (but not CXCR2) has been shown to cause the cross-phosphorylation and desensitisation of both CCR5 and CXCR4 (Richardson *et al*., 2003). However, CXCR1 activation leads to the internalisation of CCR5 but not CXCR4, and can thus inhibit HIV-1 infection using CCR5 only (Richardson *et al*., 2003).

1.5 Genetics of resistance / disease progression

The fact that host genetic factors play a major role in susceptibility to HIV-1 infection is suggested firstly by the occurrence of uninfected individuals engaged in high-risk behaviour, and uninfected infants born to HIV-1-infected mothers ("exposed uninfected") and secondly by differing immunological responses to infection (Shrestha *et al*., 2006).

The importance of genetic factors in HIV-1 and AIDS is also inferred by the time of progression from HIV-1 infection to AIDS-defining illnesses – while median time to AIDS-defining pathologies is 10 years, rapid progressors can develop AIDS within 1-5 years (Figure 10). "Long-term non-progressors", in contrast, can remain healthy for up to 20 years, while "elite controllers" are known to maintain viral loads at undetectable levels (< 50 copies per ml) for many years, even in the absence of antiretroviral therapy. These "HIV-1 controllers" show potent constitutive anti-HIV-1 activity by $CD8^+$ T cells (Saez-Cirion *et al*., 2007).

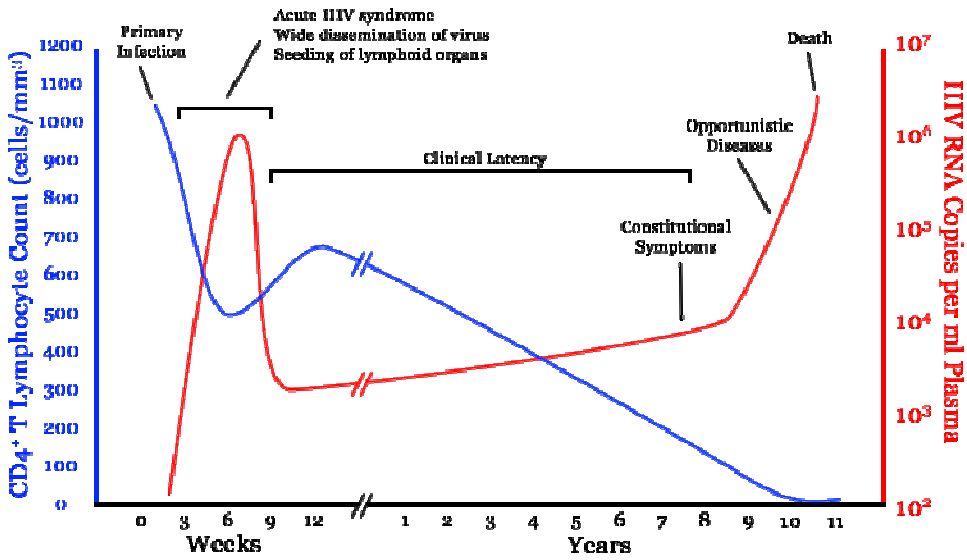


Figure 10
A generalized graph of the relationship between HIV-1 copies (viral load) and CD4 counts over the average course of untreated HIV-1 infection.
Any particular individual's disease course may vary considerably.
—— $CD4^+$ T cell count (cells per µl)
—— HIV-1 RNA copies per ml of plasma
(from http://en.wikipedia.org/wiki/Hiv)

It has been pointed out that the more genetic factors that are identified as being associated with high-risk for HIV-1 and AIDS, the "closer we come to giving patients a virtual death sentence" (Griffiths, 2005), due to insufficient potent and specific antiretroviral drugs. However, any advances in our understanding of both susceptibility to HIV-1 infection and progression to AIDS can only assist in the identification of new drug targets and possibly prevention strategies, so should be actively pursued.

Statistics based on the mutual information between disease and causative factors (the "explained fraction") can be used to estimate the contribution of known genetic factors to epidemiological data (Nelson & O'Brien, 2006). Nelson and O'Brien (Nelson & O'Brien, 2006) have shown that 9% of slow progression to AIDS can be explained cumulatively by 13 genetic factors (polymorphisms in CCR5, CCR2, SDF-1, IL10 and RANTES, HLA-B and KIR-3DS1 alleles, and combinations of these). They suggest that more host genetic factors yet to be discovered may further explain AIDS progression.

Appendix D provides a summary of genes involved in resistance to HIV-1 and TB. Genetic determinants of resistance to both HIV-1 and TB are well documented, but vary depending on the population studied. Given our focus on certain chemokine molecules, namely CCL3 and CXCL8, these are discussed in more detail below.

1.5.1 Genetics of CCL3

In humans, the CCL3[1] protein is encoded by two functional genes, *CCL3* (present at 2 copies per diploid genome) and *CCL3L1* (present at variable copy number) (Irving *et al.*, 1990, Nakao *et al.*, 1990) (Figure 11). Although *CCL3* and *CCL3L1* share 94% nucleotide sequence similarity (refer to Appendix E for genomic DNA sequences), *CCL3L1* encodes a biologically distinct protein that

[1] "CCL3" denotes the combined protein products of both the *CCL3* and *CCL3L1* genes, unless otherwise specified.

binds CCR3 in addition to CCR5, and is a more potent agonist of CCR5. The CCL3L1 protein, unlike CCL3, can be cleaved by CD26 (a membrane-associated serine protease dipeptidyl peptidase) to generate a –2 variant that has even higher affinity for CCR1 and CCR5 (Proost *et al.*, 2000), making it the most potent known inhibitor of HIV-1 entry by a factor of 30 (Menten *et al.*, 2002).

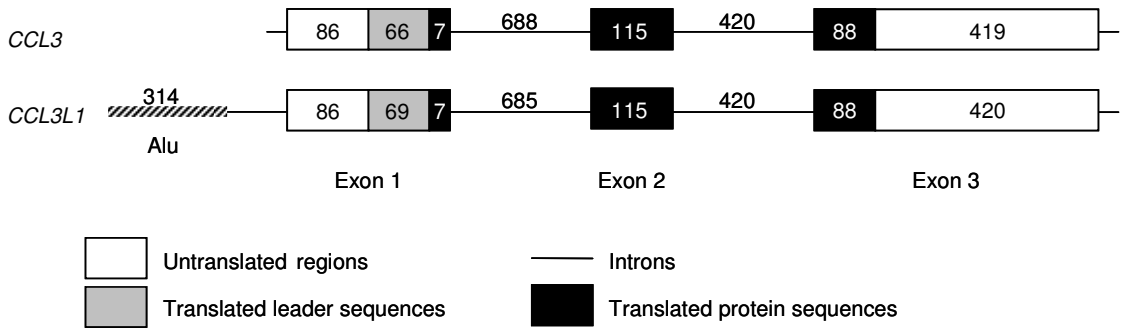


Figure 11
CCL3 and *CCL3L1* gene structure.
(adapted from (Nakao *et al.*, 1990) and (Menten *et al.*, 2002))

An increase in *CCL3L1* copy number is associated with increased CCL3 protein production (Townson *et al.*, 2002), and is negatively associated with the proportion of $CD4^+$ T cells expressing CCR5 (Gonzalez *et al.*, 2005). *CCL3L1* copy number less than the population specific average is strongly associated with HIV-1/AIDS susceptibility (Gonzalez *et al.*, 2005). It is therefore clear that the study of the genetics of CCL3 production and the associated phenotypes will contribute greatly to an understanding of susceptibility to HIV-1 infection within our study populations.

The *CCL3/CCL3L1* genes (also known as *LD78α* and *LD78β*) are clustered on chromosome 17q11.2 with other CC chemokine genes, including *CCL4/CCL4L1* (Naruse *et al*., 1996, Nomiyama *et al*., 2001). In addition, there is a non-functional pseudogene (*LD78γ*), which is truncated at the 5' end and therefore not expressed.

It is likely that gene duplication (estimated to have occurred 10 million years ago (Nakao *et al*., 1990)) generated the ancestral forms of *CCL3*, *CCL3L1*, *CCL4* and *CCL4L1*, which were then preserved due to the evolution of distinct biological function; the variable copy number of *CCL3L1* (and *CCL4L1*) is probably due to an association with a repeat unit which is highly susceptible to duplication, although *CCL3L1* and *CCL4L1* appear to duplicate independently (Shao *et al*., 2007).

Median *CCL3L1* gene copy number differs in different population groups (Gonzalez *et al*., 2005), leading to the hypothesis that gene dose relative to the average in each population group influences HIV/AIDS susceptibility, rather than copy number *per se*. Low *CCL3L1* copy number is a major determinant of enhanced HIV-1 susceptibility in both mother-to-child and adult-to-adult infection (Gonzalez *et al*., 2005). In addition, in HIV-1 infected adults, lower *CCL3L1* gene copy numbers have been shown to be associated with an increased risk of rapid progression to AIDS or death (Gonzalez *et al*., 2005).

Many copy number polymorphisms (CNPs) contribute to human genetic diversity (Sebat *et al.*, 2004) – representational oligonucleotide microarray analysis (ROMA) of 20 healthy individuals showed CNPs widely distributed throughout the genome, comprising 70 genes within a total of 221 CNPs. This is perhaps not surprising, since it seems that copying, duplication and multiplication processes are important in the evolution of protein families, and that new gene functions evolve by exploiting pre-existing genes (Britten, 2005).

Other genes which have been shown to be present in variable copy can result in distinct phenotypes – e.g. Fcgr3, in which a low copy number of the *FCGR3B* gene is linked to susceptibility to immunologically mediated glomerulonephritis (Aitman *et al.*, 2006).

1.5.2 Genetics of CXCL8

A common single nucleotide polymorphism (SNP), located 251 base pairs (bp) upstream of the *IL8* transcription start site (refer to Appendix E for genomic DNA sequences), has been shown to be associated with changes in CXCL8 production by LPS-stimulated whole blood (Hull *et al.*, 2000). The IL8-251A allele (associated with increased CXCL8 production) is significantly related to greater severity of respiratory syncytial virus (RSV) bronchiolitis (Hull *et al.*, 2000), although IL8-251A may not be the functional allele itself, but may lie in linkage disequilibrium with the functional variant (Hull *et al.*, 2001). Another study has shown a significant association between the IL8-251A allele and TB

susceptibility (Ma *et al*., 2003). Interestingly, the IL8-251T→A SNP is located upstream of the promoter binding sites for transcription factors (Ma *et al*., 2003), which raises the question of how its effects are exerted. It has been suggested that if CXCL8 expression was increased, an excess of leukocytes may be attracted to the site of *Mtb* infection, causing extensive tissue damage and increasing the risk of clinical TB (Ma *et al*., 2003).

1.6 Aims of study

This study addressed the production of a group of chemokines (CCL3, CCL4, CCL5, CXCL8 and CXCL12) that are known to play an important role in the pathogenesis of either HIV-1 or TB. These were evaluated in the context of single infections (HIV-1 and TB groups) and of coinfection with both organisms (HIV-1/TB group). Circulating levels of chemokines and the *ex vivo* cellular ability to produce these chemokines were monitored.

Altered production profiles of two chemokines, CCL3 and CXCL8, have been associated with MTCT of HIV-1 (Meddows-Taylor *et al*., 2006) and adult TB (Ma *et al*., 2003), respectively. We hypothesised that a known genetic determinant of altered CCL3 production (*CCL3L1* gene copy number (Townson *et al*., 2002)) would be associated with maternal-infant HIV-1 transmission, and that a genetic polymorphism (IL8-251T→A), associated with enhanced CXCL8 production (Hull *et al*., 2000) and susceptibility to TB in African Americans (Ma *et al*., 2003), would be highly represented in the South African Black population.

Specific objectives

1. To evaluate circulating chemokine levels (CCL3, CCL4, CCL5, CXCL8 and CXCL12) in the presence of HIV-1 infection, pulmonary TB, and coinfection with HIV-1 and *Mtb*.

2. To monitor unstimulated and PHA-stimulated PBMC capacity to produce CCL3, CCL4, CCL5, CXCL8 and CXCL12, in groups divided as above.

3. To establish methodologies for determining the presence of genetic determinants that may affect CCL3 (*CCL3L1* gene copy number) and CXCL8 (IL8-251T→A SNP) production phenotypes, and to relate these genetic polymorphisms to circulating levels of CCL3 and CXCL8.

4. To examine *CCL3L1* gene copy numbers within South African population groups, and within the context of MTCT of HIV-1.

5. To determine the distribution of the IL8-251T→A SNP within South African population groups, and to investigate this polymorphism in the context of pulmonary TB.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study cohorts

2.1.1 HIV-TB cohorts

Samples from two separate “HIV-TB” cohorts were utilised in this study.

Characteristics of the first cohort of individuals (HIV-TB coh1), all from the Johannesburg area and grouped as control (HIV-1 and TB negative), HIV-1 (HIV-1 positive, TB negative), TB (HIV-1 negative, TB positive) and HIV-1/TB (HIV-1 positive, TB positive) groups are shown in Table 2. Some analyses (Chapter 3) were restricted to Black antiretroviral-naïve individuals within these cohorts. Characteristics of these individuals are therefore presented separately in Table 3.

Plasma and DNA samples from HIV-TB coh1 were used for quantitating chemokine levels (Chapter 3.3.1), and for determining *CCL3L1* gene copy number (Chapter 4), respectively. DNA samples from the TB groups (TB and HIV-1/TB) were in addition used for the IL8-251T→A polymorphism study (Chapter 5).

Table 2
Characteristics of HIV-TB coh1.

Grouping	Control n=82	HIV-1 n=48	TB n=28	HIV-1/TB n=62
Gender (n (%))				
Male	49 (60)	28 (58)	12 (43)	19 (31)
Female	32 (39)	20 (42)	16 (57)	40 (65)
Unknown	1 (1)	0 (0)	0 (0)	3 (5)
Population group (n (%))				
Black	49 (60)	26 (54)	28 (100)	62 (100)
Caucasian	30 (37)	19 (40)	0 (0)	0 (0)
Asian	2 (2)	1 (2)	0 (0)	0 (0)
Mixed	0 (0)	2 (4)	0 (0)	0 (0)
Unknown	1 (1)	0 (0)	0 (0)	0 (0)
Age (years)				
Mean ± sd	44±11	36±8	36±13	34±9
Range	24-65	24-59	18-66	22-56
HIV-1 disease status (n (%))				
Asymptomatic	Not relevant	26 (54)	Not relevant	0 (0)
AIDS		13 (27)		62 (62)
Unknown		9 (19)		0 (0)
Antiretroviral therapy (n (%))				
ARV +	Not relevant	19 (40)	Not relevant	0 (0)
ARV -		29 (60)		62 (100)
Antituberculosis therapy (n (%))				
Anti-TB +	Not relevant	Not relevant	24 (86)	59 (95)
Anti-TB -			2 (7)	0 (0)
Unknown			2 (7)	3 (5)

Table 3
Characteristics of Black, antiretroviral-naïve individuals HIV-TB coh1.

Grouping	Control n=49	HIV-1 n=17	TB n=28	HIV-1/TB n=62
Gender (n (%))				
Male	40 (82)	6 (35)	12 (43)	19 (31)
Female	9 (18)	11 (65)	16 (57)	40 (65)
Unknown	0 (0)	0 (0)	1 (0)	3 (5)
Age (years)				
Mean ± sd	43±11	30±6	36±13	34±9
Range	24-65	24-50	18-66	22-56
HIV-1 disease status (n (%))				
Asymptomatic		9 (53)		0 (0)
AIDS	Not relevant	3 (18)	Not relevant	62 (62)
Unknown		5 (29)		0 (0)
Antituberculosis therapy (n (%))				
Anti-TB +			24 (86)	59 (95)
Anti-TB -	Not relevant	Not relevant	2 (7)	0 (0)
Unknown			2 (7)	3 (5)

The second "HIV-TB" cohort (HIV-TB coh2) consisted of Black individuals from Johannesburg hospitals with groupings as above (16 of each of normal donor, HIV-1 positive, TB positive and HIV-1/TB coinfected individuals). All TB and HIV-1/TB patients were receiving standard anti-TB therapy; none of the HIV-1 infected individuals were receiving antiretroviral therapy (ART) (Meddows-Taylor *et al.*, 1999a). PBMCs were previously isolated from these individuals and cultured as described by Meddows-Taylor *et al.* (Meddows-Taylor *et al.*, 1999a). The stored supernatants were used for comparisons of levels of chemokines by ELISA (Chapter 3.3.2).

2.1.2 MTCT cohort

MTCT samples consisted of blood samples collected from Black HIV-1 infected (n=267) and uninfected (n=13) mothers, and infants born to HIV-1 infected mothers (n=207; remaining were lost to follow-up). Samples were drawn from 3 separate mother-infant groups which formed part of prevention of mother-to-child transmission (PMTCT) studies (Table 4): Group 1 from Chris Hani Baragwanath Hospital (96 mothers received single-dose nevirapine (NVP); 98 mothers had no antenatal ART) (Gray *et al.*, 2005, Kuhn *et al.*, 2007); Group 2 from Coronation Hospital (all mothers received single-dose NVP except for 3 who received triple-drug therapy) (Kuhn *et al.*, 2007); and Group 3, also from Chris Hani Baragwanath Hospital (mothers received one of 3 ART regimens of zidovudine and lamivudine) (Team, 2002).

Blood samples were collected into EDTA Vacutainers (Becton Dickinson, San Jose, California, USA; refer to Appendix F for a full list of suppliers); peripheral blood samples were collected from all mothers, while blood samples were collected from infants (at birth, 6 weeks and 3 months) born to the HIV-1 infected mothers. Infant samples were divided into groups as follows: exposed-uninfected (EU) (HIV-1 DNA PCR negative at birth and at 6 weeks); HIV-1 infected *in utero* (IU) (HIV-1 DNA PCR positive at birth and at 6 weeks); and HIV-1 infected intrapartum (IP) (HIV-1 DNA PCR negative at birth but positive at 6 weeks).

Table 4
Maternal and infant samples from the MTCT cohort.

Group	Mothers	Infants				
		Infected *in utero*	Infected intrapartum	Infected (timing unknown)	Exposed uninfected	Total
1	194	6	14	44	69	133
2	42	-	-	7	36	43
3	31	-	-	8	23	31
Total	**267**	**6**	**14**	**59**	**128**	**207**

DNA was extracted from stored cell pellets/whole blood from the MTCT cohort and used for determining *CCL3L1* gene copy number (Chapter 4.3.3) and the presence of the IL8-251T→A polymorphism (HIV-1 positive mothers only; Chapter 5.4.4).

2.1.3 Eskom study cohort

DNA samples from the Eskom study cohort (part of an HIV-1 prevalence study among Eskom staff members) were used for the population-based study of *CCL3L1* gene copy numbers, and for the IL8-251T→A polymorphism study (Table 5).

Table 5
Population subgroups within the Eskom study cohort according to HIV-1 status.

Population group	Number (percent) of individuals		
	HIV-1 positive	HIV-1 negative	HIV-1 unknown
Black (n=282)	**26 (9)**	**240 (85)**	**16 (6)**
Ndebele (n=17)	2 (12)	15 (88)	0 (0)
Pedi (n=23)	1 (4)	20 (87)	2 (9)
South Sotho (n=20)	3 (15)	17 (85)	0 (0)
Swazi (n=27)	3 (11)	21 (78)	3 (11)
Tsonga (n=5)	0 (0)	5 (100)	0 (0)
Tswana (n=25)	0 (0)	23 (92)	2 (8)
Venda (n=22)	0 (0)	22 (100)	0 (0)
Xhosa (n=46)	4 (9)	42 (91)	0 (0)
Zulu (n=97)	13 (13)	75 (77)	9 (9)
Caucasian (n=102)	**0 (0)**	**102 (100)**	**0 (0)**
English (n=52)	0 (0)	52 (100)	0 (0)
Afrikaans (n=50)	0 (0)	50 (100)	0 (0)

2.2 Sample preparation

2.2.1 Samples for enzyme-linked immunosorbent assays (ELISA)

Plasma and PBMCs were previously isolated from whole blood samples collected by venipuncture into EDTA Vacutainers (Becton Dickinson). Samples were processed immediately upon collection.

Plasma was collected by centrifugation at 200 x *g* for 10 minutes at room temperature, and stored at -70°C.

Supernatants from PBMC cultures used in an earlier study (Meddows-Taylor *et al*., 1999a) were used for further testing of chemokines by ELISA. Briefly, PBMCs were isolated from buffy coats by centrifuging phosphate-buffered saline (PBS) diluted whole blood (1:1) on a Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) gradient at 1000 x *g* for 30 minutes at room temperature. Cells were counted by trypan blue exclusion, and diluted to 1.5 x 10^6 cells/ml for plating in RPMI medium containing 1% L-glutamine (Invitrogen Corporation, Carlsbad, California, USA). PBMCs were either unstimulated or stimulated with 12.5 µg/ml PHA (Sigma Chemical Co., St Louis, Missouri, USA), 10% human serum (obtained from the South African Blood Transfusion Service, Johannesburg, South Africa) was added to each well, and cells were incubated for 24 hours at 37°C in a moist, 5% CO_2 atmosphere. Supernatants were then harvested and stored at -70°C.

2.2.2 DNA extraction

DNA was extracted from PBMCs that were previously isolated from blood samples and stored as cell pellets at -70°C, and from whole blood. PBMC cell pellets (<5 x 10^6 cells) were thawed and 200 µl of PBS (at room temperature) added; alternatively, 200 µl of whole blood was equilibrated to room temperature. Genomic DNA was extracted from PBMCs or whole blood using a QIAamp DNA Blood Minikit (QIAgen, Hilden, Germany) according to the manufacturer's protocol. Briefly, cells were lysed by incubation at 56°C for 10 minutes with 20 µl of the kit protease and 200 µl of lysis buffer. Two hundred µl

of 96% ethanol (EtOH) (Merck Chemicals (Pty) Ltd, Nottingham, UK) was added, and the entire sample applied to a spin column (containing a silica-gel membrane to which DNA binds specifically) and centrifuged. Following washing steps to remove proteins and PCR inhibitors, the DNA was eluted from the column with 200 µl elution buffer by incubation at room temperature for 1 minute, followed by centrifugation.

2.2.3 DNA quantitation

Extracted genomic DNA samples were tested using a NanoDrop (NanoDrop Technologies, Wilmington, Detroit, USA). DNA samples with A_{260}<0.1 were considered to be of low concentration, and with $A_{260/230}$<0.5 of poor quality. These samples were excluded.

2.3 ELISAs

CCL3, CCL4 and CCL5 levels were determined using DuoSet ELISA Development Systems, while CXCL12 and CXCL8 levels were quantified using Quantikine ELISA kits (both from R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's directions. Minimum detectable levels for these assays were <10 pg/ml for CCL3, 4 pg/ml for CCL4, 2 pg/ml for CCL5, 1.0 pg/ml for CXCL12 and 1.5 pg/ml for CXCL8.

In these ELISAs, a capture antibody specific to each chemokine was immobilised on a plate, which was then washed of unbound antibody. Remaining binding sites on the plate were blocked with non-specific protein (bovine serum albumin, BSA). The plasma or supernatant sample (diluted if necessary) was then added to each well, and the chemokine of interest bound to the capture antibody. After washing to remove non-specific proteins, a detection antibody with the same specificity of the capture antibody was added, and any excess removed by a further wash step. This detection antibody is conjugated to biotin, which was then bound by streptavidin conjugated to horseradish peroxidase (HRP). The HRP enzyme catalyses a colour-change reaction (using a substrate oxidised by HRP using hydrogen peroxide as the oxidising agent), the absorbance of which was measured by spectrophotometer (measurement A_{450} – reference A_{540}) and used to calculate the amount of target by reference to a standard curve generated for each plate (using a serial dilution of a control of known concentration).

2.4 *CCL3L1* gene copy number determination

2.4.1 Real-time PCR analysis

Real-time PCR was performed using an ABI PRISM 7500 (Applied Biosystems, Foster City, California, USA) according to the protocol supplied, using primers and probes as detailed in Table 6. Briefly, each PCR reaction contained forward and reverse primers at 0.9 μM each, probe at 0.25 μM, 1x TaqMan

Universal PCR Master Mix (Applied Biosystems) and approximately 20 ng of genomic DNA. PCR reactions were carried out using the manufacturer's suggested profile of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Table 6
Primers and probes synthesised (University of Cape Town, South Africa) for determination of *CCL3* and *CCL3L1* gene copy number.

Primer / probe	Sequence (5'->3')	Reference
β-globin gene (forward)	ggcaaccctaaggtgaaggc	(Townson *et al*., 2002)
β-globin gene (reverse)	ggtgagccaggccatcacta	(Townson *et al*., 2002)
β-globin gene (probe)*	catggcaagaaagtgctcggtgcct	(Townson *et al*., 2002)
CCL3L1 gene (forward)	tctccacagcttcctaaccaaga	(Townson *et al*., 2002)
CCL3 gene (forward)	tctccacagcttcctaaccaagc	
CCL3 / *CCL3L1* genes (reverse)	ctggacccactcctcactgg	(Townson *et al*., 2002)
CCL3 gene (probe)*	aagccggcaggtctgtgctga	
CCL3L1 gene (probe)*	aggccggcaggtctgtgctga	(Townson *et al*., 2002)
CCL3 / *CCL3L1* genes (primer A)	ccgagtcacagctcagaaga	Dr M Paximadis, unpublished data
CCL3 / *CCL3L1* genes (primer B)	ccacagcatcagcccat	Dr M Paximadis, unpublished data

* All probes were labelled with a 5' FAM (6-carboxyfluorescein) reporter dye and a 3' TAMRA (carboxytetramethyl- rhodamine) quencher.

For each sample, the *β-globin*, *CCL3* and *CCL3L1* genes were amplified in duplicate. The *CCL3* and *CCL3L1* genes were distinguished on the basis of a SNP at the 3' terminal position of the forward primer site (Figure 12). *CCL3*

gene copy number was confirmed at 2 copies per diploid genome (pdg) for each sample, calculated using the Relative Quantification method (as per the protocol supplied) and using *β-globin* (present at 2 copies pdg) as the endogenous control. Due to the similarity of the primers for *CCL3* and *CCL3L1*, *CCL3* was preferentially used as the endogenous control to calculate *CCL3L1* gene copy number, again using the Relative Quantification method.

Figure 12
CCL3 and *CCL3L1* real-time PCR primer and probe sites.
Nucleotide site allowing PCR discrimination between *CCL3* and *CCL3L1* is in bold.

DNA samples giving a result of *CCL3* gene copy ≠ 2 were found to be of low concentration (A_{260}<0.1) and/or poor quality ($A_{260/230}$<0.5), and were excluded from analyses.

2.4.2 PCR method for *CCL3* and *CCL3L1* gene detection

PCR primers A and B (Table 6) were synthesised to amplify the promoter and exon 1 of the *CCL3* and *CCL3L1* genes. Due to a 312 bp Alu element insertion within the 5' region of *CCL3L1* compared to *CCL3* (Figure 13), *CCL3* and *CCL3L1* were able to be distinguished on the basis of PCR product size by agarose gel electrophoresis.

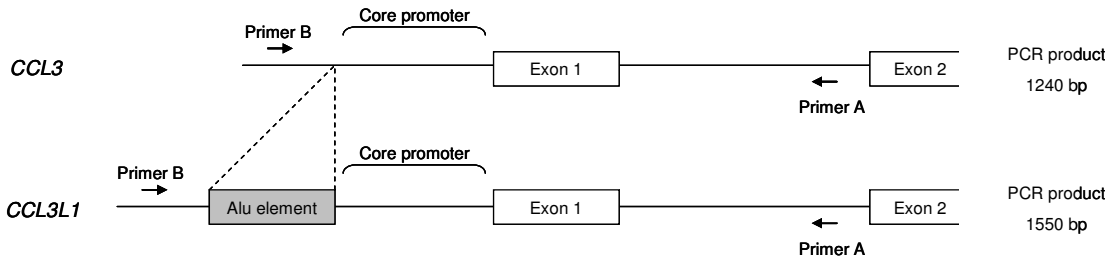


Figure 13
CCL3 and *CCL3L1* promoter and partial gene structure, showing Alu element insertion in *CCL3L1* and primer positions.

Briefly, each PCR reaction contained forward and reverse primers at 300 nM each, 200 µM of each dNTP (Promega Corporation, Madison, Wisconsin, USA), 200 ng of template DNA, 1x Expand High Fidelity Buffer and 2.6 U of Expand High Fidelity enzyme mix (Roche Diagnostics GmbH, Mannheim, Germany), made up to 50 µl with sterile dH_2O. PCR reactions were carried out using the Expand High Fidelity protocol of 94°C for 2 minutes, 10 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 68°C for 1.5 minutes, 20 cycles of 94°C for

15 seconds, 60°C for 30 seconds and 72°C for 45 seconds to 8 minutes (increasing by 5 seconds each cycle), followed by a final elongation of 72°C for 7 minutes. PCR products were run on 1% agarose (Bioline, London, UK) containing 0.5 µg/ml ethidium bromide (CLP, San Diego, California, USA), at 100V for 1 hour, in 0.5x TBE buffer (Invitrogen Corporation).

2.4.3 DNA sequencing

PCR products were extracted using the QIAquick PCR Purification Kit (QIAgen), according to the manufacturer's instructions. Briefly, PCR products were bound specifically to the silica-gel membrane of a spin column. Following washing steps to remove unincorporated PCR components and primers, PCR products were eluted with 30 µl buffer (10 mM Tris-Cl, pH 8.5).

Sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Sequencing reactions consisted of approximately 20 ng purified PCR product (estimated from the intensity of the PCR band seen on ethidium bromide-stained agarose gel, in comparison to size standards of known concentration – for a bright band, 1 µl of PCR product was used, for a fainter band, 2 µl), 3.2 pmol primer and 4 µl BigDye® reaction mix, made up to 10 µl with dH_2O. Reactions were carried out in a 96-well plate on a GeneAmp PCR System 9700 (Applied Biosystems) by denaturing at 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

Sequencing products were purified, according to the manufacturer's protocol, by precipitation with 25 µl of freshly mixed 3M sodium acetate (NaOAc) pH 4.6 (Merck) and absolute EtOH (Merck) (1:25), and centrifuged at room temperature for 30 minutes at 2000 x *g*. Supernatant was removed by inverting the plates onto paper towels and centrifuging for 1 minute at 150 x *g*. Sequenced products were washed with 50 µl of 70% EtOH, centrifuged for 5 minutes at 2000 x *g*, and supernatant removed as before. Plates were placed open at 63°C for 3 minutes to dry completely before the addition of deionised formamide for running on an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Sequences were analysed using the proprietary software installed.

2.5 IL8-251T→A SNP determination

2.5.1 IL8-251T→A SNP PCR and agarose gel electrophoresis

The following primers were synthesised (University of Cape Town) (Figure 14):

(1) IL8-251 consensus 1 5'-tgccccttcactctgttaac-3' (Hull *et al.*, 2001)

(2) IL8-251A 5'-ccacaatttggtgaattatcaat-3' and

IL8-251T 5'-ccacaatttggtgaattatcaaa-3' (Hull *et al.*, 2001)

(3) IL8-251 consensus 2 5'-gctggcttatcttcaccatc-3'

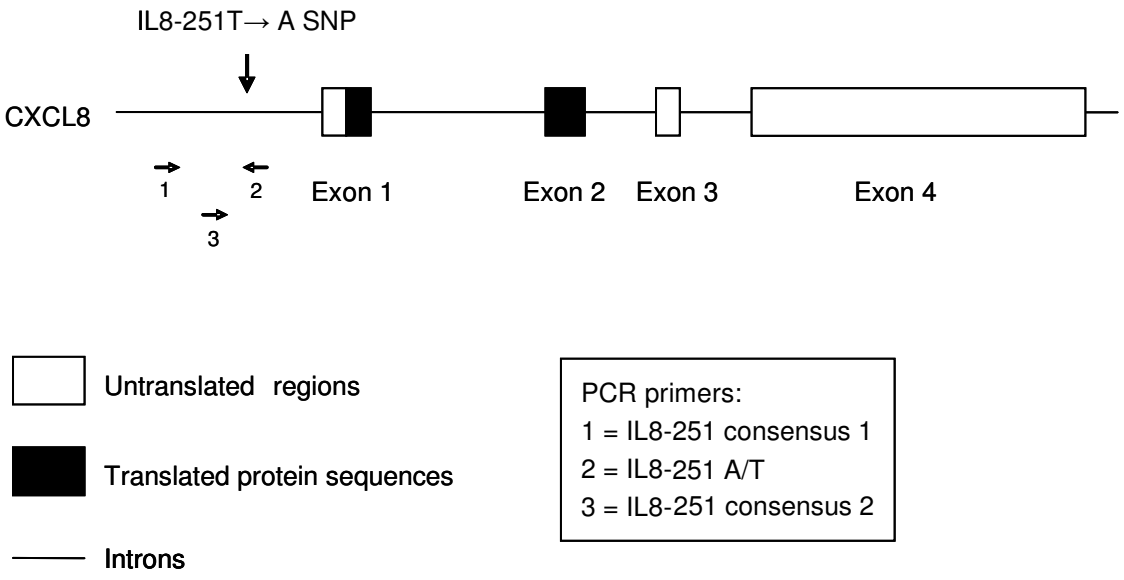


Figure 14
CXCL8 gene structure showing position of PCR primers and -251 SNP.

PCR was initially performed as described (Hull *et al.*, 2001), with the annealing temperature modified as described below. The PCR mix consisted of 1x PCR buffer and 5 U Taq DNA polymerase (Promega Corporation), with 1.5 mM $MgCl_2$ (Promega Corporation), 200 µM of each dNTP (Promega Corporation), 1 µM of each primer, and approximately 50 ng of genomic DNA in a final volume of 50 µl. Thermal cycling conditions were 94°C for 2 minutes, followed by 10 cycles of 94°C for 15 seconds, 68°C (decreasing by 1°C each cycle) for 30 seconds, 72°C for 30 seconds, followed by 20 cycles of 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 30 seconds, followed by 72°C for 3 minutes and a 4°C hold. PCR products were run on 2% agarose (Bioline) containing 0.5 µg/ml ethidium bromide (CLP), at 70V for 1 hour, using the Electro-Fast Stretch

108 Gel Tank System (ABgene Ltd., Epsom, UK), in 1x TBE buffer (Invitrogen Corporation). This system allows high-throughput agarose-based detection of PCR products – 96 PCR amplification reactions were carried out in a 96-well plate format, in parallel, with primers specific for the T- and A-alleles (combinations of primer 1 and each primer 2, separately. Primer 3 was used for confirmation purposes).

2.5.2 IL8-251T→A SNP real-time PCR

Although the system described above allows high-throughput agarose gel-based detection of PCR products, real-time PCR systems provide faster throughput as PCR products are detected during amplification as opposed to subsequently, by electrophoresis. Real-time PCR was therefore developed for the detection of the IL8-251T→A polymorphism, using the ABI PRISM 7500 (Applied Biosystems). The same primers as described before were used, with incorporation of SYBR Green as a reporter dye (Applied Biosystems). Thermal cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds, 60°C for 1 minute. Thermal cycling was followed by a dissociation stage of 95°C for 15 seconds, 60°C for 1 minute, then 95°C for 15 seconds (as recommended by the manufacturer) to ensure single PCR product.

2.6 Statistical methods

All statistical analyses were performed using SPSS version 15.0 software (SPSS Inc., Chicago, Illinois, USA). Differences in levels of chemokines and in *CCL3L1* gene copy numbers between groups were compared using the nonparametric Mann Whitney U test. Correlations between chemokine levels were calculated by bivariate Spearman's rank coefficients, while Fisher's Exact test was used to compare proportions of individuals with detectable chemokine levels, and with the IL8-251T→A SNP. For all statistical tests, significance was considered to be $P<0.05$.

CHAPTER 3

INFLUENCE OF HIV-1 AND TB ON CHEMOKINE PRODUCTION

3.1 Introduction

The chemokines CCL3, CCL4, CCL5 and CXCL12 (previously known as MIP-1α, MIP-1β, RANTES and SDF-1α, respectively) are of particular interest in HIV-1 research, since they are the natural ligands of the HIV-1 coreceptors CCR5 (CCL3, CCL4 and CCL5) and CXCR4 (CXCL12). CCL3, CCL4 and CCL5 are all inhibitors of HIV-1 binding, partly due to steric hindrance through the blocking of their receptor CCR5 (Cocchi *et al.*, 1995). In addition, desensitisation and internalisation of CCR5 through ligand binding also plays a role in reducing the targets for HIV-1 binding (Amara *et al.*, 1997). Most important, although less well understood, is the complex interaction of immune system components in chemokine-induced immune cell recruitment and regulation, including (but not limited to) the development of Th1 and Th2 responses.

According to one report (Jennes *et al.*, 2004), intracellular levels of the chemokines CCL3 and CCL4 tend to be increased in lymphocytes during HIV-1 infection, with a corresponding decrease in their PHA-stimulated secretion into lymphocyte culture supernatants; CCL5 tends to be increased both intracellularly and in culture supernatant. In the same patients, after more than

3 years of HAART, intracellular levels of CCL4 and CCL5, and CCL3 and CCL4 in supernatant, reached normal levels, coincident with higher $CD4^+$ T cell counts (Jennes *et al*., 2004). In contrast, data from our laboratory has shown that amongst a cohort of antenatal ART-naïve HIV-1 infected women, PHA-stimulated production of CCL3, CCL4 and CCL5 by PBMCs was increased by HIV-1 infection (Meddows-Taylor *et al*., 2006).

CXCL8 (previously known as IL-8) is a CXC chemokine important in the inflammatory process, and is produced predominantly by monocytes/ macrophages (Yoshimura *et al*., 1987) in response to a wide variety of microorganisms (Chensue, 2001). CXCL8 is involved in recruiting leukocytes to inflammatory sites (Pace *et al*., 1999), but if overproduced can cause bystander damage (Chensue, 2001).

The role of CXCL8 in TB is of interest, as elevated levels of CXCL8 are associated with more severe disease (Friedland *et al*., 1995, Mastroianni *et al*., 1994). However, CXCL8 is up-regulated in macrophages as part of the macrophage activation program in response to *Mtb* infection (Ribeiro-Rodrigues *et al*., 2002), and seems to be essential for host defence to *Mtb* (Larsen *et al*., 1995). It is therefore essential for efficient control of infection by the immune system that CXCL8 expression is tightly regulated.

CXCL8 levels are upregulated in both serum (Matsumoto *et al*., 1993) and plasma (Meddows-Taylor *et al*., 1999a, Thea *et al*., 1996) of HIV-1 infected adults, although not in HIV-1 infected children (Meddows-Taylor *et al*., 2001b).

CXCL8 can either decrease or increase HIV-1 replication in promonocytic U1 cells on induction by proinflammatory cytokines, depending on the nature of the cytokine and CXCL8 concentration (Tiemessen *et al*., 2000a). The down-regulation of CXCL8 receptors seen in HIV-1, and in coinfection with HIV-1 and *Mtb*, may be modulated by the up-regulation of CXCL8 itself, or alternatively by altered recycling of the receptors (Meddows-Taylor *et al*., 1998).

In this section of the study, we aimed to examine the relationships between levels of the chemokines CCL3, CCL4, CCL5, CXCL12 and CXCL8, with particular relevance to infection by HIV-1 and *Mtb*. The study comprised two components, namely examination of plasma chemokine levels using samples from HIV-TB coh1, and the study of previously stored PBMC supernatants from HIV-TB coh2 (Chapter 2.1.1).

3.2 Results

3.2.1 Chemokine levels in plasma samples

3.2.1.1 Influence of age, gender and race

As the groups within the HIV-TB coh1 study cohort (Chapter 2.1.1, Table 2) were not matched for age, gender or race, the influence of these variables on plasma levels of CCL3, CCL4, CCL5, CXCL12 and CXCL8 was first examined in the control group (n=82) of this cohort. The aim of these comparisons was to ensure that groups used for further analyses could be meaningfully compared.

Age and gender had no effect on any of the studied chemokines (data not shown). However, in comparisons of Black and Caucasian individuals, race group was seen to influence plasma CCL3 levels (Figure 15), although levels of the other chemokines were unaffected (data not shown). Circulating levels of CCL3 were significantly elevated in Caucasians, with the majority of Black individuals having undetectable levels of this chemokine. For this reason, Caucasians were omitted from further analyses in this Chapter, due to their smaller numbers in the control and HIV-1 groups within HIV-TB coh1.

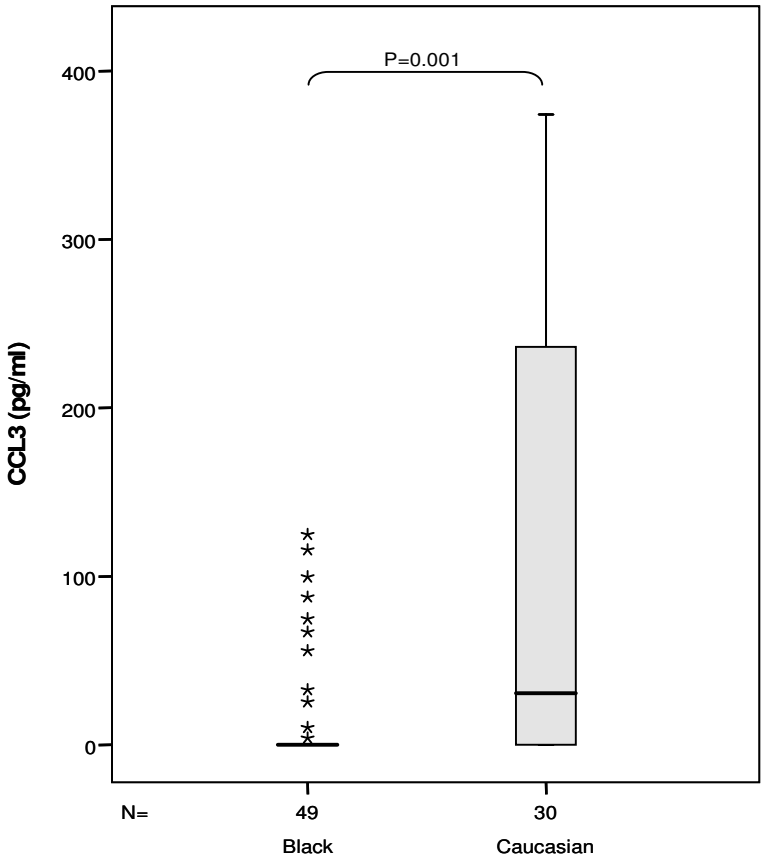


Figure 15
CCL3 plasma levels in Black and Caucasian individuals from the control group. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars) and extremes (*).

3.2.1.2 Influence of clinical status and treatment on plasma chemokine levels

In Black individuals from the HIV-1 group of HIV-TB coh1 (Table 2), no significant differences in plasma chemokine levels were seen when comparing those patients who remained asymptomatic (n=26) with those with AIDS-defining illnesses (n=13) (data not shown; P>0.05).

Within the TB groups (TB and HIV-1/TB), the influence of antituberculosis therapy on plasma chemokine levels could not be tested, since only 2 individuals were not treated, and 5 were of unknown treatment status.

The effect of ART on plasma chemokine levels in Black individuals from the HIV-1 group (n=26) of HIV-TB coh1 was compared. ART-positive individuals had a trend towards dimished levels of CCL3 (P=0.095), and had significantly decreased levels of CXCL12 relative to untreated HIV-1 infected individuals (P=0.034) (Figure 16). For this reason, further analyses were restricted to ART-naïve HIV-1 positive patients (Table 3).

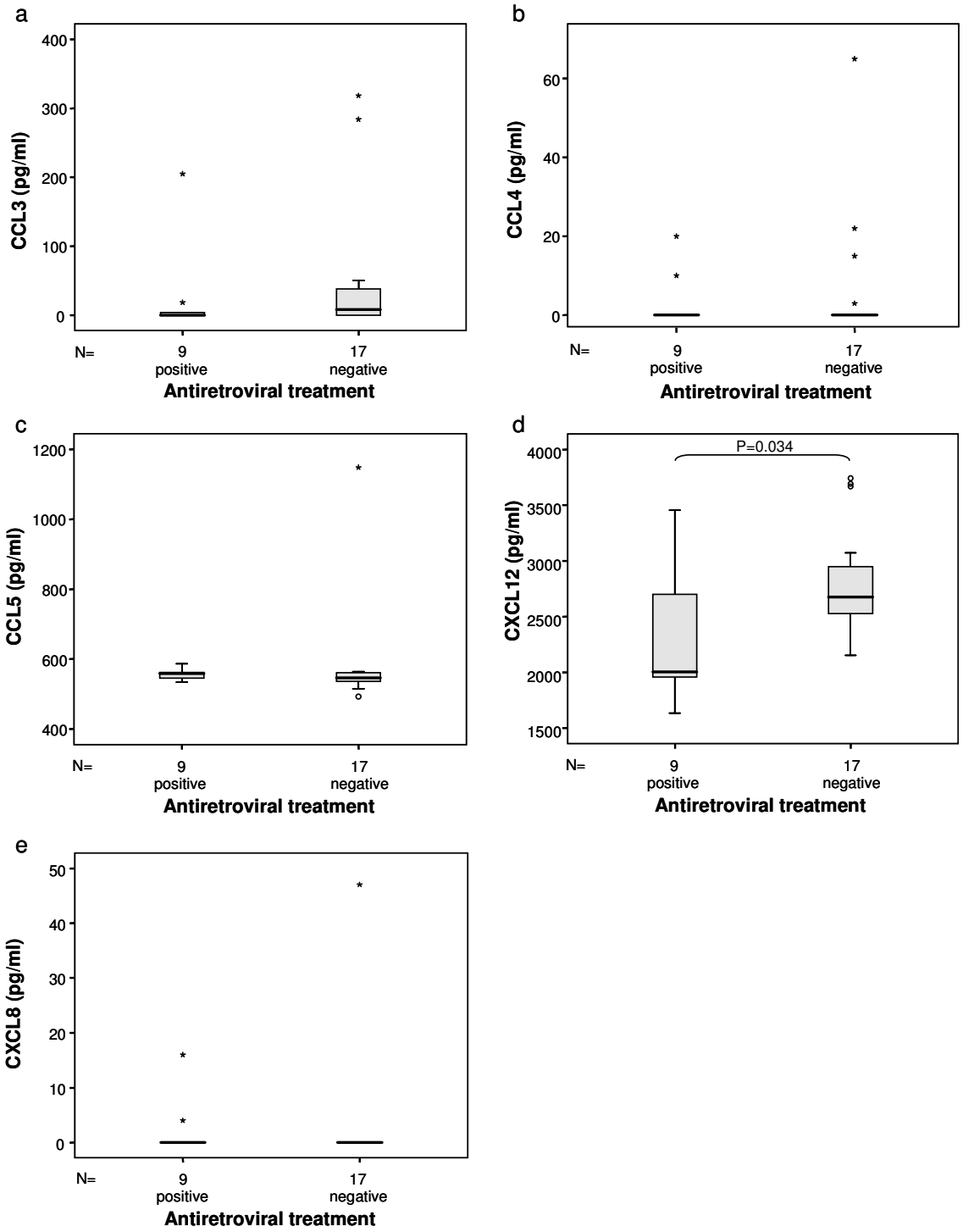


Figure 16
CCL3 (a), CCL4 (b), CCL5 (c), CXCL12 (d) and CXCL8 (e) levels in plasma samples from Black HIV-1 positive patients stratified according to ART status. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars), outliers (o) and extremes (*).

3.2.1.3 Plasma chemokine levels in HIV-1 and TB

Chemokine levels in plasma samples from Black ART-negative patients with HIV-1, TB, or HIV-1/TB coinfection were compared with uninfected controls (Chapter 2.1.1, Table 3) (Figure 17). The following results were noted:

- CCL3 levels (Figure 17a) were significantly raised by infection with HIV-1, *Mtb*, or both (P=0.002, P=0.001, P<0.001 respectively).
- CCL4 levels (Figure 17b) in the HIV-1, TB and HIV-1/TB groups were significantly different from controls, in that infection (by HIV-1 or *Mtb*, or both) significantly increased levels (P=0.004, P=0.003, P=0.007 respectively), the statistical finding clearly driven by the small proportions of individuals with detectable levels. CCL4 was detectable in 1/49 (2%) controls, 4/17 (24%) HIV-1, 6/27 (22%) TB and 11/61 (18%) HIV-1/TB individuals.
- CCL5 levels (Figure 17c) were significantly raised by HIV-1 and *Mtb* infections alone or together (P<0.001 in each case).
- CXCL12 levels (Figure 17d) were significantly increased in all infection groups, with HIV-1 (in both HIV-1 and HIV-1/TB groups) driving higher levels than *Mtb* infection alone (P<0.001 in each case).
- CXCL8 (Figure 17e) was detectable in more HIV-1 infected individuals (HIV-1 and HIV-1/TB groups 13/78; 17%) than HIV-1 uninfected (control and TB 1/76; 1.3%) (P=0.001; Fisher's Exact test), while more individuals in the HIV-1/TB group had detectable levels of CXCL8 (12/61, 20%) than in the control group (0/49) (P=0.001; Fisher's Exact test). In the HIV-1/TB group, CXCL8 levels were significantly raised relative to controls (P=0.001; Mann Whitney *U* test).

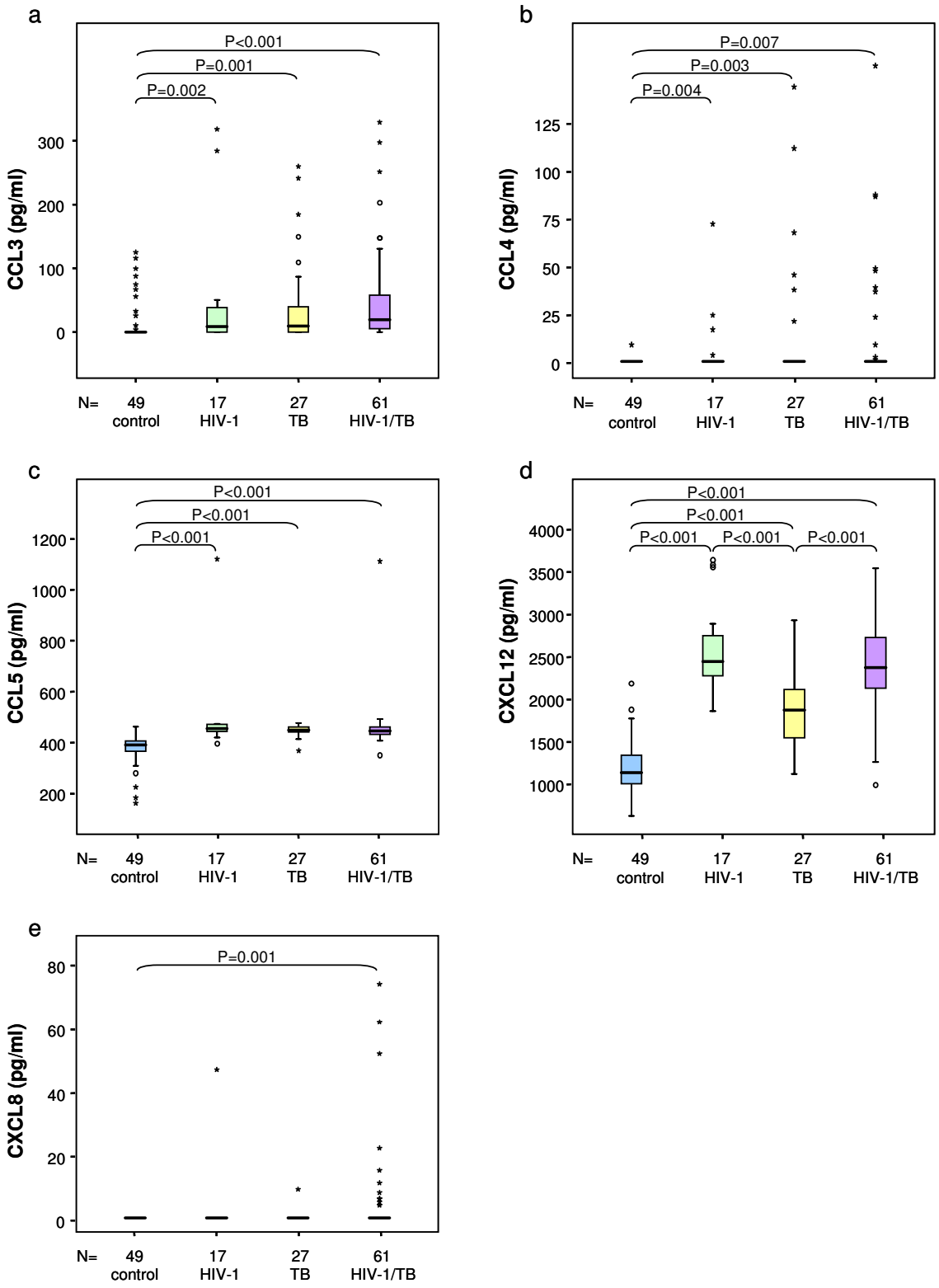


Figure 17
CCL3 (a), CCL4 (b), CCL5 (c), CXCL12 (d) and CXCL8 (e) levels in plasma samples from Black uninfected control, HIV-1, TB and HIV-1/TB groups. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars), outliers (o) and extremes (*).

3.2.2 Chemokine levels in PBMC culture supernatants

Chemokine levels in PBMC culture supernatants from patients in HIV-TB coh2 (Chapter 2.1.1) infected with HIV-1, TB, or HIV-1 and TB (HIV-1/TB) were compared with uninfected controls. Unstimulated cell culture supernatants were tested, together with PHA-stimulated cells to determine chemokine production capacity. Note that 16 individuals from each group were tested; where the numbers within each group drop below 16 (sample numbers as shown in the Figures), this indicates that there was insufficient sample for testing.

In unstimulated PBMCs, levels of CCL3 were marginally but significantly increased by HIV-1 infection only (P=0.021). In PHA-stimulated PBMCs, *Mtb* infection significantly reduced the capacity of cells to produce CCL3, with (P=0.002) or without (P=0.035) coinfection with HIV-1. HIV-1 infection alone reduced PHA-induced production of CCL3, although statistical significance was not reached (P=0.086) (Figure 18).

CCL4 levels in unstimulated PBMC cultures were unchanged by infection in any of the groups. PHA-induced CCL4 production was unaffected by HIV-1 infection, but was significantly reduced from controls in HIV-1/TB coinfected individuals (P=0.007). There was a trend to reduced CCL4 production in the presence of TB alone (P=0.067) (Figure 19).

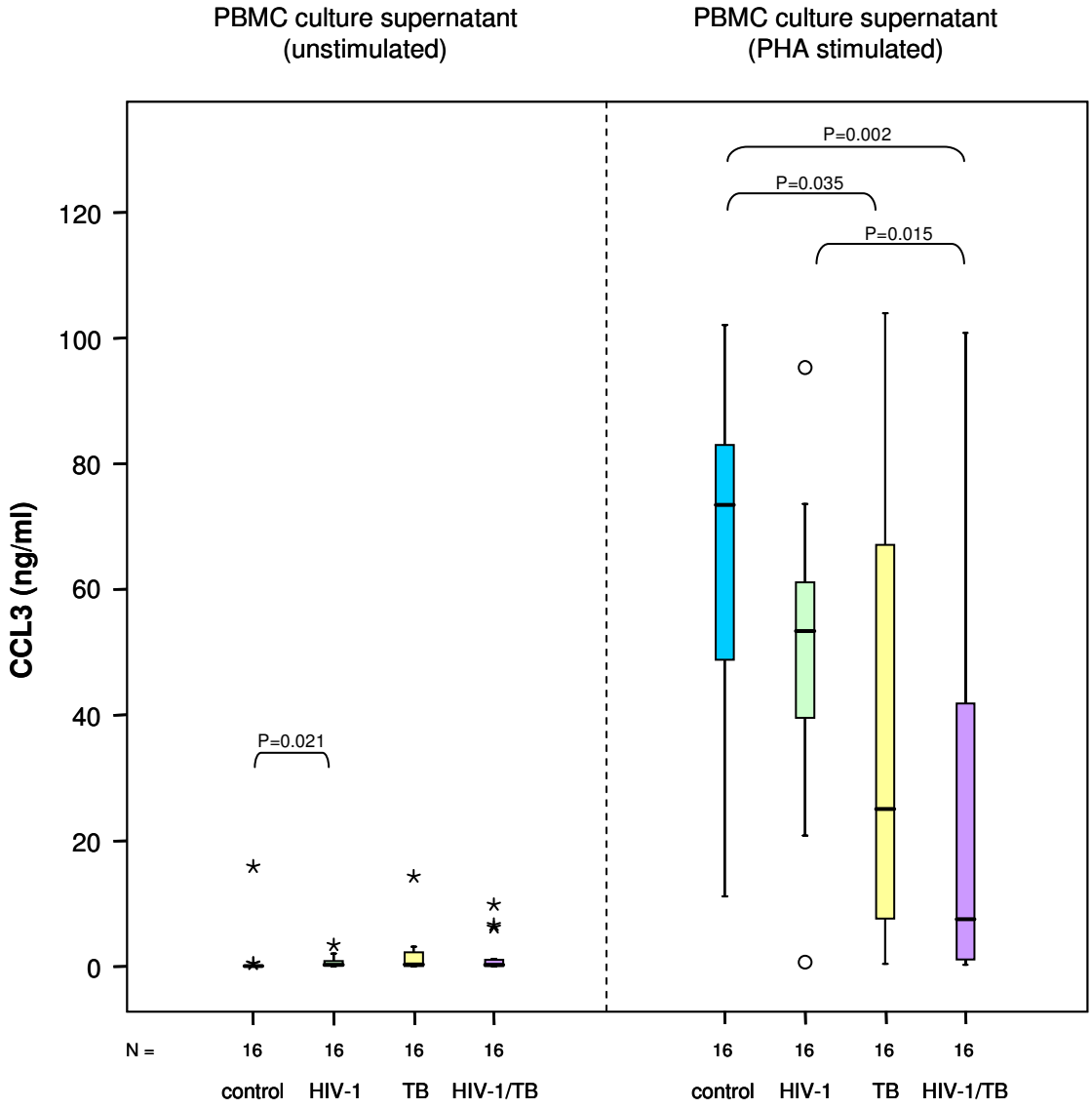


Figure 18
CCL3 levels in PBMC culture supernatants (unstimulated and PHA-stimulated) from uninfected control, HIV-1, TB and HIV-1/TB groups.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars), outliers (o) and extremes (*).

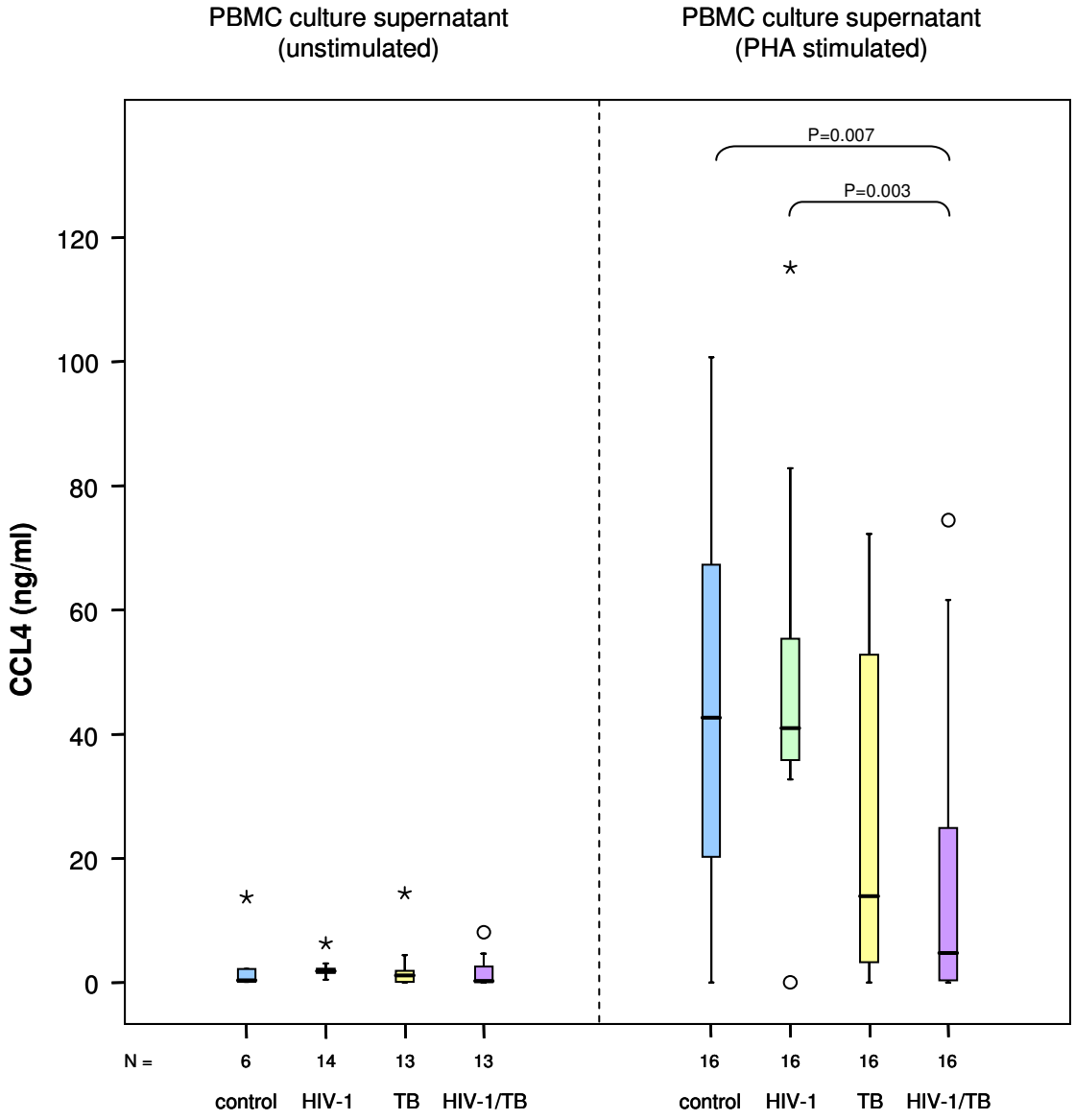


Figure 19
CCL4 levels in PBMC culture supernatants (unstimulated and PHA-stimulated) from uninfected control, HIV-1, TB and HIV-1/TB groups.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars), outliers (o) and extremes (*).

In unstimulated PBMC culture, levels of CCL5 were reduced in response to TB infection regardless of HIV-1 status; significant differences were only seen when comparing the HIV-1 group with the HIV-1/TB coinfected group (P=0.031). PHA-induced production of CCL5 was significantly increased by HIV-1 infection (P=0.026), but significantly reduced in HIV-1/TB coinfection (P=0.014), while showing a trend towards reduction in TB alone (P=0.733) (Figure 20).

Unstimulated CXCL8 production was not significantly affected by infection in any of the groups. There was a trend towards reduced PHA-stimulated CXCL8 production in the TB group (P=0.086), with significant reductions in HIV-1 infection (P=0.012). The most significant reduction in CXCL8 production was seen in dual HIV-1/TB infection (P=0.001) (Figure 21).

There were no detectable levels of CXCL12 in any of the PBMC culture supernatant samples, whether unstimulated or PHA-stimulated (data not shown).

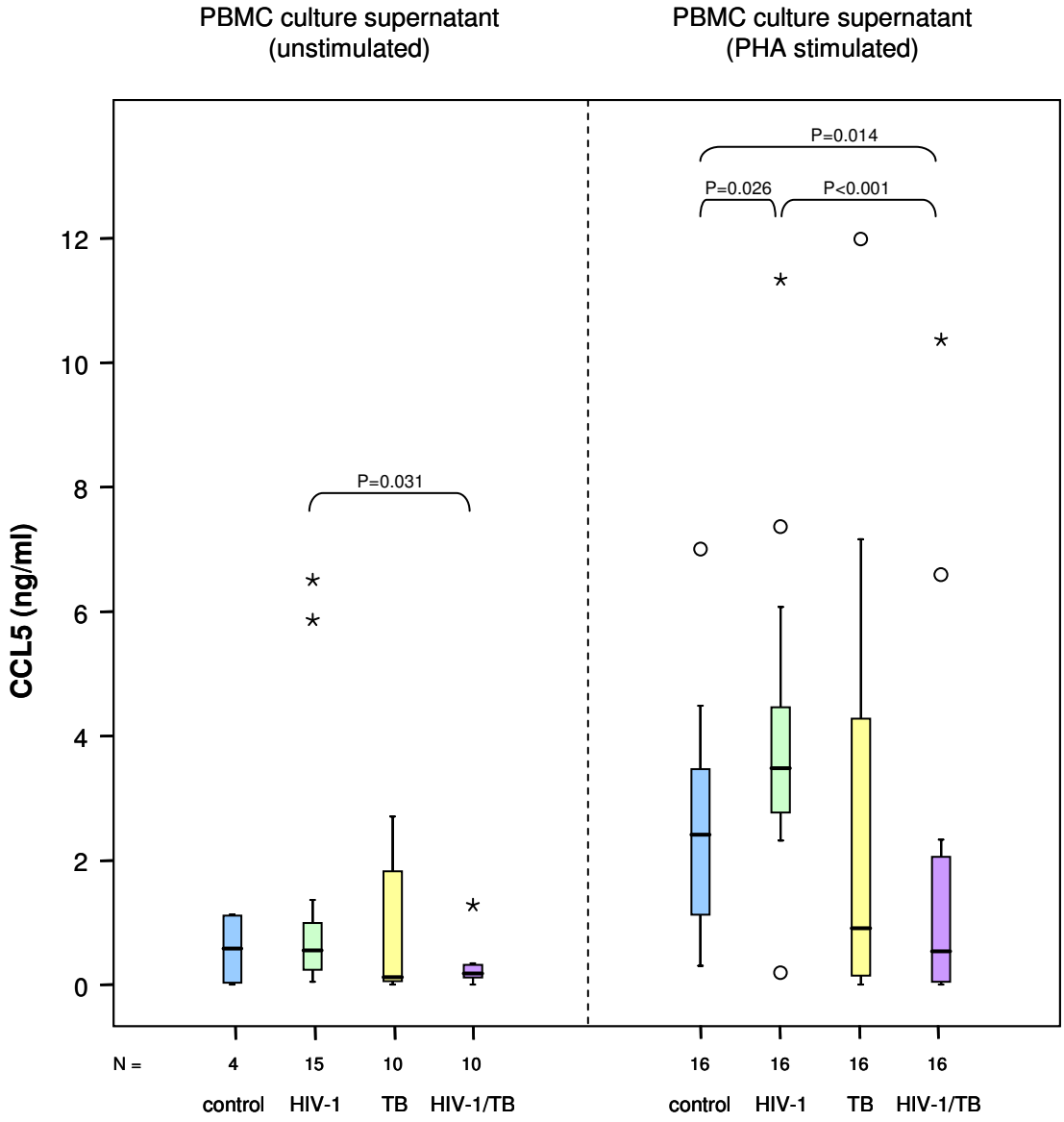


Figure 20
CCL5 levels in PBMC culture supernatants (unstimulated and PHA-stimulated) from uninfected control, HIV-1, TB and HIV-1/TB groups.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars), outliers (o) and extremes (*).

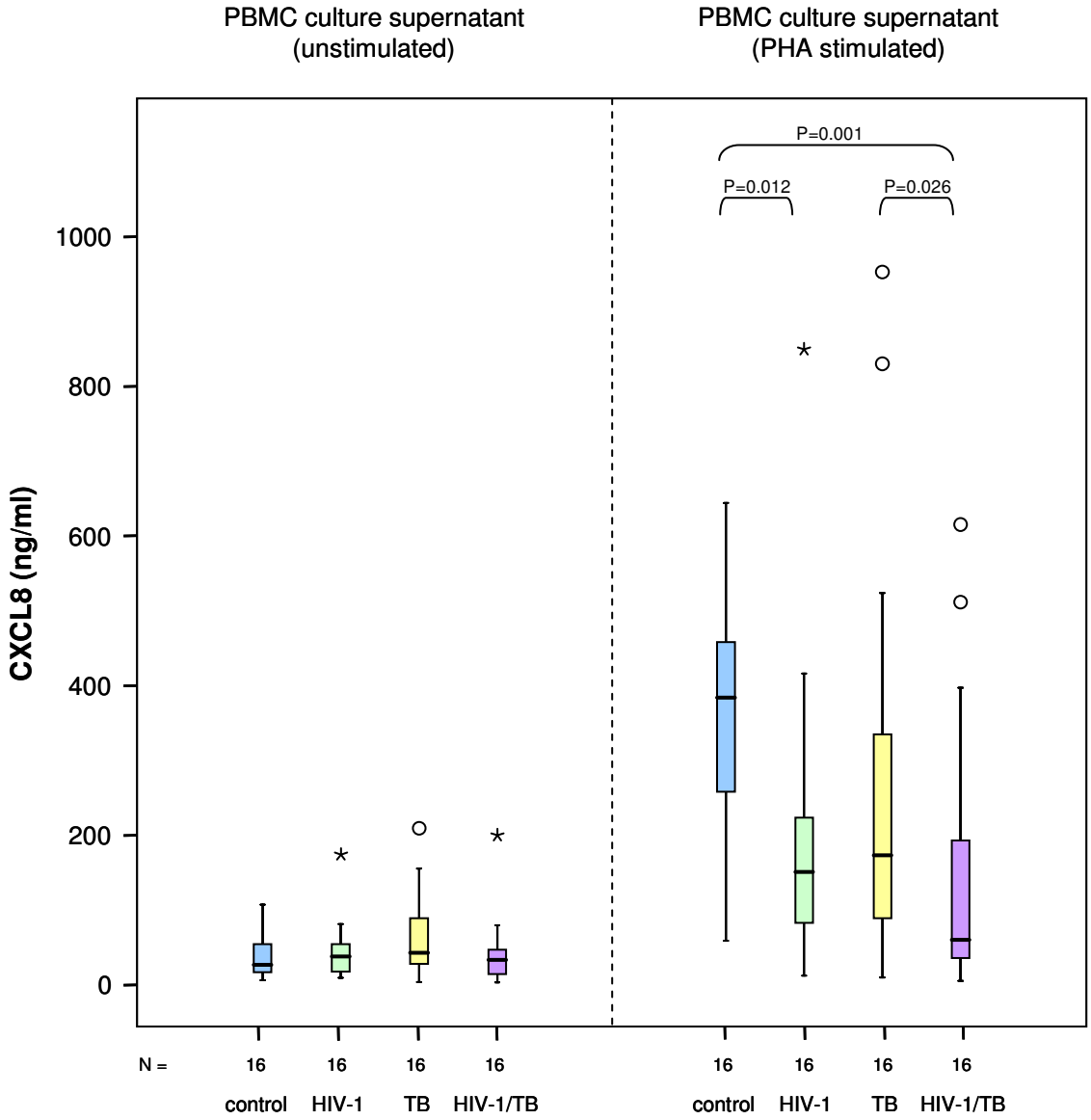


Figure 21
CXCL8 levels in PBMC culture supernatants (unstimulated and PHA-stimulated) from uninfected control, HIV-1, TB and HIV-1/TB groups.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars), outliers (o) and extremes (*).

3.2.3 Relationships between chemokines

3.2.3.1 Chemokine levels in the periphery

Plasma samples tested included all Black, ART-naïve individuals from HIV-TB coh1 (Chapter 2.1.1, Table 3). Relationships between each of the chemokines tested were examined (Table 7); CCL4 was excluded from comparisons as only a small proportion of the total number of samples (n=22/154; 14%) had detectable plasma levels of CCL4. CXCL8 was similarly excluded, as even fewer samples (n=15/154; 10%) had detectable plasma levels of CXCL8.

The strongest correlation noted was a positive correlation between the levels of CCL5 and CXCL12 ($P<0.001$). There were weaker correlations between CCL3 and CCL5 ($P=0.004$), and CCL3 and CXCL12 ($P<0.001$), only when all samples were included. No correlations were observed when any of the groups were analysed separately, indicating that no group in particular was responsible for the relationships detected, but that increased sample number gave rise to the relationships noted when the groups were combined.

Table 7
Correlations between levels of plasma chemokines in Black, ART-negative individuals from HIV-TB coh1.

Comparison	All samples	Controls	HIV-1	TB	HIV-1/TB
CCL3 vs. CCL5	n=153 R=0.233** P=0.004	n=49 R=-0.036 P>0.05	n=17 R=0.085 P>0.05	n=27 R=-0.081 P>0.05	n=60 R=-0.019 P>0.05
CCL3 vs. CXCL12	n=154 R=0.399** P<0.001	n=49 R=-0.207 P>0.05	n=17 R=0.253 P>0.05	n=27 R=0.173 P>0.05	n=61 R=0.227 P>0.05
CCL5 vs. CXCL12	n=153 R=0.497** P<0.001	n=49 R=0.012 P>0.05	n=17 R=0.176 P>0.05	n=27 R=0.117 P>0.05	n=60 R=-0.089 P>0.05

** Correlation is significant at the 0.01 level (2-tailed).

3.2.3.2 *Ex vivo* cellular chemokine production

PBMC culture supernatants (unstimulated and PHA-stimulated) from all groups within HIV-TB coh2 (Chapter 2.1.1) were tested for relationships between the levels of each of the chemokines. CXCL12 was excluded from these comparisons, as no detectable levels of CXCL12 were produced by unstimulated or PHA-stimulated PBMCs.

In unstimulated PBMCs (Figure 22), there was a significant positive correlation between the levels of CCL3 and CCL4 in all groups combined ($P<0.001$). The cohort was not sub-divided into the separate groups (control, HIV-1, TB and HIV-1/TB) for all correlations due to the small sample numbers in each individual group. However, despite the smaller numbers in each group, the positive relationships noted between these chemokines remained, although statistical significance was lost (data not shown). No relationship between CCL3 and CCL5 was seen, contrasting with the weak correlation seen for these chemokines in plasma samples, while CCL5 and CXCL8 showed an inverse relationship ($P=0.018$). There were weak but significant correlations between CCL4 and CCL5 ($P=0.010$), and CCL3 and CXCL8 ($P=0.020$).

In PHA-stimulated PBMCs from all groups, strong positive correlations between production capacities of CCL3, CCL4 and CCL5 were observed ($P<0.001$ in each case) (Figure 23a-c). In addition, positive, but weaker, correlations were noted for CCL3-CXCL8 ($P<0.001$) and CCL4-CXCL8 ($P=0.001$) (Figure 23d,e).

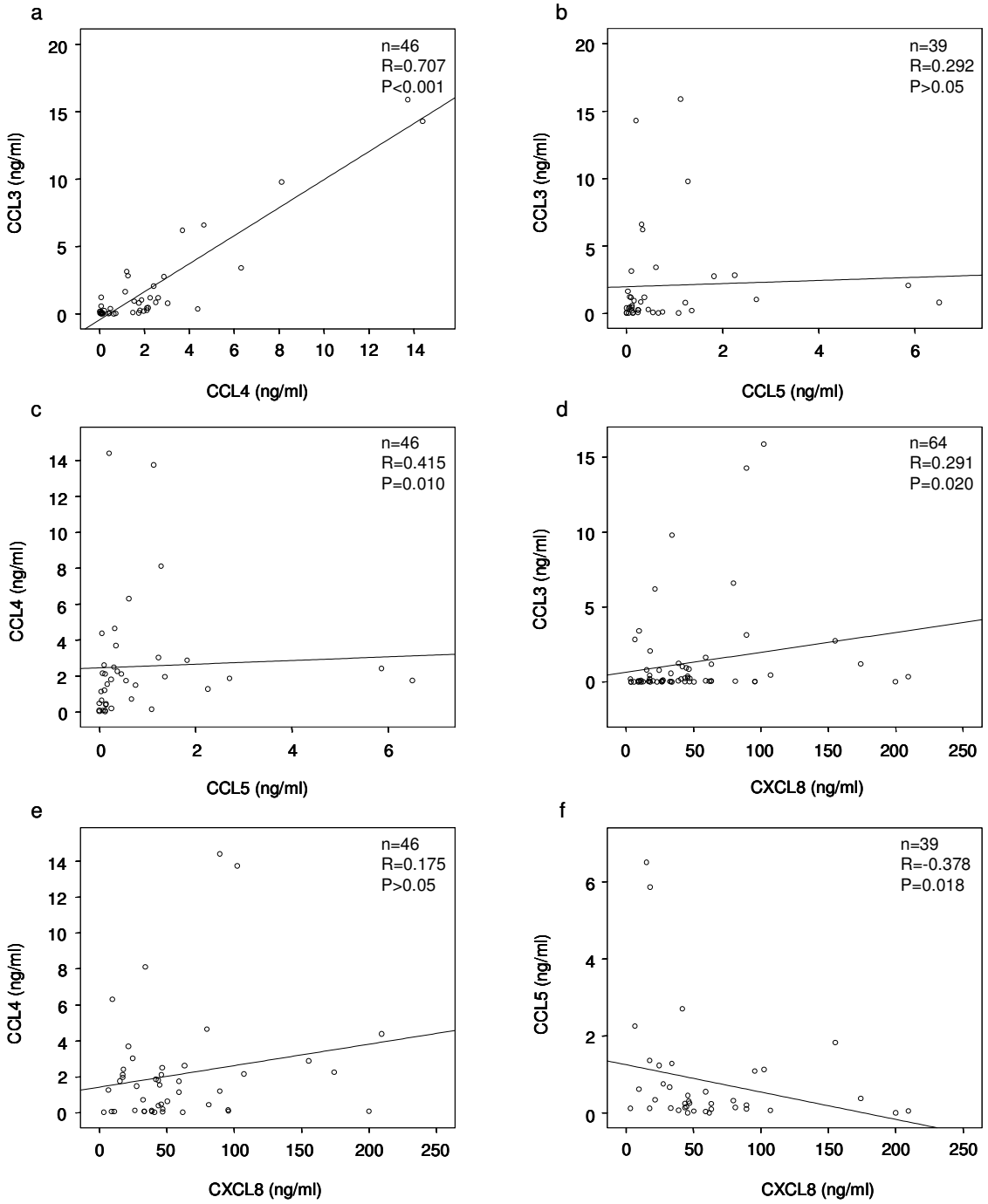


Figure 22
Correlations between levels of chemokines in unstimulated PBMC supernatant samples from all groups.
CCL3 vs. CCL4 (a), CCL3 vs. CCL5 (b), CCL4 vs. CCL5 (c), CCL3 vs. CXCL8 (d), CCL4 vs. CXCL8 (e) and CCL5 vs. CXCL8 (f).

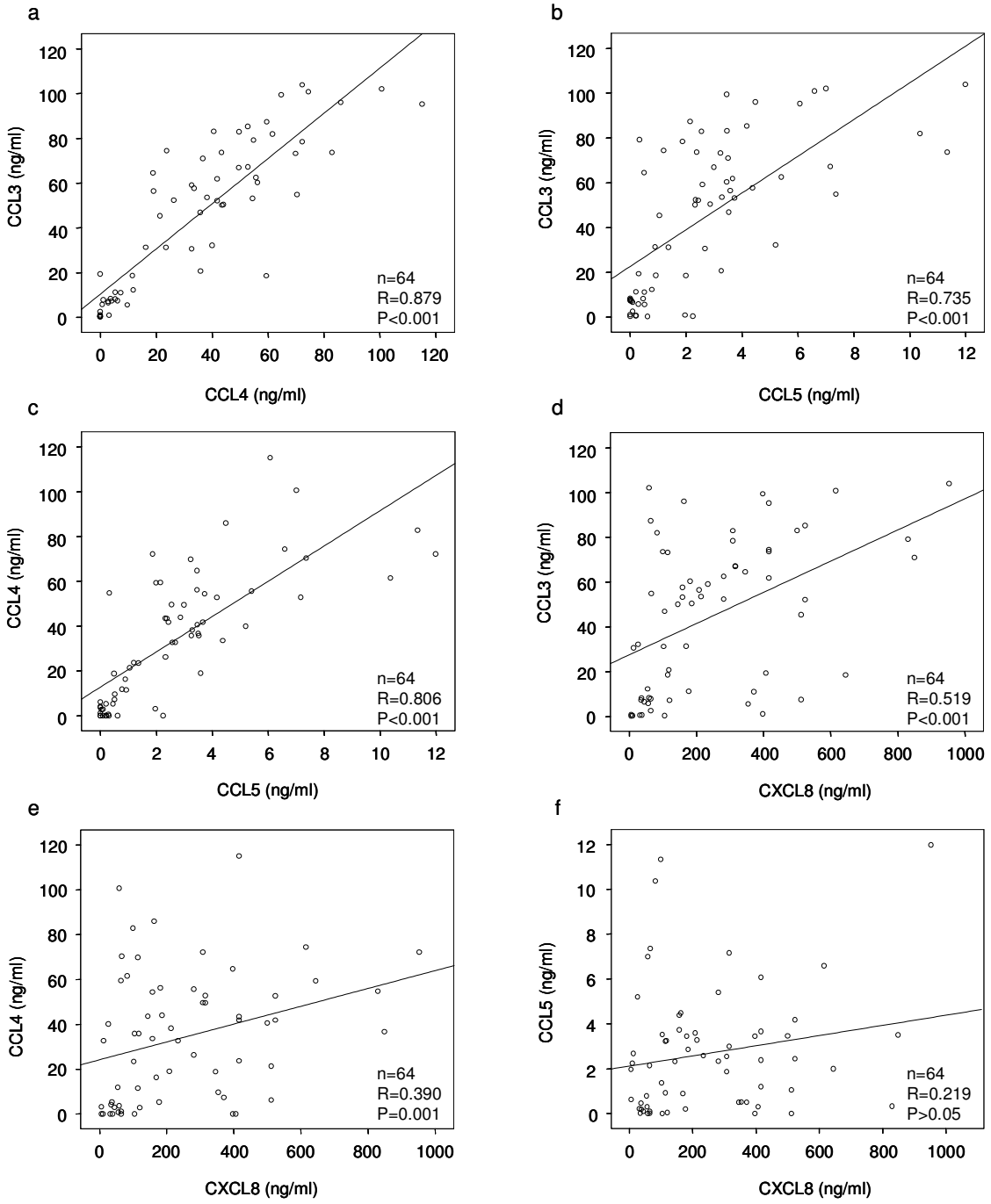


Figure 23
Correlations between chemokine production in PHA-stimulated PBMC supernatant samples from all groups.
CCL3 vs. CCL4 (a), CCL3 vs. CCL5 (b), CCL4 vs. CCL5 (c), CCL3 vs. CXCL8 (d), CCL4 vs. CXCL8 (e) and CCL5 vs. CXCL8 (f).

When PHA-stimulated production of chemokines was examined in each of the groups within HIV-TB coh2, it became clear which *in vivo* stimuli (infecting organism/s) were predominantly contributing to the relationships noted (Table 8). Comparisons between CCL3-CCL4, CCL4-CCL5 and CCL3-CCL5 showed contributions by all groups, although the presence of TB influenced CCL4 comparisons to the greatest extent. CCL3-CXCL8 comparisons showed greatest contributions from HIV-1 and TB groups, while CCL4-CXCL8 and CCL5-CXCL8 relationships were driven primarily by TB.

Table 8
Correlations between levels of PHA-stimulated chemokines in HIV-TB coh2.

Comparison	All samples	Controls	HIV-1	TB	HIV-1/TB
CCL3 vs. CCL4	n=64 R=0.879** P<0.001	n=16 R=0.682** P=0.004	n=16 R=0.636** P=0.008	n=16 R=0.929** P<0.001	n=16 R=0.964** P<0.001
CCL3 vs. CCL5	n=64 R=0.735** P<0.001	n=16 R=0.688** P=0.003	n=16 R=0.556* P=0.025	n=16 R=0.650** P=0.006	n=16 R=0.658** P=0.006
CCL4 vs. CCL5	n=64 R=0.806** P<0.001	n=16 R=0.668** P=0.005	n=16 R=0.693** P=0.003	n=16 R=0.739** P=0.001	n=16 R=0.744** P=0.001
CCL3 vs. CXCL8	n=64 R=0.519** P<0.001	n=16 R=-0.468 P>0.05	n=16 R=0.671** P=0.004	n=16 R=0.776** P<0.001	n=16 R=0.497 P>0.05
CCL4 vs. CXCL8	n=64 R=0.390** P=0.001	n=16 R=-0.402 P>0.05	n=16 R=0.194 P>0.05	n=16 R=0.834** P<0.001	n=16 R=0.419 P>0.05
CCL5 vs. CXCL8	n=64 R=0.219 P>0.05	n=16 R=-0.446 P>0.05	n=16 R=0.018 P>0.05	n=16 R=0.547* P=0.028	n=16 R=0.018 P>0.05

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

3.3 Discussion

Establishing the levels of chemokines is of great importance in the study of HIV-1 and TB, due to their effect on immunity (both innate and acquired) and hence on susceptibility to and progression of disease. Our study focussed on the relationships between 5 chemokines which are of particular relevance to HIV-1 and TB, namely the CCR5 ligands CCL3, CCL4 and CCL5, CXCR4 ligand CXCL12, and CXCL8. We examined these chemokines in plasma in order to establish their *in vivo* levels (which reflect immune events that have already occurred), in unstimulated PBMCs (a measure of spontaneous *ex vivo* production, which is likely to be influenced by prior *in vivo* immune events), and in PHA-stimulated PBMCs (a measure of *ex vivo* induced cellular production).

The determination of chemokine levels in plasma samples showed firstly that both race group and ART had an influence (on CCL3 and CXCL12 levels, respectively), which led us to restrict the cohort used to Black ART-naïve individuals. The influence of antimycobacterial drugs could not be determined in TB and HIV-1/TB patients due to the fact that most of the individuals with TB were on antimycobacterial therapy.

In general, plasma levels of all the chemokines tested were raised in response to infection with HIV-1, *Mtb* and both organisms, suggesting that increased immune activation that accompanies these infections results in higher peripheral circulating chemokine levels. CXCL8 has been shown to be increased in active TB infection (Friedland *et al.*, 1995, Sahiratmadja *et al.*,

2007), but here was only significantly increased in HIV-1/TB patients, with very few TB-positive individuals having detectable levels (possibly due to the fact that all were on anti-TB therapy - proinflammatory cytokines are known to be increased in active TB, while anti-inflammatory cytokines increase during therapy (Sahiratmadja *et al*., 2007)).

In general, chemokine production by unstimulated PBMCs was not strongly influenced by infection; however, large changes were seen in the *ex vivo* induced cellular production of chemokines when PHA was used as a mitogen. The presence of HIV-1 infection was accompanied by decreased CCL3 and CXCL8 production, did not affect CCL4 but increased CCL5 production. On the other hand, the presence of pulmonary TB decreased PHA-induced production of all the CC chemokines and CXCL8; this was further exacerbated (for most chemokines) by coinfection with HIV-1. PBMC cultures, both unstimulated and PHA-stimulated, did not produce detectable levels of CXCL12. This can be explained by the fact that CXCL12 is produced primarily by endothelial cells and is not expressed constitutively by PBMCs (Pablos *et al*., 2003). The elevated levels found in plasma were therefore most likely produced by activated endothelial cells.

There were strong positive correlations noted between levels produced of each of the CC chemokines in PHA-stimulated PBMC culture supernatants (independent of infection status), possibly because they are all are produced by similar cell types, for example $CD8^+$ T cells, and are likely to be regulated in a

coordinated fashion. It was interesting that TB influenced the CCL4 and CXCL8 comparisons to the greatest extent.

It should be noted that although the same numbers of cells were used in each culture, composition of the different cell types can be altered by infection – for example, HIV-1 infection tends to increase the numbers of $CD8^+$ T cells, and decreases numbers of $CD4^+$ T cells with disease progression. This could have an influence on the results obtained. Aside from possible alterations in cell numbers, an explanation for the reduced *ex vivo* cellular responsiveness to PHA can be provided by chronic immune activation (Hazenberg *et al*., 2003) (as reflected by increased plasma levels of chemokines in HIV-1 and TB), which causes persistent immune stimulation *in vivo* with resulting T cell anergy (Bangs *et al*., 2006, Empson *et al*., 1999).

It would be interesting in the future to identify the specific populations of cell types that are producing these particular chemokines and relating these to markers of disease progression, such as viral load and $CD4^+$ T cell count in HIV-1 infected patients.

Future work should involve larger groups of individuals, particularly in studies of plasma chemokine levels between HIV-1, TB and HIV-1/TB groups for which our numbers were low; it is becoming difficult to obtain prospective samples from TB patients who are not HIV-1 infected. In addition, chemokine levels in individuals during the acute phase of HIV-1 infection, and during reactivation of latent TB, would be of great interest. The numbers of supernatant samples

from HIV-TB coh2 were too low for many comparisons, and insufficient sample volume for some reduced numbers even further. Further studies using larger cohorts would strengthen the statistical power to draw conclusions from chemokine measurements and correlations.

The chemokines we have studied might be expected to be linked in their regulation since they form integral parts of similar immune pathways. These results have demonstrated the influence of infection, both retroviral and mycobacterial, on the regulation of chemokine production, which in turn may influence disease progression and the immune response to further opportunistic infections. What is clear from this study is that HIV-1 and TB result in dysregulated chemokine production, the consequences of which would be altered chemokine-induced functions such as chemotaxis (Matsukawa *et al.*, 2000). Altered chemokine production would be expected to result in changes in chemokine gradients that could affect both cell numbers and composition of cell types entering into an immune response to further challenge.

Studies of the regulation of chemokines in infection are of great importance to the development of effective therapies, since the design of these depends on gaining an understanding of both the correlates of protective immunity, and the dysregulation of the immune system seen in chronic HIV-1 and *Mtb* infections.

CHAPTER 4

CCL3L1 GENE COPY NUMBER IN SOUTH AFRICAN POPULATION GROUPS, AND AS A FACTOR CONTRIBUTING TO SUSCEPTIBILITY TO HIV-1

4.1 Introduction

In humans, the chemokine CCL3 is encoded by two functional genes, *CCL3* (present at 2 copies pdg) and *CCL3L1*, present at variable copy number (Irving *et al*., 1990, Nakao *et al*., 1990). In addition, there is a non-functional pseudo-gene, *LD78γ*. Despite 94% nucleotide homology between the two functional genes (Nakao *et al*., 1990), CCL3L1 is a biologically distinct protein in that, in addition to binding CCR1 and CCR5, it can bind to receptor CCR3. CCL3L1 is also a more potent agonist of CCR5 than CCL3 in that, unlike CCL3, it can be cleaved by CD26 (a membrane-associated serine protease dipeptidyl peptidase) to generate a –2 variant that has 30-fold higher affinity for CCR1 and CCR5 (Proost *et al*., 2000). This makes CCL3L1 the most potent known natural inhibitor of HIV-1 entry.

It is likely that gene duplication (estimated to have occurred 10 million years ago (Nakao *et al*., 1990)) generated the ancestral forms of the *CCL3*, *CCL3L1*, *CCL4* and *CCL4L1* genes, which were then preserved due to the evolution of distinct biological function. The variable copy number of *CCL3L1* (and *CCL4L1*)

is probably due to an association with a repeat unit which is highly susceptible to duplication.

It has been reported that median *CCL3L1* gene copy number differs in different population groups (Gonzalez *et al*., 2005), leading to the hypothesis that gene dose relative to the average in each population group influences HIV-1/AIDS susceptibility, rather than copy number *per se*. Low *CCL3L1* copy number is a major determinant of enhanced HIV-1 susceptibility in both mother-to-child and adult-to-adult infection (Gonzalez *et al*., 2005). It has been further shown that the phenotype of deficient production of CCL3 (i.e. total production of CCL3 and CCL3L1) is associated with increased risk of intrapartum maternal-infant HIV-1 transmission (Meddows-Taylor *et al*., 2006). In addition, in HIV-1 infected adults, lower *CCL3L1* gene copy numbers have been shown to be associated with an increased risk of rapid progression to AIDS or death (Gonzalez *et al*., 2005). A recently-published study has shown that variations in *CCL3L1* gene copy number, together with *CCR5* polymorphisms, influence the risk of HIV-1 transmission and disease progression independently of HIV-1 viral load (Dolan *et al*., 2007).

The first aim of this study was to determine median *CCL3L1* gene copy numbers among HIV-1 uninfected adult South Africans within various population groups, and subgroupings within these groups. Once population-specific median *CCL3L1* copy numbers had been established, *CCL3L1* copy numbers in HIV-1 infected Black South Africans were investigated.

In addition, *CCL3L1* copy number polymorphisms were examined within the context of MTCT of HIV-1, with the aim of establishing whether lower *CCL3L1* gene copy numbers could be independently associated with transmission of HIV-1.

4.2 Results

4.2.1 *CCL3L1* copy number determination

A real-time PCR assay to determine *CCL3L1* gene copy number was established and optimised, based on a previously published method (Townson *et al.*, 2002). As additional confirmation of results obtained, those samples determined to have zero or single *CCL3L1* gene copies pdg were further examined.

Samples giving a result of zero *CCL3L1* gene copies were confirmed using alternate PCR primers (A and B; Chapter 2.4.2) designed to amplify a different region of the *CCL3* and *CCL3L1* genes. These primers amplify PCR products of approximately 1240 bp for *CCL3* and 1550 bp for *CCL3L1* (due to a 312 bp Alu element deletion within the 5' region of *CCL3* compared to *CCL3L1*), allowing for their separation using agarose gel electrophoresis (Figure 24).

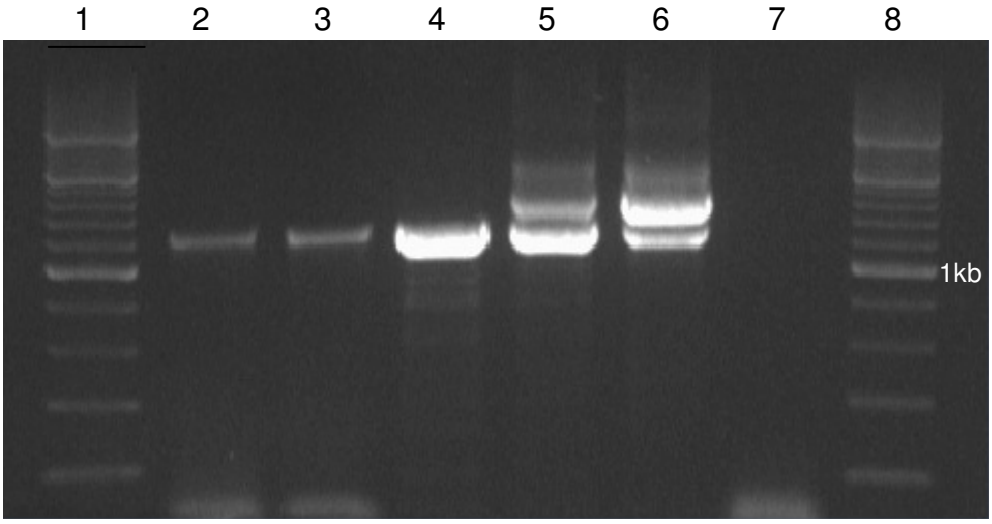


Figure 24
CCL3 (bottom band; 1240 bp) and *CCL3L1* (top band; 1550 bp) PCR products. Lanes 1 and 8: 200 bp molecular weight markers; lanes 2-4: samples with 0 copies of *CCL3L1*; lane 5: sample with 1 copy of *CCL3L1*; lane 6: sample with 8 copies of *CCL3L1*; lane 7: PCR negative control.

Samples giving a result of a single *CCL3L1* gene copy pdg were confirmed by sequencing to ensure homozygosity. Sequencing was performed using primers A and B described above, covering intron 1 (Chapter 2.4.2, Table 6), which contains SNPs known to be highly polymorphic within our study population (M. Paximadis, unpublished data). An example of one such site is shown in Figure 25; Samples 2 and 3 show a single peak at the position marked, demonstrating homozygous TT genotypes, while Sample 1 shows a double peak, demonstrating a heterozygous CT genotype.

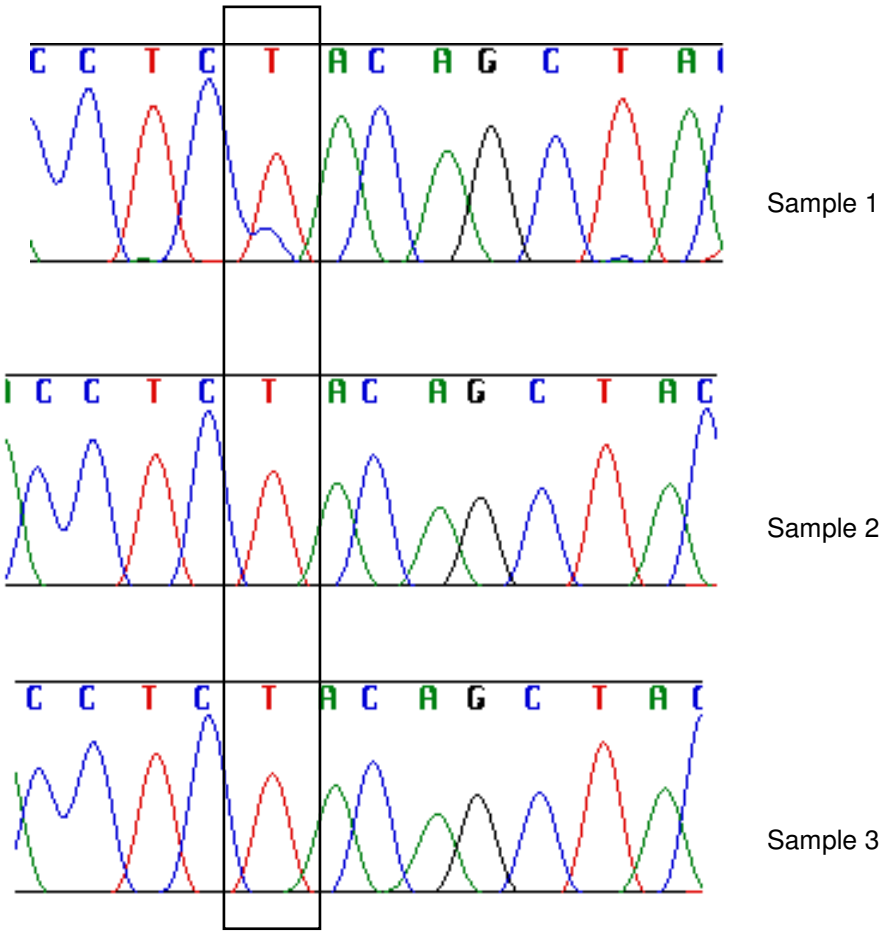
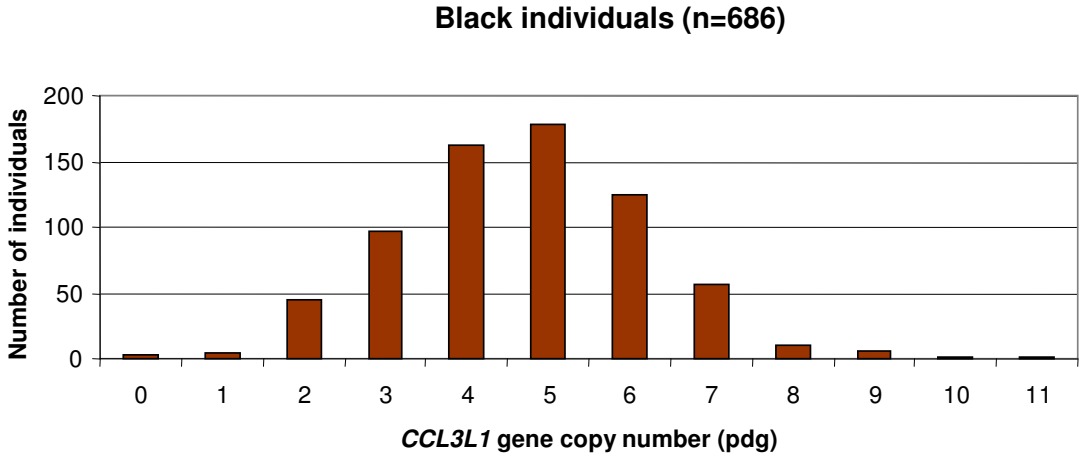


Figure 25
Sequencing chromatograms showing heterozygosity (Sample 1; multiple copies of *CCL3L1*) and homozygosity (Samples 2 and 3; single copies of *CCL3L1*) at the highlighted position in the *CCL3L1* gene.

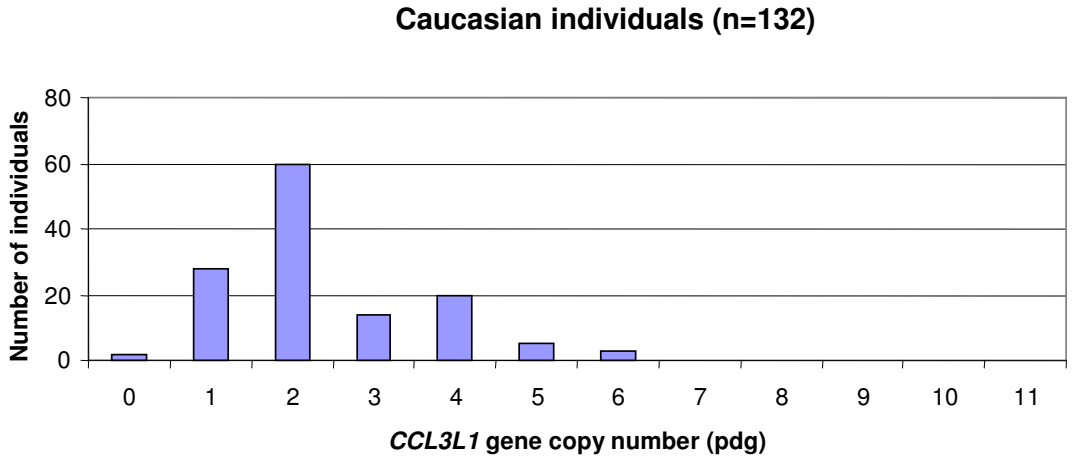
4.2.2 Median *CCL3L1* copy numbers determined in South African populations

Median *CCL3L1* gene copy numbers have been shown to vary depending on the population studied (Gonzalez *et al*., 2005). With this in mind, before any conclusions can be drawn from *CCL3L1* copy number studies within South African population groups, it was important first to establish the extent of *CCL3L1* copy number variation among different populations. Individuals studied were from all cohorts described in Chapter 2.1.1, for whom both DNA was available and race group documented (HIV-TB coh1, MTCT and Eskom). 11/829 (1.3%) of the DNA samples (10 from the MTCT cohort, 1 from the TB group of HIV-TB coh1) were inadequate for accurate copy number determination due to low DNA concentration and poor quality (Chapter 2.3.3).

In groups of all infection status, *CCL3L1* copy number pdg was significantly different between Black (median 5, range 0-11) and Caucasian (median 2, range 0-6) individuals ($P<0.001$) (Figure 26).



%	0.4	0.6	6.5	14.1	23.7	25.8	18.0	8.3	1.5	0.9	0.1	0.1



%	1.5	21.2	45.5	10.6	15.2	3.8	2.3	0	0	0	0	0

Figure 26
CCL3L1 gene copy numbers pdg amongst Black and Caucasian South African adults.

These data show that within the Black population of South Africa, significantly (P<0.001) more individuals (45.3%) have *CCL3L1* copy numbers below their population-specific median than do Caucasian South Africans (22.7%) (Figure 27).

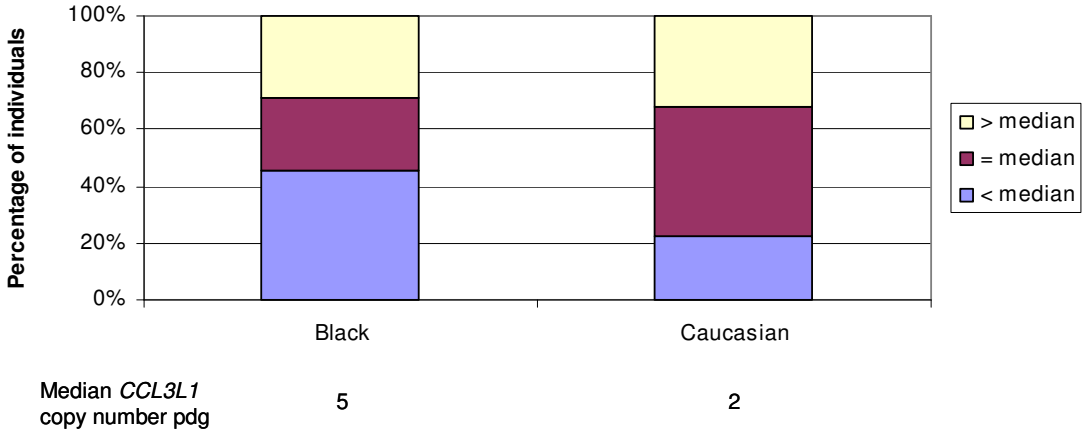


Figure 27
Black and Caucasian South African adults stratified on the basis of their *CCL3L1* gene copy numbers pdg greater than, equal to, or less than their respective population-specific medians.

Population subgrouping data was available for individuals within the Eskom cohort (Chapter 2.1.3, Table 5). Figure 28 shows these subgroups, along with groups of Caucasian and Black individuals for whom subgrouping data was unavailable (HIV-TB coh1 and MTCT), with Total Caucasian and Total Black groups. Subgroups within each of the Total Caucasian and Total Black groups did not differ significantly from each other in median *CCL3L1* copy number (P>0.05), supporting that population stratification need not be based on such subgroupings.

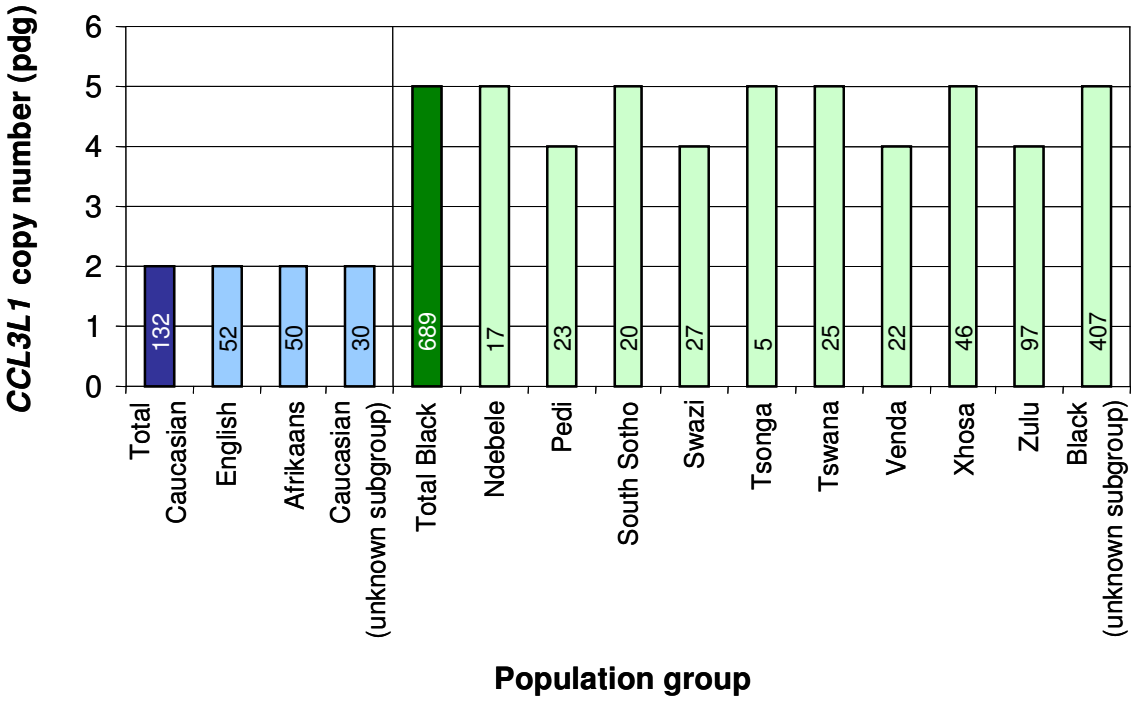


Figure 28
Median *CCL3L1* copy number pdg within South African populations and subgroups.
Numbers within bars indicate the number of individuals in each group.
Dark bars represent total groups, blue and green Caucasian and Black respectively; light bars represent subgroups, blue and green Caucasian and Black respectively.

4.2.3 *CCL3L1* copy number within HIV-1 positive and negative populations

Having established median *CCL3L1* gene copy numbers for the South African population groups comprising our study cohorts, we next questioned whether *CCL3L1* copy number plays a role in susceptibility to HIV-1 within the Black population.

HIV-1 positive and HIV-1 negative groups have similar median *CCL3L1* copy numbers (Figure 29); this was largely independent of study cohort (Figure 30).

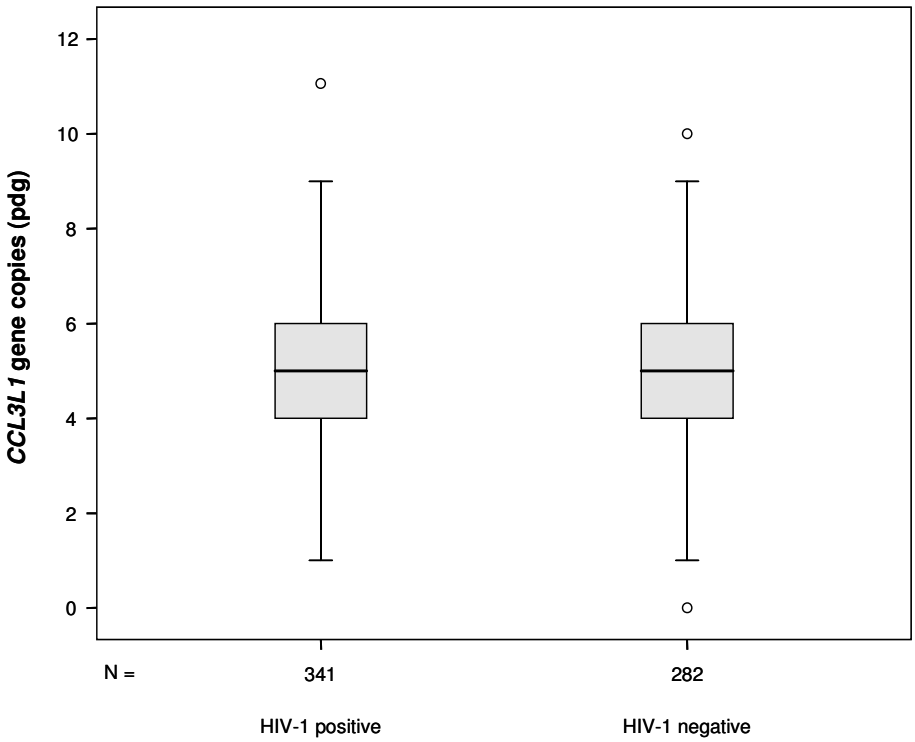


Figure 29
CCL3L1 gene copy numbers amongst HIV-1 positive and HIV-1 negative Black South African adults (from all cohorts with known HIV-1 status).
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars), and outliers (o).

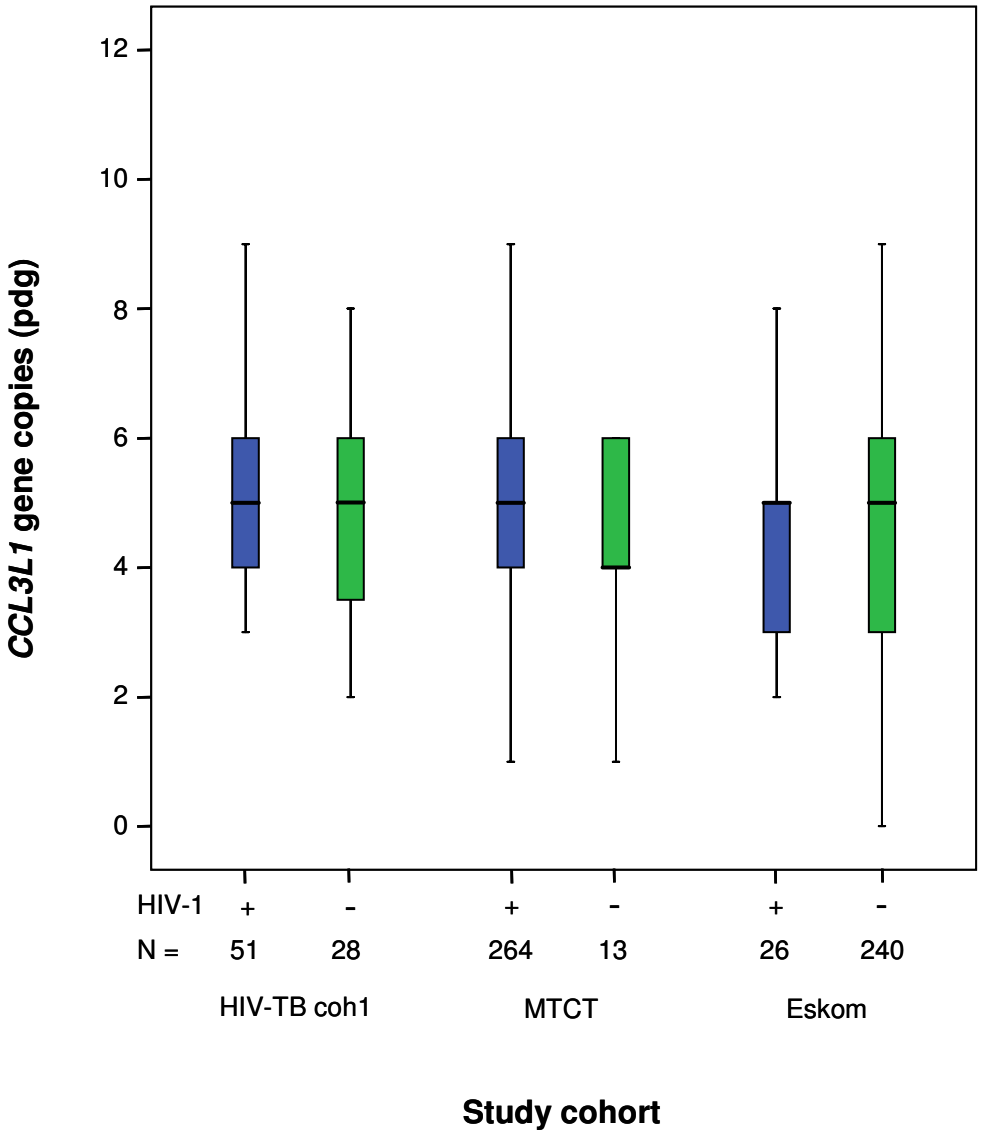
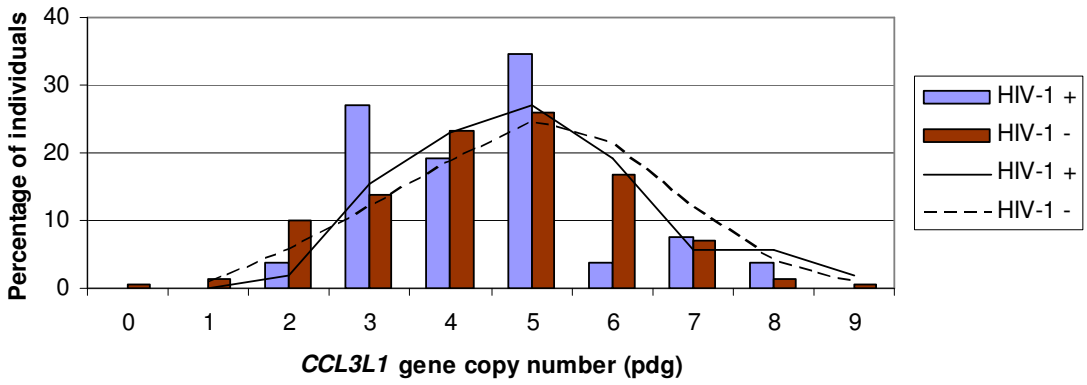


Figure 30
CCL3L1 gene copy numbers amongst HIV-1 positive and HIV-1 negative Black South African adults, divided according to study cohort.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars).

If the HIV-1 positive and negative groups of Black South Africans from the Eskom cohort were stratified according to *CCL3L1* gene copy number, a non-significant (P=0.838) trend towards lower copy number in HIV-1 positive individuals was seen, with a higher proportion of HIV-1 negative individuals (26% vs. 15% for HIV-1 positive individuals) having a *CCL3L1* gene copy number greater than the population-specific median of 5 (P=0.340) (Figure 31).

a



b

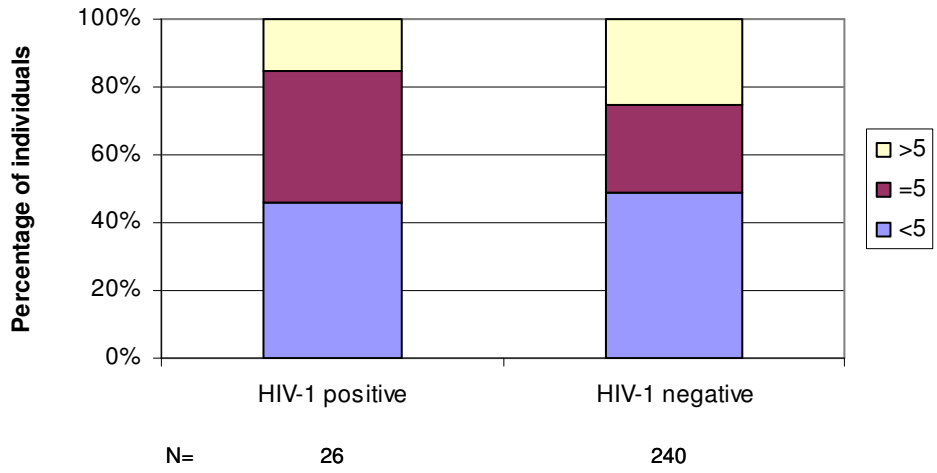


Figure 31
CCL3L1 gene copy numbers pdg amongst HIV-1 positive and HIV-1 negative Black South African adults from the Eskom cohort (a), and individuals from the same cohort grouped according to *CCL3L1* gene copy number distribution around the population-specific median of 5 (b).

4.2.4 *CCL3L1* copy number and mother-to-child transmission of HIV-1

MTCT of HIV-1 provides a good model for the study of susceptibility or resistance to HIV-1, in that all infants born to HIV-1-infected mothers are known to have been exposed to HIV-1 infection during pregnancy and birth, and the levels of exposure (as quantified by viral load) can be determined.

A deficient production of the CCL3 chemokine has been demonstrated in those infants who become infected with HIV-1 intrapartum; importantly, a similar deficiency (although not statistically significant) has been shown in their mothers (Meddows-Taylor *et al.*, 2006). This suggests that a genetic determinant may underlie this deficiency, leading us to question whether *CCL3L1* gene copy number may play a role.

Samples used in this section of the study were from the MTCT study cohort described in Chapter 2.1.2; 3 samples from the cohort of HIV-1 infected mothers and 3 from the infant cohort were insufficient for genotyping.

There was no difference in median *CCL3L1* copy number between HIV-1 infected mothers (n=264) and their uninfected counterparts (n=13), although the number of uninfected controls was too low for accurate comparison.

When mothers transmitting HIV-1 to their infants were compared with non-transmitting mothers, there was a trend towards reduced *CCL3L1* copy number; however, this was not significant (P=0.099) (Figure 32).

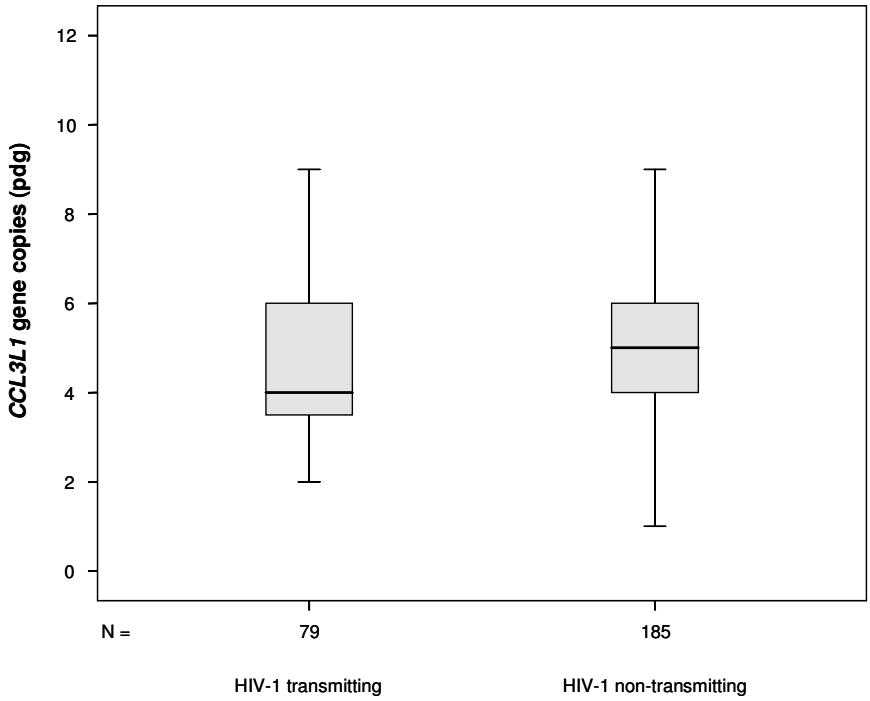


Figure 32
CCL3L1 copy numbers pdg in HIV-1 positive mothers comparing those that transmitted HIV-1 to their infants with those that did not.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars).

However, when *CCL3L1* copy numbers were examined in HIV-1-exposed infants, those who became infected with HIV-1 had significantly lower *CCL3L1* copy numbers (P=0.018) than those who remained uninfected (Figure 33). *CCL3L1* copy numbers did not differ according to the timing of infection where this was known (whether intrapartum or *in utero*).

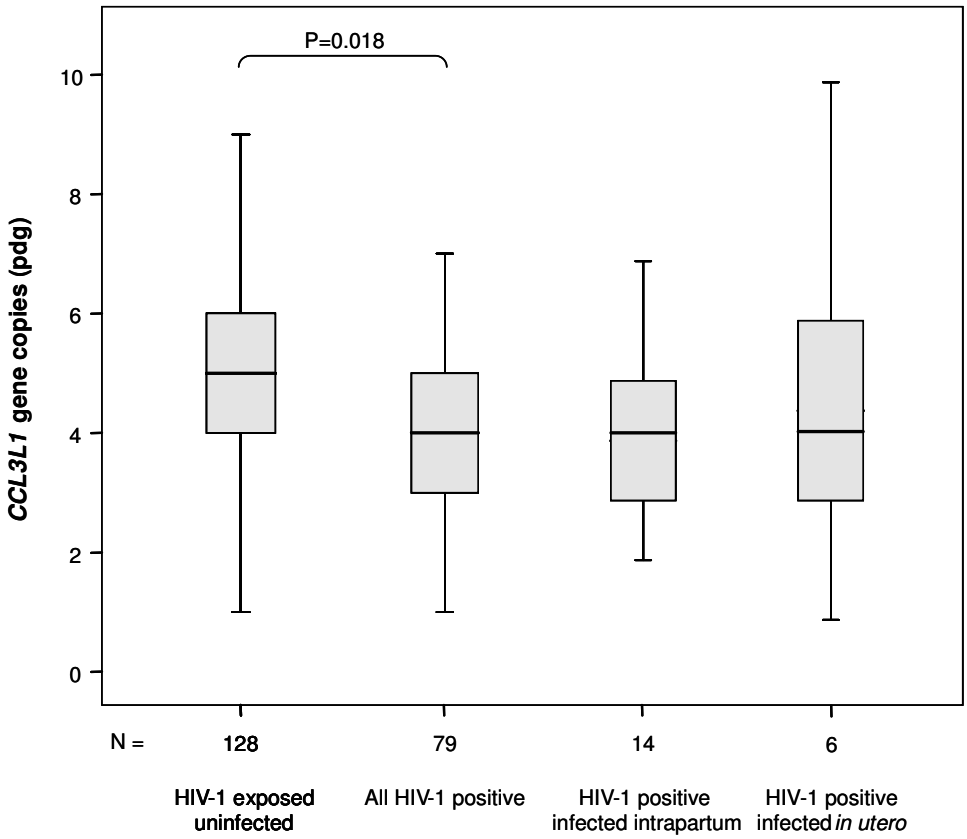


Figure 33
CCL3L1 copy numbers pdg in infants born to HIV-1 positive mothers comparing those that became infected with those that did not, and comparing those HIV-1 positive infants known to have become infected intrapartum vs. *in utero*.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars).

4.2.5 *CCL3L1* copy number and plasma levels of CCL3

CCL3L1 copy number has been related to *ex vivo* LPS-stimulated monocyte production of CCL3 (Townson *et al.*, 2002), and to PHA-stimulated CCL3 production in CBMCs of HIV-1-exposed uninfected infants and PBMCs of HIV-1 infected mothers (Meddows-Taylor *et al.*, 2006). Cells from individuals with higher *CCL3L1* gene copy numbers would therefore in general be expected to produce more CCL3 upon stimulation. However, to date there have been no reports on how *CCL3L1* gene copy number might relate to peripheral circulating levels of CCL3.

Previously tested MTCT study cohorts have demonstrated lower peripheral levels of CCL3 at comparable *CCL3L1* gene copy numbers in mothers transmitting HIV-1 to their infants intrapartum compared to those not transmitting HIV-1 (Meddows-Taylor *et al.*, 2006), mirroring results from PHA-stimulated PBMCs and deficient infant CCL3 production. As seen in Chapter 3, circulating CCL3 levels are increased in the presence of HIV-1 infection. Understanding the relationship between *CCL3L1* gene copy number and peripheral levels of CCL3 is therefore confounded by existing HIV-1 infection. For this reason, peripheral plasma CCL3 levels were examined in uninfected individuals (Caucasian and Black individuals from the control group of HIV-TB coh1), to assess the relationship between these levels and *CCL3L1* gene copy number in population groups that we have shown to have significantly different *CCL3L1* copy numbers.

We have shown (Chapter 3.3.1.1) that Black individuals within our control group tend to have lower peripheral blood levels of CCL3 than Caucasians. Earlier in this Chapter we showed that Black individuals have significantly higher *CCL3L1* copy numbers than Caucasians. Figure 34 shows the contrasting data for protein levels and *CCL3L1* gene copy number in these two population groups.

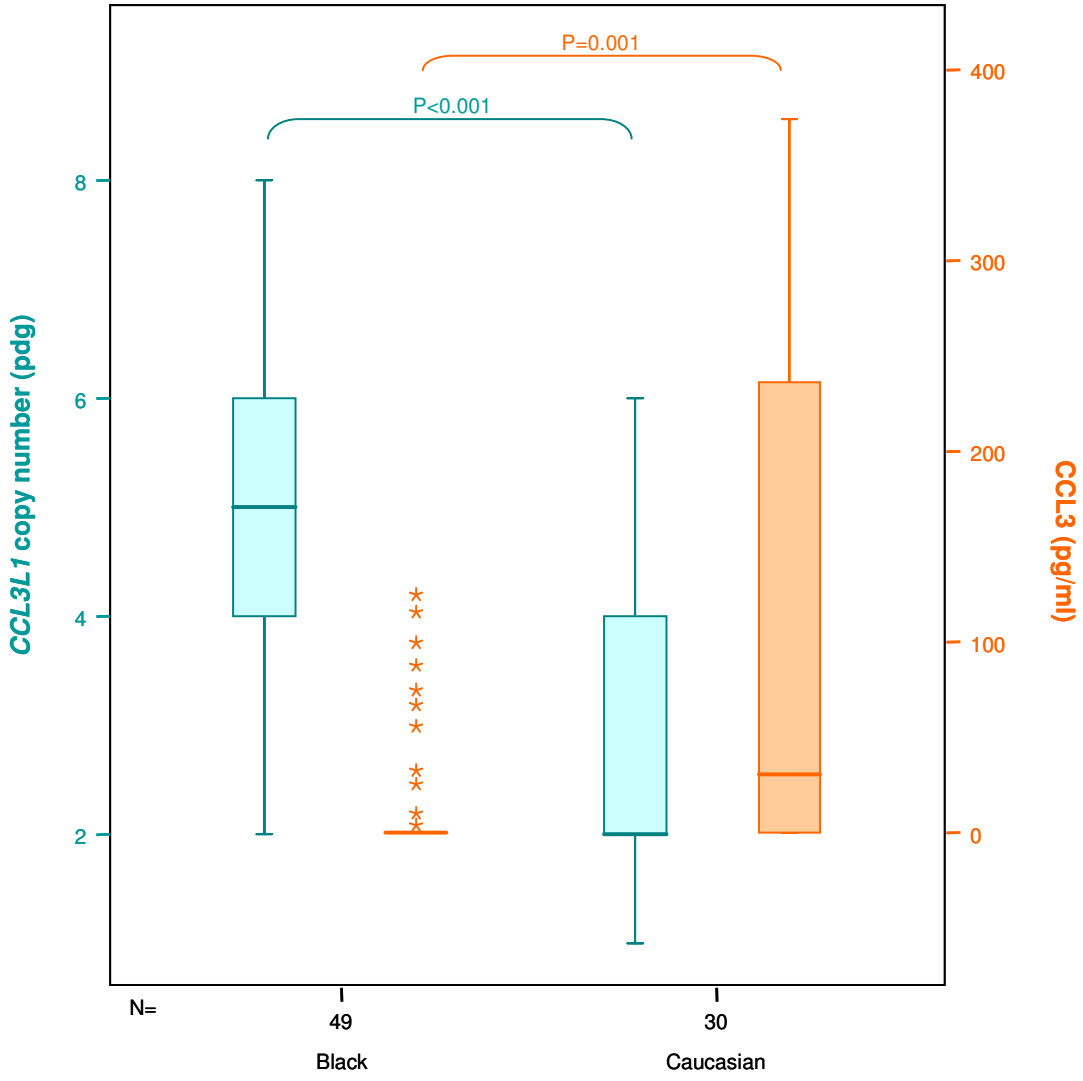


Figure 34
CCL3L1 gene copy number pdg (in blue) and plasma CCL3 levels (in orange) in uninfected Black and Caucasian individuals from the HIV-TB coh1 control group.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars) and extremes (*).

Within these population groups (in the HIV-TB coh1 control group), *CCL3L1* gene copy number was weakly correlated with plasma CCL3 levels in Caucasian individuals only (P=0.021) (Figure 35). In contrast to what might be expected, among Black individuals, those with detectable plasma levels of CCL3 (n=11) did not present with higher *CCL3L1* gene copy numbers when compared to those with undetectable CCL3 levels (n=38) (Figure 36).

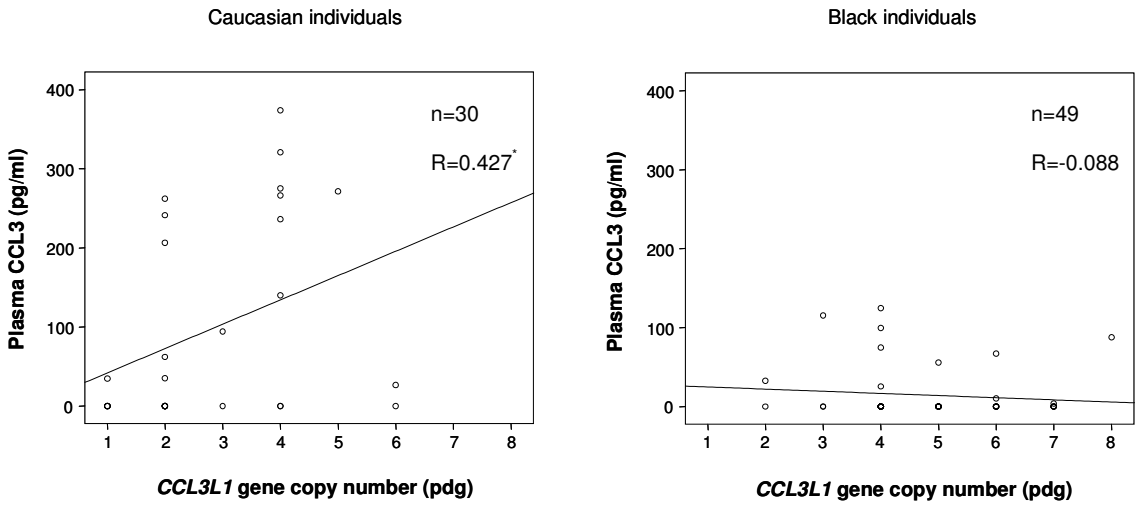


Figure 35
Scatter plot of plasma CCL3 levels against *CCL3L1* gene copy number pdg in uninfected Caucasian and Black individuals from the HIV-TB coh1 control group.
* correlation is significant at the 0.05 level (2-tailed)

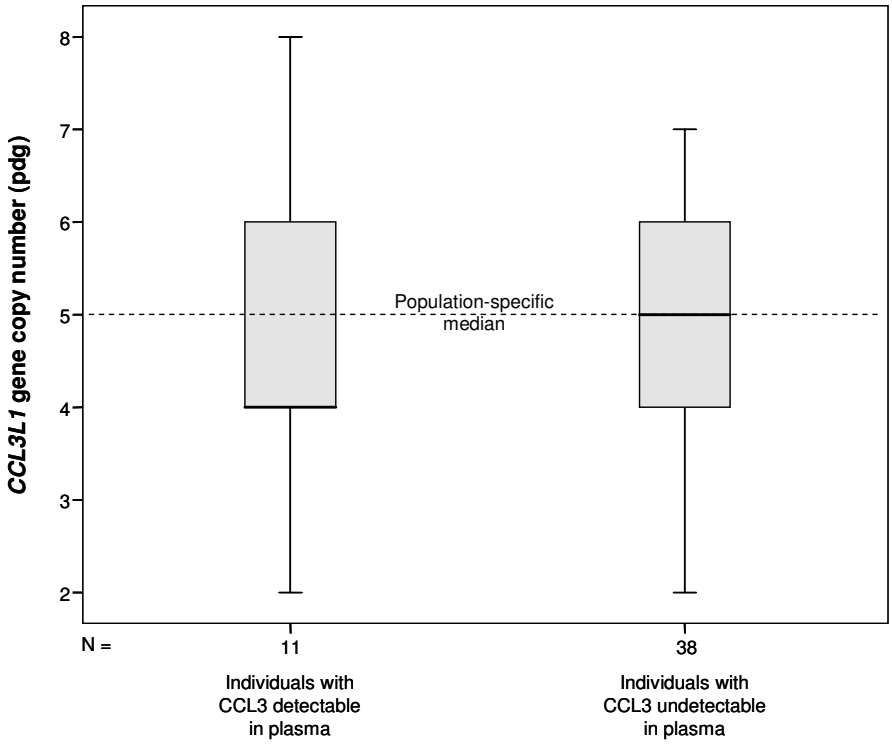


Figure 36
CCL3L1 gene copy number pdg in uninfected Black individuals from the HIV-TB coh1 control group, comparing those with detectable plasma CCL3 to those without.
Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes) and 10th and 90th percentiles (bars).

When Caucasian individuals from the control group of HIV-TB coh1 were stratified according to *CCL3L1* gene copy number less than, equal to, or greater than the population-specific mean of 2, a trend towards increasing CCL3 production with increasing *CCL3L1* gene copy number was noted (Figure 37).

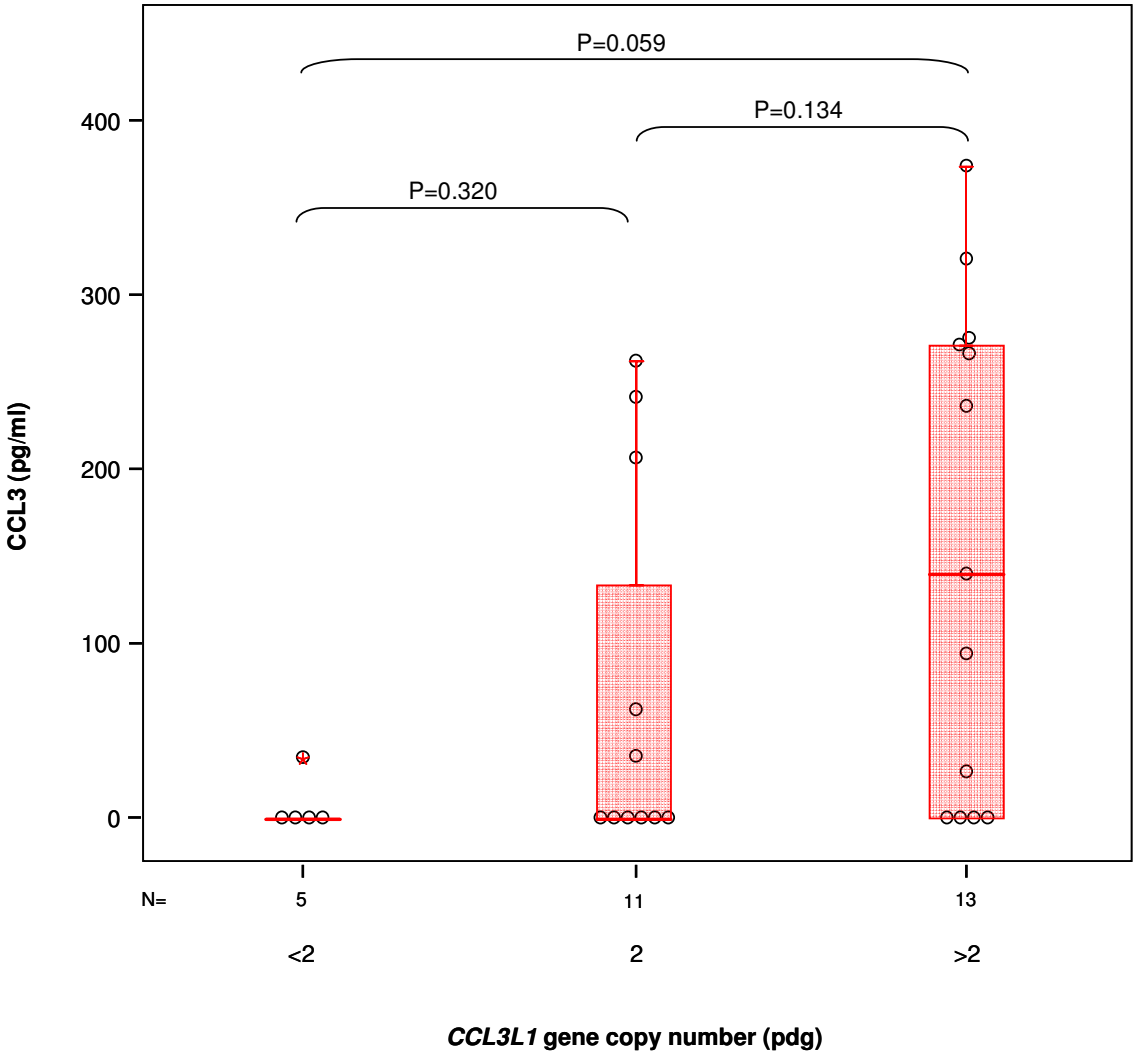


Figure 37
CCL3 levels in plasma in uninfected Caucasian individuals from HIV-TB coh1 control group grouped according to *CCL3L1* gene copy number pdg distribution around the population-specific median of 2.
Data (in red) are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars) and extremes (*). Black circles represent individual data points.

Plasma CCL3 levels in ART-naïve HIV-1 positive Black individuals from HIV-TB coh1 were examined, since HIV-1 infection provides an *in vivo* stimulus for increased chemokine production (Chapter 3.2.1.3, Figure 17). No correlation with *CCL3L1* gene copy number was evident, when either HIV-1 and HIV-1/TB groups together were studied (Figure 38), or the HIV-1 group alone was analysed (data not shown).

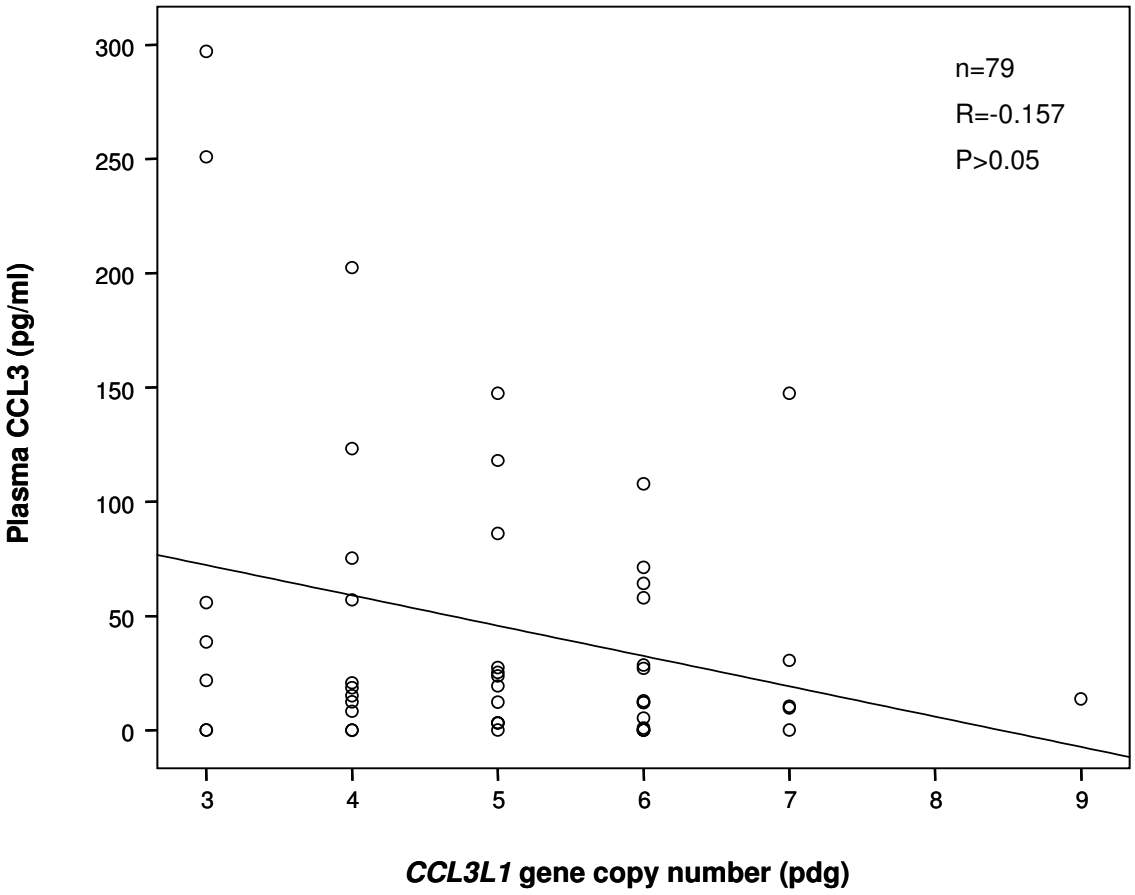


Figure 38
Scatter plot of plasma CCL3 levels against *CCL3L1* gene copy number pdg in HIV-1 infected Black individuals (ART-naïve) from the HIV-1 and HIV-1/TB groups within HIV-TB coh1.

4.3 Discussion

Previously published results have shown that median copy numbers of the *CCL3L1* gene differ depending on the population group studied (Gonzalez *et al*., 2005). Our results agree with these findings, with figures for median *CCL3L1* copy numbers in South African Caucasian and Black individuals (2 and 5, respectively) matching the published data. These results highlight the importance of controlling for population grouping when undertaking genetic association studies and when attempting to understand the relationships between specific genetic determinants and phenotypic outcomes (based on immunological, transmission and disease progression data).

Gonzalez *et al*. (Gonzalez *et al*., 2005) demonstrated a clear link between reduced *CCL3L1* copy number and susceptibility to HIV-1 within their study cohorts (adults of European American, African American and Hispanic American descent, and Argentinean mothers and children); most importantly, the effects of reduced *CCL3L1* copy number were relevant only when compared to the population-specific medians. However, another study has been unable to show any correlation between *CCL3L1* gene copy number and susceptibility to HIV-1 or progression to AIDS in a smaller cohort comprising mainly adolescent African Americans (Shao *et al*., 2007).

We have demonstrated that more Black individuals (45.3%) in South Africa have lower *CCL3L1* copy numbers than their population-specific median, compared to Caucasian individuals (22.7%), which according to the criterion

described by Gonzalez *et al.* (Gonzalez *et al*., 2005), would suggest that as a population more Black than Caucasian individuals may be at increased risk for acquiring HIV-1 infection (at least with respect to this particular factor).

In Black mothers from antenatal clinics within Gauteng, we were unable to draw conclusions about any association between *CCL3L1* gene copy numbers below median and susceptibility to HIV-1, due to the very low numbers of HIV-1 uninfected controls from the MTCT group. In Black adults from the Eskom cohort, we have shown a non-significant trend towards lower *CCL3L1* gene copy numbers in HIV-1 positive individuals, but this requires further verification. It was not the intention of this study to address susceptibility to HIV-1 in adults, but rather to obtain some insight into the distribution of *CCL3L1* gene copy numbers in our population for the design of future studies.

It may prove to be the case that since individuals with lower *CCL3L1* copy numbers seem to have a more rapid disease progression (Gonzalez *et al*., 2005), they may have died earlier during the South African HIV-1/AIDS epidemic, which has only recently begun to be addressed with the roll-out of antiretroviral therapy. It remains possible that our sample selection may show bias in that it does not comprise a random case controlled study. Ideally, a prospective study cohort could be examined in which exposure to HIV-1 infection is well-documented, such as sex workers or haemophiliacs. It should however be noted that the risk of infection with HIV-1 varies with the route of infection (Table 9), which needs to be taken into account.

Table 9
Risk of infection with HIV-1 depending on route of infection.
(from (Cohen, 2004))

Route of infection	Risk of infection
Sexual transmission	
Female-to-male	1:700 to 1:3000
Male-to-female	1:200 to 1:2000
Male-to-male	1:10 to 1:1600
Fellatio	0 to 6%
Parenteral transmission	
Transfusion of infected blood	95:100
Needle sharing	1:150
Needle stick	1:200
Needle stick / AZT PEP	1:10000
MTCT	
Without AZT treatment	1:4
With AZT treatment	< 1:10

Of the infection routes described above, we directly addressed the question of the possible association of *CCL3L1* gene copy number and maternal-infant HIV-1 transmission. HIV-1 positive mothers who transmitted HIV-1 to their infants (*in utero* or intrapartum) showed a trend towards lower *CCL3L1* copy numbers compared to mothers who did not. *CCL3L1* gene copy numbers were however significantly reduced in those infants who became infected with HIV-1 (regardless of the timing of transmission, i.e. *in utero* vs. intrapartum) compared with those infants exposed to HIV-1 who remained uninfected. This demonstrated that the genetic factor(s) underlying MTCT of HIV-1 in our cohorts was primarily due to susceptibility of the infants that become infected rather than due to a transmissibility factor in their mothers. Therefore, the trends noted amongst mothers were reflecting their genetic relatedness to their infants. These results have been published (Meddows-Taylor *et al.*, 2006). A further

extension of this current study (with larger numbers) has since demonstrated that higher infant *CCL3L1* gene copy number is strongly associated with reduced susceptibility to HIV-1 only in the absence of maternal nevirapine (this association was attenuated at low maternal viral loads) (Kuhn *et al*., 2007).

It is known that an increase in *CCL3L1* gene copy number corresponds to an increase in cellular production of CCL3 chemokine, in both LPS-activated monocytes (Townson *et al*., 2002) and in PHA-stimulated PBMCs (Meddows-Taylor *et al*., 2006); this led us to question whether a similar relationship might exist between *CCL3L1* gene copy number and peripheral CCL3 levels in uninfected individuals (since the presence of HIV-1 infection serves as a confounder given that circulating CCL3 levels are increased, and are likely to be affected by stage of disease progression).

There are clear and contrasting differences in median *CCL3L1* copy number and circulating levels of CCL3 between uninfected South Africans of Black or Caucasian descent – higher median copy number in Black groups did not correspond to higher peripheral levels of chemokine product, which suggests that the Black population may be even more at risk for HIV-1 infection than would be suggested by *CCL3L1* copy number data alone, if circulating levels of CCL3 are indeed a meaningful measure of likely susceptibility. In contrast, within the Caucasian uninfected individuals, the expected trend towards increased CCL3 production at *CCL3L1* gene copy numbers greater than the population-specific median of 2 was clearly evident. These latter findings provide evidence that increased circulating levels of CCL3 do relate to

increased *CCL3L1* copy number, but this would likely only apply to populations with largely detectable levels of this chemokine.

Genetic factors contributing to changes in CCL3 regulation, in terms of polymorphisms within chemokine genes themselves, or in other genes controlling chemokine production, remain to be elucidated. It is likely that copy number *per se* will not prove to be the only factor controlling CCL3 production and therefore susceptibility to HIV-1, but that polymorphisms within both active genes (*CCL3* and *CCL3L1*) may also play a role, along with polymorphisms in the genes of other factors that may be involved in the regulation of expression of the CCL3 chemokine. For this reason, further analysis of SNPs within the *CCL3* and *CCL3L1* genes, together with an investigation of *CCL3* and *CCL3L1* gene expression patterns within our study cohorts would be of great interest for future studies.

Factors other than those with a genetic basis that can impact on levels of CCL3 *in vivo* include (i) infectious agents, for example HTLV-2 (Pilotti *et al*., 2007) and hepatitis G (GBV-C) (Xiang *et al*., 2004), both of which increase CCL3 levels, and (ii) the maturation state of the immune system. In the latter regard, infants are known to produce higher levels of CCL3 than adults, despite no differences in *CCL3L1* gene copy number (Tiemessen & Kuhn, 2006). These points further highlight the complex nature of the regulation of chemokine production.

Findings using the maternal-infant HIV-1 transmission model (Meddows-Taylor *et al*., 2006) suggest that it is the abundance of CCL3 upon initial encounter

with HIV-1 that may be important in protection vs. susceptibility in infants, in that infants with deficient *ex vivo* cord blood cell production of CCL3 were susceptible to HIV-1 infection. The levels of CCL3 may be key to the development of an effective innate immune response (including the inflammatory process) and subsequent adaptive immune responses (Tiemessen & Kuhn, 2007), and repeated exposure to HIV-1 may provide a "booster effect" on acquired immune responses developed in response to HIV-1 encounter.

It remains to be established how circulating levels of CCL3 compare to unstimulated or PHA-stimulated production for the same individuals (for example uninfected Black and Caucasian individuals), and how these measures compare to *CCL3L1* gene copy number and mRNA expression. Understanding these relationships is central to our understanding of how this important chemokine is produced and regulated in different population groups and in different individuals within these groups.

CHAPTER 5

IL8-251T→A POLYMORPHISM AND SUSCEPTIBILITY TO TB IN SOUTH AFRICANS

5.1 Introduction

There is a common single nucleotide polymorphism (SNP), located 251 bp upstream of the *IL8* transcription start site (IL8-251T→A), which affects CXCL8 expression in response to LPS stimulation of whole blood (Hull *et al*., 2000). Hull *et al.* (Hull *et al*., 2000) demonstrated that CXCL8 production in whole blood (from healthy donors in the United Kingdom) on stimulation with LPS was greatest in AA homozygotes, then AT heterozygotes, with lowest production in TT homozygotes. This SNP may not be a functional change in itself, since it is located upstream of the promoter binding sites for transcription factors (Ma *et al*., 2003), but may lie in linkage disequilibrium with the functional variant (Hull *et al*., 2001).

It has been suggested that increased expression of CXCL8, related to the IL8-251A allele, may attract an excess of leukocytes to the site of *Mtb* infection, causing extensive tissue damage and increasing the risk of severe vs. latent TB (Ma *et al*., 2003).

Given these findings, we hypothesised that the IL8-251T→A SNP may be contributing to the TB epidemic in South Africa, so this was a preliminary study which aimed to investigate the prevalence of the IL8-251A allele within different South African population groups.

In addition, we aimed to compare the prevalence of this allele (known to be associated with differences in CXCL8 expression and susceptibility to *Mtb* infection) between groups comprising uninfected controls, individuals who are singly infected with HIV-1 or *Mtb*, and individuals coinfected with HIV-1 and *Mtb*.

With these aims in mind, the first objective was to develop a real-time PCR-based assay for analysis of the IL8-251T→A SNP.

5.2 Results

DNA samples used in this section of the study were selected from the following groups (for details of study cohorts, refer to Chapter 2.1):

(1) Caucasian adults (HIV-1 negative n=88) from the Eskom study cohort (English n=44, Afrikaans n=44).

(2) Black HIV-1 positive adults (total n=135) from the MTCT cohort (n=70), Eskom cohort (Xhosa n=4, Zulu n=12) and HIV-1/TB individuals from HIV-TB coh1 (n=49).

(3) Black HIV-1 negative adults (total n=113) from the Eskom cohort (Venda n=2, Xhosa n=42 Zulu n=43) and TB individuals from HIV-TB coh1 (n=26).

Where sample numbers are lower than the total number of samples in each cohort, this reflects the fact that this section of the project is designed as a preliminary study simply to assess the variation in the IL8-251T→A SNP within South African populations.

5.2.1 IL8-251T→A SNP real-time PCR

We first tested the published method (Hull *et al*., 2000) for determination of the IL8-251T→A polymorphism. This involved PCR amplification using 2 sets of primers designed to amplify separately the IL8-251T and IL8-251A alleles

(Chapter 2.5.1), followed by detection of the 347 bp PCR products by agarose gel electrophoresis (Figure 39). This method was then optimised for high-throughput using the Electro-Fast Stretch 108 Gel Tank System, but was still not conducive to rapid high-throughput screening (Figure 40). For this reason, a real-time PCR-based method was developed which would allow rapid and accurate SNP determination.

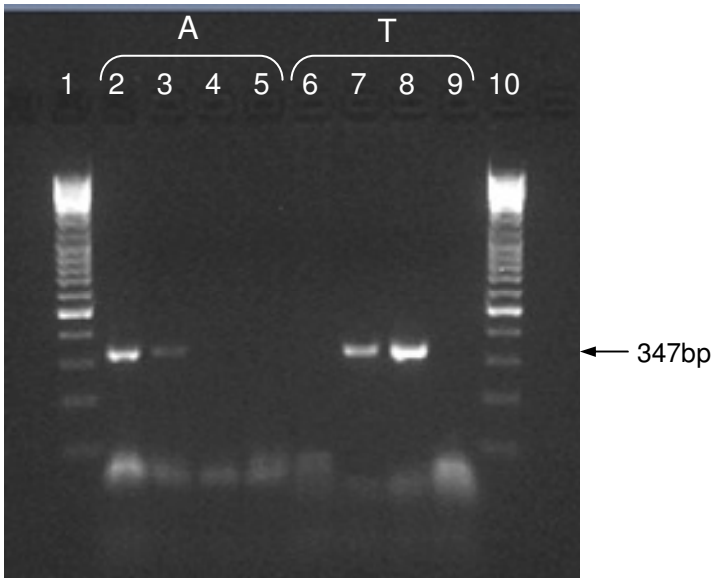


Figure 39
2% agarose gel showing IL8-251A and IL8-251T PCR products.
Lanes 1 and 10: standards (100 bp DNA ladder); lanes 2 and 6: AA homozygous; lanes 3 and 7: AT heterozygous; lanes 4 and 8: TT homozygous; lanes 5 and 9: negative controls.

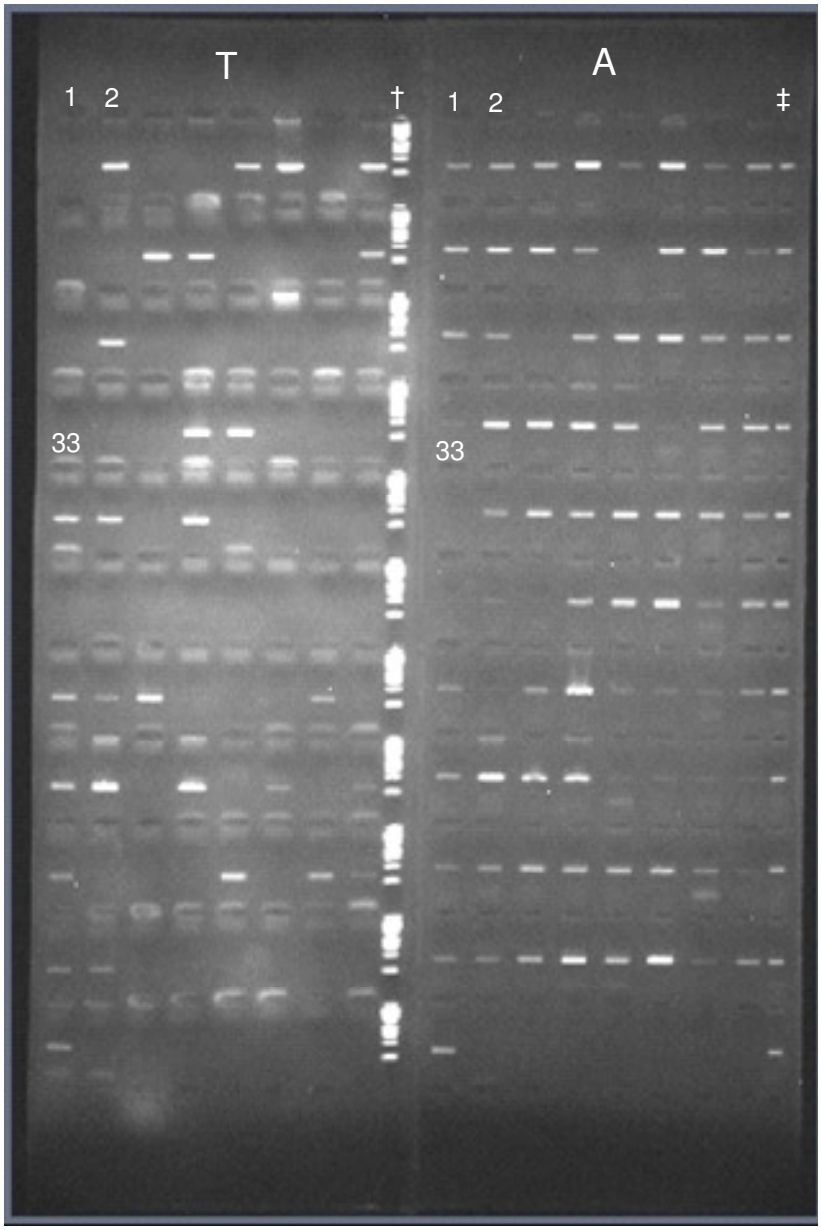


Figure 40
2% agarose gels showing IL8-251A and IL8-251T SNP PCRs run on the high-throughput Electro-Fast Stretch 108 Gel Tank System.
Samples were amplified using T-allele and A-allele specific primers and run in corresponding lanes on parallel agarose gels. E.g. lane 1=AA, lane 2=AT, lane 33=TT. Standards: 100 bp DNA ladder (†) and PCR positive control (‡).

Real-time PCR results take the form of amplification plots, which allow rapid and simple assignment of genotypes (homozygous AA or TT, or heterozygous AT) (Figure 41). Figures show the point during PCR amplification at which fluorescence (due to incorporation of SYBR Green into double-stranded PCR products) rises above background levels, indicating the presence of target sequence. The apparent presence of low concentrations of the opposite allele in homozygous individuals indicates a low degree of cross-priming which can be clearly distinguished from a true positive.

Each PCR reaction can be checked by dissociation curve analysis to ensure single specific PCR product amplification (Figure 42). Samples are heated to 95℃ to denature double-stranded DNA, then annealed slowly. Single PCR product (indicating specific PCR reactions) shows a single peak indicating the temperature at which SYBR Green (which binds only double-stranded DNA) becomes incorporated.

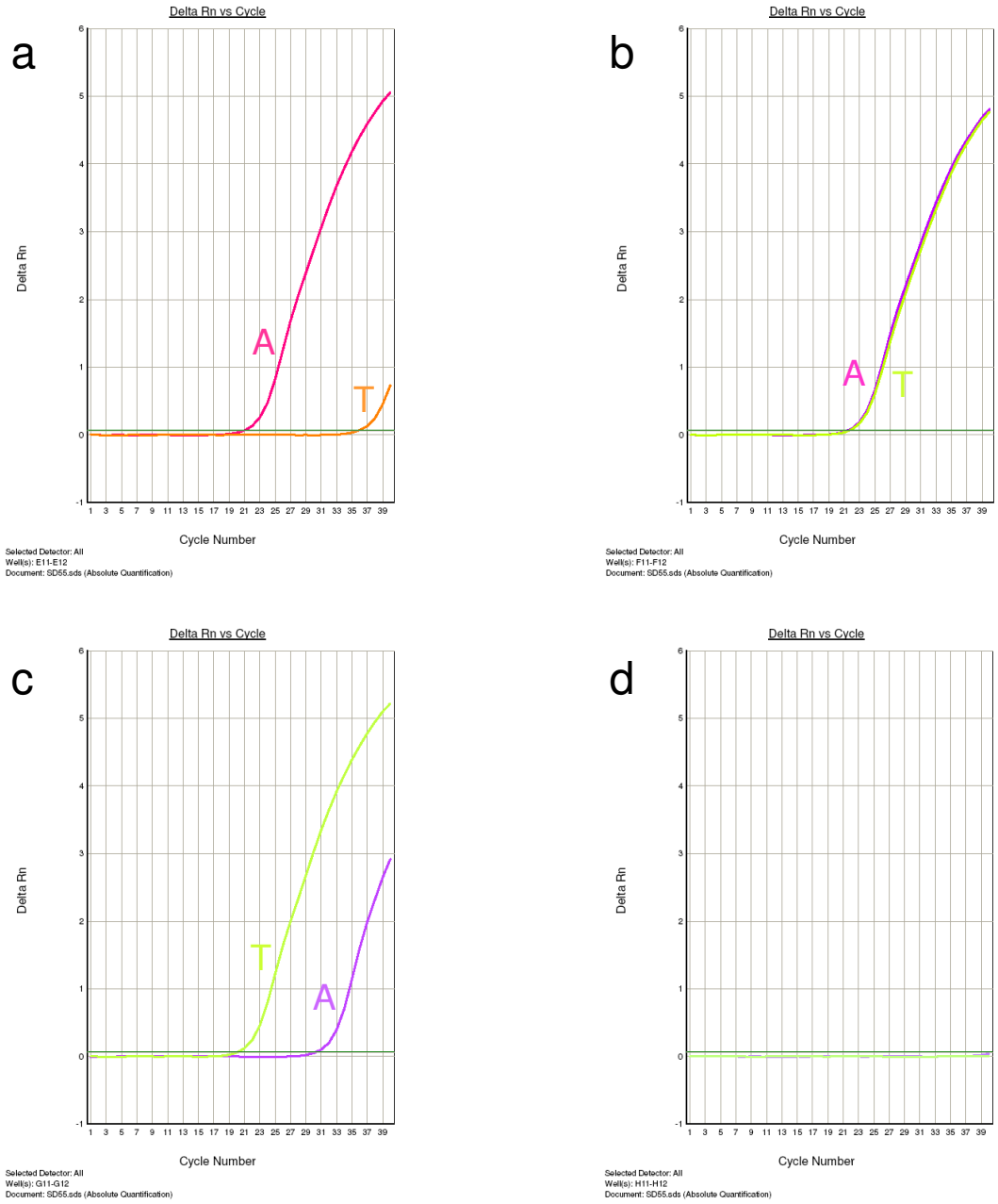


Figure 41
Amplification plots showing IL8-251T and IL8-251A real-time PCRs.
(a) AA homozygous, (b) AT heterozygous, (c) TT homozygous, (d) negative control.

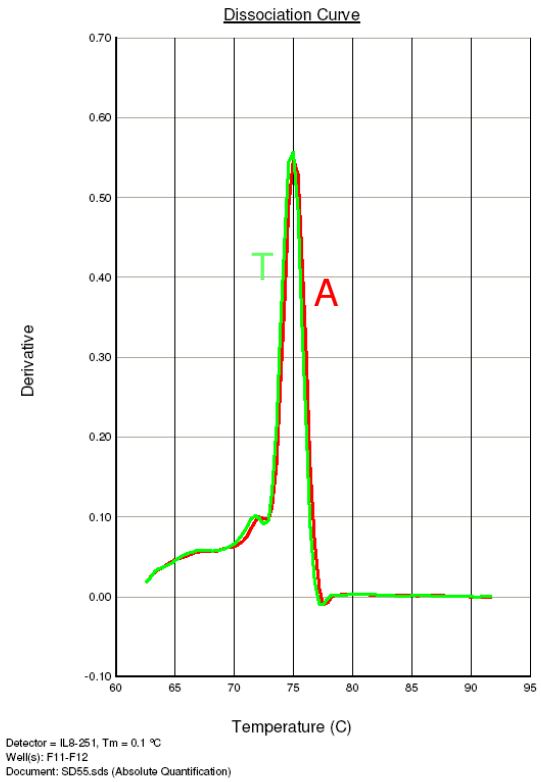


Figure 42
Dissociation curves showing single PCR products for IL8-251T and A real-time PCRs.

To test the sensitivity and efficiency of the real-time PCR method, 90 samples which included representatives of each variation (i.e. AA homozygous, AT heterozygous and TT homozygous) were run in parallel using both the conventional agarose gel and real-time methods. In each case exact concordance was achieved, demonstrating the high specificity of the real-time method; the sensitivity of the real-time PCR method was superior to the agarose gel-based method in that samples which produced no visible PCR bands on agarose (9/90 samples; 10%) were successfully amplified by real-time PCR.

Using the real-time PCR method, 96 samples can be analysed in less than 2 hours, compared to a minimum of 4 hours using agarose-gel based systems, and with a dramatic reduction in manual operations.

5.2.2 Genotype and population group

We first addressed the question of whether population groups within South Africa showed differing proportions of the IL8-251T→A genotype, as this has the potential to influence susceptibility to TB, which is known to comprise a significant health risk in South Africa. Ma *et al.* (Ma *et al*., 2003) have shown that AA homozygosity was linked to an increased risk of TB compared to TT homozygosity, with AT heterozygosity linked to an intermediate risk. For this reason, we examined both the frequency of the A allele in groups, as well as the frequency of heterozygosity vs. homozygosity.

Among Caucasian individuals, significantly greater proportions of AT (44%; P=0.005) and TT (36%; P<0.001) compared to AA (19%) genotypes were found, while among Black individuals, there was a greater proportion of AA (71%; P<0.001) than AT (27%) and TT (2%) genotypes (Figure 43a). These proportions were independent of population subgrouping (Figure 43b). In addition, IL8-251T or A alleles were significantly associated with population group, with Caucasian individuals showing a greater proportion of T alleles (59%), and Black individuals a greater proportion of A alleles (85%; P<0.001)

(Figure 44a). These proportions did not change significantly if population groups were broken down into subgroupings as indicated (P>0.05) (Figure 44b).

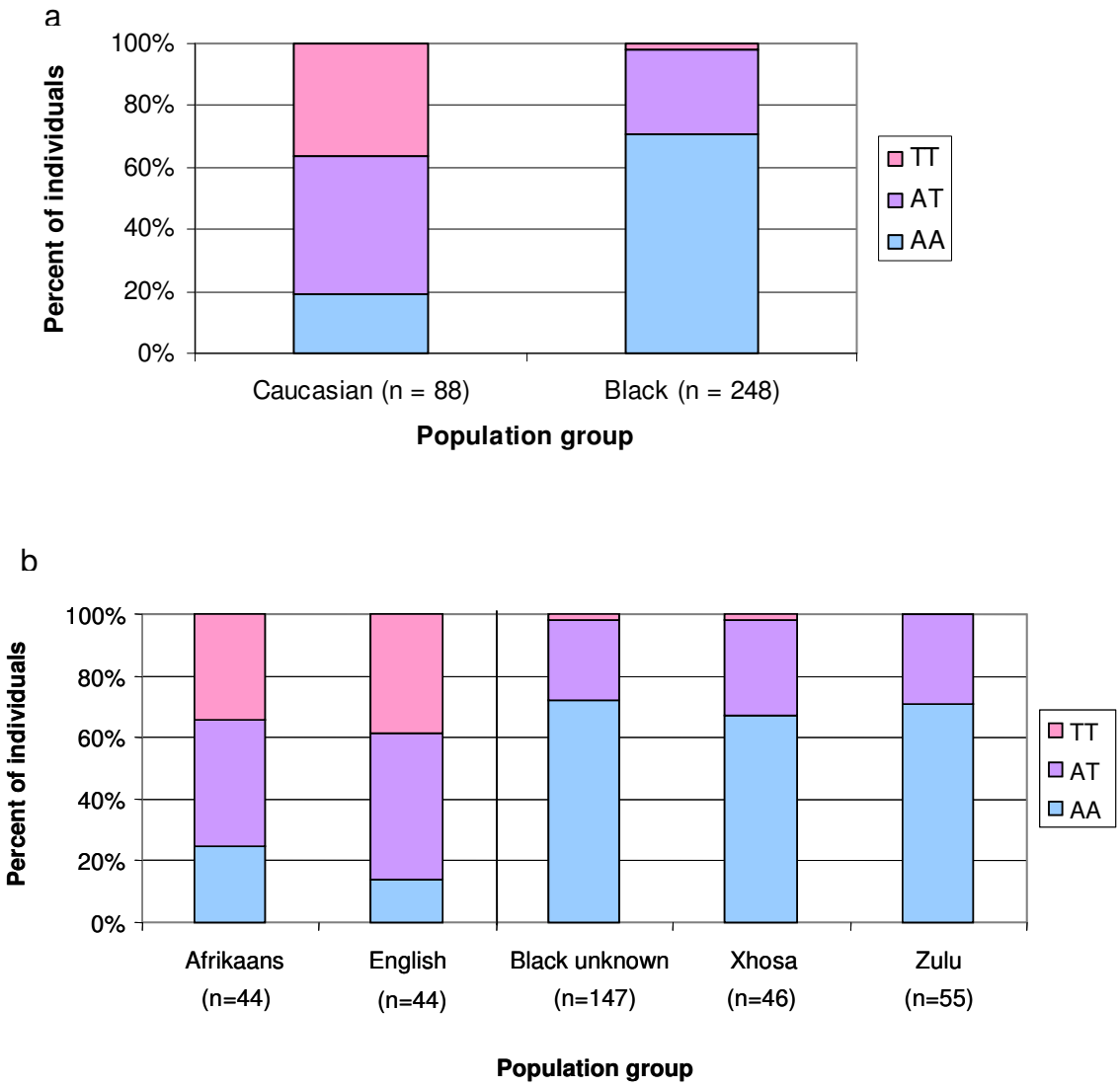


Figure 43
Percentages of IL8-251T→A genotype in study populations (a) and in population subgroups (b).

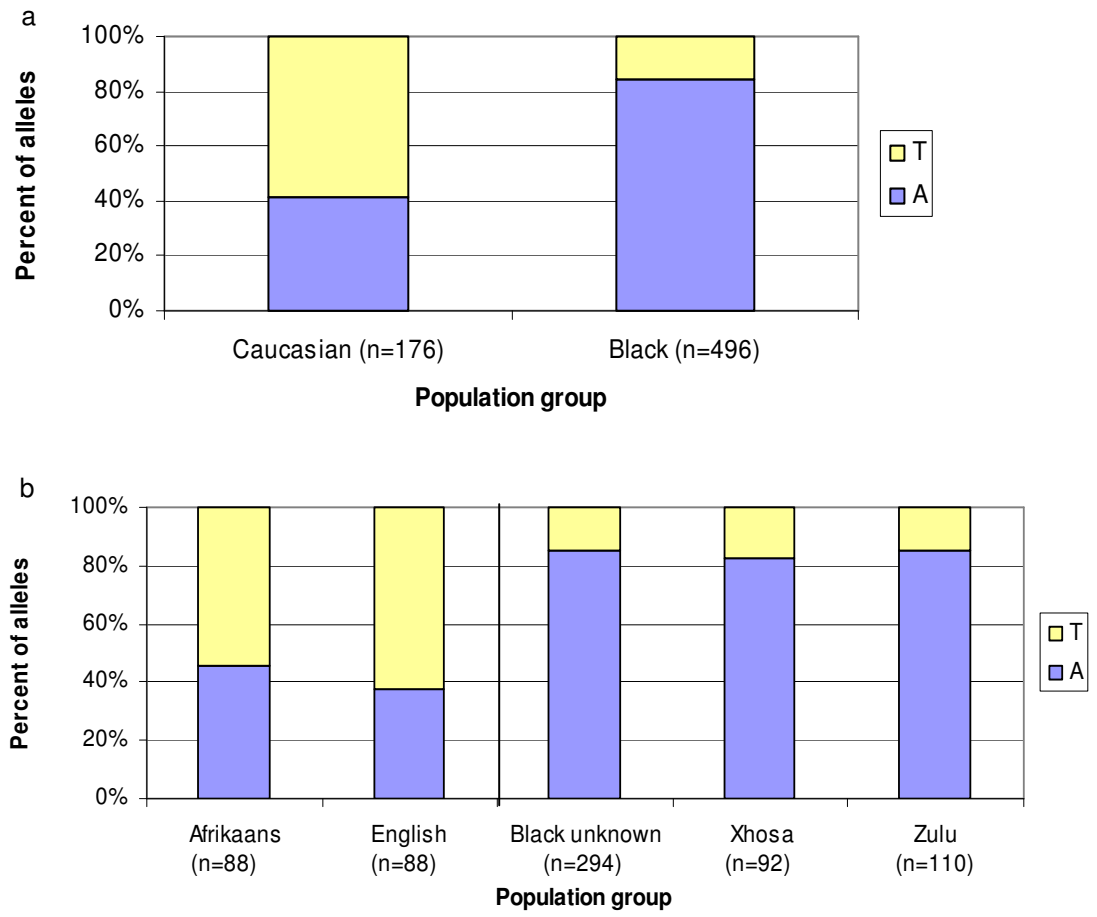


Figure 44
Percentages of IL8-251 alleles in study populations (a) and in population subgroups (b).
(x alleles = 2n individuals)

5.2.3 Genotype and TB status

Having demonstrated that Black South Africans have very high proportions of the IL8-251A allele, potentially increasing their risk for severe TB, we next examined this allele in the context of *Mtb* infection in Black South Africans, firstly as a single group, and then stratified on the basis of HIV-1 infection.

There was no statistically significant difference between proportions of individuals with either the IL8-251T→A genotypes or IL8-251 alleles in TB positive and negative groups within the Black population, regardless of HIV-1 status (P>0.05) (Figure 45a and b).

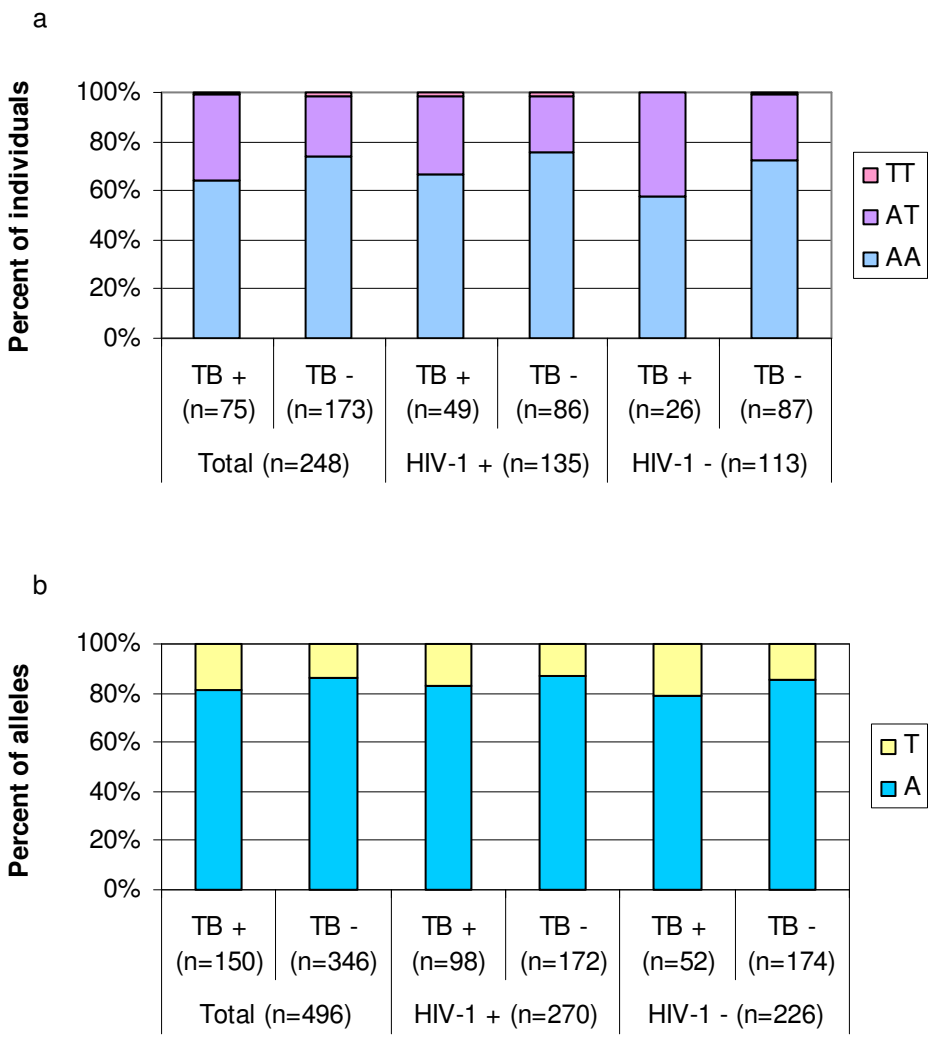


Figure 45
Percentages of (a) IL8-251T→A genotypes and (b) IL8-251 alleles in TB positive and negative Black individuals stratified according to HIV-1 status. (x alleles = 2n individuals)

5.2.4 Genotype and HIV-1 status

With the IL8-251A genotype showing no association with TB infection in our study cohort, we next examined it within the context of HIV-1 infection, both as a group and then specifically within those HIV-1 positive patients coinfected with TB.

There was no statistically significant difference between proportions of individuals with either the IL8-251T→A genotypes or IL8-251 alleles in HIV-1 positive and negative groups within the Black population, regardless of TB status ($P>0.05$) (Figure 46a and b).

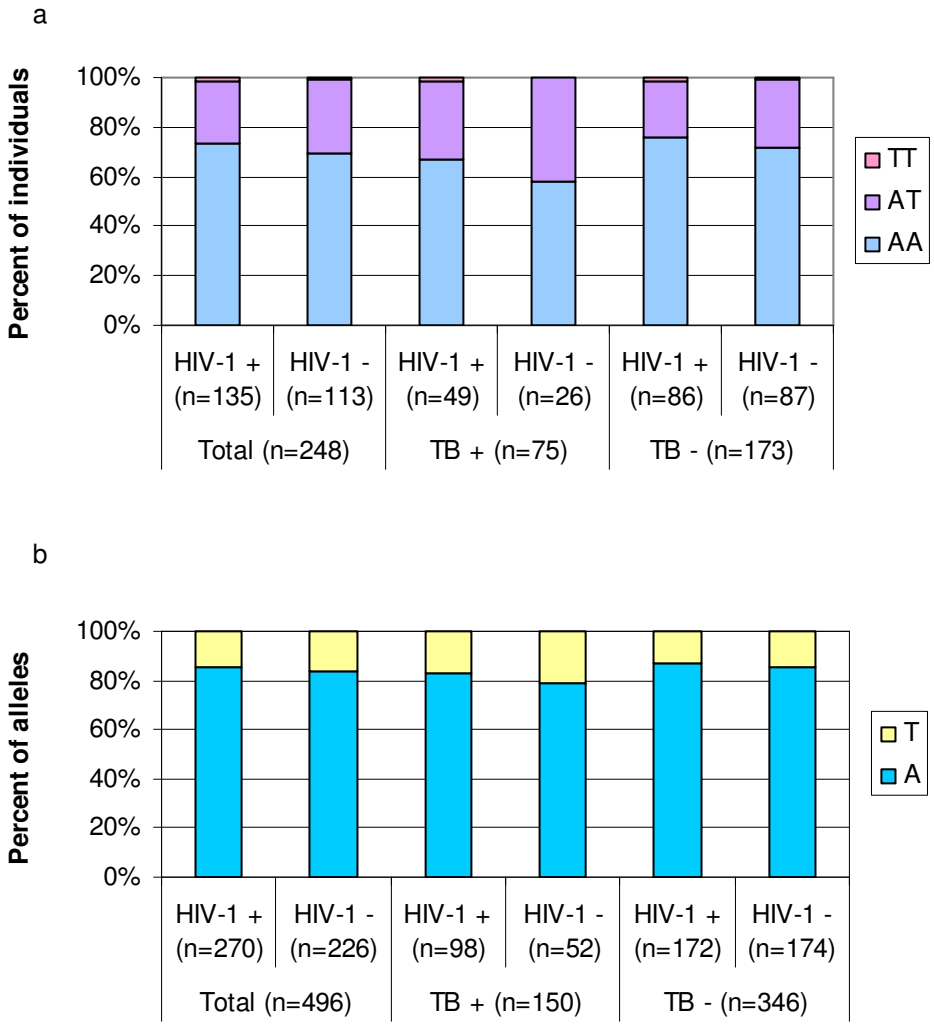


Figure 46
Percentages of (a) IL8-251T→A genotypes and (b) IL8-251 alleles in HIV-1 positive and negative Black individuals stratified according to TB status.
(x alleles = 2n individuals)

5.2.5 Genotype and peripheral CXCL8 levels

Given the very small numbers of samples showing detectable plasma CXCL8 levels (n=1/17 of the HIV-1 group, n=1/27 of the TB group, and n=10/61 of the HIV-1/TB group), most of which were from HIV-1/TB coinfected individuals (Chapter 3.2.1.3, Figure 17e), we could not address the relationship between circulating levels of CXCL8 and the IL8-251T→A genotype.

5.3 Discussion

The IL8-251A allele has been shown to be present in European (United Kingdom) and African (The Gambia) individuals at frequencies of 0.47 and 0.89, respectively (Hull *et al.*, 2001), and in White American and African American individuals at 0.37 and 0.75, respectively (Ma *et al.*, 2003). In South African population groups, a similar pattern was seen, with IL8-251A allele frequencies of 0.41 for Caucasian groups, and 0.85 for Black groups. The differences in allele distribution between population groups suggest that selection pressure may have been a factor at this locus during human evolution.

Published data have shown an association between the IL8-251T→A genotype and risk for severe TB infections in HIV-1 seronegative TB patients (106 White and 188 African American individuals), with control HIV-1 negative, TB negative groups (107 White and 167 African American individuals) (Ma *et al.*, 2003). Our study was not powered to address this question, but we nonetheless stratified

Black individuals on the basis of known disease outcomes. As expected, no associations could be seen. With so few Black individuals within our study cohort that are homozygous TT (n=4/248; 2%), and no TT homozygous individuals with TB alone (out of 26 Black TB patients) a far larger cohort would be needed to show disease associations.

The possibility remains that the sheer magnitude of the TB epidemic in this country, and the large majority of the IL8-251A allele seen in the population group at greatest risk of acquiring TB, have combined to overwhelm any such genetic predisposition. In addition, South Africa has one of the largest HIV-1 epidemics in the world, and HIV-1 is a major risk factor for acquiring TB; the study linking TB to the IL8-251T→A polymorphism was limited to HIV-1 seronegative TB patients and controls, whereas it is becoming increasingly difficult in South Africa to obtain prospective samples from HIV-1 negative TB patients.

In addition, changes in CXCL8 levels dependent on the IL8-251T→A polymorphism have been demonstrated only in LPS-stimulated whole blood, rather than as circulating levels determined in plasma (Hull *et al.*, 2000). Whether a similar relationship might exist between CXCL8 circulating levels and genotype is unknown. In our study, there were too few DNA samples with corresponding plasma samples which had detectable levels of CXCL8 to allow for a comparison of peripheral blood CXCL8 levels with genotype. Further studies on large numbers of individuals with TB only (preferably at the time of acute TB infection, prior to the commencement of antituberculosis therapy), that

include determining CXCL8 levels in stimulated PBMC culture supernatants and comparison with genotyping data at the IL8-251 locus, would enable testing of the hypothesis that changes in allele frequency (within a population group) affect susceptibility to TB, due to their impact on changes in CXCL8 levels.

Future studies would benefit from including a population group in South Africa not studied to date with respect to the IL8-251T→A genotype, namely those of mixed ancestry. The so-called "Coloured" communities in the Western Cape of South Africa are at extremely high risk of TB (Berman *et al*., 1992), and studies of the proportions of IL8-251T→A genotypes within these groups could shed greater light on the subject. In a population of mixed ancestry a greater proportion of T alleles should be present (compared to Black population groups), which would allow any association of the IL8-251T→A SNP with susceptibility to TB to be more easily studied.

The IL8-251A allele is significantly more prevalent amongst Black individuals, both those infected with HIV-1 and/or TB and those uninfected, than amongst Caucasian individuals. Given the data linking TB susceptibility to this allele (Ma *et al*., 2003), this suggests that South African Black population groups are genetically predisposed to be at greater risk for TB, as seems to be borne out by the demographics of the TB epidemic. TB demographics are of course complicated by other factors, including (but not limited to) socio-economics and polymorphisms in other genes, neither of which has been taken into account in this study.

There are many factors, both genetic and environmental, which contribute to susceptibility to both *Mtb* and HIV-1, and which clearly need to be considered within each population group individually. CXCL8 is one of these factors which no doubt influences both susceptibility to and progress of TB disease, but which cannot be considered in isolation as a means of determining groups at risk.

CHAPTER 6

CONCLUDING REMARKS

The regulation of chemokine levels is of paramount importance in the development of protective immunity to infectious disease. We have focussed on those chemokines (CCL3, CCL4, CCL5, CXCL8 and CXCL12) known to play an important role in the pathogenesis of HIV-1 and TB, which together pose the largest public health challenge in South Africa.

CCL3 has generated much interest recently in the literature as a major determinant of susceptibility to HIV-1 and AIDS. We have shown that in Black South African adults, higher numbers of *CCL3L1* gene copies are not linked to correspondingly higher plasma CCL3 levels, implying the presence of other genetic polymorphisms which may result in lowered gene expression (in contrast to Caucasian individuals, in whom *CCL3L1* gene copy number higher than the population-specific median does correspond to higher CCL3 production). This, coupled to the finding that more Black individuals have *CCL3L1* gene copy numbers lower than their population-specific median, suggests that Black South Africans may be at a particularly high risk for acquiring HIV-1 (at least with respect to this factor). The high rate of HIV-1/TB coinfection in South Africa may serve to exacerbate this problem.

We have demonstrated that a polymorphism within the *IL8* gene (encoding CXCL8), which has been shown to be linked to susceptibility to TB (in White and African American individuals), is far more prevalent within Black South African individuals than in Caucasians. This suggests that if the IL8-251T→A polymorphism is found to be linked to TB susceptibility in South African populations, South African Black individuals may be genetically predisposed to be at greater risk for infection with *Mtb*.

There are many factors, both genetic and environmental, which contribute to susceptibility to both *Mtb* and HIV-1, and which clearly need to be considered within each population group individually. Further studies should investigate larger cohorts (to include all groups available) to examine any possible differences in chemokine levels and genetic polymorphisms more closely.

Future studies, besides including larger study cohorts, would benefit from an investigation into other polymorphisms (within the genes encoding these chemokines) known to affect chemokine production. In addition, *in vitro* expression studies to characterise the polymorphisms we have studied would improve our understanding of genotype-phenotype relationships.

Only once we have gained an understanding of the host response to infection, and the mechanisms by which innate and acquired immunity are generated (and, most importantly, the reasons for failure of the immune system to mount an effective response), can scientifically appropriate interventions be designed to combat infections such as HIV-1 and TB. A “one size fits all” approach may

be of little use, since we have demonstrated that different individuals do not necessarily have identical immune responses to the same pathogens. An understanding of the effects of host genotype will be key in the design of efficient therapies and prevention strategies, which are so desperately needed.

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Appendix A

Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ART	antiretroviral therapy
BCG	Bacille Calmette-Guerin
bp	base pairs
BSA	bovine serum albumin
CBMCs	cord-blood mononuclear cells
CNP	copy number polymorphism
CO_2	carbon dioxide
DC	dendritic cell
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
EtOH	ethanol
EU	exposed-uninfected
FAM	6-carboxyfluorescein
GPCR	guanosine nucleotide-protein-coupled receptor
HAART	highly active antiretroviral therapy
HIV-1	Human Immunodeficiency Virus type 1
HRP	horseradish peroxidase
HTLV-2	human T-lymphotropic virus
IFN-γ	interferon gamma
IL-8/CXCL8	interleukin-8
IP	intrapartum
IU	*in utero*
LPS	lipopolysaccharide
LTNP	long-term non-progressor
MDR-TB	multi-drug resistant tuberculosis
$MgCl_2$	magnesium chloride
MIP-1α/CCL3	macrophage inflammatory protein-1 alpha
MIP-1β/CCL4	macrophage inflammatory protein-1 beta
ml	millilitre

mM	millimolar
Mtb	*Mycobacterium tuberculosis*
MTCT	mother-to-child transmission
NaOAc	sodium acetate
ng	nanogram
NK	natural killer cell
nM	nanomolar
NSI	non-syncitia-inducing
NVP	nevirapine
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pdg	per diploid genome
pg	picogram
PHA	phytohaemagglutinin
PMNL	polymorphonuclear leukocyte
pmol	picomole
PMTCT	prevention of mother-to-child transmission
PRR	pattern recognition receptor
RANTES/CCL5	regulated upon activation, normal T-cell expressed, and secreted
ROMA	representational oligonucleotide microarray analysis
RPMI medium	Roswell Park Memorial Institute medium
RSV	respiratory syncytial virus
SDF-1α/CXCL12	stromal cell-derived factor-1
SI	syncitia-inducing
SIV	Simian Immunodeficiency Virus
SNP	single nucleotide polymorphism
STD	sexually-transmitted disease
TAMRA	carboxytetramethyl-rhodamine
TB	tuberculosis
TBE	Tris/Borate/EDTA
Th	T-helper cell
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
U	units
WHO	World Health Organisation
XDR-TB	extremely/extensively drug resistant tuberculosis
µg	microgram
µl	microlitre
µM	micromolar

Appendix B

Chemokine nomenclature

(from http://en.wikipedia.org/wiki/Chemokine)

CC chemokines

Name	Gene	Other name(s)
CCL1	Scya1	I-309, TCA-3
CCL2	Scya2	MCP-1
CCL3	Scya3	MIP-1α
CCL4	Scya4	MIP-1β
CCL5	Scya5	RANTES
CCL6	Scya6	C10, MRP-2
CCL7	Scya7	MARC, MCP-3
CCL8	Scya8	MCP-2
CCL9/CCL10	Scya9	MRP-2, CCF18, MIP-1γ
CCL11	Scya11	Eotaxin
CCL12	Scya12	MCP-5
CCL13	Scya13	MCP-4, NCC-1, Ckβ10
CCL14	Scya14	HCC-1, MCIF, Ckβ1, NCC-2
CCL15	Scya15	Leukotactin-1, MIP-5, HCC-2, NCC-3
CCL16	Scya16	LEC, NCC-4, LMC, Ckβ12
CCL17	Scya17	TARC, dendrokine, ABCD-2
CCL18	Scya18	PARC, DC-CK1, AMAC-1, Ckβ7, MIP-4
CCL19	Scya19	ELC, Exodus-3, Ckβ11
CCL20	Scya20	LARC, Exodus-1, Ckβ4
CCL21	Scya21	SLC, 6Ckine, Exodus-2, Ckβ9, TCA-4
CCL22	Scya22	MDC, DC/β-CK
CCL23	Scya23	MPIF-1, Ckβ8, MIP-3, MPIF-1
CCL24	Scya24	Eotaxin-2, MPIF-2, Ckβ6
CCL25	Scya25	TECK, Ckβ15
CCL26	Scya26	Eotaxin-3, MIP-4α, IMAC, TSC-1

CCL27	Scya27	CTACK, ILC, Eskine, PESKY, skinkine
CCL28	Scya28	MEC

CXC chemokines

Name	Gene	Other name(s)
CXCL1	Scyb1	Gro-α, GRO1, NAP-3
CXCL2	Scyb2	Gro-β, GRO2, MIP-2α
CXCL3	Scyb3	Gro-γ, GRO3, MIP-2β
CXCL4	Scyb4	PF-4
CXCL5	Scyb5	ENA-78
CXCL6	Scyb6	GCP-2
CXCL7	Scyb7	NAP-2, CTAPIII, β-Ta, PEP
CXCL8	Scyb8	IL-8, NAP-1, MDNCF, GCP-1
CXCL9	Scyb9	MIG, CRG-10
CXCL10	Scyb10	IP-10, CRG-2
CXCL11	Scyb11	I-TAC, β-R1, IP-9
CXCL12	Scyb12	SDF-1, PBSF
CXCL13	Scyb13	BCA-1, BLC
CXCL14	Scyb14	BRAK, bolekine
CXCL15	Scyb15	Lungkine, WECHE
CXCL16	Scyb16	SRPSOX
CXCL17	VCC-1	DMC, VCC-1

C chemokines

Name	Gene	Other name(s)
XCL1	Scyc1	Lymphotactin α, SCM-1α, ATAC
XCL2	Scyc2	Lymphotactin β, SCM-1β

CX3C chemokines

Name	Gene	Other name(s)
CX3CL1	Scyd1	Fractalkine, Neurotactin, ABCD-3

Appendix C

Cytokines involved in HIV-1 infection

(from (Alfano & Poli, 2005))

Cytokine/chemokine	Producer cell	Effect on HIV infection/replication
IL-2	Activated T cells, mDC	Upregulation via induction of pro-inflammatory cytokines
IL-4	Activated T cells, Th2 cells	Bimodal: inhibition of cytokine-mediated virus replication (monocytes); enhancement of HIV expression (macrophages)
IL-7	Epithelial cells	Enhancement of HIV transcription
IL-10	DC, activated T cells	Bimodal: inhibition of cytokine-mediated virus replication (macrophages); enhancement of HIV transcription
IL-12	MDC, APC	Enhancement of HIV replication (PBMC)
IL-13	Macrophages	Post-transcriptional inhibition of HIV replication (macrophages)
IL-15	Macrophages, PDC, NK	Upregulation via induction of pro-inflammatory cytokines
IFN-α/β	Monocytes, macrophages, PDC	Inhibition of multiple steps of virus life cycle
IFN-γ	NK, NKT, γδT, CTL, PDC, activated T cells, Th1 cells	Bimodal: enhancement of HIV transcription; inhibition of R5 virus entry and virion release
TGF-β	Hemopoietic & endothelial cells, connective tissue, macrophages, Th3 cells	Bimodal: enhancement and suppression of HIV replication as a function of time of stimulation vs. infection (macrophages)
CCL3, CCL4, CCL5	NK, DC, γδ, activated T cells, macrophages	Inhibition of R5 virus entry
CCL2	Monocytes, macrophages	Enhancement of HIV replication
CXCL8	Monocytes, activated T cells, NK, fibroblasts, endothelial & epithelial cells	Enhancement of HIV replication

Appendix D

Genes involved in resistance to HIV-1/TB

Genetic polymorphisms involved in resistance/susceptibility to HIV-1.

Gene	Polymorphism	Effect on infection with HIV	Ref
CCL2-CCL7-CCL11 gene cluster	H7 haplotype	Higher frequency in multiply-exposed uninfected individuals	(Modi *et al.*, 2003)
CCL3L1	Copy number < pop.-specific average	Susceptibility	(Gonzalez *et al.*, 2005)
CCL4L1	G590A	Higher incidence in HIV+	(Colobran *et al.*, 2005)
	SCYA4*L2	Susceptibility	(Colobran *et al.*, 2005)
CCL5	-28G	Slow disease progression	(Liu *et al.*, 1999)
	-403A	Increased frequency of infection	(An *et al.*, 2002)
	In1.1C	Increased frequency of infection; rapid disease progression	(An *et al.*, 2002)
	3' 222C	Increased frequency of infection	(An *et al.*, 2002)
CCR2	V64I	Delayed onset of AIDS	(Smith *et al.*, 1997)
CCR5	Δ32 (het)	Slow disease progression	(Samson *et al.*, 1996), (Dean *et al.*, 1996)
	Δ32 (hom)	Protection against infection	(Liu *et al.*, 1996), (Dean *et al.*, 1996)
	CCR5P1 (hom)	Rapid progression to AIDS	(Martin *et al.*, 1998)
CXCL12	SDF1-3'A (hom)	Delayed onset of AIDS	(Winkler *et al.*, 1998)
		Accelerated progression (late-stage AIDS)	(Brambilla *et al.*, 2000)
CX3CR1	I249/M280 (hom)	Rapid progression to AIDS	(Faure *et al.*, 2000)
CXCR1	CXCR1-Ha	Protects against rapid progression to AIDS	(Vasilescu *et al.*, 2007)

DCSIGN	-336T→C	Higher risk for parenteral HIV infection	(Martin *et al.*, 2004)
HLA class I	Heterozygosity	Delayed onset of AIDS	(Carrington *et al.*, 1999)
	HLA-B*35	Rapid progression to AIDS	(Carrington *et al.*, 1999), (Gao *et al.*, 2005)
	HLA-Cw*04	Rapid progression to AIDS	(Carrington *et al.*, 1999), (Gao *et al.*, 2005)
	HLA-B57	Slow disease progression	(Gao *et al.*, 2005)
	HLA-B27	Delayed onset of AIDS	(Gao *et al.*, 2005)
IFNG	-179T	Rapid progression to AIDS	(An *et al.*, 2003)
IL10	-592A	Increased risk of infection; rapid progression to AIDS	(Shin *et al.*, 2000)
	-592C/C	Long term non-progression	(Shin *et al.*, 2000)
IL4R	V50 (hom)	Slow disease progression	(Soriano *et al.*, 2005)
KIR3DS1	with HLA-B Bw4-80Ile	Delayed progression to AIDS	(Martin *et al.*, 2002)
	without HLA-B Bw4-80Ile	Rapid progression to AIDS	(Martin *et al.*, 2002)

Genes and polymorphisms tested for association with TB and other mycobacterial diseases.

Gene	Polymorphism	Population studied	Disorder	Effect of polymorphism	Ref
2q33-q37 (incl. NRAMP1)	markers	Mexican	TB/Lsh/leprosy	no linkage	(Shaw *et al.*, 1993)
5q (incl. IL4, IL5, IL9, IRF1, CD14)	microsatellite	Brazilian	TB/Lsh/leprosy	no linkage; linkage to ability to mount immune response to mycobacterial antigens	(Blackwell *et al.*, 1997)
17q (incl. NOS2A, SCYA2-5)	microsatellite	Brazilian	TB	linkage	(Blackwell *et al.*, 1997)
17q (NOS2A, CCL18, CCL4, STAT5B)	SNPs	Brazilian	TB/leprosy	linkage	(Jamieson *et al.*, 2004)
CD209 (encodes DC-SIGN)	-871G	S African	TB	protection	(Barreiro *et al.*, 2006)
	-336A	S African	TB	protection	(Barreiro *et al.*, 2006)
CXCR1&2		White & AfrAm	TB	no significant associations	(Ma *et al.*, 2003)
FUT-2	SNP	Gambian	TB	no association	(Bellamy *et al.*, 1998a)
HLA	HLA-DQ		leprosy	associated with multi-bacillary lepromatous forms	(Lagrange & Abel, 1996)
	HLA-DQA	S Indian	TB	no association	(Sanjeevi *et al.*, 1992)
	HLA-DQB	S Indian	TB	no association	(Sanjeevi *et al.*, 1992)
	DQB1*0503	Cambodian	TB	associated with susceptibility	(Goldfeld *et al.*, 1998)
	HLA-DR		leprosy	associated with protective response	(Lagrange & Abel, 1996)
	HLA-DRB	S Indian	TB	no association	(Sanjeevi *et al.*, 1992)
	HLA-DR2		TB	susceptibility	(Singh *et al.*, 1983)
			leprosy	susceptibility to tuberculoid leprosy (not lepromatous)	(van Eden *et al.*, 1980)

	HLA-B52	Indian	pulmonary TB	protective	(Vijaya Lakshmi *et al.*, 2006)
	HLA-B51	Indian	pulmonary TB	susceptibility	(Vijaya Lakshmi *et al.*, 2006)
HLA complex (incl. TNFA/B	microsatellite	Brazilian	leprosy	association	(Blackwell *et al.*, 1997)
		Brazilian	leishmaniasis	weak association	(Blackwell *et al.*, 1997)
		Brazilian	TB	no association	(Blackwell *et al.*, 1997)
	haplotype HLA/TNF		TB	association with protection & susceptibility to relapse	(Selvaraj *et al.*, 2001)
Hp (haptoglobin)	Hp1 & 2	Zimbabwe	TB	equal susceptibility; Hp2-2 higher risk of mortality	(Kasvosve *et al.*, 2000)
IFNG	+874T/A	Spanish	TB	A allele ↑ risk of TB; AA homozygotes ↓ stimulated prodn of IFN- γ	(Lopez-Maderuelo *et al.*, 2003)
		S African	TB	T allele over-represented in controls	(Rossouw *et al.*, 2003)
IFNGR1	$(CA)_n$ intron 6	Croatian	TB	weak association	(Fraser *et al.*, 2003)
	inactivating mutations		mycobacteria	↑ susceptibility in homozygous children	(Hill, 1998)
IL1A	microsatellite	Gambian	TB	marginal assoc	(Bellamy *et al.*, 1998a)
IL1B	SNP	Gambian	TB	no association	(Bellamy *et al.*, 1998a)
	-511	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	3953	Cambodian	TB	no association	(Delgado *et al.*, 2002)
IL1RA	minisatellite	Gambian	TB	marginal assoc	(Bellamy *et al.*, 1998a)
	intron 2 tandem repeats	Cambodian	TB	no association	(Delgado *et al.*, 2002)
IL8	-251A	White & AfrAm	TB	homozygosity associated with TB	(Ma *et al.*, 2003)

		Caucasian	RSV	associated with disease severity (IL-8 production by LPS-stimulated blood)	(Hull *et al.*, 2000)
IL10	SNP	Gambian	TB	no association	(Bellamy *et al.*, 1998a)
	microsatellite	Gambian	TB	no association	(Bellamy *et al.*, 1998a)
	-1082	Cambodian	TB	heterozygosity assoc with susceptibility	(Delgado *et al.*, 2002)
	-819	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	-592	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	-1082G/A	Spanish	TB	no association	(Lopez-Maderuelo *et al.*, 2003)
IL12	A/C 3'UTR	Russian	TB	no association	(Puzyrev *et al.*, 2002)
IL12RB1	1: Q214-M365-G378 / 2: R214-T365-R378	Japanese	TB	allele 2 assoc with susceptibility & ↓ levels of IL-12-induced signalling	(Akahoshi *et al.*, 2003)
mannose-binding lectin	polymorphisms		TB	association	(Bellamy, 2000)
MCP1	-2518G	Mexican	TB	association	(Flores-Villanueva *et al.*, 2005)
NAT2	NAT2 "fast" allele	African	TB	high prevalence	(Adams *et al.*, 2003)
NLI-IF (close to NRAMP1)	204C→A, 402T→C, 472-42G→A	Caucasian	TB	no association	(Ma *et al.*, 2002b)
NOS2	NOS2(-/-)	mice	TB	highly susceptible	(MacMicking *et al.*, 1997)
NRAMP1 / SLC11A1	D543N	Japanese	TB	(A) TB patients more likely to develop cavitary lesion	(Abe *et al.*, 2003)
		Cambodian	TB	heterozygosity assoc with resistance	(Delgado *et al.*, 2002)
			tuberculous pleurisy	no association	(Kim *et al.*, 2003)
		Taiwanese	TB	no association	(Liaw *et al.*, 2002)

		Danish	TB	no association	(Soborg *et al.*, 2002)
		Gambian	TB	association	(Bellamy *et al.*, 1998b)
	TGTG del 3' untrans. (1729+55del4, 3'UTR)	Cambodian	TB	heterozygosity assoc with resistance	(Delgado *et al.*, 2002)
			tuberculous pleurisy	association	(Kim *et al.*, 2003)
		Gambian	TB	association	(Bellamy *et al.*, 1998b)
		Korean	TB	association	(Ryu *et al.*, 2000)
		W Africa	leprosy	assoc with leprosy type	(Meisner *et al.*, 2001)
		Danish	TB	no association	(Soborg *et al.*, 2002)
		Guinea-Conakry	TB	no association	(Cervino *et al.*, 2000)
		Taiwanese	TB	no association	(Liaw *et al.*, 2002)
		Bengali	leprosy	no association	(Roy *et al.*, 1999)
	469+14G/C (INT4)		tuberculous pleurisy	association	(Kim *et al.*, 2003)
		Guinea-Conakry	TB	significant association	(Cervino *et al.*, 2000)
		Danish	TB	↑ mycobacterial replication	(Soborg *et al.*, 2002)
		Gambian	TB	association	(Bellamy *et al.*, 1998b)
		Russian	TB	no association	(Puzyrev *et al.*, 2002)
		W African	leprosy	no association	(Meisner *et al.*, 2001)
		Bengali	leprosy	no association	(Roy *et al.*, 1999)
		Cambodian	TB	uncommon in cohort	(Delgado *et al.*, 2002)
	5'(CA)n (influences expression)	Danish	TB	↑ mycobacterial replication	(Soborg *et al.*, 2002)
		AfrAm	sarcoidosis	protective	(Maliarik *et al.*, 2000)

		Gambian	TB	association	(Bellamy *et al.*, 1998b)
		Caucasian (Texas)	TB	association (HIV- and HIV+)	(Ma *et al.*, 2002a)
		Gambian	TB	allele 2 assoc with susceptibility & LPS-induced production of IL-10	(Bellamy *et al.*, 1998b)
		W African	leprosy	no association	(Meisner *et al.*, 2001)
		Bengali	leprosy	no association	(Roy *et al.*, 1999)
		Guinea-Conakry	TB	no association	(Cervino *et al.*, 2000)
	274C/T	Taiwanese	TB	no association	(Liaw *et al.*, 2002)
		Russian	TB	no association	(Puzyrev *et al.*, 2002)
	-236C/T	Cambodian	TB	uncommon in cohort	(Delgado *et al.*, 2002)
	microsatellite (exon 2)	Bengali	leprosy	no association	(Roy *et al.*, 1999)
	577-18G/A	Taiwanese	TB	no association	(Liaw *et al.*, 2002)
	1465-85G/A	Russian	TB	no association	(Puzyrev *et al.*, 2002)
	A318V	Taiwanese	TB	no association	(Liaw *et al.*, 2002)
	microsatellite	Brazilian	TB/Lsh	no linkage	(Blackwell *et al.*, 1997)
	coding region	Japanese	MAC	no variations found assoc with susceptibility	(Tanaka *et al.*, 2000)
	Gly105->Asp105	mice	BCG	sensitive	(Malo *et al.*, 1994)
	polymorphism	rhesus macaque	TB	no association	(Deinard *et al.*, 2002)
	3'UTR (SSCP analysis)	cattle	*M bovis*	no association with resistance/susceptibility	(Barthel *et al.*, 2000)
P2RX7	-1269, -1140, -838, -298	Gambian	TB	no association	(Li *et al.*, 2002)
	-762	Gambian	TB	protective	(Li *et al.*, 2002)

	1513A->C	Gambian	TB	CC-loss of function; no association	(Li *et al.*, 2002)
SP-A			RSV & TB	implicated in susceptibility	(Haataja & Hallman, 2002)
	SP-A1C1416T	Indian	TB	association	(Madan *et al.*, 2002)
	SP-A2C1382G	Indian	TB	association	(Madan *et al.*, 2002)
	SNPA1660G + SP-A2G1649C	Indian	TB	association	(Madan *et al.*, 2002)
SP-D			RSV & TB	implicated in susceptibility	(Haataja & Hallman, 2002)
TNFA	-308	Bashkorto-stan	TB	allele 2 ↑ in TB patients	(Bikmaeva *et al.*, 2002)
	-1030	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	-862	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	-856	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	-375	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	-307	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	-243	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	-237	Cambodian	TB	no association	(Delgado *et al.*, 2002)
	RFLP	Cambodian	TB	no association	(Goldfeld *et al.*, 1998)
	-238		TB	no association	(Selvaraj *et al.*, 2001)
	-308		TB	no association	(Selvaraj *et al.*, 2001)
TNFB	NcoI		TB	no association	(Selvaraj *et al.*, 2001)
TNFSF5 (CD40 ligand)	6 SNPs & microsatellite.	W African	TB	no association	(Campbell *et al.*, 2003)
UBE3A	7 bp del promoter region	W African; S African	TB	association	(Cervino *et al.*, 2002)

VDR	polymorphisms		TB	association	(Bellamy & Hill, 1998); (Bellamy, 2000)
	codon 352	Gambian	TB	TT underrepresented	(Bellamy *et al*., 1999)
		Bengali	leprosy	association with leprosy type	(Roy *et al*., 1999)
	C/T polymorphism	Chinese Han	TB	VDR-ff genotype might be assoc with susceptibility	(Liu *et al*., 2003)
	TaqI/FokI	Cambodian	TB	no association	(Delgado *et al*., 2002)
	TaqI/BsmI/FokI	Gujarati	TB	polymorphisms may contribute to susceptibility (in combination with 25-hydroxycholecalciferol deficiency)	(Wilkinson *et al*., 2000)

Appendix E

Genomic DNA sequences

CCL3 and *CCL3L1* genomic DNA sequences (see following page). Genbank accession numbers D90144 (α; *CCL3*) and D90145 (β; *CCL3L1*) (adapted from (Nakao *et al*., 1990)).

Exons are in capitals. Where *CCL3L1* sequences are identical to *CCL3*, nucleotides are marked with dashes. Transcription initiation site is +1, termination site is Ter. The TATA box and polyadenylation signal are boxed. PCR primer sites are highlighted in yellow, probe in green.

```
α                                   acccagggacctatcacacaaatataagaactattcattctttaaggcatgtatttccaagcctttgtatttttttccatgcttagggtt  -979

α  ggcaaggaatatatatatatttgtacaaatatatatgtgtatatgtacaaatacatgtatatatagtacaaatatatatatatatttgtacaattcttcagactttgtagaatttgtata  -859

α  atgtcgtatcttgctttttttaaccactgatgttataagcatatttatgccacttcattcattttagagacttaataataaatgatctagtggataatttatcattccctgatggagaaa  -739
β                                                   ------------------g-----t-----------------------a------ -1055

α  aatttagctttgtttattttagagttataaacgatgctgggtcaggtatctttatgtttgaagatggctccatatttgggttgtttccacagaactctttcctagaaatgctttttctag  -619
β  ------------c------------------t-------------------------------------------------------------------c-----------------  -935

α  gttaatggctacagatatttctaggcacctgacatattgacacccacctctaaagtatttttatgatccacaactagcgtttaacacagcgccctagtcactacatgactaataaataga  -499
β  -------------c----------------------c-------------------------------------------------------------c-------c-ga--------------  -815
                                                                                                                Primer B
α  caaatgactgaaacatgacctcatgctttctattcctccagctttcattcagttctttgcctctgggaggaggaaggggttgtgcagccctccacagcatcagcccatcaaccctatccct  -379
β  --------------g---------------------------------g-----c--t------------ctgg-------ct-----------------------tg---------t-  -695

α  gtggttatagcagctgaggaagcagaattgcagctctgtgggaaggaatggggctggagagttcatgcacagaccagttc*************************************
β  ----------------------------a-------------------------------------t------a---tttttttttttttttttgagatggagtttcacttttgtt  -575

α  ****************************************************************************************************************
β  gcccaggctggagtgcaatggcatgatctcagctcaccacagcccccacctcctgggttcaagcgattctcctgccctcagcctcccgagtagctgggattacaggcatgtgccaccacg  -455

α  ****************************************************************************************************************
β  cctgactacttttgtatttttagtagagatggagtttctctttcttggtcaggttggtctcaaactcctgacctcaggtgatccgcagcctcggcctcccaaagtgttgggattacaggt  -335

α  *********************************ttatgagaagggactgactaagaatagccttgggttgacatatacccctcttcacactcacaggagaaaccatttccctatgaaac  -213
β  gtgagcgaccatgcctggctgcatagaccagttc-------------tca------------------------c-c---------------------------******---c-----g-  -215

α  tataacaagtcatgagttgagagctgagagttagagaatagctcaaagatgctattcttggatatcctgagcccctgtggtcaccagggaccctgagttgtgcaac**ttagcatgacag  -95
β  --g---c-----------------------------g-------g-------------------------------------------------------------ac-c----------  -95
                                                                                                  +1
α  catcactacgcttaaaaatttccctcctcacccccagattccatttccccatccgccagggctgcctataaagaggagagctggtttcagacttCAGAAGGACACGGGCAGCAGACAGTG  26
β  ---------a-------------------------------------------------------------------a---c-------a----------G-A-------A-G---A  26

                                                                M  Q  V  S  T  A  A  L  A  V  L  L  C  T  M  A  L  C  N  Q
α  GTCAGTCCTTTCTTGGCTCTGCTGACACTCGAGCCCACATTCCGTCACCTGCTCAGAATCATGCAGGTCTCCACTGCTGCCCTTGCTGTCCTCCTCTGCACCATGGCTCTCTGCAACCAG  146
β  --------C---------------------------------A----------CC-------------------------------C-----------------------------  146
                                                                M  Q  V  S  T  A  A  L  A  V  L  L  C  T  M  A  L  C  N  Q
   F     S  A  S  L
α  TTC***TCTGCATCACgtgagtctgagtt*tcgttgtgggtatcaccactctctggccatggttagaccacatcaatcttttcttgtggcctaaaagcccccaagagaaaaga**gaact  260
β  G--CTC------C----------cat---g-t-----------------------------------------g------t---c-----g-g------g----------ag---g-  266
   V  L  S  A  P  L

α  tcttaaagggctgccaaacatcttggtctttctctttaagacttttatttttatctctagaaggggtcttagcc*ccctagtctccaggtatgagaatctaggcaggggcaggggagtta  379
β  --------c----------c----------t----c-ca-------------------------------------ct-------------------------------------  386

α  cagtccctttacagatagaaaaacagggttcgaaacgaatcagttagcaagaggcagaatccagggctgcttacttcccagtggggtatgttgttcactctccagctcactctaggtct  499
β  ---------g---------------------a------------t-------------------------------------c----------------------c--------  506

α  cccaggagctctgtcccttggatgtcttatgagagatgtccaaggcttctcttgggttggggtatgacttcttgaaccagacaaaattccctgaagagaactgagataagagaacagtcc  619
β  ---------c-------------------------------g-------------c--------------------*-----------a------*****---c--------------  620

α  gttcaggtatctggatcacacagagaaacagagaacccactatgaagagtcaaggagaaagaaggatacagacagaaacaaagagacatttctcagcaaaaatgcccaaatgccttccag  739
β  a-------------------t--------------------------------g------g-a---t-------------------------t------ccc------------g---  740

                                                                                                                A  A  D  T
                                                Primer A
α  tcacttggtctgagcaagcctgccttcctcaactgctcggggatcagaagctgcctggcctttctctctgagctgtgactcggGctcattctcttcctttctccacagTTGCTGCTGACA  859
β  ------------------------c--------ca---a-------------------------------------------t------*----------g---------------  859
                                                                                                                A  A  D  T
    P  T  A  C  C  F  S  Y  T  S  R  Q  I  P  Q  N  F  I  A  D  Y  F  E  T  S  S  Q  C  S  K  P  G  V  I
α  CGCCGACCGCCTGCTGCTTCAGCTACACCTCCCGGCAGATTCCACAGAATTTCATAGCTGACTACTTTGAGACGAGCAGCCAGTGCTCCAAGCCCGGTGTCATgtaagtgccagtcttcc  979
β  ---------------------------------A-------------------------------------------------------------------A-----------------  979
    P  T  A  C  C  F  S  Y  T  S  R  Q  I  P  Q  N  F  I  A  D  Y  F  E  T  S  S  Q  C  S  K  P  S  V  I

α  tgctcacctctatggaggtagggagggtcaggttggggcagagacaggccagaaggctatcctggaaaggcccagccttcaggagcctatcggggatacaggacgcagggctccgaggt  1099
β  ------------g------------t--------g--------a--------------c-------------------------------------------------------a-t-----  1099

α  gtgacctgacttggagctggagtgaggcatgtgttacagagtcaggaagggctgccccagcccagaggaaagggacaggaagaaggaggcagcgggacactctgagggccacccctact*  1218
β  --------------g------------tgg-----------------------------g------------a-------------------a----------------c--ttgc--g  1219

α  gagtcactgagagaagctctctagacagagataggcaggggggccctgaaagaggagcaagccctgagctgcccaggacagagagcagaatggtggggccatggtgggcccaggattccc  1338
β  -------------------------g----------------------g---------g---t-----------------------g---*-ca-----------------------  1338
                                                                                                                                
                                Real-time PCR primer F        Real-time PCR probe        Real-time PCR primer R
                                                F  L  T  K  R  S  R  Q  V  C  A  D  P  S  E  E  W  V  Q  K  Y  V  S  D  L
α  ctgctggattccccagtgcttaactcttcctccctctctccacagCTTCCTAACCAAGCCAAGCCGGCAGGTCTGTGCTGACCCCCAGTCAGCAGTGGGTCCAGAAATATGTCAGCGACCTG  1458
β  -g-------------------------------------------------------A--G-----------------------------------------------C-----T------  1458
                                                F  L  T  K  R  G  R  Q  V  C  A  D  P  S  E  E  W  V  Q  K  Y  V  S  D  L
   E  L  S  A  Ter
α  GAGCTGAGTGCCTGAGGGGTCCAGAAGCTTCGAGGCCCAGCGACCTCGGTGGGCCCAGTGGGGAGGAGCAGGAGCCTGAGCCTTGGGAACATGCGTGTGACCTCCACAGCTACCTCTTCT  1578
β  ---------------------------------------------A-------------------------------------------------------T---------------  1578
   E  L  S  A  Ter

α  ATGGACTGGTTGTTGCCAAACAGCCACACTGTGGGACTCTTCTTAACTTAAATTTTAATTTATTTATACTATTTAGTTTTTGTAATTTATTTTCGATTTCACAGTGTGTTTGTGATTGTT  1698
β  -----------A-------------------------------------------------------------------------------A-----------T-------------------------  1698

α  TGCTCTGAGAGTTCCCC*TGTCCCCTCCCCCTTCCCTCACACCGCGTCTGGTGACAACCGAGTGGCTGTCATCAGCCTGTGTAGGCAGTCATGGCACCAAAGCCACCAGACTGACAAATG  1817
β  -----------------C----------A------------GT-T----------G-----------------G-------------------------------------------  1818

α  TGTATCGGATGCTTTTGTTCAGGGCTGTGATCGGCCTGGGGAAATAATAAACATGCTCTTTTAAAAGGTAAAccagtattgagtttggttttgtttttctggcaaatcaaaatcactggt  1937
β  ------A-----------------------------------------------T---------C-------------------------------------------------a--  1938

α  taagaggaatcataggcaaagattaggaagaggtgaaatggagggaaattgggagagatggggagggctaccacagagttatccactttacaacggagacacagttctggaacattgaaa  2057
β  ----------------------------------------------c----------------c---

α  ctacgaatatgttataactcaaatcataacatgcatgctctaggagaattc
```

IL8 genomic DNA sequence (encoding CXCL8)

(Genbank accession number AF385628).

Exons are in capitals; IL8-251A/T polymorphism and start codon are in bold type. Real-time PCR primer and probe sites are marked.

```
2761 cctcccaccc caaccaagca gctccagtgc accactttct ggagcataaa catacccttaa
2821 ctttacaact tgagtggcct tgaatactgt tcctatctgg aatgtgctgt tctctttcat
2881 cttcctctat tgaagccctc ctattcctca atgccttgct ccaactgcct ttggaagatt
2941 ctgctcttat gcctccactg gaattaatgt cttagtacca cttgtctatt ctgctatata
3001 gtcagtcctt acattgcttt cttcttctga tagaccaaac tctttaagga caagtaccta
3061 gtcttatcta tttctagatc ccccacatta ctcagaaagt tactccataa atgtttgtgg
                                   IL8-251 cons1     →
3121 aactgatttc tatgtgaagc acatgtgccc cttcactctg ttaacatgca ttagaaaact
3181 aaatcttttg aaaagttgta gtatgccccc taagagcagt aacagaaact aagagttcct
3241 agaaactctc taaaatgctt agaaaaagat ttattttaaa ttacctcccc aataaaatga
           IL8-251 cons2    →
3301 ttggctggct tatcttcacc atcatgatag catctgtaat taactgaaaa aaaataatta
3361 tgccattaaa agaaaatcat ccatgatctt gttctaacac ctgccactct agtactatat
                                                          ← IL8-251A/T
3421 ctgtcacatg gtactatgat aaagttatct agaaataaaa aagcatacaa ttgataattc
3481 accaaattgt ggagcttcag tattttaaat gtatattaaa attaaattat tttaaagatc
3541 aaagaaaact ttcgtcatac tccgtatttg ataaggaaca aataggaagt gtgatgactc
3601 aggtttgccc tgaggggatg ggccatcagt tgcaaatcgt ggaatttcct ctgacataat
3661 gaaaagatga gggtgcataa gttctctagt agggtgatga tataaaaagc caccggagca
3721 ctccataagg cacaaacttt cagagacAGC AGAGCACACA AGCTTCTAGG ACAAGAGCCA
3781 GGAAGAAACC ACCGGAAGGA ACCATCTCAC TGTGTGTAAA CATGACTTCC AAGCTGGCCG
3841 TGGCTCTCTT GGCAGCCTTC CTGATTTCTG CAGCTCTGTG TGAAGgtaag cacatctttc
3901 tgacctacag cgttttccta tgtctaaatg tgatccttag atagcaaagc tattcttgat
3961 gctttggtaa caaacatcct ttttattcag aaacagaata taatcttagc agtcaattaa
4021 tgttaaattg aagatttaga aaaaactata tataacactt aggaaagtat aaagtttgat
4081 caatatagat attctgcttt tataatttat accatgtagc atgcatatat ttaacgtaaa
4141 taagtaattt atagtatgtc ctattgagaa ccacggttac ctatattatg tattaatatt
4201 gagttgagca aggtaactca gacaattcca ctccttgtag tatttcattg acaagcctca
4261 gatttgtcat taattcctgt ctggtttaaa gataccctga ttatagacca ggcatgtata
4321 acttatttat atatttctgt taattctttc tgaaggcaat ttctatgctg gagagtctta
4381 gcttgcctac tataaataac actgtggtat cacagaggat tatgcaatat tgaccagata
4441 aaaataccat gaagatgttg atattgtaca aaaagaactc taactcttta tataggaagt
4501 cgttcaatgt tgtcagttat gactgttttt taaaacaaag aactaactga ggtcaagggc
4561 taggagaata ttcaggaatg agttcactag aaacatgatg ccttccatag tctccaaata
4621 atcatattgg aattagaaag gaagtagctg gcagagctgt gcctgttgat aaaatcaatc
4681 cttaatcact ttttccccca acaggTGCAG TTTTGCCAAG GAGTGCTAAA GAACTTAGAT
4741 GTCAGTGCAT AAAGACATAC TCCAAACCTT TCCACCCCAA ATTTATCAAA GAACTGAGAG
4801 TGATTGAGAG TGGACCACAC TGCGCCAACA CAGAAATTAT GTAAgtactt taaaaaagat
4861 tagatatttt gttttagcaa acttaaaatt aaggaaggtg gaaatattta ggaaagttcc
4921 aggtgttagg attacagtag taaatgaaac aaaacaaaat aaaaaatatt tgtctacatg
4981 acatttaaat atggtagctt ccacaactac tataaatgtt attttggact tagactttat
5041 gcctgactta aggaatcatg atttgaatgc aaaaactaaa tattaatctg aaccatttct
5101 ttcttatttc agtgtaaagC TTTCTGATGG AAGAGAGCTC TGTCTGGACC CCAAGGAAAA
5161 CTGGGTGCAG AGGGTTGTGG AGAAGTTTTT GAAGAGgtaa gttatatatt ttttaattta
5221 aatttttcat ttatcctgag acatataatc caaagtcagc ctataaattt ctttctgttg
5281 ctaaaaatcg tcattaggta tctgcctttt tggttaaaaa aaaaaggaat agcatcaata
5341 gtgagtttgt tgtactcatg accagaaaga ccatacatag tttgcccagg aaattctggg
5401 tttaagcttg tgtcctatac tcttagtaaa gttctttgtc actcccagta gtgtcctatt
5461 ttagatgata atttctttga tctccctatt tatagttgag aatatagagc atttctaaca
5521 catgaatgtc aaagactata ttgacttttc aagaacccta ctttccttct tattaaacat
5581 agctcatctt tatattttta atTTTATTTT AGGGCTGAGA ATTCATAAAA AAATTCATTC
5641 TCTGTGGTAT CCAAGAATCA GTGAAGATGC CAGTGAAACT TCAAGCAAAT CTACTTCAAC
5701 ACTTCATGTA TTGTGTGGGT CTGTTGTAGG GTTGCCAGAT GCAATACAAG ATTCCTGGTT
5761 AAATTTGAAT TTCAGTAAAC AATGAATAGT TTTTCATTGT ACCATGAAAT ATCCAGAACA
5821 TACTTATATG TAAAGTATTA TTTATTTGAA TCTACAAAAA ACAACAAATA ATTTTTAAAT
5881 ATAAGGATTT TCCTAGATAT TGCACGGGAG AATATACAAA TAGCAAAATT GAGGCCAAGG
5941 GCCAAGAGAA TATCCGAACT TTAATTTCAG GAATTGAATG GGTTTGCTAG AATGTGATAT
6001 TTGAAGCATC ACATAAAAAT GATGGGACAA TAAATTTTGC CATAAAGTCA AATTTAGCTG
```

6061 GAAATCCTGG ATTTTTTTCT GTTAAATCTG GCAACCCTAG TCTGCTAGCC AGGATCCACA
6121 AGTCCTTGTT CCACTGTGCC TTGGTTTCTC CTTTATTTCT AAGTGGAAAA AGTATTAGCC
6181 ACCATCTTAC CTCACAGTGA TGTTGTGAGG ACATGTGGAA GCACTTTAAG TTTTTTCATC
6241 ATAACATAAA TTATTTTCAA GTGTAACTTA TTAACCTATT TATTATTTAT GTATTTATTT
6301 AAGCATCAAA TATTTGTGCA AGAATTTGGA AAAATAGAAG ATGAATCATT GATTGAATAG
6361 TTATAAAGAT GTTATAGTAA ATTTATTTTA TTTTAGATAT TAAATGATGT TTTATTAGAT
6421 AAATTTCAAT CAGGGTTTTT AGATTAAACA AACAAACAAT TGGGTACCCA GTTAAATTTT
6481 CATTTCAGAT AAACAACAAA TAATTTTTTA GTATAAGTAC ATTATTGTTT ATCTGAAATT
6541 TTAATTGAAC TAACAATCCT AGTTTGATAC TCCCAGTCTT GTCATTGCCA GCTGTGTTGG
6601 TAGTGCTGTG TTGAATTACG GAATAATGAG TTAGAACTAT TAAAACAGCC AAAACTCCAC
6661 AGTCAATATT AGTAATTTCT TGCTGGTTGA AACTTGTTTA TTATGTACAA ATAGATTCTT
6721 ATAATATTAT TTAAATGACT GCATTTTTAA ATACAAGGCT TTATATTTTT AACTTTAAGA
6781 TGTTTTTATG TGCTCTCCAA ATTTTTTTTA CTGTTTCTGA TTGTATGGAA ATATAAAAGT
6841 AAATATGAAA CATTTAAAAT ATAATTTGTT GTCAAAGTAA tcaagtgttt gtctttttt
6901 tagttttagc ttattgggat tctctttgtt tatatttaaa attatacttt gatttagaaa
6961 acataaatgc ttccccttag cattttgttt atggaaaatt acaaactttt atttttagaa
7021 aacagaactc ctttccagaa ataggttaca aacagtagtg tcctccacag aatgttggaa
7081 atgttttcaa ctccccactg tatactatct tgctaataag tctgtcttca gatttcgatt
7141 aaccggtttg tatgtc

Appendix F

List of suppliers

ABI Prism 3100 Genetic Analyser	Applied Biosystems, Foster City, California, USA
ABI PRISM 7500	Applied Biosystems
agarose	Bioline, London, UK
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
dNTPs	Promega Corporation, Madison, Wisconsin, USA
DuoSet ELISA Development Systems	R&D Systems, Minneapolis, Minnesota, USA
EDTA Vacutainers	Becton Dickinson, San Jose, California, USA
Electro-Fast Stretch 108 Gel Tank System	ABgene Ltd., Epsom, UK
ethanol	Merck Chemicals (Pty) Ltd, Nottingham, UK
ethidium bromide	CLP, San Diego, California, USA
Expand High Fidelity enzyme and buffer	Roche Diagnostics GmbH, Mannheim, Germany
Ficoll-Paque	Amersham Biosciences, Uppsala, Sweden
GeneAmp PCR System 9700	Applied Biosystems
$MgCl_2$	Promega Corporation
NanoDrop	NanoDrop Technologies, Wilmington, Detroit, USA
PCR primers and probes	University of Cape Town, South Africa
PHA	Sigma Chemical Co., St Louis, Missouri, USA
QIAamp DNA Blood Minikit	QIAgen, Hilden, Germany
QIAquick PCR Purification Kit	QIAgen
Quantikine ELISA kits	R&D Systems
RPMI medium	Invitrogen Corporation, Carlsbad, California, USA
sodium acetate	Merck
SPSS version 15.0	SPSS Inc., Chicago, Illinois, USA
SYBR Green	Applied Biosystems
Taq DNA polymerase and buffer	Promega Corporation
TaqMan Universal PCR Master Mix	Applied Biosystems
TBE	Invitrogen Corporation
trypan blue	Sigma

Appendix G

Ethical clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Donninger

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M071162**
PROJECT	Chemokine Production in HIV-1 Infection and Pulmonary Tuberculosis
INVESTIGATORS	Ms SL Donninger
DEPARTMENT	Virology & Comm Diseases
DATE CONSIDERED	07.11.30
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

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

DATE 07.12.07 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof C Tiemessen

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES