THE ROLE OF INTERLEUKIN-8 IN THE IMMUNOPATHOGENESIS OF HIV-1 DISEASE AND TUBERCULOSIS

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ABSTRACT

Interleukin-8 (IL-8), a member of the C-X-C chemokine subfamily, is an important chemoattractant and cellular activator. This study was conducted to determine the role of IL-8 in the immunopathogenesis of HIV-1 disease and tuberculosis.

The first section involved determining the effect of infection with HIV-1, *Mycobacterium tuberculosis* and co-infection with both of these organisms on IL-8 production *in vivo*. This was monitored by the determination of levels of serum or plasma IL-8 and peripheral cell-associated IL-8, assessing peripheral mononuclear (PBMC) and polymorphonuclear (PMN) cell capacity to produce IL-8 spontaneously or in response to various stimuli, and the detection of constitutive IL-8 mRNA expression in purified subsets of mononuclear cells. Results show that whereas there is evidence of detectable levels of cell-associated IL-8 (mRNA and protein) in peripheral cells of healthy individuals, this is largely lost in the disease states studied. Coupled with this was significantly increased circulating levels of IL-8 in serum and plasma found in HIV-1 infected individuals with or without concomitant pulmonary TB. On the other hand, the capacity of PBMC to produce IL-8 spontaneously *ex vivo* was enhanced in HIV-1 and TB patients and many of the HIV/TB group, but their corresponding capacities to respond to various stimuli was significantly diminished when compared to that of the normal donors. The release of IL-8 from PMN in the presence of an agonist was diminished mainly in individuals with pulmonary TB, which was further exacerbated by the presence of HIV-1 infection.

HIV-1-infected individuals have an increased incidence of bacterial infections which could be related to defective functioning of PMN. The second section was aimed at detecting PMN abnormalities in HIV and HIV/TB patients by monitoring IL-8-induced β-glucuronidase release and PMN chemotaxis in response to IL-8. IL-8-induced β-glucuronidase release from PMN of normal individuals and TB patients occurred in a dose-dependent manner. In contrast, PMN from

HIV-1 infected individuals, whether co-infected with *M. tuberculosis* or not, showed a reciprocal response in that increasing IL-8 concentrations resulted in decreased enzyme release. This reciprocal slope of the IL-8 dose-response curve was altered for the majority of HIV-1 positive individuals tested irrespective of their $CD4^+$ cell counts. In addition, PMN chemotaxis in response to IL-8 was also found to be significantly impaired in a group of HIV-1 infected patients co-infected with *M. tuberculosis* when compared to healthy individuals.

The third section of the study involved analysing the expression of the PMN cell surface markers, FcγRIII (CD16), and the two human IL-8 receptors, designated IL-8RA and IL-8RB. FcγRIII (CD16) expression on the surface of PMN was significantly reduced in HIV-1 seropositive patients with pulmonary tuberculosis when compared to those individuals with either disease alone or healthy blood donors. A significant reduction in the percentage of PMN expressing IL-8RA and IL-8RB and in their respective fluorescence intensities was found in TB, HIV, and HIV/TB groups when compared to that obtained for the ND group. IL-8RA intensity of fluorescence was significantly decreased in the HIV/TB group when compared to the TB and HIV groups indicating a further down-regulation of IL-8RA expression owing to dual infection. On the other hand, IL-8RB fluorescence intensity was substantially reduced on PMN from patients with pulmonary TB and to a greater degree in those patients co-infected with HIV-1 and *M. tuberculosis*. Having found a reduction in the expression of both IL-8 receptors on PMN in all the infection groups, cellular events following the binding of IL-8 to IL-8 receptors on PMN isolated from dually infected patients, the group which showed the greatest reduction in IL-8 expression was analysed. Results indicated that the impairment of IL-8-dependent PMN functions such as degranulation and chemotaxis was associated with the reduced expression of IL-8 receptors on these cells.

Increased circulating levels of IL-8 in HIV-1 infection and a diminished cellular capacity to produce IL-8 as shown in this study may have important implications for antimicrobial defences and normal immune processes. A dysregulated production of IL-8 *in vivo* is likely to play a role in the pathogenesis of HIV-1 disease, pulmonary tuberculosis, and dual infections with both organisms. In addition, cellular responses dependent on specific receptor engagement and the subsequent translation of signal transducing events that lead to phagocyte effector functions are

clearly impaired in IL-8 receptor deficient phagocytes. Abnormal PMN functioning in HIV-1 infected individuals, as shown here by defective degranulation and chemotactic responses, have important implications in the pathogenesis of HIV-1 infection in terms of their ability to clear secondary microbial infections. Future attempts should be aimed at defining the mechanisms that bring about these changes in order to contribute to a greater understanding of the mechanisms that lead to an enhanced risk of superinfections in immunosuppressed individuals.

DECLARATION

I declare that this thesis 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.

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CHAPTER ONE

INTRODUCTION

1.1 HIV-1 infection and tuberculosis

Infection with human immunodeficiency virus type 1 (HIV-1) has been shown to be the largest known risk factor for the development of tuberculosis (Murray, 1989) which is typically an early complication of HIV infection, occurring before acquired immunodeficiency syndrome (AIDS)-defining illness in 50%-67% of HIV-1 infected patients (Ellner, 1990). This presumably occurs because *Mycobacterium tuberculosis* is more virulent than other HIV-1 associated pathogens, such as *Pneumocystis carinii* and *Mycobacterium avium* complex (Barnes *et al.*, 1991). The development of tuberculosis in HIV-1 infected individuals may be the result of reactivation of a latent mycobacterial infection (Selwyn *et al.*, 1989), a re-infection with *M. tuberculosis* (Small *et al.*, 1993), or the rapid progression of a recently acquired infection (Edlin *et al.*, 1992).

During primary infection of immunocompetent persons with *M. tuberculosis*, macrophages phagocytose the organisms and present mycobacterial antigens to T-cells. The capacity of macrophages to ingest and kill mycobacteria is enhanced by lymphokines which are secreted by CD4 cells. Infection with HIV-1 has a profound effect on cell-mediated immunity, where there is a progressive depletion and dysfunction of CD4 cells, coupled with defects in macrophage and monocyte function (Bender *et al.*, 1988). Since macrophages and CD4 cells play a central role in anti-mycobacterial defences, dysfunction of these cells place patients infected with HIV-1 at a high risk for primary infection or reactivation with tuberculosis.

Studies have shown that in active tuberculosis, patients who are co-infected with HIV-1 have a reduced survival rate compared to patients without HIV-1 infection (Nunn *et al.*,1992;

Stoneburner *et al.*, 1992). Individuals infected with both organisms also have an increased risk of acquiring new opportunistic infections when compared to those infected with HIV-1 alone (Whalen *et al.*, 1995). Although the cellular immune activation that occurs in a host infected with HIV-1 and *M. tuberculosis* may be beneficial in terms of the mycobacterial infection, it may be deleterious in terms of the HIV-1 disease. The cellular production of tumor necrosis factor-α (TNF-α) may be particularly important in this regard, since this cytokine is produced in response to mycobacterial products (Valone *et al.*, 1988; Wallis *et al.*, 1990), is produced in increased amounts in active tuberculosis (Fujiwara *et al.*, 1986; Takashima *et al.*, 1990; Ogawa *et al.*, 1991), and is important in granuloma formation (Kindler *et al.*, 1989). On the other hand, TNF-α promotes the expression of HIV-1 in both lymphocytes and monocytes (Kinter *et al.*, 1990; Latinan *et al.*, 1990; Potts *et al.*, 1990; Griffin *et al.*, 1991; Lederman *et al.*, 1994), and thus the increased expression of TNF-α in tuberculosis may have the undesirable effect of promoting HIV-1 expression. Several other cytokines have been demonstrated to modulate HIV-1 expression *in vitro*, including interleukin (IL)-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) which up-regulate expression, and interferon (IFN)-γ, IL-10, and transforming growth factor (TGF)-β which have been shown to have a bi-functional activity on HIV-1 replication (Rosenburg and Fauci, 1990; Pantaleo *et al.*, 1993; Fauci, 1996).

The proposal that *M. tuberculosis* accelerates the progression of HIV-1 infection is supported by both clinical studies (Isaksson *et al.*, 1988; Pape *et al.*, 1993; Toossi *et al.*, 1993; Wallis *et al.*, 1993; Martin *et al.*, 1995; Whalen *et al.*, 1995) and *in vitro* studies, which have demonstrated that phagocytosis of tubercle bacilli by HIV-1 infected monocyte lines induces viral expression (Gollapudi *et al.*, 1994; Shattock *et al.*, 1994; Zhang *et al.*, 1995b). Other *in vitro* studies have shown the activation of latent HIV-1 by *M. tuberculosis* and its purified protein derivative in alveolar macrophages from HIV-1-infected individuals (Toosi *et al.*, 1997). During tuberculosis, large amounts of cytokines such as TNF-α, IL-6, IFN-γ, and GM-CSF are accumulated in the pleural compartment (Barnes *et al.*, 1990; Barnes *et al.*, 1993; Maeda *et al.*, 1993), and studies with pleural fluids have indicated that this milieu generates a micro-environment which enhances the productive infection of local lymphocytes by HIV-1 (Garrait *et al.*, 1997). Studies by Mancino *et al.*(1997) indicated that *M. tuberculosis* infection can up-regulate both HIV-1 infection and replication within human monocyte-derived macrophages

(MDM). In addition, it was found that MDM infected with *M. tuberculosis* are able to drive antigen specific T-cell proliferation, thereby facilitating the transmission of the virus to T-cells.

1.2 The chemokines

The chemokines are a family of secreted proteins which mediate a variety of inflammatory processes including activation and chemotaxis of neutrophils, monocytes and lymphocytes to sites of infection (Oppenheim *et al.*, 1991; Schall, 1991; Miller and Krangel, 1992). The chemokines have a molecular mass of between 8-kDa and 10-kDa and demonstrate 20-50% sequence homology at the amino acid level. The combined effect of multiple chemokines are assumed to be responsible for the cellular composition at inflammatory sites. Members of this family share a highly conserved cysteine motif, the arrangement of which forms the basis of dividing the family into four subfamilies. In the first subfamily, the first two cysteine residues are adjacent (C-C), whereas in the second subfamily, the first cysteine pair is separated by an intervening amino acid (C-X-C) (Balwit and Haak-Frendscho, 1993). In the third subfamily, the second cysteine is absent (C) (Kelner *et al.*, 1994), and in the fourth, the first pair of cysteines is separated by three amino acids (C-X_3-C) (Bazan *et al.*, 1997; Pan *et al.*, 1997). The C and C-X_3-C chemokine subfamilies have only one member each described to date, those being lymphotactin and neurotactin, respectively. Lymphotactin is principally chemotactic for $CD8^+$ T lymphocytes (Kelner *et al.*, 1994), while neurotactin or fractalkaline has been reported to act as a chemoattractant for neutrophils, monocytes, and T-cells (Bazan *et al.*, 1997; Pan *et al.*, 1997). The best characterized of the subfamilies are the C-C and C-X-C chemokines.

1.2.1 C-C chemokines

The C-C chemokines (ß-chemokines) act on monocytes and some on T-lymphocyte subsets (Schall *et al.*,1993; Tanaka *et al.*, 1993; Taub *et al.*, 1993). Members of this subfamily include macrophage inflammatory protein (MIP-1α) and MIP-1ß, RANTES (regulation upon activation normal T-expressed and secreted), I-309 and MCAF (Balwit and Haak-Frendscho, 1993).

1.2.2 C-X-C chemokines

The C-X-C chemokines, which act predominantly on neutrophils, can be further subdivided into ELR^+ and ELR^- groups, based on the presence or absence of a Glu-Leu-Arg (ELR) sequence motif in the adjacent position N-terminal to the C-X-C group (Baggiolini *et al.*, 1994). Chemokines belonging to the ELR^+ group include IL-8, growth regulated oncogene (GRO)α, GROβ, GROγ, neutrophil activating peptide-2 (NAP-2), epithelial cell derived neutrophil activating peptide-78 (ENA-78), and granulocyte chemoattractant protein-2. Members from the ELR^+ group, including NAP-2, IL-8, and ENA-78 have been shown to exhibit various neutrophil stimulating functions, including oxygen radical formation (Thelen *et al.*, 1988; Brandt *et al.*, 1989; Walz *et al.*, 1991b), degranulation (Walz and Baggiolini, 1989; Moser *et al.*, 1990), chemotactic migration (Schröder *et al.*, 1987; Walz *et al.*, 1989; Balentien *et al.*, 1990; Leonard *et al.*, 1991; Walz *et al.*, 1991a), and the upregulation of adhesion molecules (Detmers *et al.*, 1991).

1.2.2.1 Interleukin-8

Interleukin-8 (IL-8), the best characterized of the C-X-C chemokines, is an 8-kDa polypeptide which has been described as a neutrophil chemotactic factor which is produced by stimulated human blood mononuclear leukocytes (Yoshimura *et al.*, 1987). It is a polypeptide which is synthesized as a 99-amino acid precursor which is processed to a 8-kDa mature form of 72-amino acids. Cloning and sequencing data for human IL-8 suggest that the full length mRNA consists of a 101 base 5' untranslated region, an open reading frame of 297 bases and a 1.2 kilobase 3' untranslated region (Matsushima *et al.*, 1988). Using nuclear magnetic resonance analysis (Clore *et al.*, 1990) and X-ray crystallography (Baldwin *et al.*, 1991), the three-dimensional structure of IL-8 has been determined, which consists of a triple-stranded anti-parallel sheet, connected with loops and a long carboxyl-terminal α-helix. The structural arrangement of the two anti-parallel α-helices suggest a specific receptor interaction site, similar to a region in the human class I major histocompatability (MHC) antigen, which is involved in both antigen and T-cell receptor binding (Clore *et al.*, 1990). The mature IL-8 contains four cysteine residues, which form disulphide bridges between Cys-7 and Cys-34 and between Cys-9 and Cys-50, resulting in folding of the peptide (Baldwin *et al.*, 1991). IL-8 has also previously been known by a variety of descriptive

terms including monocyte-derived neutrophil chemotactic factor (MDNCF), granulocyte chemotactic protein (GCP), neutrophil activating factor (NAF) and T-lymphoctye chemotactic factor (TCF).

IL-8 has been shown to be produced by a number of cell types including T-lymphocytes, endothelial cells, keratinocytes, fibroblasts (Liles and Van Hoorhis, 1995), and neutrophils (Cassatella, 1995) although the most prominent source is monocytes/macrophages (Yoshimura *et al.*,1987; Colditz *et al.*, 1989). Biologic actions of IL-8 include the induction of respiratory burst (Walz *et al.*, 1991b), induction of chemotaxis of neutrophils (Matsushima and Oppenheim, 1989), T-cells (Larsen *et al.*, 1989; Jinquan *et al.*, 1993), and basophils (Leonard *et al.*,1991), promoting release of lysosomal enzymes from neutrophils, and enhanced killing of various microorganisms including *M. tuberculosis* (Liles and Van Hoorhis, 1995).

It has been shown that IL-8 production is elevated in a number of diseases including *Plasmodium falciparum* malaria (Friedland *et al.*, 1993), influenza virus A infection (Choi and Jacoby, 1992), respiratory syncytial virus nasal infection (Becker *et al.*, 1993; Noah and Becker, 1993), rheumatoid arthritis (Endo *et al.*, 1991), Epstein-Barr virus infection (McColl *et al.*, 1997), human herpesvirus-6 infection (Inagi *et al.*, 1996), and severe sepsis associated with *Pseudomonas pseudomallei* infection (Friedland *et al.*, 1992b). With regard to IL-8 levels in HIV-1 infected patients, Matsumoto *et al.* (1993) have reported elevated serum levels of IL-8. Levels of IL-8 have also been reported to be significantly elevated in cerebrospinal fluid (CSF) of HIV-1 infected patients with cryptococcal meningitis (Chaka *et al.*, 1997) and in the plasma of asymptomatic HIV-1 infected individuals, although not in AIDS patients (Thea *et al.*, 1996).

1.3 Leukocyte receptors

1.3.1 Adhesion molecules and intergrins

As a result of tissue injury or infections, neutrophils migrate to sites of inflammation, elicited by a variety of inflammatory mediators. Neutrophil recruitment begins with an increased endothelial expression of the adhesion molecules E-selectin and P-selectin, which bind weakly to the

neutrophil ligand sialylated Lewis-X (Mayadas *et al.*, 1993; Vestweber, 1993). Selectin bonds are rapidly established and reversed, but are resistant to shear, and thus are well suited for the initial tethering event in flowing blood, which manifests as the rolling of neutrophils along the endothelial surface (Zimmerman *et al.*, 1992; McEver *et al.*, 1995). This weak interaction, coupled with exposure to inflammatory mediators such as complement fragments and cytokines such as IL-8 and TNF, results in an increased expression of members of the β_2 intergrin family, such as MAC-1 (CD11b/CD18)(Carveth *et al.*, 1989; Kishimoto *et al.*, 1989; Detmers *et al.*, 1990; Huber *et al.*, 1991), allowing tight binding to endothelial intercellular adhesion molecule-1 (ICAM-1), and migration into the tissues toward a chemotactic gradient. Soluble chemoattractant gradients cannot however persist on the blood-endothelial cell interface since they are likely to be washed away by the blood flow (Rot, 1992b; Tanaka *et al.*, 1993). Various observations, including the *in situ* binding of IL-8 to endothelial cells in human skin (Rot, 1992a), and the ability of immobilized IL-8 to attract neutrophils *in vitro* (Rot, 1993), led to suggestions that *in vivo*, IL-8 and other chemokines may promote neutrophil-endothelial cell adhesion more effectively while immobilized on the endothelial cell membrane (Rot, 1992b; Tanaka *et al.*, 1993). This has recently been shown by Middleton *et al.* (1997), using ^{125}I-IL-8 and electron microscopy, that endothelial cells internalize IL-8 and transcytose it to the luminal surface, where it is presented to adherent leukocytes on the tips of endothelial cell microvilli.

1.3.2 Fc receptors

Fcγ receptors are cell surface molecules, found on phagocytic cells such as neutrophils, monocytes and macrophages, which bind the Fc region of IgG class antibodies, providing an essential link between cellular effector mechanisms and humoral immunity. The triggering of Fcγ receptors induces phagocyte activation, and in addition, Fcγ receptors are required for the binding, ingestion, and destruction of foreign particles opsonized by IgG antibodies (Fanger *et al.*, 1989; Van de Winkel and Anderson, 1991; Van de Winkel and Capel, 1993; Ravetch, 1994). Three subclasses of Fcγ receptor have been identified, which are subdivided according to their cellular distribution, molecular weight and ligand binding specificity. These are FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) (Van de Winkel and Anderson, 1991).

FcγRI is a 72kDa glycoprotein, found mainly on monocytes and macrophages, which has a high avidity for monomeric IgG. The receptor has a short charged cytoplasmic tail, and contains three Ig domains in its extracytoplasmic portion (Fridman, 1991).

FcγRII by comparison, is not a single receptor, but a family of closely related 40-kDa transmembrane glycoproteins which have a low avidity for monomeric IgG, although all FcγRII receptors avidly bind IgG aggregates or immune complexes (Van de Winkel and Anderson, 1991). At least six isoforms of FcγRII have been identified, with one or other being found on monocytes, macrophages, neutrophils, and B-cells (Qui *et al.*, 1990; Van de Winkel and Anderson, 199[illegible] In humans, the structural and functional diversity of FcγRII is due to alternative mRNA splicing of intracellular regions and the existence of several genes (Hulett and Hogarth, 1994). FcγRII is normally expressed on the surface of cells, but has also been detected within the cytoplasm of normal human peripheral blood lymphocytes (Sandilands *et al.*, 1995). It is thought this "occult" CD32 may represent an internal receptor pool which is up-regulated upon cell activation. Following FcγR-ligand interactions, receptor cross-linking occurs, and certain isoforms of FcγRII (eg. FcγRIIa$_1$ and FcγRIIb$_2$) become internalized and are recycled back to the plasma membrane (Gergely *et al.*, 1994). This FcγR cross-linking by ligand is required to initiate function, and may be an important mechanism in signal transduction pathways (Heyman, 1990).

FcγRIII, which is found on neutrophils, macrophages, and NK cells is a receptor with low affinity for monomeric IgG, and preferentially binds immune complexes. Two homologous FcγRIII genes (hFcγRIIIA and -B) have been found for this receptor, which differ in only nine nucleotides in the coding regions. FcγRIIIA codes for the receptor on macrophages and NK cells, while FcγRIIIB encodes the receptor found on neutrophils. Although both receptors have two Ig domains in their extracytoplasmic portion, FcγRIIIa has a typical hydrophobic transmembrane domain and cytoplasmic tail, which differs from FcγRIIIb where the receptor is attached to the plasma membrane via a glycosyl-phosphatidylinositol (GPI) linkage (Kurosaki and Ravetch, 1989; Lanier *et al.*, 1989; Scallon *et al.*, 1989; Selveraj *et al.*, 1989). In addition to being found on the surface of neutrophils, FcγRIIIb has been detected intracellularly, within the cytoplasm (Jost *et al.*, 1991). Soluble FcγRIII has also been detected in plasma, and is thought to be derived from FcγRIII receptors being released from the surface of neutrophils (Huizinga *et al.*, 1990a).

1.3.3 Chemokine receptors

1.3.3.1 IL-8 receptors

Studies have identified two types of IL-8 receptor, namely IL-8RA or CXCR-1 (Holmes *et al.*, 1991) and IL-8RB or CXCR-2 (Murphy and Tiffany, 1991), which have a 29-34% sequence homology with the complement 5a (C5a) and N-formylmethionyl-leucylphenylalanine (FMLP) receptors and share 77% amino acid identity (Holmes *et al.*, 1991). These two receptors are members of the superfamily of seven transmembrane, G-protein coupled receptors (Taylor, 1990) which exist in a variety of forms, ranging from the IL-8 receptors which have a narrow ligand specificity, to the multi-specific erythrocyte chemokine receptor (duffy group antigen) which has a broad chemokine binding specificity (Horuk, 1994). Although IL-8RA and IL-8RB share considerable amino acid sequence identity within the seven transmembrane domains and connecting loops, the amino terminal domains of the two IL-8 receptors show low amino acid identity (24%), which confers the typical ligand specificity of IL-8RA and IL-8RB (LaRossa *et al.*, 1992; Gayle *et al.*, 1993). IL-8 has been shown to bind with high affinity (K_d values between 0.5 to 3nM) to both receptors, whereas all the other C-X-C chemokines have a high affinity for IL-8RB only (Moser *et al.*, 1991; Schumacher *et al.*, 1992; Cerretti *et al.*, 1993). The ELR site has been shown to be important for the binding of IL-8 to each of its receptors, although additional regions such as the carboxyl terminal α-helical region of IL-8 binding to the β-receptor, appear necessary for high affinity binding (Schraufstätter *et al.*, 1993).

Specific receptors for IL-8 have been identified on human neutrophils, monocytes, and several myeloid cell lines (Samanta *et al.*, 1989; Grob *et al.*, 1990; Leonard *et al.*, 1990). In addition to these cell types, IL-8 has been reported to be chemotactic for T-lymphocytes (Bacon *et al.*,1989; Larsen *et al.*, 1989), basophils (Leonard *et al.*, 1990), natural killer (NK) cells (Sebok *et al.*, 1993), and melanocytes (Wang *et al.*, 1990; Moser *et al.*, 1993), which suggests the expression of IL-8 receptors on these cells. Chuntharapai *et al.* (1994) carried out a series of experiments with monoclonal antibodies to investigate the expression of IL-8RA and IL-8RB on various cell types. They found that the receptors for IL-8 are present on all neutrophils, monocytes, and 5-25% of total lymphocytes. The highest levels of IL-8RA and IL-8RB were

found on neutrophils, with IL-8RA and IL-8RB expression occurring at an approximately equal ratio. On monocytes and IL-8 receptor positive lymphocytes, higher levels of IL-8RB were expressed than that of IL-8RA. With regard to the IL-8 receptor positive lymphocytes, 7-42% of $CD8^+$ T-cells, and 39-76% of $CD56^+$ NK cells were shown to express IL-8 receptors. These results indicate that only certain subpopulations of $CD8^+$ T-cells and $CD56^+$ NK cells express the IL-8 receptors, which could suggest different stages of cell activation. $CD4^+$ T-cells and $CD20^+$ B-cells were found not to express IL-8 receptors. In contrast to the $CD4^+$ T-cell results described by Chuntharapai *et al.* (1994), $CD4^+$ T-helper (Th) cells have been shown to migrate in response to IL-8, suggesting the presence of IL-8 receptors on this cell population (Xu *et al.*, 1994). A recent report by Bonecchi *et al.* (1998), looking at the differential expression of chemokine receptors on $CD4^+$ T-cells found no detectable IL-8RA or IL-8RB on Th1 or Th2 $CD4^+$ T-cells, confirming the observations by Chuntharapai *et al.* (1994).

Jinquan *et al.* (1997) have recently described IL-8 and GRO-α induced chemotactic migration and IL-8 receptor expression on B-cells from normal (N-B cells) and HIV-infected (HIV-B cells) subjects. In this study, IL-8 receptors were detected on purified $CD19^+$ N-B cells and HIV-B cells, in comparison to other studies (Chuntharapai *et al.*, 1994; Morohashi *et al.*, 1995) carried out on total mononuclear cells in peripheral blood, which reported a failure in detecting IL-8 receptors on N-B cells. The chemotactic migration of N-B cells and HIV-B cells was shown to occur via stimulation of IL-8RB. Th1 and Th2-like cytokines can regulate N-B cell and HIV-B cell chemotaxis towards GRO-α and IL-8 by regulating the expression of IL-8RB on these cells. The chemotaxis of N-B cells was augmented by IL-4 and IL-13 (Th2 cytokines), inhibited by IFNγ and IL-2 (Th1 cytokines), whereas TNFα and IL-10 had no influence. A different pattern was observed for the HIV-B cells, where chemotaxis was augmented by TNFα, IL-4, and IL-10, inhibited by IFNγ and IL-2, with IL-13 exerting no influence. Another interesting observation was that IL-8 receptors were expressed more abundantly on freshly isolated HIV-B cells (51%) compared to N-B cells (15%) (Jinquan *et al.*, 1997).

Studies carried out with two monoclonal antibodies raised against both IL-8 receptors of human neutrophils, indicate that IL-8RA and IL-8RB are functionally different (Jones *et al.*, 1996). Granule enzyme release is a characteristic response of neutrophils to chemokines such as

IL-8 (Peveri *et al.*, 1988), NAP-2 (Walz *et al.*, 1989), and the GRO proteins (Geiser *et al.*, 1993). Elastase release from neutrophils, stimulated with IL-8 and GRO-α was measured in the presence of anti-IL-8RA and/or anti-IL-8RB, and the results indicated that both IL-8 receptors can signal independently for granule enzyme release. Binding studies with ^{125}Iodine (I)-labelled IL-8 showed that changes of cytosolic free Ca^{2+} are also mediated by both receptors. Other functions however, such as the activation of phospholipase D (PLD), and superoxide production via NADPH oxidase are triggered exclusively by IL-8RA (Jones *et al.*, 1996). Chemotaxis has been shown to be independently mediated by both IL-8 receptors (Loetscher *et al.*, 1994), although Hammond *et al.* (1995) found neutrophil chemotaxis was predominantly mediated via IL-8RA. IL-8 has been shown to be more potent than NAP-2 in attenuating neutrophil chemotaxis, which can be related to their divergent capabilities to phosphorylate IL-8RB (Ben-Baruch *et al.*, 1997).

On human neutrophils, IL-8 has been shown to dynamically regulate its own receptor expression. IL-8 very rapidly down-regulates its receptor expression, but the down-regulated receptor can be rapidly recycled to the surface of neutrophils, which may be essential for normal cellular chemotaxis (Samanta *et al.*, 1990). Other studies have indicated that IL-8RA and IL-8RB on T-lymphocytes can be up-regulated by IFNγ, TNFα, and IL-2 (Jinquan *et al.*, 1995). Receptor densities have been calculated at 20000 receptors/cell on neutrophils and 1040 receptors/cell for peripheral blood mononuclear cells. The chromosonal location of the IL-8RA and IL-8RB genes, as well as a pseudogene for IL-8RB, have been mapped to position 2q35 in the human genome (Ahuja *et al.*, 1992; Lloyd *et al.*, 1993).

1.3.3.2 C-C chemokine receptors and HIV-1 infection

Considerable interest has recently been focussed on the chemokines following work indicating that certain chemokines suppress the production of HIV-1. These inhibitors of HIV-1 replication have been characterized as the ß-chemokines RANTES, MIP-1α and MIP-1ß (Cocchi *et al.*, 1995). With the receptor for these chemokines, CCR-5, being identified as a co-receptor for entry of macrophage-tropic strains of HIV-1, results indicate that RANTES, MIP-1α and MIP-1ß inhibit HIV-1 infection by interfering with the virus-cell fusion reaction (Dragic *et al.*, 1996). The actual mechanism of chemokine blocking remains unclear however, although it is thought it may involve

binding site competition or desensitization of the receptor through conformational changes or downregulation (Deng *et al.*, 1996). Although HIV-1 predominantly uses one, or occasionally both, of the major co-receptors CCR-5 or CXCR-4 to infect target cells, other C-C chemokine receptors, including CCR-2 and CCR-3, have been shown to function as minor co-receptors (Choe *et al.*, 1996; Doranz *et al.*, 1996; Rana *et al.*, 1997). Studies have recently identified a conservative substitution in the coding region of CCR-2, causing a valine to isoleucine switch in the first transmembrane region of the CCR-2 receptor (Smith *et al.*, 1997). Although the functional consequences of this transition are unclear, it was found to delay HIV-1 disease progression, but not reduce the risk of infection (Smith *et al.*, 1997; Kostrikis *et al.*, 1998). In addition, mutant CCR-5 alleles, which will not support HIV-1 fusion have been reported (Dean *et al.*, 1996; Lui *et al.*, 1996; Samson *et al.*, 1996), where a 32 base- pair deletion results in a mutant protein which lacks three of the seven transmembrane regions, as well as the sequence involved in G-protein coupling.

1.3.3.3 C-X-C chemokine receptors and HIV-1 infection

HIV viruses infect T-helper cells and $CD4^+$ macrophages (Sattentau and Weiss, 1988) and although HIV-1 entry into target cells requires cell surface expression of CD4, studies have indicated that additional host cell factors are required for entry of HIV-1 virions into $CD4^+$ cell lines. A member of the seven transmembrane G-protein coupled receptors, originally named Fusin, and recently designated CXCR-4, was shown to be a co-receptor required for HIV-1 infection in T-cell lines (Feng *et al.*, 1996). CXCR-4, is most closely related to the IL-8 receptor, having 39% homology in the transmembrane domains (Jazin *et al.*, 1993). Further studies identified the lymphocyte chemoattractant, stromal cell-derived factor-1 (SDF-1), which is a member of the C-X-C chemokine subfamily, as being the ligand for CXCR-4. SDF-1 inhibited the infection of HeLa CD4-expressing cells, CXCR-4 transfectants and peripheral blood mononuclear cells (PBMC) by T-tropic HIV-1 strains, but had no effect on the CCR-5 mediated infection by macrophage-tropic and dual-tropic primary HIV-1 (Bleul *et al.*, 1996). Recently, a mutant form of the gene coding for SDF-1 has been discovered (Winkler *et al.*, 1998), which delays the progress of HIV-1-infected patients to AIDS. It is thought that the mutation protects individuals by increasing the production or availability of SDF-1, thereby blocking the virus from entering T-cells.

1.4 Neutrophil function in HIV-1 infection and tuberculosis

Polymorphonuclear neutrophils (PMN) are key effector cells in non-specific host defence (Sandborg and Smolen, 1988), and as a result of infections or tissue injury are recruited to sites of inflammation. Large numbers of neutrophils are rapidly recruited from the bloodstream to an inflamed site (Roitt *et al.*, 1985), where they avidly ingest and efficiently kill a variety of microbial pathogens. The migration of neutrophils occurs by a coordinated cascade, with chemokines serving to attract cells to the site of injury. The five principal neutrophil chemotactic factors are IL-8, FMLP, platelet activating factor (PAF), C5a, and leukotriene B_4 (LTB_4). In addition to being involved in the extravasion of neutrophils, these agents act in conjunction with other cytokines and chemotactic agonists in priming neutrophils to respond more effectively once at the site of inflammation (Atkinson *et al.*, 1988; Dahinden *et al.*, 1988; Sullivan *et al.*, 1989; McColl *et al.*, 1990; Stewart *et al.*, 1991; Daniels *et al.*, 1992).

Opsonized micro-organisms are phagocytosed by neutrophils and killed by either an oxygen-dependent or oxygen-independent mechanism. The oxygen-dependent mechanisms occur when the neutrophils undergo respiratory burst which results in the production of a series of toxic oxygen intermediates. In this case, the enzyme NADPH oxidase, which is inactive until the engagement of various receptors, catalyses the formation of the superoxide radical (O_2^-), which can be rapidly converted into hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and other toxic species (Babior, 1978; Badwey and Karnovsky, 1980). The non-oxidative armature of neutrophils, on the other hand, involves the action of potent antimicrobial polypeptides contained within cytoplasmic granules (Lehrer and Ganz, 1990), which are released into phagolysosomes. Primary granules, also referred to as azurophilic granules (Damiano *et al.*, 1988), are first seen in the promyelocyte during the process of neutrophil maturation. These granules, which bud from the concave surface of the golgi complex, contain lysozyme, myeloperoxidase, proteases and acid hydrolases (eg. β-glucuronidase and elastase), which disrupt microbial functions or structural components. As the myelocyte divides, the number of primary granules per cell progressively declines, as no further primary granules are produced at this stage. Only one third of granules in mature neutrophils are primary granules, with the balance being either secondary (specific) or tertiary granules. Secondary granules, first seen in myeloctes and metamyelocytes also harbour

lysozyme, in addition to containing lactoferrin, cytochrome b and collagenase (Kanwar and Cairo, 1993). The killing of *M. tuberculosis* by PMN occurs via non-oxidative means (Jones *et al.*, 1990), with cytokines such as interleukin-8 (Nibbering *et al.*, 1993) and interferon-γ (Geertsma *et al.*, 1990) further enhancing mycobacterial killing.

Patients infected with HIV-1 display a variety of immune abnormalities, including various defects in the microbicidal response of phagocytic cells, which could contribute to the impaired host defence against various opportunistic pathogens that characterise AIDS. Infection with HIV-1 has been shown to be the largest known risk factor for the development of tuberculosis (Murray, 1989), and individuals infected with both organisms also have an increased risk of acquiring new opportunistic infections (Whalen *et al.*, 1995). In addition to tuberculosis, opportunistic infections frequently found in HIV-1 infected patients include other bacterial pathogens such as *Streptococcus pneumoniae, Salmonella* sp., and *Pseudomonas aeruginosa*, fungal infections such as cryptococcal meningitis and *Pneumocystis carinii* pneumonia, and parasitic pathogens like *Toxoplasma gondii* (Jentsch, 1997).

In addition to the functional abnormalities reported in monocytes/macrophages from HIV-1 infected individuals, including defective chemotaxis (Smith *et al.*, 1984; Poli *et al.*, 1985), impaired phagocytosis (Capsoni *et al.*, 1992), and the decreased intracellular killing of various microorganisms (Estevez *et al.*, 1986; Roux-Lombard *et al.*, 1986), functional defects in neutrophils from HIV-1 infected patients have also been observed. Similar to the abnormalities seen in monocytes/macrophages, these include defects in phagocytosis, (Lazzarin *et al.*, 1986), chemotaxis (Valone *et al.*, 1984; Ellis *et al.*, 1988; Roilides *et al.*, 1990), oxidative burst (Chen *et al.*, 1993; Pitrak *et al.*, 1993), and bacterial killing (Ellis *et al.*, 1988; Murphy *et al.*, 1988; Roilides *et al.*, 1990). However, not all studies are in agreement as to whether phagocytosis and/or oxidative burst are in fact impaired in HIV-1 infected persons (Pos *et al.*, 1992; Bandres *et al.*, 1993; Wenisch *et al.*, 1996).

As for HIV-1 infection, there is also conflicting data on the capacity of neutrophils from patients with pulmonary tuberculosis to undergo phagocytosis or oxidative burst. On the one hand, an increased capacity for respiratory burst (Mandell and Fuller, 1972) and phagocytosis

(Rieger *et al.*, 1979) has been reported, whereas another study by Antonaci *et al.* (1991) indicated that neutrophils from patients with either active or chronic pulmonary tuberculosis had depressed chemotaxis, phagocytosis and bacterial killing. Similarly, Shalekoff *et al.* (1998) found that neutrophils from individuals with pulmonary tuberculosis, with or without concomitant infection with HIV-1, have an impaired ability to undergo oxidative burst and to phagocytose *Escherichia coli.*

Depressed neutrophil responses are thought to play a role in the greater susceptibility of HIV-1 infected individuals to secondary infections (Chaves *et al.*, 1989; Schuchat *et al.*, 1991), and the impairment of various neutrophil functions, including phagocytosis, chemotaxis, oxidative burst, and degranulation (Haslett *et al.*, 1991; Whyte *et al.*, 1993) have been associated with apoptosis of these cells. Recently, a study examining neutrophil apoptosis in patients with AIDS, showed that neutrophil apoptosis was markedly accelerated in these patients, which may contribute to the risk of secondary infections (Pitrak *et al.*, 1996). Apoptosis, or programmed cell death, is a physiological suicide mechanism that preserves homeostasis by removing individual cells that are no longer needed, or that function abnormally. Apoptosis is characterised by distinct morphological features, including cell shrinkage and the compaction of chromatin against the nuclear membrane. Various metabolic events can also activate latent enzymes such as endonuclease, which cleaves DNA, leading to characteristic DNA fragmentation (Wyllie *et al.*, 1980; Ellis *et al.*, 1991). In addition to these morphologic and nuclear changes, neutrophil apoptosis studies have indicated that alterations in the expression of surface receptors may also be associated with apoptosis, where a reduction in CD16 (FcγRIII) expression correlated with enhanced neutrophil apoptosis *in vitro.* (Dransfield *et al.*, 1994). In normal individuals, ageing neutrophils spontaneously undergo apoptosis within approximately 24 hours of leaving the bone marrow. Neutrophils undergoing apoptosis are phagocytosed by macrophages and monocytes (Savill *et al.*, 1989b) to prevent further tissue damage at the site of infection (Cohen, 1991), and this occurs via a specific recognition mechanism involving the macrophage vitronectin receptor (Newman *et al.*, 1982; Savill *et al.*, 1989a).

The rate of neutrophil apoptosis can be modulated by a variety of pro-inflammatory mediators. Host-derived cytokines such as IL-1, IFN-γ (Colotta *et al.*, 1992), GM-CSF (Brach

et al., 1992; Cox *et al.*, 1992; Yamamoto *et al.*, 1993), granulocyte colony-stimulating factor (G-CSF) (Brach *et al.*, 1992; Lee *et al.*, 1993; Gottlieb *et al*., 1995), and IL-2 (Pericle *et al.*, 1994), glucocorticoid treatment (Cox, 1995; Meagher *et al.*, 1996), and circulating bacterial products such as lipopolysaccharide (LPS) (Colotta *et al.*, 1992) have been shown to retard neutrophil apoptosis. A recent study has also indicated that an interaction between platelets and neutrophils can inhibit neutrophil apoptosis (Andonegui *et al.*, 1997). On the other hand, IL-6 (Afford *et al.*, 1992), TNF-α (Takeda *et al.*, 1993), various proteolytic enzymes (Trevani *et al.*, 1996), and the generation of intracellular reactive oxygen intermediates following the ingestion of *E. coli* (Watson *et al*, 1996) have been shown to induce neutrophil apoptosis.

CHAPTER TWO

IL-8 PRODUCTION *IN VIVO*

2.1 Introduction

IL-8 has been shown to be produced by a variety of cell types including T-lymphocytes (Liles and Van Hoorhis, 1995) and monocytes/macrophages (Yoshimura *et al.*,1987; Colditz *et al.*, 1989), the latter being a major source of IL-8 production *in vitro*. The release of IL-8 from monocytes has been shown to be induced by two independent FcγR-mediated pathways, where stimulation can either occur indirectly through an FcγRIII stimulated soluble lymphocyte factor, or directly via FcγRI cross linking (Marsh *et al.*, 1996).

The capacity of neutrophils to produce cytokines is approximately 100-fold less than that produced by an equivalent number of monocytes (Cassatella, 1995). With regard to IL-8 production by neutrophils, IL-8 has been reported to be released by PMN after stimulation (Bazzoni *et al.*, 1991; Cassatella *et al.*, 1992; Strieter *et al.*, 1992; Fujishima *et al.*, 1993; Wei *et al.*, 1994) as well as constitutively (Arnold *et al.*, 1994). Where zymosan was used to stimulate IL-8 production, it was found that this occurred via the CD11b/CD18 receptor, with an endogenous platelet activating factor acting as an autocrine modulator (Au *et al.*, 1994). Altstaedt *et al.* (1996), working with non-prestimulated neutrophils, concluded that the production of proinflammatory cytokines by neutrophils is limited to only IL-8, which is in contrast to the findings of other groups who have reported the production of IL-1β (Tiku *et al.*, 1986; McColl *et al.*, 1992; Beaulieu and McColl, 1994), IL-6 (Cicco *et al.*, 1990), IL-12 (Gasperini *et al.*, 1998) and TNF-α (Djeu *et al.*, 1990; Dubravec *et al.*, 1990) by stimulated PMN.

IL-8 levels are reported to be elevated in the peripheral circulation of HIV-1 infected patients (Matsumoto *et al.*, 1993; Thea *et al.*, 1996), and significantly so in the CSF of HIV-1 infected patients with cryptococcal meningitis (Chaka *et al.*, 1997). When infected *in vitro* with HIV-1, monocytes/macrophages give rise to increased IL-8 production (Esser *et al.*, 1991; Tsai *et al.*, 1991). Other *in vitro* studies have shown IL-8 secretion by human monocyte cell lines following phagocytosis of *M. tuberculosis* (Friedland *et al.*, 1992a) and an enhanced IL-8 gene expression and release from macrophages after exposure to *M. tuberculosis* and its cell wall components lipomannan, lipoarabinomannan, and phosphoinositolmannoside (Zhang *et al.*, 1995a). The monitoring of IL-8 levels has been reported to be of clinical prognostic significance in patients with tuberculosis (Friedland *et al.*, 1995).

The aim of this chapter was to determine the effect of infection with HIV-1, *M. tuberculosis* and co-infection with both of these organisms on IL-8 production *in vivo*. This was monitored by determining the levels of patient serum or plasma IL-8 as well as peripheral cell-associated IL-8. The capacity of their peripheral mononuclear and polymorphonuclear cells to produce IL-8 spontaneously or in response to various stimuli was assessed, in addition to the detection of constitutive IL-8 mRNA expression in purified subsets of mononuclear cells.

2.2 Materials and methods

2.2.1 Patient samples

Serum samples previously collected and immediately processed and stored for two other studies (Schoub *et al.*, 1992; Martin *et al.*, 1994) were used. These were from 4 groups and included 40 healthy blood donors (ND), individuals diagnosed with pulmonary tuberculosis (TB), patients seropositive for HIV-1 (HIV), and individuals with both HIV-1 disease and pulmonary TB (HIV/TB). There were approximately equivalent numbers of male and female subjects all aged between 25 and 40 years in each group. Apart from age, sex and disease diagnosis, no other information was available for these individuals. A further four subject groups consisting of 16 individuals in each of the ND, TB, HIV, and HIV/TB groups were recruited for further evaluations, including plasma and peripheral cell-associated IL-8 levels, and the capacity of their peripheral mononuclear and polymorphonuclear cells to produce IL-8 spontaneously or in response to various stimuli. All TB and HIV/TB patients were receiving standard 4-drug anti-TB therapy including rifampicin, isoniazid, pyrazinamide and ethambutol. The mean duration of anti-TB treatment did not differ significantly between the TB and HIV/TB groups ($p>0.05$), and was 25.5 and 27 weeks respectively. Blood was collected by venipuncture into EDTA vacutainers (Becton Dickinson, San Jose, CA, USA). The blood was processed immediately upon collection and plasma stored at -70°C until testing. Three part differential counts were determined using a Coulter Onyx (Coulter Corp., Hialeah, FL, USA) cell counter. The immunological characteristics of the recruited individuals within each group are shown in Table 2.1. For mononuclear cell fractionation studies, blood was drawn separately from another 4 individuals belonging to each of the 4 groups. Their CD4 and CD8 cell counts are shown in Figure 2.5

2.2.2 IL-8 ELISA

Plasma and serum IL-8 levels were determined using the Biotrak Interleukin-8 [(h) IL-8] human, ELISA system (Amersham, Buckinghamshire, England). Briefly, 50μl of sample was added to the microtitre plate coated with antibody against IL-8. After 60 minutes incubation at room temperature, the wells were washed, and 50μl biotinylated antibody reagent was added to each

well. Following a second 60 minute incubation at room temperature, the plate was again washed, 100μl strepavidin horseradish peroxidase (HRP) conjugate added to each well, and the plate was incubated for 30 minutes at room temperature. After a final wash step, 100μl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to each well, the plate was incubated for 30 minutes at room temperature in the dark, and 100μl stop solution was added to each well. The optical density at 450nm of each well was then determined using a spectrophotometer. The minimum detectable dose of IL-8 was 0.5 pg/well. For determination of IL-8 levels in cell lysates, the control IL-8 titration was carried out using lysing solution (1% Triton X-100 in phosphate buffered saline (PBS) as diluent). There were no significant differences between the IL-8 titration curves determined in the presence of the supplied serum control diluent, cell culture growth medium or the lysing solution.

2.2.3 HIV-1 Viral load quantitation

HIV-1 viral load determinations for the patients in the HIV and HIV/TB groups were determined using the Amplicor HIV-1 Monitor test (Roche Diagnostic Systems, Branchburg, NJ, USA). Briefly, HIV-1 RNA was isolated from 200μl of patient plasma by lysis of viral particles and precipitation of the RNA with ethanol. Extracted RNA was then added to a polymerase chain reaction (PCR) master mix, with the reverse transcription and amplification reactions being carried out using a Perkin-Elmer GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA). The amplified PCR products were then hybridized to specific oligonucleotide probes in a microwell plate, followed by the addition of Avidin HRP conjugate and TMB substrate. The optical density at 450nm of each well was determined using a spectrophotometer

2.2.4 Isolation of peripheral blood leukocytes

PBMC were isolated from whole blood by centrifugation on Histopaque Ficoll. PMN were isolated by layering the Ficoll-granulocyte-red blood cell (RBC) interface onto a second Ficoll gradient, centrifuging at 1000g for 30 minutes and again removing the same interface. RBC were lysed using a solution of 0.15M NH_4Cl, 10mM $KHCO_3$, and 1mM sodium EDTA (pH 7.0).

2.2.5 Preparation of PBMC, PMN, and RBC lysates

Purified PBMC and PMN lysates were prepared by resuspending 10^6 cells in 100μl 1% Triton X-100/PBS lysis buffer and incubated on ice for 40 minutes. Erythrocytes were isolated by washing the RBC pellet 4 times with PBS, centrifuging at 180g for 10 minutes. RBC were adjusted to the original hematocrit for confirmation of the removal of leukocytes (<200/μl) and platelets (<1000/μl) using a Coulter Cell Counter (Coulter Corp., Hialeah, FL, USA). The RBC concentration was then adjusted to $5x10^6$ cells/100μl lysis buffer and incubated as for the PBMC and PMN lysates.

2.2.6 *In vitro* stimulation of primary cell cultures

PBMC and PMN were adjusted to a concentration of 1 x 10^6 cells/ml in RPMI 1640 medium supplemented with 10mM Hepes, 100μg/ml penicillin, 100μg/ml streptomycin, and 10% (v/v) foetal calf serum (FCS). PBMC were either untreated or stimulated with phytohaemagglutinin (PHA), purified protein derivative (PPD), or LPS at final concentrations of 5μg/ml, 50μg/ml, and 1μg/ml, respectively. One hundred microlitre aliquots (10^5 cells) of each treatment were then placed into 4 separate wells of a 96-well microtitre plate and incubated at 37°C. After 2 days the culture supernatants were harvested and the replicates pooled. Levels of IL-8 in each of the supernatants were determined in duplicate using the Biotrak™ IL-8 ELISA system. PMN cultures were either untreated or stimulated with PPD as described above.

2.2.7 Magnetic bead cell separations

Freshly isolated PBMC were immuno-magnetically separated into different cell compartments (CD14, CD4, CD8, and CD19) using the MACS magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, $5x10^6$ PBMC were resuspended in 80μl wash buffer (PBS with 5mM EDTA and 0.5% bovine serum albumin (BSA)), to which 10μl of the appropriate MACS magnetic microbead was added. After a 15 minute incubation at 4°C, 500μl wash buffer was added to the magnetically labelled cell suspension, which was then pipetted onto a MiniMACS separation column. The suspension was then allowed to run through the column,

500μl wash buffer was added to wash the column, with the effluent being collected as the negative fraction. After washing the column twice with 500μl wash buffer, the column was removed from the separation unit, 1ml wash buffer was added to the top of the column, which was then flushed through with a plunger and collected as the positive cell fraction. The negative fraction was then pelleted, resuspended in 80μl wash buffer, and the next volume of antibody-conjugated magnetic microbeads added to positively select the second cell population in a similar manner. This process was continued, allowing the sequential positive selection of CD14, CD4, CD8, and then CD19 cells. Total PBMC and the cell fraction remaining after the specific microbead isolations were also collected.

2.2.8 RNA isolation and RT-PCR

Cytoplasmic RNA was isolated from $1x10^6$ mononuclear cells using NP-40 lysis extraction (Gough, 1988). After isolation, the cells were washed and resuspended in 20μl PBS, to which 200μl NP-40 lysis buffer was added. The tubes were then vortexed and spun to pellet the debris, allowing the supernatant to be collected and transferred to a new tube. Following the addition of 200μl sodium dodecyl sulphate (SDS)-Urea buffer to the supernatant, 400μl phenol-chloroform-isoamyl alcohol (IAA) was added, and the tubes vortexed and spun. The aqueous phase containing the RNA was then harvested into a new tube, to which 1ml of 100% ethanol was added. The tubes were then placed at -20^{0}C overnight, and the following day the RNA was pelleted and washed with 70% ethanol. After briefly vacuum drying the samples, the RNA was resuspended in an appropriate volume of sterile water.

cDNA was synthesized using 60 pmol primer $p(dT)_{15}$ and 8U AMV RT in the presence of 40U RNAsin and 500μM each of dATP, dCTP, dGTP, and dTTP, 50mM Tris-HCl (pH 8.3), 50mM KCl, 10mM $MgCl_2$, and 10mM dithiothreitol (DTT) in a final reaction volume of 20μl. Five microlitres of cDNA (which corresponds to approximately 10^4 cells) was amplified by PCR using 25 pmol of IL-8-specific primers or control glyceraldehyde phosphate dehydrogenase (GAP-DH) primers and 2.5U of *Taq* DNA polymerase in a reaction mix containing 50mM KCl, 10mM Tris-HCl (pH 9), 0.1% Triton X-100, 850μM $MgCl_2$ and 50μM each of dATP, dCTP, dGTP, and dTTP. Template was denatured at 98°C for 10 min prior to addition of enzyme, buffer

and $MgCl_2$ at 55°C. Thirty five cycles of 93°C, 30s ; 55°C, 30s and 72°C, 1 min was followed by 10 min primer extension at 72°C. PCR products were then analysed by electrophoresis on 6% polyacrylamide gels and stained with ethidium bromide (1μg/ml).

2.3 Results

2.3.1 IL-8 levels in the peripheral circulation

A preliminary screen was first conducted to quantitate IL-8 levels in stored serum samples from 160 individuals, 40 within each of the four study groups described in Section 2.2.1. The range of IL-8 values obtained for the ND group using a commercial ELISA was in keeping with the range determined by the manufacturer. The median concentrations of IL-8 in the serum of the HIV and HIV/TB groups were both significantly elevated above that of the ND and TB groups which in turn did not differ from each other (Figure 2.1). Although not significant, there was a trend towards reduced IL-8 levels in the HIV/TB group when compared to the HIV group.

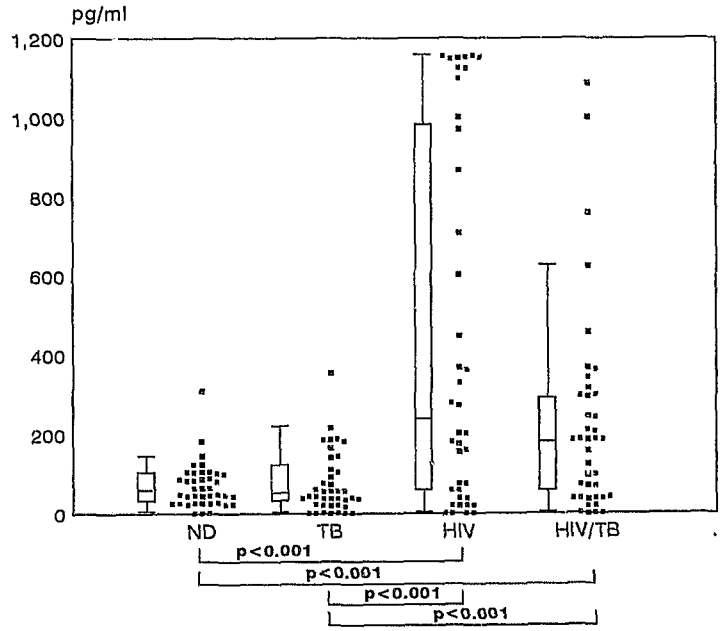

Figure 2.1 Circulating IL-8 levels determined by ELISA in the serum of 40 individuals in each of the ND, TB, HIV, and HIV/TB study groups. Data are presented as the individual values (solid squares), medians (horizontal bars), 25th and 75th percentiles (boxes) and the 10th and 90th percentiles (bars). Significant differences between groups are indicated. Differences between groups were compared using the non-parametric Mann-Whitney U-test.

It was shown by a relatively large sample size that elevated IL-8 levels are associated with HIV-1 infection, but because no additional information other than diagnosis was available, a further 16 individuals in each of the ND, TB, HIV, and HIV/TB groups were recruited in order that a number of aspects relating to IL-8 production *in vivo* were able to be studied in more detail. These included the determination of circulating IL-8 plasma levels together with their respective levels of cell-associated IL-8 and cellular capacities to secrete IL-8 *ex-vivo*.

The immunological characteristics of these groups of individuals are shown in Table 2.1. The median HIV viral load determinations in the plasma of the HIV and HIV/TB groups were 10,064 copies/ml (range 240-284,308 copies/ml) and 34,788 copies/ml (range 549-308,832 copies/ml), respectively. Both CD4 cell counts and viral load determinations were not significantly different between the HIV and HIV/TB groups ($p>0.05$). For this group of individuals, only plasma samples were available for IL-8 quantitation, and the sensitivity of the ELISA test used for IL-8 detection has been shown to be less in plasma than in serum. However, as for results obtained with serum, the median IL-8 values for the HIV and HIV/TB groups were significantly increased above the ND group whose median value was below the level of detection of the assay (Table 2.1). The HIV group also showed higher IL-8 levels than the TB group. No significant correlations were found between IL-8 plasma levels and HIV-1 RNA copies/ml in either the HIV or HIV/TB groups. Similarly, no correlations were found between the IL-8 plasma levels within each group and their corresponding differential cell counts, CD4 or CD8 T-cell counts. An interesting correlation found was a negative one between absolute CD8 count and viral load in the HIV/TB group ($p<0.005$; $r = -0.735$). Furthermore, the majority of TB (7 of 11, 63.6%) and HIV/TB (5 of 7, 71.4%) patients with undetectable levels of IL-8 were on anti-TB therapy for more than 6 months.

Table 2.1 Immunological status and plasma IL-8 levels of study groups

	Study Group			
	ND	TB	HIV	HIV/TB
n	16	16	16	16
White blood cells*				
count : x $10^3/\mu l$	5.4 ± 0.2	7.16 ± 0.74	5.53 ± 0.57	6.12 ± 0.43
Monocytes*				
count : x $10^3/\mu l$	0.32 ± 0.02	1.12 ± 0.25	0.48 ± 0.06	0.56 ± 0.08
Granulocytes*				
count : x $10^3/\mu l$	2.8 ± 0.2	3.18 ± 0.34	3.03 ± 0.46	2.96 ± 0.38
percentage of cells	50.6 ± 2.0	44.8 ± 2.4	51.9 ± 2.9	48.6 ± 4.9
CD4 cells*				
count : x $10^3/\mu l$	982 ± 46	1229 ± 128	446 ± 61	387 ± 115
percentage of cells	44.8 ± 1.7	43.1 ± 1.9	21.6 ± 2.3	12.2 ± 2.7
CD8 cells*				
count : x $10^3/\mu l$	580 ± 36	869 ± 113	1079 ± 117	1556 ± 256
percentage of cells	27.8 ± 1.7	30.3 ± 2.0	53.1 ± 3.4	57.1 ± 3.9
CD4 : CD8 Ratio*	1.7 ± 0.2	1.54 ± 0.14	0.47 ± 0.08	0.25 ± 0.06
Plasma IL-8 (pg/ml)#	<5.0 (<5.0-19.05)	<5.0 (<5.0-34.67)	12.94 (<5.0-407.38)	12.62 (<5.0-63.09)

*Results are expressed as the mean ± SEM

Results are expressed as the median with the range in parentheses

2.3.2 Peripheral cell-associated levels of IL-8

In addition to cell-free detection of IL-8, it was important to quantitate levels of IL-8 protein that may be associated with specific cell compartments *in vivo*. Peripheral cells isolated from whole blood were fractionated into mononuclear cells, PMN, and RBC using Ficoll density centrifugation and these populations were enumerated, lysed, and associated IL-8 quantitated by ELISA (Figure 2.2). In general, the highest cell-associated levels were found for PMN in all the subject groups (Figure 2.2B). Within the ND group, there was wide variability with a strong correlation between levels of IL-8 associated with each compartment. Although some ND had higher PBMC-associated levels of IL-8 when compared to the other groups, the medians were not significantly different ($p>0.05$) (Figure 2.2A). However, IL-8 levels associated with RBC and PMN in the ND group were significantly higher than those of the TB, HIV, and HIV/TB groups (Figure 2.2B and 2.2C).

2.3.3 Spontaneous and induced secretion of IL-8 by PBMC cultures

The ability of peripheral mononuclear cells to spontaneously produce IL-8 and their subsequent capacity to produce IL-8 in response to various agonists was determined by culturing PBMC either, without any stimulus or by including various stimuli such as PHA, PPD or LPS, respectively. In general, the levels of IL-8 released spontaneously by PBMC cultures of infected individuals (TB, HIV, HIV/TB) were elevated above those cultures of individuals in the ND control group, although only attaining significance in the HIV and TB groups (Figure 2.3A).

PHA and PPD, both potent inducers of IL-8, showed a similar pattern of stimulation in PBMC cultures from the different subject groups (Figure 2.3B and 2.3C). The reduction in the ability of PBMC from TB, HIV, and HIV/TB groups to respond was, however, more evident with PHA. On the other hand, LPS stimulation showed a different response between the different infected groups of individuals in that those with pulmonary TB produced greater amounts of IL-8 in response to LPS than did HIV-1 seropositive individuals with or without concurrent pulmonary TB (Figure 2.3D). Although no direct relationships were noted between duration of anti-TB treatment and either spontaneous or induced IL-8 production in the TB and HIV/TB groups, there

was a trend that longer times of treatment did result in a recovery of the capacity to produce IL-8 in response to PHA and PPD. Those patients in either group that had the highest IL-8 levels induced by PHA or PPD were all on anti-TB treatment for > 6 months. A number of these, in fact, produced higher levels of IL-8 than stimulated PBMC cultures from the majority of healthy normal donors.

2.3.4 Spontaneous and induced secretion of IL-8 by PMN cultures

Levels of IL-8 spontaneously produced in PMN cultures of ND (Figure 2.4A) were approximately 100-fold less than that obtained for the equivalent number of unstimulated ND PBMC in culture (Figure 2.3A). There were no differences noted between the levels of IL-8 produced spontaneously by PMN cultures between the different study groups (Figure 2.4A). However, when PMN cultures were stimulated with PPD, the amount of IL-8 produced was significantly reduced only in the HIV/TB group (Figure 2.4B). The TB group showed a trend towards decreased and the HIV group towards increased IL-8 induction although this was not statistically significant ($p>0.05$). PPD induced IL-8 secretion was however significantly lower in the HIV/TB group when compared to the HIV group. The duration of anti-TB treatment had no apparent effect on spontaneous or PPD induced IL-8 production in PMN cultures from TB and HIV/TB patients.

Levels of IL-8, produced spontaneously or when induced in PBMC or PMN cultures, showed no correlations with mononuclear cell counts (CD4, CD8) and PMN cell counts, or with plasma IL-8 levels ($p>0.05$). Furthermore, there were also no significant correlations between levels of IL-8 produced spontaneously or those induced in PBMC or PMN cultures, and viral load determinations in the HIV and HIV/TB groups ($p>0.05$).

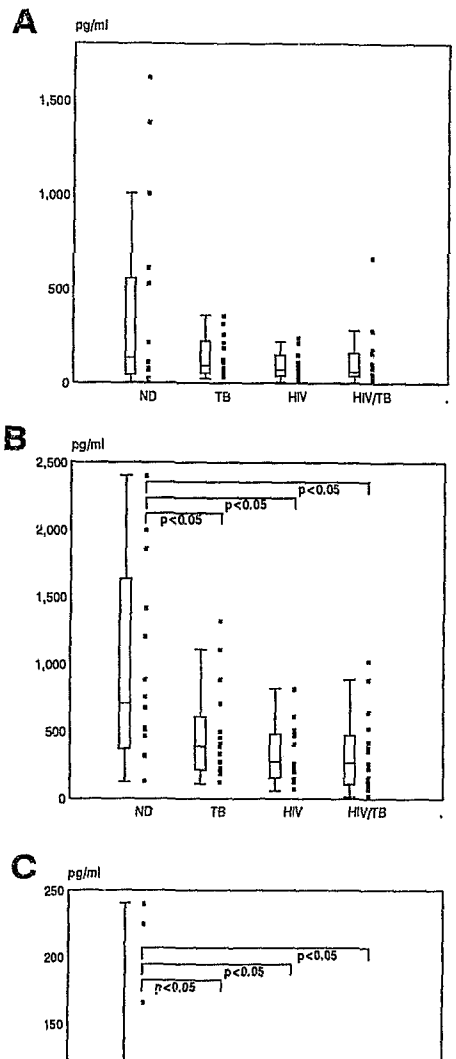

Figure 2.2 Peripheral cell-associated levels of IL-8 from the ND, TB, HIV, and HIV/TB study groups. Cells were isolated and lysed as described in Section 2.2.4 and Section 2.2.5. IL-8 was quantitated in cell lysates of PBMC (A), PMN (B), and RBC (C) by ELISA. Data are presented as the individual values (solid squares), medians (horizontal bars), 25th and 75th percentiles (boxes) and the 10th and 90th percentiles (bars). Significant differences between groups are indicated.

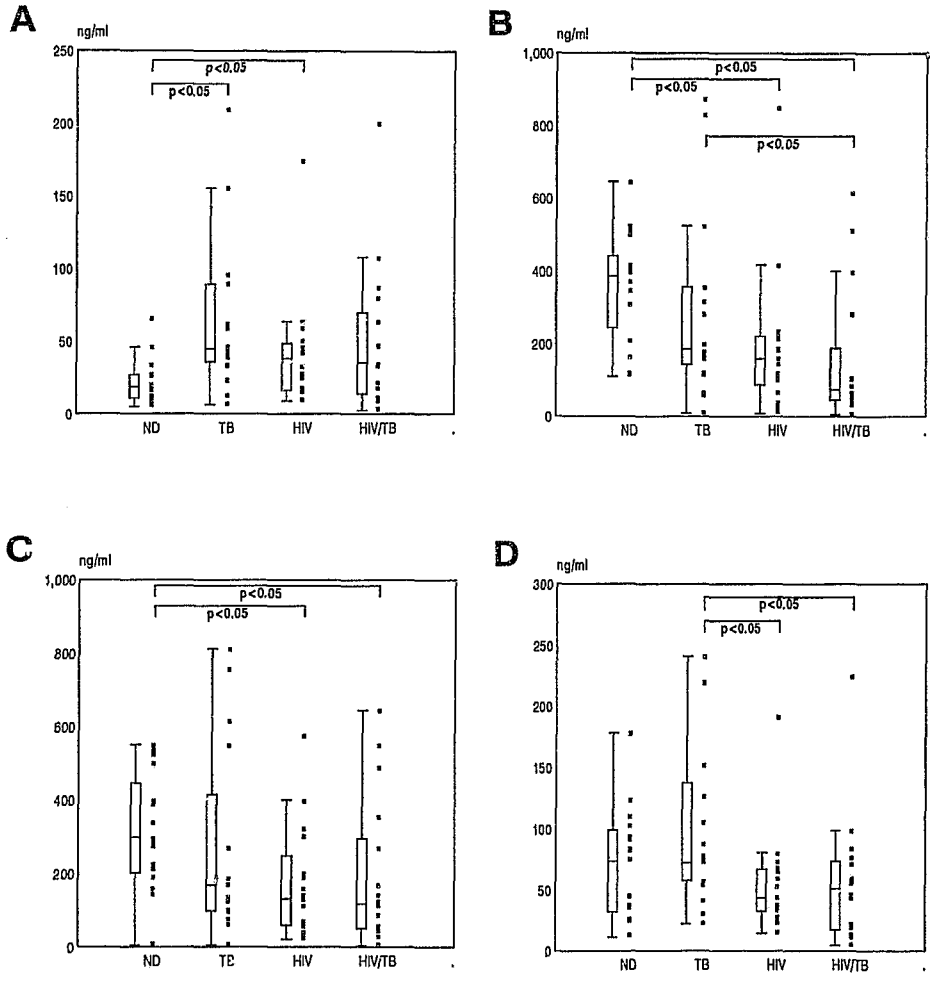

Figure 2.3 Spontaneous and induced production of IL-8 by PBMC *ex vivo*. PBMC from the 16 individuals in each study group were cultured either without an agonist to determine spontaneous IL-8 release (A), or stimulated with PHA (B), PPD (C), or LPS (D) for 48 hours, and then supernatant IL-8 levels were determined by ELISA. Data are presented as the individual values (solid squares), medians (horizontal bars), 25th and 75th percentiles (boxes) and the 10th and 90th percentiles (bars). Significant differences between groups are indicated.

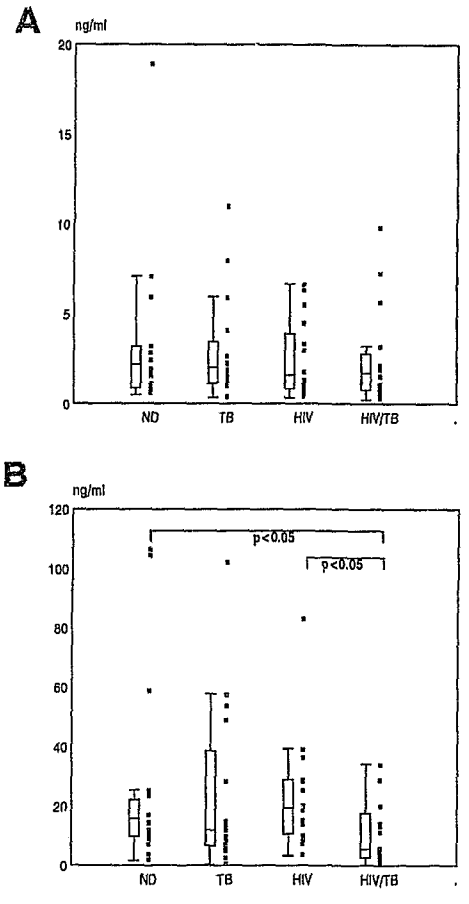

Figure 2.4 Spontaneous and induced production of IL-8 by PMN *ex vivo*. PMN from the 16 individuals in each study group were cultured either without an agonist to determine spontaneous IL-8 release (A), or stimulated with PPD (B) for 48 hours, and then supernatant IL-8 levels were determined by ELISA. Data are presented as the individual values (solid squares), medians (horizontal bars), 25th and 75th percentiles (boxes) and the 10th and 90th percentiles (bars). Significant differences between groups are indicated.

2.3.5 Detection of IL-8 mRNA in mononuclear cell fractions

As there were no significant differences in cell-associated IL-8 levels detected between PBMC lysates of the individual groups but clear differences seen in production of IL-8 *in vitro*, it was decided to use RT-PCR to determine the presence of IL-8 expression in specific mononuclear cell

subsets. PBMC from each of 4 individuals in the four subject groups, were separated into different cell fractions using antibody-conjugated magnetic beads. Prior to embarking on RT-PCR assays on subject material, the magnetic bead isolation method was optimised to allow for the positive selection of approximately similar numbers of input cells. As the efficiency of positive selection is dependent on the microbead:cell ratio employed, this was achieved by adjusting these ratios in such a way as to allow less than optimal selection of the total number of cells within each cell population. This approach ensured that similar cell numbers would be isolated even though specific cell numbers in some compartments differed between the groups, the compromise therefore being on total selection and not purity of the individual cell fractions. Cell purity was monitored after each positive isolation by flow cytometry using double-staining (FITC/PE) with CD3/CD4, CD3/CD8, CD45/CD14, and one-colour staining with CD19 (FITC). The only concern with respect to purity was within the CD4 cell fraction in the event that CD14 monocytes may not have been efficiently removed from total PBMC, as monocytes also display the CD4 receptor. This however was found not to interfere with the positive isolation procedure as the affinity of CD4 microbeads for CD4 T cells is much greater than for CD4 on monocytes, and at the microbead input used, this completely excluded contaminating monocytes. Furthermore, contaminating monocytes that were not positively isolated in the first fractionation step were retrieved in equivalent numbers in the peripheral cell fraction remaining at the end of all the positive selections.

RT-PCR was carried out on mRNA extracted from PBMC, CD14, CD4, CD8, CD19, and the cell fractions remaining at the end of the positive selections. GAP-DH was used as a control for mRNA integrity and end-point titrations of the cDNAs were carried out to ensure that cell input (particularly of CD4 cells in patients with low CD4 cell count) was approximately similar throughout. The PCR assay as used allows for a semi-quantitative analysis of mRNA results, that is, the accumulation of GAP-DH and IL-8-specific PCR products did not reach saturation levels within the number of amplification cycles used. The lack of detectable IL-8 mRNA expression within the CD4 cell fractions of most individuals in the HIV and HIV/TB groups was not due to lower cell input resulting from lower absolute CD4 cell counts, as end-point titrations of input cDNA for GAP-DH were found to be similar to those obtained for ND samples.

IL-8 was detected in mainly the CD4 and CD8 cell fractions (Figure 2.5) and in only one CD14 sample (TBc) and one CD19 cell fraction (NDd) (data not shown). Only one total PBMC fraction was found positive for IL-8 mRNA (NDa), which also had detectable IL-8 mRNA in the cell fraction remaining after the positive cell selections. Cells in this latter fraction would include predominantly NK cells and monocytes, and smaller numbers of each of the other cell fractions positively selected for. Figure 2.5 shows results obtained for the CD4 and CD8 cell fractions for individuals within each group as well as their immunological characteristics. For the 4 individuals in the ND group, all were positive for IL-8 mRNA in the CD4 and CD8 cell fractions (Figure 2.5A). For the patients with pulmonary TB (Figure 2.5B), 3 out of 4 CD4 fractions were IL-8 mRNA positive, and 2 out of 4 of the CD8 cell fractions. In the HIV group, CD4 cells from 2 of the 4 individuals were IL-8 mRNA positive, whereas CD8 cells from only one individual contained IL-8 mRNA (Figure 2.5C). In the HIV/TB group, no IL-8 mRNA was detected in any of the cell fractions for any of the individuals (Figure 2.5D).

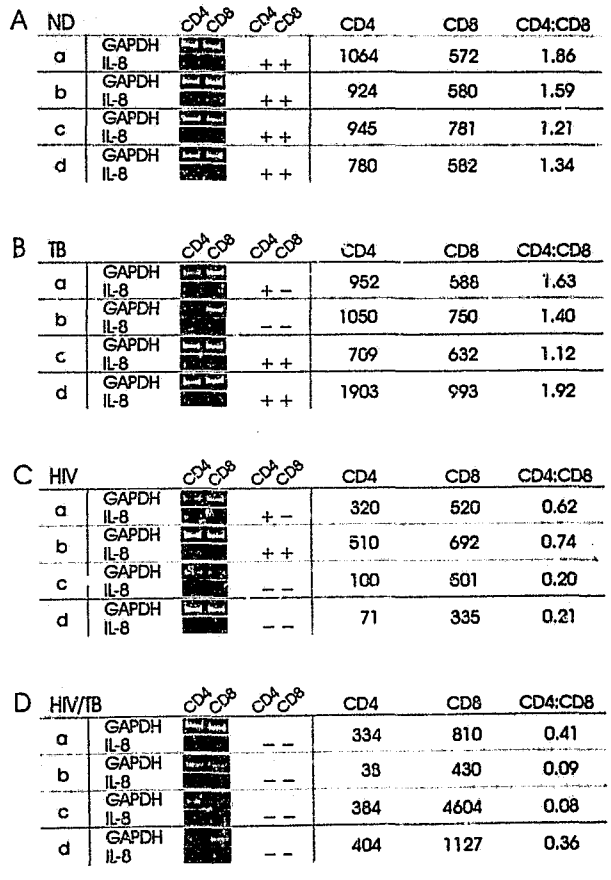

Figure 2.5 IL-8 mRNA expression in CD4 and CD8 mononuclear cell subsets of ND (A), TB (B), HIV (C), and HIV/TB (D) individuals. Mononuclear cells from 4 individuals (a-d) within each group, their immunological characteristics shown at the right of the figure, were fractionated into total PBMC, CD14, CD4, CD8, CD19, and a remaining cell population. mRNA was isolated and RT-PCR performed using GAP-DH and IL-8-specific primers as described in Section 2.2.8. Results are shown for the cell fractions that were consistently positive for IL-8 mRNA in ND, namely CD4 and CD8. Duration of anti-TB treatment : TBa to TBd : 8, 15, 26, and 2 weeks, respectively; HIV/TBa to HIV/TBd : 7, 10, 13, and 8 respectively.

2.4 Discussion

The production of IL-8, an important chemoattractant and cellular activator, if altered is likely to play an important role in the pathogenesis of HIV-1 disease and tuberculosis particularly in the context of secondary microbial infections. The purpose of the initial part of this study was intended to confirm the presence of elevated circulating levels of IL-8 in individuals with HIV-1, as has been reported by others (Matsumoto *et al.*, 1993; Thea *et al.*, 1996) and further to determine peripheral levels of IL-8 in the presence of pulmonary tuberculosis. Circulating IL-8 levels were raised in HIV-1 infected individuals with or without concurrent pulmonary TB. In contrast, those with pulmonary TB in the absence of HIV-1 co-infection showed IL-8 levels similar to those of control subjects. Increased circulating levels of IL-8 associated with HIV-1 infection is suggestive of dysregulated *in vivo* production of this chemokine. Whether this is a peripheral event or reflects the increased production of IL-8 in other tissue compartments remains uncertain. In this regard, increased concentrations of IL-8 have been reported in the bronchoalveolar lavage (BAL) fluid from symptomatic as well as asymptomatic HIV-1 infected individuals (Lipschik *et al.*, 1993) and a dysregulated release of IL-8 by alveolar macrophages from HIV-1 infected subjects has been shown, where asymptomatic subjects and patients with nonspecific interstitial pneumonitis spontaneously released elevated IL-8, IL-10, and IL-12 compared to normal volunteers. Cells from AIDS patients infected with *Pneumocystis carinii* released elevated levels of IL-8 and IL-10, but low levels of IL-12 (Denis and Ghadirian, 1994). In monocytes/macrophages infected *in vitro* with HIV-1, a constitutive production of IL-8, IL-6, and TNF-α was induced by HIV-1 infection at day 8 of culture, indicating that HIV-1-infected macrophages may be in a pro-inflammatory state. When HIV-1-infected macrophages were stimulated with LPS, there was an increased release of IL-8, IL-6, and TNF-α compared to mock-infected cultures, indicating a priming effect of HIV-1 infection (Esser *et al.* 1991). In a similar set of experiments conducted by Tsai *et al.* (1991), it was also found that *in vitro* infection of monocytes/macrophages by HIV-1 induced significant increases in the mRNA levels of IL-8, IL-6, TNF-α, and IL-1β, although in contrast, the levels of LPS-induced mRNAs for IL-8, IL-6, TNF-α, and IL-1β were decreased compared to uninfected LPS-stimulated cells.

In the second section a closer analysis was done on 16 individuals within each of the same 4 study groups : ND, TB , HIV, and HIV/TB. They were monitored for their plasma IL-8 levels, cell-associated IL-8 levels, and the capacity of their peripheral leukocytes to produce IL-8 spontaneously or in response to particular stimuli, and constitutive IL-8 mRNA expression in fractionated mononuclear cells. The presence of IL-8 found associated with the PBMC and PMN compartments in healthy individuals may represent either preformed intracellular IL-8 or IL-8 bound to IL-8 receptors on the surface of these cells or both. IL-8 binding to these cell surfaces in ND individuals would not be expected to occur as any excess IL-8 is thought to be bound to the promiscuous chemokine receptor, the duffy antigen on RBC (Darbonne *et al.*, 1991), or forms complexes with free IgG anti-IL-8 antibody (Sylvester *et al.*, 1992), thereby removing IL-8 from the circulation thus preventing peripheral cell activation. The corresponding increased levels of IL-8 associated with RBC may therefore represent normal IL-8 turnover *in vivo*. From the results obtained, the reduced binding of IL-8 to RBC of infected individuals may suggest impaired clearance of circulating IL-8. Increased circulating levels of IL-8, particularly in HIV-1 infection, could then be the result of this deficit and excess IL-8 may then instead bind to IL-8 receptors on leukocytes giving rise to their activation. This possibility is consistent with the immune activation described in both HIV-1 infection and TB, and has implications for the *in vivo* regulation of chemokines in general.

Levels of IL-8 associated with cells upon direct isolation were low in comparison to levels secreted by leukocytes *in vitro*. PBMC from both HIV and TB patients shared similar cellular responses *in vitro* as measured by their respective abilities to produce IL-8 spontaneously or when induced. In general, the spontaneous release of IL-8 was higher in PBMC cultures from the infected groups, with a reduced release in response to various stimuli, when compared to cultures from healthy individuals. This suggests that cells may be in an activated state *in vivo* and therefore are primed for subsequent IL-8 production *in vitro*. However, upon receiving a second stimulus the cells were defective in their ability to respond as measured by IL-8 secretion. Gasperini *et al.* (1998) found spontaneous release of IL-8 from PBMC cultures from patients with AIDS to be very similar to normal donor control PBMC, but HIV-1-positive PBMC cultures stimulated with LPS showed reduced release of IL-8 compared to LPS-stimulated cultures from normal donors.

Whereas PMN cultures from all the groups showed similar levels of IL-8 produced spontaneously, a significantly diminished response to PPD was noted for PMN from the HIV/TB group and a similar trend in TB patients without HIV-1 infection. Although not significant, the median IL-8 levels induced by PPD in PMN cultures from HIV-1 infected individuals was increased relative to that of the ND group. Similar results were observed by Gasperini *et al.* (1998), who found equivalent levels of IL-8 being spontaneously produced by PMN cultures of HIV-1 infected patients and healthy individuals, but significantly higher production of IL-8 by LPS-stimulated HIV-1-infected cultures compared to normal donor PMN cultures stimulated with LPS. This is suggestive of PMN being in a primed state *in vivo* and necessitating a subsequent stimulus *ex vivo* for increased activity. This possibility is supported by a number of findings, including the enhanced phagocytosis of *Candida* sp. (Wenisch *et al.*, 1996) and *E. coli* (Shalekoff *et al.*, 1998) by PMN from HIV-1 infected individuals and increased CD11b expression (Palmer *et al.*, 1993). Although cell-associated levels are lower in infected individuals, spontaneous release is increased relative to untreated cells from uninfected donors, suggesting some prior activation or priming event *in vivo*. The intracellular stores of IL-8 may be what is limiting as subsequent stimulation *in vitro* gives rise to reduced levels of secreted IL-8 when compared to stimulated cells of normal individuals. It is also possible that the triggering stimulus may rather be provided by *in vitro* culture, one that is sufficient to drive IL-8 secretion from cells from infected individuals but is insufficient to trigger a similar response in cells from healthy individuals.

Furthermore, these results show that the mononuclear cell types *in vivo* that predominantly contain IL-8 mRNA are those identified by the CD4 and CD8 cell surface markers and not, as one might expect, monocytes which are potent IL-8 producers *in vitro*. Associated with HIV-1 infection or pulmonary TB was the loss of this constitutive IL-8 mRNA expression in some patients, with a total abrogation thereof in individuals that were co-infected with both organisms. The presence or absence of IL-8 mRNA was not related to the extent of immune deficiency as determined by CD4 cell count, as some individuals with low CD4 cell count had detectable mRNA and others with much higher counts were negative for IL-8 mRNA. The presence of IL-8 mRNA in CD4 and CD8 cells from healthy individuals may form part of a process of normal IL-8 turnover as suggested earlier, or alternatively these cells may be poised for a subsequent post-transcriptional stimulus for IL-8 translation and secretion. These results support IL-8 being regulated differently in different cell populations. Consistent with this possibility is the finding that

T cells upon direct isolation from blood have detectable IL-8 mRNA which is lost upon *in vitro* culture within 24 hours (C.Tiemessen, unpublished observations) whereas freshly isolated monocytes have undetectable expression but rapidly produce IL-8 mRNA upon adherence to plastic in culture (Kasahara *et al.*, 1991). This suggests that post-transcriptional processes or regulation of secretion of preformed protein may be operational in T cells, whereas monocytes require transcriptional activation for IL-8 production. However regulated, it is clear that in the presence of HIV-1 infection or pulmonary TB or both, mechanisms underlying constitutive IL-8 expression in T cells are interfered with.

A longitudinal study carried out by Friedland *et al.* (1996) has shown a role for antibiotic therapy in tuberculosis, where both *in vivo* and LPS-stimulated *ex vivo* plasma IL-8 concentrations were increased following antibacterial therapy. This suggests that both peripheral levels of IL-8 and peripheral cell capacity to produce IL-8 are increased during anti-TB therapy. Results presented here, however, support an increase in cellular capacity to produce IL-8 with time of anti-TB treatment but not of an increase in circulating plasma levels. It is not unreasonable to consider a recovered capacity to produce IL-8 as a positive effect of the drugs coinciding with the resolution of TB bacteremia. A contrasting role for IL-8 in TB is however shown by the finding that IL-8 plasma levels upon patient admission were found to be higher in TB patients, with or without HIV-1 infection, who died compared to those who survived (Friedland *et al.*, 1995).

Increased circulating levels of IL-8 in HIV-1 infection and a diminished cellular capacity to produce IL-8 as shown in this study may have important implications for antimicrobial defences and normal immune processes. These results suggest that the production of IL-8 is altered *in vivo*, which is likely to play a role in the disease pathogenesis of HIV-1, *M. tuberculosis* and co-infection with both organisms.

CHAPTER THREE

IL-8-INDUCED NEUTROPHIL FUNCTIONS

3.1 Introduction

Patients with HIV-1 infection have an increased susceptibility to infection with various opportunistic pathogens and this is thought to be due to an impaired microbicidal response of phagocytic cells. Such functional defects have been reported to occur in neutrophils from HIV-1 infected patients and include defects in phagocytosis, (Lazzarin *et al.*, 1986), chemotaxis (Valone *et al.*, 1984; Ellis *et al.*, 1988; Roilides *et al.*, 1990), oxidative burst (Chen *et al.*, 1993; Pitrak *et al.*, 1993), and bacterial killing (Ellis *et al.*, 1988; Murphy *et al.*, 1988; Roilides *et al.*, 1990). In the presence of pulmonary tuberculosis, an increased capacity for respiratory burst (Mandell and Fuller, 1972) and phagocytosis (Rieger *et al.*, 1979) has been found. In contrast, Antonaci *et al.* (1991) demonstrated that neutrophils from patients with either active or chronic pulmonary tuberculosis had depressed chemotaxis, phagocytosis and bacterial killing. Similar results have been observed by Shalekoff *et al.* (1998) who assessed neutrophil phagocytosis and oxidative burst by flow cytometry, and found that neutrophils from individuals with pulmonary tuberculosis, with or without concomitant infection with HIV-1, have an equivalent impaired ability to undergo oxidative burst and to phagocytose *E. coli.*

In a study by Wenisch *et al.* (1996), neutrophils from HIV-1 infected individuals were shown to have an inability to kill *Candida* sp. despite enhanced phagocytosis and unimpaired oxidative burst. These results suggest that defective microbial killing by neutrophils of HIV-1 infected individuals is likely to be the result of an ineffective non-oxidative defence armature. In addition, the killing of *M. tuberculosis* by PMN has been shown to occur by non-oxidative means (Jones *et al.*, 1990), with IL-8 further enhancing mycobacterial killing (Nibbering *et al.*, 1993).

Results from the previous chapter showed that IL-8 production is dysregulated in the different disease states studied. This chapter, in turn, addresses the integrity of PMN effector functions that are dependent on IL-8. This was carried out by evaluating degranulation in response to IL-8 as a measure of the capacity of non-oxidative killing mechanisms, and chemotaxis in response to IL-8 as a measure of PMN migratory capacity.

3.2 Materials and methods

3.2.1 Patient samples

Four subject groups were recruited for studies on PMN degranulation, and included 7 ND as controls, 11 TB, 11 HIV, and 9 HIV/TB patients. The immunological characteristics of these individuals are shown in Table 3.1. For all other analyses relating to PMN degranulation, the groups and numbers of individuals involved are outlined in Results. A further 11 ND and 22 HIV/TB patients were recruited for studies looking at PMN chemotaxis. The immunological status of these individuals was similar to the ND controls and HIV/TB patients shown in Table 3.1. Blood was collected by venipuncture into EDTA vacutainers, and processed immediately for assays of PMN function. All TB and HIV/TB patients were receiving standard 4-drug anti-TB therapy.

3.2.2 Separation of neutrophils

Anticoagulated blood was centrifuged at 200g for 10 minutes at room temperature and the plasma removed, aliquoted and frozen at -20°C. PMN were isolated from buffy coats by first centrifuging PBS-diluted whole blood (1:1) on a primary Histopaque-Ficoll gradient at 1000g for 30 minutes at room temperature. After removal of the mononuclear cell layer, the remaining Ficoll and PMN layer were over-layered onto a secondary Ficoll gradient and centrifuged as before. The PMN layer was then removed and the residual erythrocytes lysed with a solution of 0.15 M NH_4Cl, 10 mM $KHCO_3$, and 1 mM sodium EDTA (pH 7.0). Purified PMN suspensions were >98% viable as determined by trypan blue exclusion.

3.2.3 β-glucuronidase bioassay

PMN degranulation was measured in response to IL-8 by the release of β-glucuronidase as described by Schröder *et al.* (1987). Briefly, the concentration of PMN was adjusted to 10^7/ml and cytochalasin B added to a final concentration of 5μg/ml. Aliquots of 100 μl of the cell suspension were placed in a 96-well round-bottom plate and incubated for 15 minutes at 37°C.

Human IL-8 test samples (in 2-fold serial dilutions for dose-response curves, final concentrations 7.8 to 500 ng/ml; or at one low (15.63 ng/ml) and one high (500 ng/ml) input concentration), each in a total volume of 100 µl, were added to separate wells and incubated for a further 30 minutes at 37°C. The cells were then pelleted at 1000 rpm (Sorvall H1000B rotor) for 10 minutes at 4°C and 100 µl of the supernatant transferred to the wells of a 96-well flat-bottom plate containing 100 µl 0.01 M p-nitrophenyl-β-D-glucuronide in 0.1 M sodium acetate pH 4.0. The plate was incubated overnight at 20°C, the reaction stopped with 100 µl 0.4 M glycine buffer (pH 10) and the absorbance read at 405 nm. For the determination of total β-glucuronidase content in PMN, 5 x 10^5 and 1 x 10^6 cells were lysed in 100µl of 0.4% Triton-X/PBS (v/v). The release of β-glucuronidase at different IL-8 concentrations was calculated as the optical density (OD) at 405 nm obtained at a particular IL-8 concentration divided by the total OD 405nm of the PMN lysate and expressed as a percentage.

3.2.4 Effect of HIV-1 positive plasma on IL-8 dose response

PMN were isolated from 2 ND and 10^7 cells, resuspended in 900µl RPMI 1640 medium supplemented with 10mM hepes, 100 µg/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) FCS, were placed into the wells of a 48-well culture plate, with 100µl of plasma obtained from 8 ND and 8 HIV/TB individuals added to duplicate wells. Cultures were incubated for 3 hours, the cells were washed and then resuspended in PBS, and used in the β-glucuronidase bioassay as described in Section 3.2.3.

3.2.5 Effect of cytokines on IL-8-induced release of β-glucuronidase

PMN were isolated from 4 ND and resuspended in RPMI with 10% FCS at a concentration of 5 x 10^6/ml. The cytokines tested and concentrations at which they were added are shown in Table 3.2. After overnight incubation at 37°C, the cells were harvested, cell concentrations adjusted to 10^7/ml, and PMN tested for the release of β-glucuronidase in response to different concentrations of IL-8.

3.2.6 Infection of PMN with HIV-1 *in vitro*

PMN cultures from 4 ND were set up in 6-well plates at a concentration of 3 x 10^6 cells/ml in RPMI containing 10% FCS. HIV-1 (strain IIIB) suspension was then added to the appropriate wells (final concentration 3 ng/ml p24 as determined by p24 antigen ELISA (Coulter Corp., Hileah, FL, USA); 6 x 10^6 RNA copies/ml, determined using the Amplicor HIV-1 Monitor test as described in Section 2.2.3 (Roche Diagnostic Systems, Branchburg, NJ, USA)), and the cultures were incubated at 37°C. Control supernatant was added to uninfected cultures. After 24 hours and 96 hours, PMN were harvested, the cells washed three times with PBS, re-adjusted to a viable cell count of 10^7 cells/ml, and assayed for degranulation in response to IL-8.

3.2.7 Chemotaxis assay

PMN chemotaxis, through polycarbonate filters with a diameter of 6.5mm and pore size of 3μm, was monitored using 24-well Costar Transwell plates. IL-8 in 500μl (100ng/ml) of RPMI 1640 medium containing 10% FCS was placed in the lower chamber (below the filter). Control wells received medium without IL-8. Purified PMN, resuspended at 5x10^6/ml in RPMI medium containing 10% FCS, were added to the top chambers in 100μl volumes. Control and test experiments were carried out in duplicate for each individual. The plates were incubated at 37°C in 5% CO_2 for 60 minutes in a humidified chamber. After incubation, PMN migrating randomly (control) or directly in response to IL-8 were counted by trypan blue exclusion. PMN viability remained unchanged over the duration of the assay.

3.3 Results

3.3.1 PMN degranulation

3.3.1.1 PMN β-glucuronidase release in response to IL-8

The induction of β-glucuronidase release from cytochalasin-treated PMN occurs in response to IL-8 in a dose-dependent manner. There is therefore an increased release of the enzyme with an increase in IL-8 concentration. The release of β-glucuronidase in response to 2-fold dilutions of IL-8 and in response to PBS (control for spontaneous release) was determined for PMN isolated from the peripheral blood of 4 study groups: 7 ND, 11 TB, 11 HIV, and 9 HIV/TB individuals. The immunological characteristics of these patient groups are shown in Table 3.1. The IL-8 dose-response graphs, determined as mean OD 405nm values of the total individuals, were similar for the ND and TB groups (Figure 3.1). PMN from HIV and HIV/TB patients, however, showed a near-reciprocal relationship between IL-8 concentration and the amount of β-glucuronidase released, in that increasing IL-8 concentrations resulted in decreased enzyme release. Figure 3.2 shows the β-glucuronidase released at a low (15.63 ng/ml) and at a high (500 ng/ml) IL-8 input concentration for all the same individuals in each of the four groups from Figure 3.1. PMN from 7 of the 11 HIV patients showed a reciprocal response to IL-8, whereas patients 508, 651 and 538 had flat responses, and patient 652 had a positive slope dose-response graph as found for the ND group. Interestingly, the latter patient had a CD4 T cell count of only 53. Overall, there was no relationship between absolute CD4 T cell counts and either the type of response or the magnitude of enzyme release obtained in the HIV or HIV/TB groups. When comparing results within the groups at the 15.63 ng/ml and 500 ng/ml IL-8 concentrations, β-glucuronidase released at 500 ng/ml was significantly higher than at 15.63 ng/ml for the ND ($p<0.02$) and TB ($p<0.01$) groups, whereas with both the HIV and HIV/TB groups, where the overall response was reciprocal, the release of β-glucuronidase was significantly lower at 500 ng/ml of IL-8 than at 15.63 ng/ml ($p<0.05$). When comparing results between the groups at the different IL-8 concentrations, significant differences in release of enzyme in response to 15.63 ng/ml IL-8 were obtained between the ND and HIV groups ($p<0.05$) and TB and HIV groups ($p<0.01$). At 500 ng/ml of IL-8, significant differences were observed between the ND and HIV ($p<0.01$), ND and HIV/TB

($p<0.01$), TB and HIV ($p=0.05$), and TB and HIV/TB ($p<0.02$) groups.

Table 3.1 Immunological status of study groups

	Study group		
	TB	HIV	HIV/TB
n	11	9	11
White blood cells			
count : $x10^3/\mu l$	10.3 ± 0.9	4.7 ± 0.5	6.1 ± 0.6
Granulocytes			
count : $x10^3/\mu l$	6.2 ± 1.2	2.2 ± 0.2	3.2 ± 0.4
percentage of cells	64.6 ± 2.5	48.4 ± 4.8	55.7 ± 4.1
CD4 cells			
count : cells/μl	776 ± 115	412 ± 109	286 ± 76
percentage of cells	42.2 ± 2.5	17.6 ± 2.9	15.3 ± 2.4
CD8 cells			
count : cells/μl	565 ± 75	1524 ± 248	994 ± 126
percentage of cells	34.5 ± 1.2	53.5 ± 3.5	61.7 ± 2.1
CD4 : CD8	1.29 ± 0.12	0.38 ± 0.09	0.26 ± 0.05

Results are expressed as the mean ± SEM
Percentile P25 and P75 values for healthy adults (absolute counts, percentage): WBC (3.6-10.0); granulocytes (1.4-6.5, 42.2-75.2); CD4 cells (700-1100, 38-46); CD8 cells (500-900, 31-40); CD4:CD8 (1.0-1.5)

In order to determine if there was any difference in the ability of PMN to spontaneously degranulate as a result of disease, we compared the amount of β-glucuronidase released from PMN of the different groups in the absence of any stimulus (Figure 3.3). This was significantly increased in the HIV group when compared to the TB group ($p<0.01$) and HIV/TB group

($p<0.01$). Although not significant ($p>0.05$), there was a definite trend towards an increase in release of enzyme in the HIV group relative to that of the ND group. The presence of IL-8 significantly increased the amount of enzyme released above that found for the corresponding PBS controls at a concentration of 15.63 ng/ml of IL-8 in the ND ($p=0.05$) and TB ($p<0.01$) groups, and at a concentration of 500 ng/ml of IL-8 in HIV ($p<0.01$) and HIV/TB ($p<0.01$) groups.

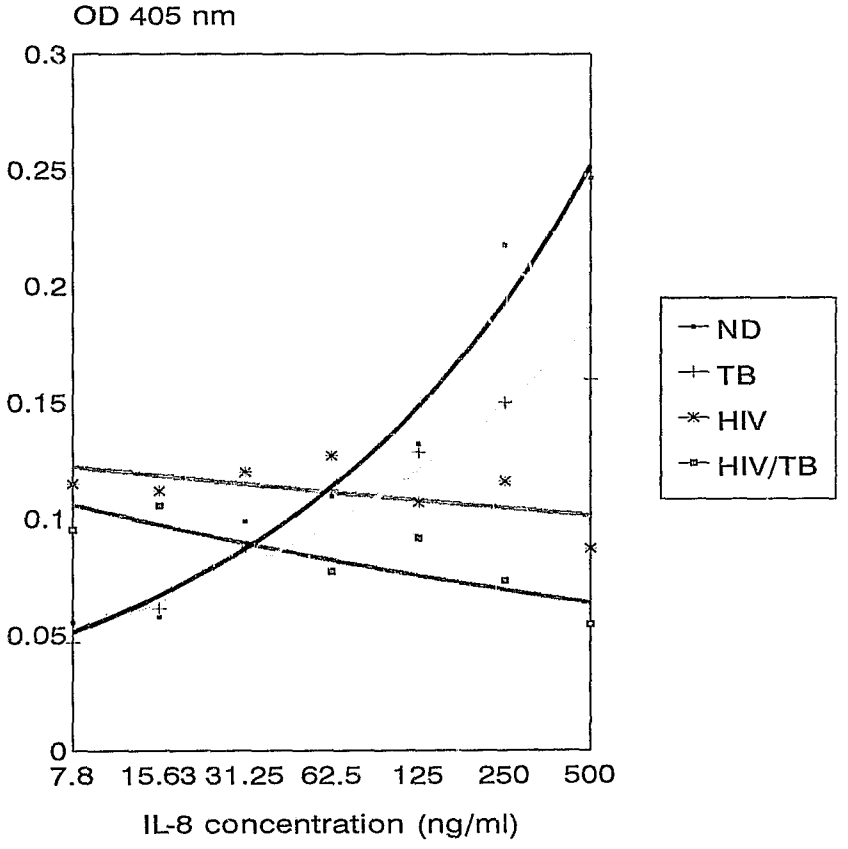

Figure 3.1 Induced release of β-glucuronidase from PMN in response to different doses of IL-8. PMN were isolated from whole blood of ND (n=7), TB (n=11), HIV (n=11) and HIV/TB (n=9) study groups, and assayed for the release of β-glucuronidase. OD 405nm values shown represent enzyme release that is due to IL-8 i.e. the total OD 405nm at a particular IL-8 input concentration minus the OD 405nm value obtained for the unstimulated control. Results are expressed as the mean OD 405nm at each IL-8 concentration for each group of individuals.

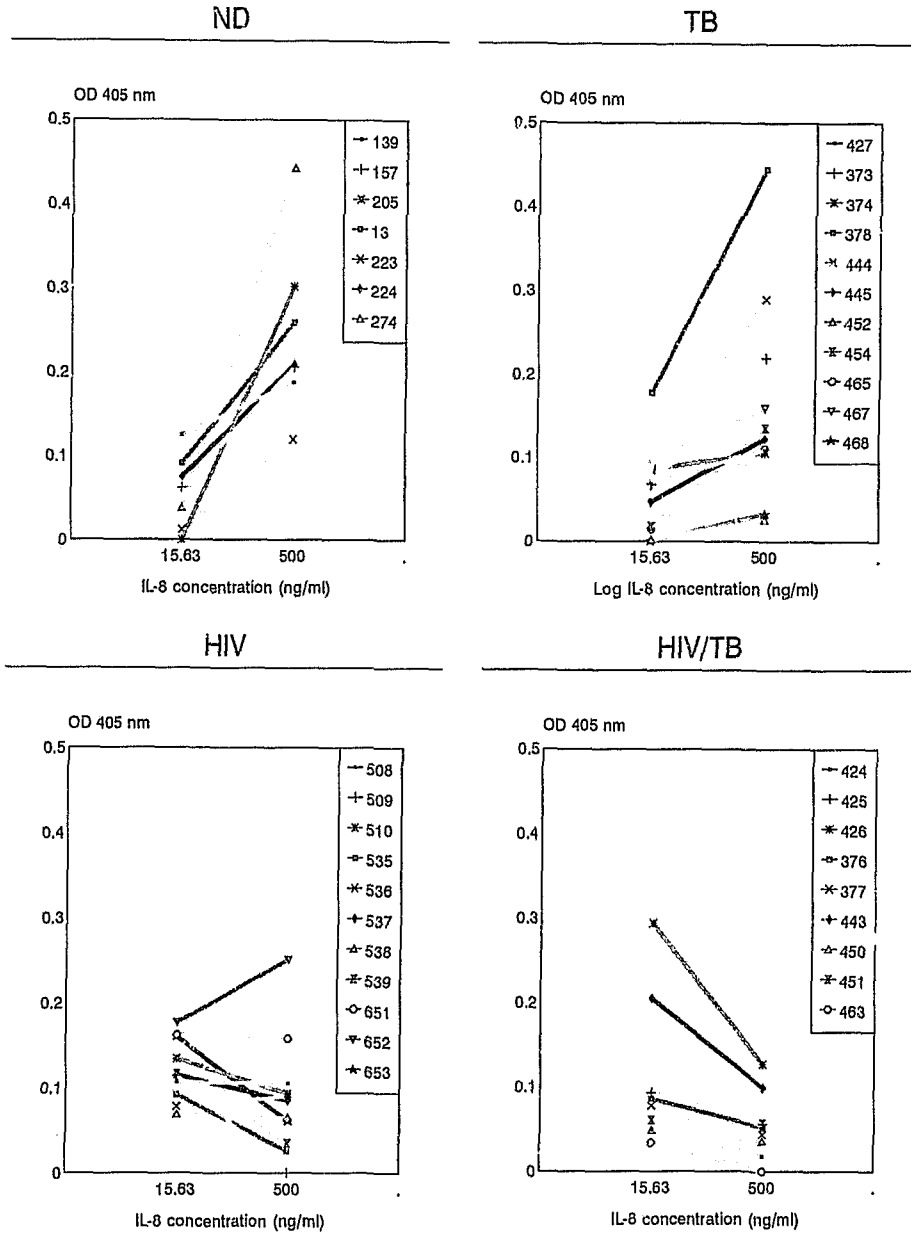

Figure 3.2 β-glucuronidase released at a low and at a high concentration of IL-8 showing degranulation responses for each individual within each of the 4 study groups described in Figure 3.1. Results are shown as the OD 405nm values due to IL-8 only i.e. values obtained for unstimulated controls are subtracted from the total enzyme released.

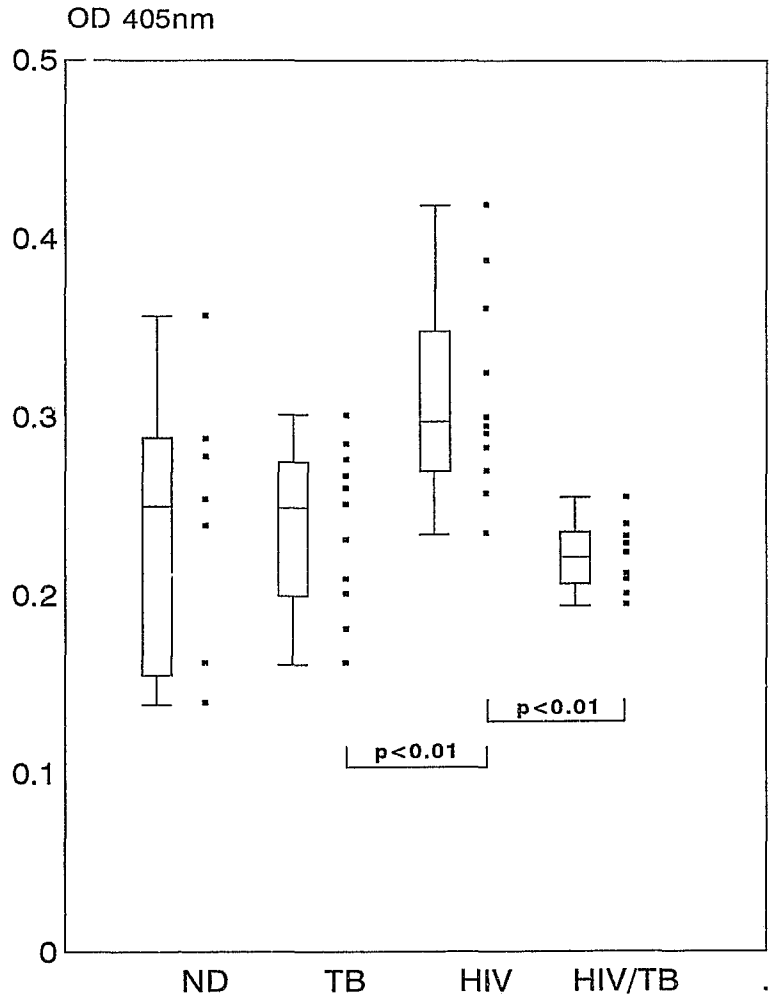

Figure 3.3 Spontaneous release of β-glucuronidase from PMN of ND, TB, HIV and HIV/TB study groups. The amount of β-glucuronidase released from PMN in the absence of any stimulus (PBS controls) was determined for each of the study groups described in Figure 3.1. Solid squares, individual values; error bars, 10th and 90th percentiles. Boxes represent values between the 25th and 75th percentiles, with the median indicated. Significant differences between groups are indicated.

3.3.1.2 PMN β-glucuronidase release in response to FMLP

As degranulation of PMN from HIV-1 infected individuals was clearly impaired in response to IL-8, it was questioned whether this was specifically an IL-8-dependent phenomenon or whether impairment of degranulation was a more generalized phenomenon in HIV-1 infection. Using the same assay system as above, the ability of PMN from 4 HIV-1 infected individuals to release β-glucuronidase in response to another agonist, FMLP, was therefore tested. Degranulation in response to FMLP (concentration range 10^{-6} to 10^{-9} M) showed similar results to that found for IL-8 in HIV-1 infected patients (data not shown).

3.3.1.3 Levels of β-glucuronidase in primary granules

As the IL-8-induced release of β-glucuronidase from PMN of HIV-1 infected individuals was decreased relative to healthy controls, it was important to question if PMN from HIV-1 infected individuals contained less granules (perhaps due to altered PMN maturation in infected patients), rather than an altered ability to exocytose granule contents. This was determined by calculating the amount of β-glucuronidase released by PMN when induced by IL-8 as a proportion of the total amount present in 10^6 PMN. PMN from healthy individuals released only 20 to 34% of their total available β-glucuronidase at the highest IL-8 concentration (500 ng/ml) used in this study. On the other hand, using the concentration of IL-8 which allowed maximum enzyme release in HIV-1 infected individuals (15.63 ng/ml), the mean percentage of enzyme released, calculated for a group of 20 HIV/TB patients, was 15.8% and ranged from 10.6 to 23%. Thus the reduced release of enzyme from PMN of HIV-1 infected individuals was not due to a limited β-glucuronidase content in primary granules.

3.3.1.4 The effect of HIV-1 seropositive plasma on the IL-8 dose response graph

The effect of plasma obtained from ND (n=8) and HIV/TB (n=8) individuals on the IL-8-induced release of β-glucuronidase from PMN was measured by adding the plasmas to PMN cultures of 2 healthy individuals for 3 hours and then monitoring degranulation responses. There was no significant difference in IL-8 dose-response graphs of ND PMN pretreated with either ND plasma

or HIV/TB plasma (Figure 3.4).

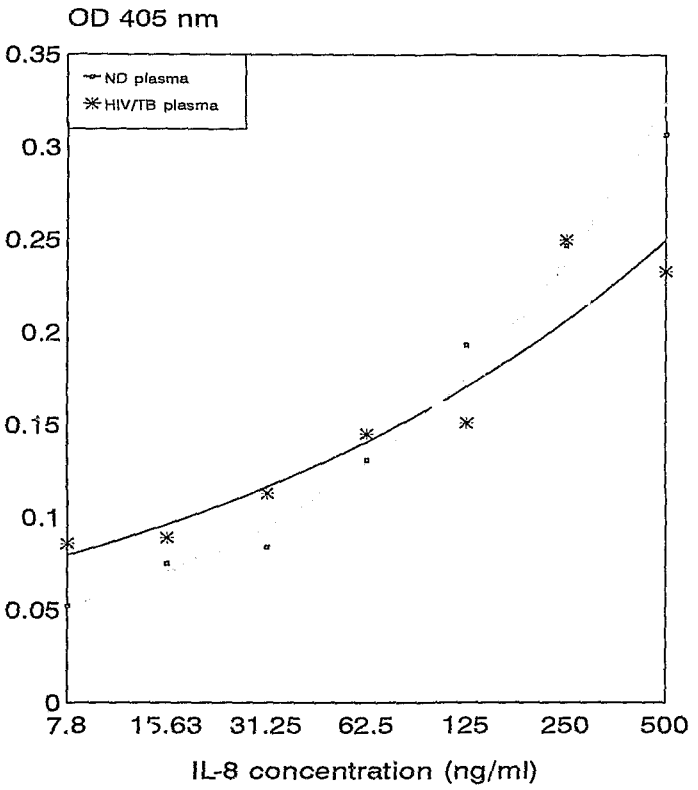

Figure 3.4 The effect of plasma obtained from ND (n=8) and HIV/TB (n=8) individuals on the IL-8-induced release of β-glucuronidase from PMN of 2 healthy individuals. The release of β-glucuronidase was measured over the range of IL-8 concentrations shown in Figure 3.1 and results are expressed as the mean OD 405nm at each IL-8 concentration for the 2ND PMN incubated with the ND and HIV/TB plasmas.

3.3.1.5 The effect of pretreatment with other cytokines on IL-8-induced release of β-glucuronidase from PMN

PMN from HIV-1 infected individuals may be exposed to elevated levels of certain cytokines *in vivo* that may contribute to the subsequent altered response to IL-8 *in vitro*. To determine if other cytokines may play a role, PMN from 3 ND were treated for 24 hours with G-CSF, IL-4, IL-1β,

TNF-α, IL-6, GM-CSF, IL-2, IFN-γ, TNF-β and IL-8, and then monitored for IL-8-induced degranulation. Dose-response curves were determined over the IL-8 concentration range indicated in Figure 3.1. Results are described in Table 3.2 as either an increase, decrease or as no change relative to the untreated control found at a high and at a low IL-8 input concentration. No significant changes at either concentration of IL-8 were noted with G-CSF, GM-CSF, IL-2, and IL-4, whereas TNF-β, TNF-α, IL-6, and IL-1β enhanced IL-8-induced enzyme release only at a lower input concentration of IL-8. IFN-γ had the greatest enhancing effect on IL-8-induced degranulation at all IL-8 concentrations. Exposure of PMN to IL-8 prior to treatment with IL-8 in the degranulation assay resulted in an increase in enzyme release at low, but a decrease at high IL-8 concentrations.

Table 3.2 Pretreatment of PMN with cytokines

Cytokine	Concentration	Effect on β-glucuronidase release *	
		15ng/ml	500ng/ml
IL-1β	500 U/ml	↑	-
IL-2	100 U/ml	-	-
IL-4	1 U/ml	-	-
IL-6	100 U/ml	↑	-
IL-8	100 ng/ml	↑	↓
GM-CSF	100 U/ml	-	-
G-CSF	100 U/ml	-	-
IFN-γ	100 U/ml	↑	↑
TNF-α	2.5 ng/ml	↑	-
TNF-β	2.5 ng/ml	↑	-

*↑: increase in enzyme release relative to untreated control; ↓: decrease; - : no change.

3.3.1.6 Infection of PMN with HIV-1 *in vitro*

In order to determine if HIV-1 was directly involved in the altered IL-8 induced β-glucuronidase response, PMN from 4 ND were incubated with HIV-1 (strain IIIB) for 24 hours and 96 hours (with or without the addition of GM-CSF), and monitored for IL-8-induced release of β-glucuronidase. Results are shown in Figure 3.5.

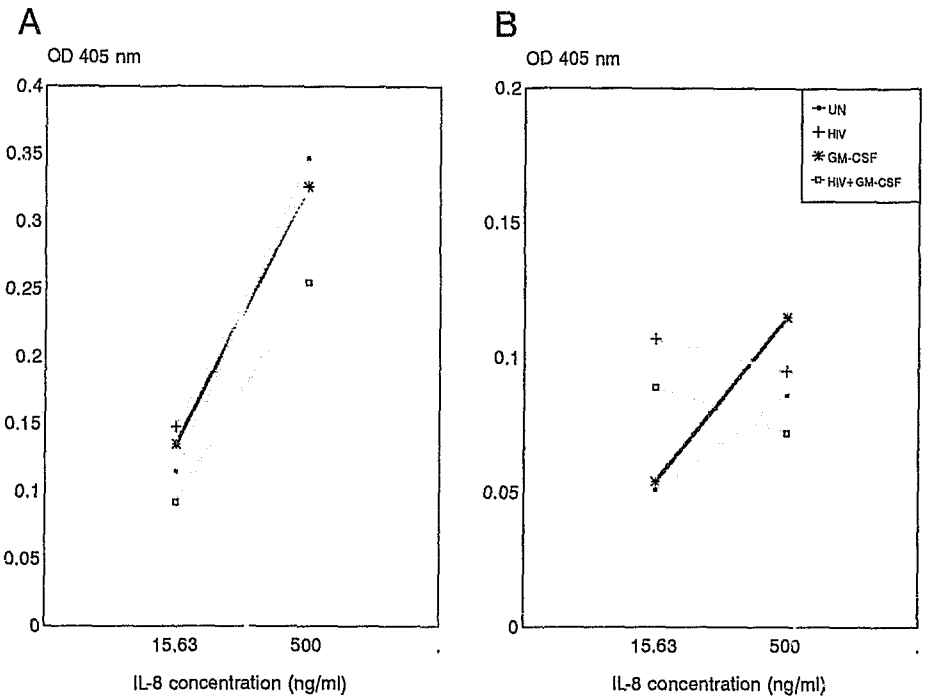

Figure 3.5 Effect of *in vitro* infection with HIV-1 on the β-glucuronidase released from PMN in response to IL-8. PMN isolated from 4 healthy individuals were exposed to HIV-1 (strain IIIB) for 24 hours (A) and 96 hours (B), with or without the addition of 100 U/ml of GM-CSF, and then assayed for their ability to degranulate in response to IL-8. Results are the mean values obtained at a low (15.63 ng/ml) and a high IL-8 input concentration (500 ng/ml) from 4 independent experiments. UN: uninfected; HIV: HIV-1 infected.

Neither HIV-1 alone or GM-CSF had any effect on IL-8-induced enzyme release in PMN cultured for 24 hours ($p>0.05$) but together synergized in decreasing IL-8-induced enzyme release ($p=0.05$) (Figure 3.5A). However, after 96 hours of culture in the presence of HIV-1, a near-

reciprocal dose-response graph (Figure 3.5B) typical of the type of response seen with PMN isolated directly from HIV-1 infected individuals was obtained for 3 of the 4 ND PMN, with no difference in response to the two IL-8 input concentrations in the other. Whereas, GM-CSF potentiated degranulation in response to IL-8 in uninfected PMN (at 500 ng/ml of IL-8 only; $p=0.05$), it decreased the release of enzyme in HIV-1 infected PMN at both IL-8 input concentrations ($p<0.05$). Figure 3.6 presents a comparison of these same IL-8-induced degranulation responses of PMN infected with HIV-1 *in vitro*, with and without the addition of GM-CSF, and of PMN from the HIV/TB group described in Table 3.1 and Figure 3.1, showing the similarity in responses.

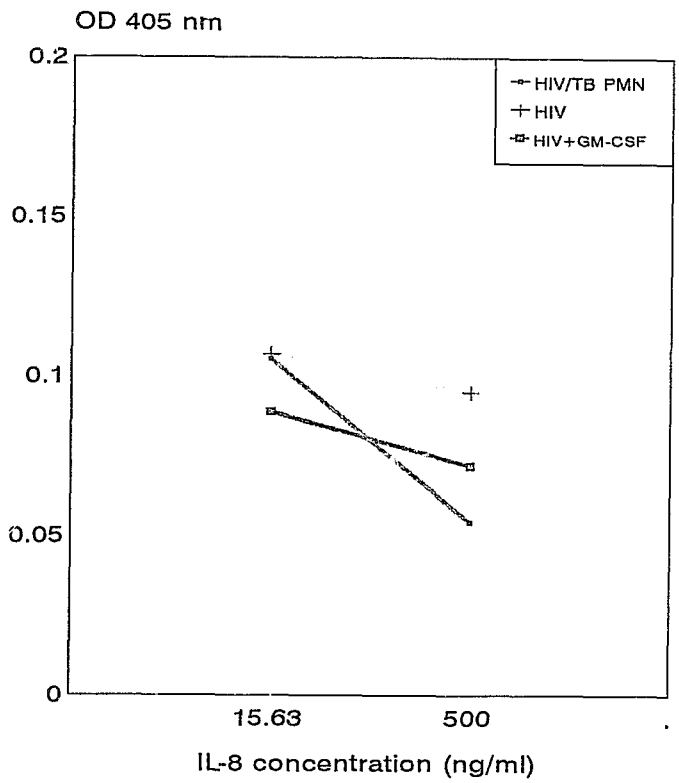

Figure 3.6 Comparison of IL-8-induced degranulation responses of PMN from ND (n=4) exposed to HIV-1 *in vitro* after 96 hours in culture with and without the addition of 100 U/ml of GM-CSF, and those of PMN isolated directly from HIV-1-infected individuals (n=9; HIV/TB group from Figure 3.1).

3.3.1.7 GM-CSF differentially modulates the degranulation response to IL-8 in PMN from ND and HIV/TB groups

In experiments carried out on PMN from 4 ND and 4 HIV/TB patients, the effect of GM-CSF on IL-8-induced release of β-glucuronidase was determined after culture for 96 hours. Due to reduced responses obtained after prolonged culture of PMN the viable cell input for β-glucuronidase assays was increased to double that used in previous experiments. In contrast to results obtained with PMN from ND, where IL-8-induced degranulation was enhanced in the presence of GM-CSF (Figure 3.7A), this response was reduced in HIV/TB patient PMN relative to their untreated controls ($p<0.05$) (Figure 3.7B).

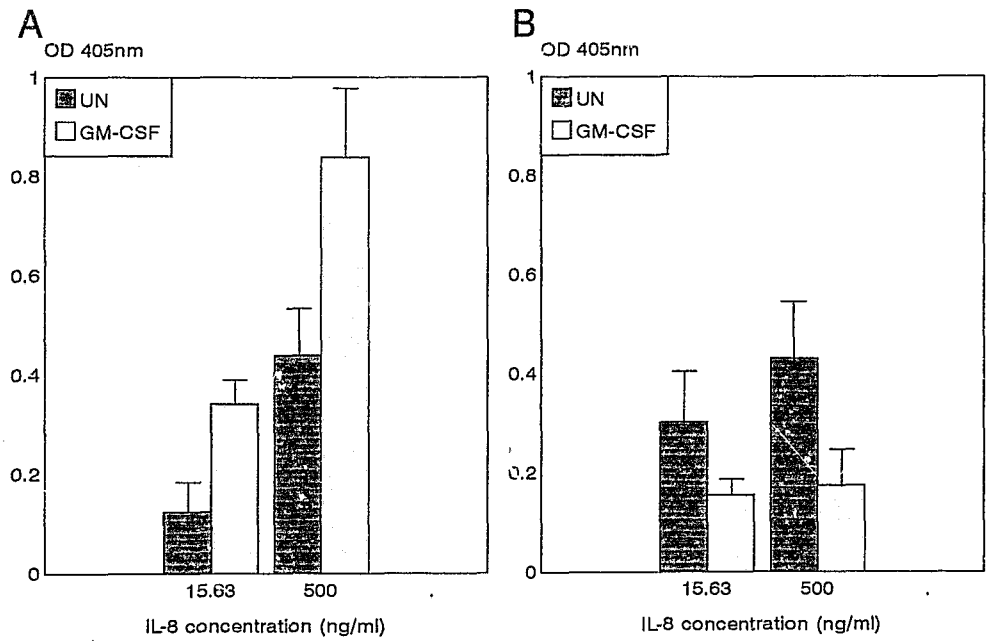

Figure 3.7 The effect of GM-CSF on the IL-8-induced β-glucuronidase response of PMN of ND (A) and HIV/TB (B) individuals after 96 hours culture. PMN were isolated from 4 ND and 4 HIV/TB individuals, incubated with or without 100 U/ml GM-CSF for 96 hours and assayed for their ability to degranulate in response to IL-8 at a concentration of 15.63 ng/ml and 500 ng/ml. Results are expressed as the mean ± standard error of the mean determined from 4 independent experiments.

Interestingly, untreated HIV/TB PMN showed a tendency toward a positive dose-response slope in 96 hours cultures although the mean enzyme release at 15.63 ng/ml was not significantly different from that at 500 ng/ml ($p>0.05$). The addition of GM-CSF to the cultures affords a growth advantage over untreated cultures and PMN survival was enhanced to the same degree for both ND and HIV/TB cultures, as determined by trypan blue exclusion (data not shown). The effect of GM-CSF on HIV/TB PMN was therefore not due to a differential effect on cell survival when compared to ND PMN.

3.3.2 PMN chemotaxis

3.3.2.1 IL-8-induced PMN chemotaxis

Directed migration in response to IL-8 was measured using a Transwell chemotaxis assay as described in Section 3.2.7. Figure 3.8 shows the chemotactic indices determined for 11 ND and 22 HIV/TB patients. When further stratified on the basis of the magnitude of the chemotactic response, indicies obtained for the HIV/TB patients could be subgrouped as either low ($CI<4.0$), intermediate (CI 4.0-6.0) or high ($CI>6.0$). These all independently were significantly different from the ND group ($p<0.05$). There were no significant correlations between CI and immunological characteristics of these patients.

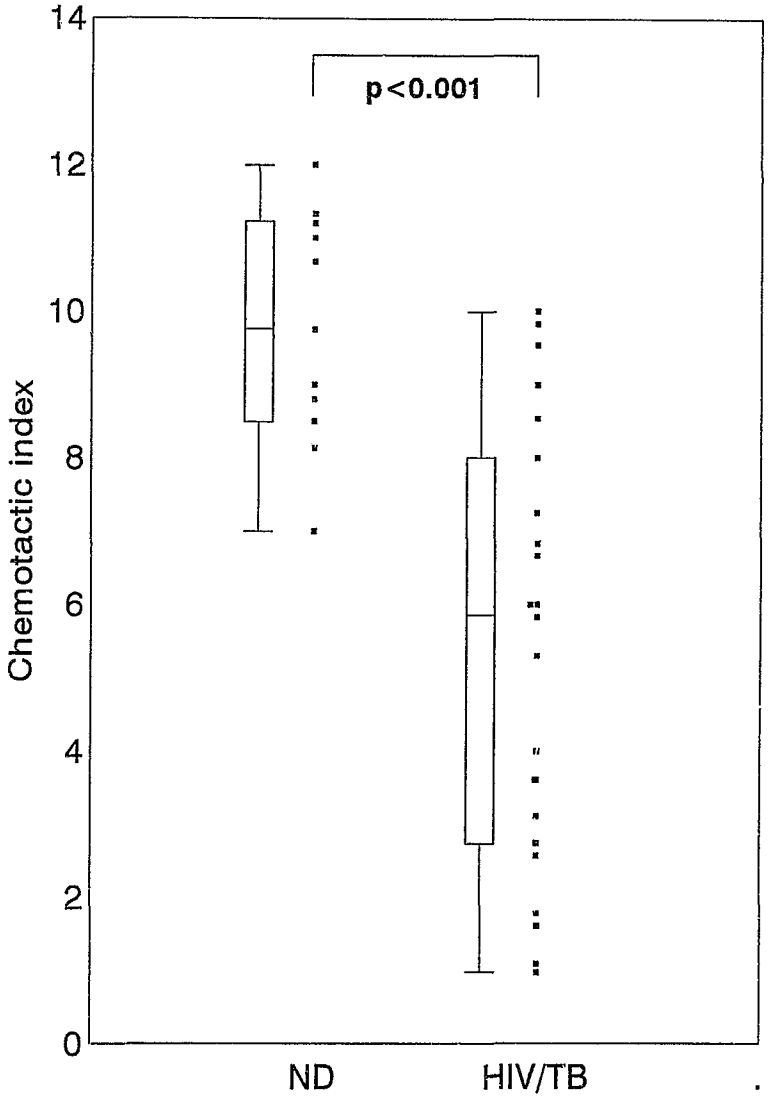

Figure 3.8 IL-8-induced chemotaxis of PMN from the ND and HIV/TB study groups. Chemotaxis in response to IL-8 (100ng/ml) was monitored by a Transwell assay. Results are expressed as the mean chemotactic index (CI) from duplicate determinations : the ratio of the number of cells migrating in response to IL-8 divided by the number of randomly migrating cells in control wells. Data are presented as individual values (solid squares), medians (horizontal bar), 25th and 75th percentiles (boxes) and the 10th and 90th percentiles (bars). Significant differences between groups are indicated.

3.4 Discussion

Defective functioning of the non-oxidative armature of PMN may be what is responsible for the reduced microbial killing seen in PMN from HIV-1 infected individuals (Wenisch *et al.*, 1996; Shalekoff *et al.*, 1998). In this section, neutrophil degranulation was measured in response to IL-8 by the release of β-glucuronidase. Results showed that PMN from HIV-1 infected individuals, whether co-infected with *M. tuberculosis* or not, had a significantly altered ability to degranulate in response to IL-8. The sensitivity of the test did not however allow determination of whether HIV/TB individuals show greater impairment in this function compared to persons infected with HIV-1 alone. Most striking was the reciprocal nature of the response of both these groups compared to normal, in that higher IL-8 concentrations induced significantly less release of β-glucuronidase than did lower input concentrations. Even at the lower IL-8 concentrations PMN from HIV-1 infected persons did not attain comparable enzyme release to normal maximal release. The type of enzyme release response or its magnitude was unrelated to the stage of disease in these patients as determined by CD4 T-cell count. Impaired degranulation was not only an IL-8-specific event as a similar impairment of degranulation in response to another agonist, FMLP, was also shown.

Ellis *et al.* (1988) conducted a series of experiments to evaluate neutrophil functions in patients with AIDS or AIDS-related complex (ARC) including degranulation which was assayed using similar methodology to that used in this study. They, however, found no difference between degranulation of PMN in response to FMLP in HIV-1 infected patients and healthy controls, although in their study release of β-glucuronidase was only measured at one concentration of FMLP (10^{-7}M). From results obtained in this study using IL-8 as the agonist, it can be seen that had only one concentration been employed in the range where there is a cross-over of the dose-response curves of the different study groups (see Figure 3.1), then the defective response in HIV-1 infected individuals would not have been detected. Similarly, Valone *et al.* (1984) studied PMN degranulation in HIV-1 infected patients with persistent generalized lymphadenopothy (PGL) using FMLP and LTB_4 as stimuli in the β-glucuronidase assay. β-glucuronidase release in response to FMLP was similar for the HIV patient PMN compared to control PMN, with a maximal net β-glucuronidase release of 26.7% for the patient PMN at 10^{-6}M FMLP. Differences were however observed when LTB_4 was used as an agonist, in that there was a significantly

reduced release of β-glucuronidase at a variety of LTB_4 input concentrations from patient PMN when compared to control PMN.

Having observed an altered degranulation response in HIV-1 infected individuals, various experiments were designed in an attempt to determine what factors could be contributing to the reciprocal IL-8 dose-response pattern. This reciprocal response was apparently not due to a factor present in the plasma of HIV-1 infected individuals or prior exposure to elevated levels of a number of cytokines tested, except for perhaps IL-8, which increased the release of β-glucuronidase at low but decreased it at high IL-8 concentrations *in vitro*. Levels of IL-8 are known to be raised in the peripheral circulation of HIV-1 infected individuals (Matsumoto *et al.*, 1993; Thea *et al.*, 1996), and as IL-8 dynamically regulates its own receptors on PMN (Samanta *et al.*, 1990), this could be one mechanism whereby non-oxidative processes of PMN could be altered. The closest parallel to the *in vivo* situation was however obtained when infection of PMN from healthy individuals with HIV-1 *in vitro* resulted in an altered dose-response graph after prolonged *in vitro* culture. Therefore, a combination of both the phenotype of ageing PMN together with exposure to HIV-1 resulted in a reversal of the normal response. In agreement with this is the fact that as one might expect, ageing PMN show impaired function associated with apoptosis (Whyte *et al.*, 1993) and PMN from HIV-1 infected patients show enhanced apoptosis upon their isolation (Pitrak *et al.*, 1996). Interestingly, GM-CSF when added to PMN cultures from healthy individuals enhanced degranulation in uninfected PMN but decreased degranulation in HIV-1 infected PMN. The same was true for the effects of GM-CSF on PMN isolated from ND and HIV/TB individuals, suggesting that GM-CSF did not have an equivalent ability to restore degranulation function in the presence of HIV-1 infection as in its absence. GM-CSF can have both direct effects on neutrophil function such as the induction of other cytokines by PMN (Lindemann *et al.*, 1988; Lindemann *et al.*, 1989; Cicco *et al.*, 1990) and PMN migration (Gasson *et al.*, 1984), and indirect effects in enhancing responses to other stimuli, a process called "priming". GM-CSF enhances antibody-dependent cellular cytotoxicity (ADCC) (Vadas *et al.*, 1983), primes neutrophils for chemotaxis and respiratory burst in response to FMLP (Weisbart *et al.*, 1986), and increases phagocytosis of bacteria (Fleischmann *et al.*, 1986). The underlying mechanism responsible for differential IL-8-mediated degranulation of PMN when exposed to GM-CSF may be related to its activity in inhibiting apoptosis (Brach *et al.*, 1992), this potential being altered in the presence of HIV-1 infection. Furthermore, direct effects of HIV-1 proteins

on PMN have been described previously (Gabrilovich *et al.*, 1993). There are several lines of evidence to suggest that PMN of HIV-1 infected individuals are primed *in vivo*, and this is born out by studies showing altered surface marker expression such as increased CD11b (Palmer and Hamblin, 1993), altered PMN functions such as enhanced phagocytosis of *E. coli* (Shalekoff *et al.*, 1998) and *Candida* sp. (Wenisch *et al.*, 1996), and the enhanced apoptosis of PMN upon their isolation (Pitrak *et al.*, 1996). A tendency towards an enhanced spontaneous release of β-glucuronidase from PMN of the HIV group shown here would further support this. Exposure to the presence of HIV-1 proteins, various pro-inflammatory mediators, circulating bacterial products, and cytokines which activate neutrophils (Colotta *et al.*, 1992; Lee *et al.*, 1993) in the peripheral circulation of HIV-1 infected persons may contribute to a primed PMN phenotype conducive to an increased rate of subsequent apoptosis.

In addition to degranulation, PMN chemotaxis in response to IL-8 was also found to be significantly impaired in a group of HIV/TB patients when compared to healthy individuals. Valone *et al.* (1984) have previously shown defective PMN chemotaxis to LTB_4 and FMLP as chemotactic stimuli in AIDS patients with persistent lymph node syndrome. Similarly, Ellis *et al.* (1988) found PMN chemotaxis in patients with ARC to be significantly less than in healthy controls, using in their study an *E. coli* filtrate as chemoattractant.

These altered IL-8-induced PMN responses may be indicative of abnormal PMN function and have important implications in the pathogenesis of HIV-1 infection in terms of their ability to clear bacterial and fungal infections. Virus-induced changes or indirect immune processes as a result of HIV-1 infection may render PMN phagocytic cells relatively ineffectual with respect to one or more cell functions or antimicrobial activities. Future attempts should be aimed at defining the mechanisms that bring about these changes in order to contribute to a greater understanding of the mechanisms that lead to an enhanced risk of superinfections in immunosuppressed individuals.

CHAPTER FOUR

RECEPTORS THAT MEDIATE NEUTROPHIL FUNCTION

4.1 Introduction

Human neutrophils possess two classes of receptors for the Fc portion of IgG molecules (FcγR), FcRII (CD32) and FcRIII (CD16) (Anderson and Looney, 1986), both which have been implicated in the clearance of circulating immune complexes. The interaction of FcγR with immune complexes triggers a variety of cellular responses, including phagocytosis, activation of the respiratory burst, ADCC, and secretion of inflammatory mediators (Rosales and Brown, 1993). The FcγRIII is expressed 5-15 times more abundantly on the plasma membrane of neutrophils than FcγRII (Huizinga *et al.*, 1989). A soluble form of the receptor has been detected in plasma and appears to be derived from PMN (Huizinga *et al.*, 1990a), either released spontaneously (Lanier *et al.*, 1989) or when neutrophils are activated (Huizinga *et al.*, 1988).

Two types of IL-8 receptor have been described, namely IL-8RA (Holmes *et al.*, 1991) and IL-8RB (Murphy and Tiffany, 1992), which are both members of the superfamily of seven transmembrane G-protein coupled receptors (Taylor, 1990). The receptors for IL-8 are present on all PMN, monocytes, and 5-25% of total lymphocytes (Chuntharapai *et al.*, 1994). The highest levels of both IL-8RA and IL-8RB are found on PMN, with IL-8RA and IL-8RB expression occurring at approximately equal ratios (Chuntharapai *et al.*, 1994). IL-8 has been shown to bind with high affinity to both receptors, whereas all the other C-X-C chemokines, including the ELR^+ C-X-C chemokines GROα, GROβ, GROγ, NAP-2, and ENA-78 are potent agonists for IL-8RB, but not IL-8RA (Moser *et al.*, 1991; Schumacher *et al.*, 1992; Ahuja *et al.*, 1996).

Not only do the IL-8 receptors on PMN bind C-X-C chemokines differentially, but they have also been shown to be functionally different (Jones *et al.*, 1996). Neutrophil responses such as the release of granule enzymes and changes in cytosolic free Ca^{2+} (Jones *et al.*, 1996) and chemotaxis (Loetscher *et al.*, 1994) are mediated by both IL-8RA and IL-8RB. The activation of phospholipase D and oxidative burst, via NADPH oxidase, are however only triggered through IL-8RA, but not IL-8RB (Jones *et al.*, 1996).

The aims of this chapter were firstly to monitor the expression of receptors belonging to two different families of receptors that mediate a variety of PMN functions. The first of these was FcγRIII (CD16), important in IgG-mediated PMN functions, and the second family with the two specific IL-8 receptors (A and B), important in IL-8 mediated PMN functions. Results described in the previous chapter demonstrated that both degranulation and chemotaxis in response to IL-8 were impaired in HIV-1 infected individuals. As IL-8 mediates its effects on PMN by high-affinity binding to these receptors, it was important to evaluate the expression of these receptors on the surface of PMN in the different study groups. Furthermore, the relationship between the degree of expression of IL-8 receptors and the capacity of the subsequent IL-8-induced functions of degranulation and chemotaxis were determined.

4.2 Materials and methods

4.2.1 Patient samples

Four subject groups were recruited for each of the studies and included 16 ND as controls, 16 TB, 16 HIV, and 16 HIV/TB patients. For the ND, TB, and HIV/TB groups, the 16 individuals in each were common to both the IL-8 receptor and CD16 studies, while only 8 of the HIV patients were common to both studies, with the remainder of each group comprising different individuals. 6 ND and 12 HIV/TB patients were recruited for the concurrent analysis of IL-8 receptor expression on their PMN, and the capacity of their PMN to degranulate in response to IL-8 as measured by the release of β-glucuronidase. A further 6 ND and 15 HIV/TB patients were recruited for studies comparing IL-8 receptor expression and chemotaxis of PMN. All TB and HIV/TB patients were receiving standard 4-drug anti-TB therapy. The mean duration of anti-TB treatment did not differ between the TB and HIV/TB groups ($p>0.05$). Blood was collected by venipuncture into EDTA vacutainers. The blood was processed and analyzed by flow cytometry within 6 hours of collection. Three part differential counts were determined using a Coulter Onyx (Coulter Corp., Hialeah, FL) cell counter. The immunological characteristics of the individuals are shown in Table 4.1 (CD16 study) and Table 4.2 (IL-8 receptor study).

4.2.2 Fluorescent labelling of cells in whole blood

Labelling of cells for CD16 expression was performed by adding 5μl of fluorescein isothiocyanate (FITC)-conjugated CD16 antibody to 50μl whole blood. As a control, 5μl IgG1-FITC was added to 50μl whole blood. Samples were incubated with the antibodies for 20 minutes at room temperature, and the red blood cells were lysed with 2ml 1X FACS lysing solution. The cells were then washed and resuspended in 200μl of fixative which was 1.5% (v/v) formaldehyde containing 2% (w/v) BSA. Samples were stored at 4°C until analysis.

Labelling of cells with IL-8RA and IL-8RB antibodies was done using an indirect staining method. The antibody dilutions for optimal fluorescent staining of cells in normal donor whole blood was first determined by primary and secondary antibody titrations. Staining was performed

on each of the samples by adding 5μl of an appropriate dilution of either IL-8RA or IL-8RB antibody to 50μl whole blood (2.5 μg/ml final concentration). As a control for IL-8RA and IL-8RB staining, 5μl of appropriately diluted mouse IgG1 or IgG2a, respectively, was added to 50μl whole blood. The samples were then incubated with the antibodies for 20 minutes at room temperature, and washed twice with 3ml wash solution. 5μl of the secondary antibody, FITC-conjugated goat anti-mouse, was then added to each of the samples, which were again incubated for 20 minutes at room temperature. Samples were then washed, the red blood cells lysed with 2ml 1X FACS lysing solution, washed again and the cells resuspended in 200μl fixative. Samples were stored at 4°C until analysis.

4.2.3 Flow cytometry

A Becton Dickinson FACSort flow cytometer with a 488 nm argon laser was used for all analyses. Forward (FSC) and side light scatter (SSC) characteristics were used in gating the granulocyte population. The data were analysed using Cellquest™ version 1.0 software and expressed as the percentage of cells expressing CD16, IL-8RA or IL-8RB and their respective fluorescence intensities or median channel shift values (median channel number of the sample stained with CD16 or IL-8 receptor antibodies minus the median channel number of the corresponding isotype antibody control sample). For comparison in the IL-8 receptor study, lymphocytes were also gated using FSC and SSC on the same samples and the proportion of these cells expressing either IL-8 receptor determined.

4.2.4 β-glucuronidase bioassay

See Section 3.2.3

4.2.5 Chemotaxis assay

See Section 3.2.7

4.3 Results

4.3.1 Expression of FcγRIII (CD16)

4.3.1.1 Immunological status of study groups

As described in Section 4.2.1, four subject groups, comprising 16 individuals in each group were recruited to analyse the expression of FcγRIII (CD16) on the surface of whole blood PMN. The immunological characteristics of the 3 patient groups and the ND control group are shown in Table 4.1.

Table 4.1 Immunological status of study groups (CD16 study)

	Study group			
	ND	TB	HIV	HIV/TB
n	16	16	16	16
White blood cells				
count: x $10^3/\mu l$	5.4 ± 0.2	10.3 ± 1.1	5.1 ± 0.5	5.7 ± 0.5
Granulocytes				
count: x $10^3/\mu l$	2.8 ± 0.2	6.7 ± 1.0	2.4 ± 0.2	3.2 ± 0.4
percentage of cells	50.6 ± 2.0	62.4 ± 3.6	49.4 ± 2.5	55.9 ± 3.4
CD4 cells				
count: cells/μl	982 ± 46	1030 ± 113	368 ± 57	296 ± 68
percentage of cells	44.8 ± 1.7	45.3 ± 2.4	15.8 ± 2.1	15.3 ± 2.1
CD8 cells				
count: cells/μl	580 ± 36	804 ± 93	1485 ± 261	1130 ± 207
percentage of cells	27.8 ± 1.7	30.7 ± 2.1	59.3 ± 4.0	60.9 ± 2.7
CD4:CD8	1.7 ± 0.2	1.6 ± 0.2	0.3 ± 0.1	0.3 ± 0.05

Results are expressed as the mean ± SEM

Significant differences were observed between the total white blood cell (WBC) counts of the TB group when compared to those of ND, HIV and HIV/TB groups ($p<0.001$) (Table 4.1). Significant differences in absolute PMN counts were found between ND and TB ($p<0.001$), TB and HIV ($p<0.001$), and TB and HIV/TB ($p<0.05$). For the PMN percentages, significant differences were only found between the ND and TB groups ($p<0.05$), and the TB and HIV groups ($p<0.05$). Highly significant differences ($p<0.001$) were observed comparing the CD4 counts of the ND group with that of the HIV and HIV/TB groups and comparisons between the TB group and the HIV and HIV/TB groups. CD4 cell counts did not differ significantly between the HIV and HIV/TB groups ($p>0.05$).

4.3.1.2 CD16 expression on peripheral blood PMN

Similar proportions of PMN expressed CD16 in the ND, TB and HIV groups, whereas there was a significant reduction in both the proportion of CD16-expressing PMN and CD16 fluorescence intensity expressed as median channel shift (MCS) in the HIV/TB group (Figure 4.1A and 4.1B, respectively). The only correlation was between the percentage of CD16-positive PMN and the CD16 fluorescence intensity and % PMN in the HIV/TB group ($r=0.77$; $p=0.003$). A lack of correlation between CD4 cell count and CD16 expression, even when stratified into subgroups that have CD16 expression within the ND group range and those that have less, suggests that reduced CD16 expression is not related to the stage of HIV-1 disease, but rather is exacerbated by the presence of TB in the HIV/TB group.

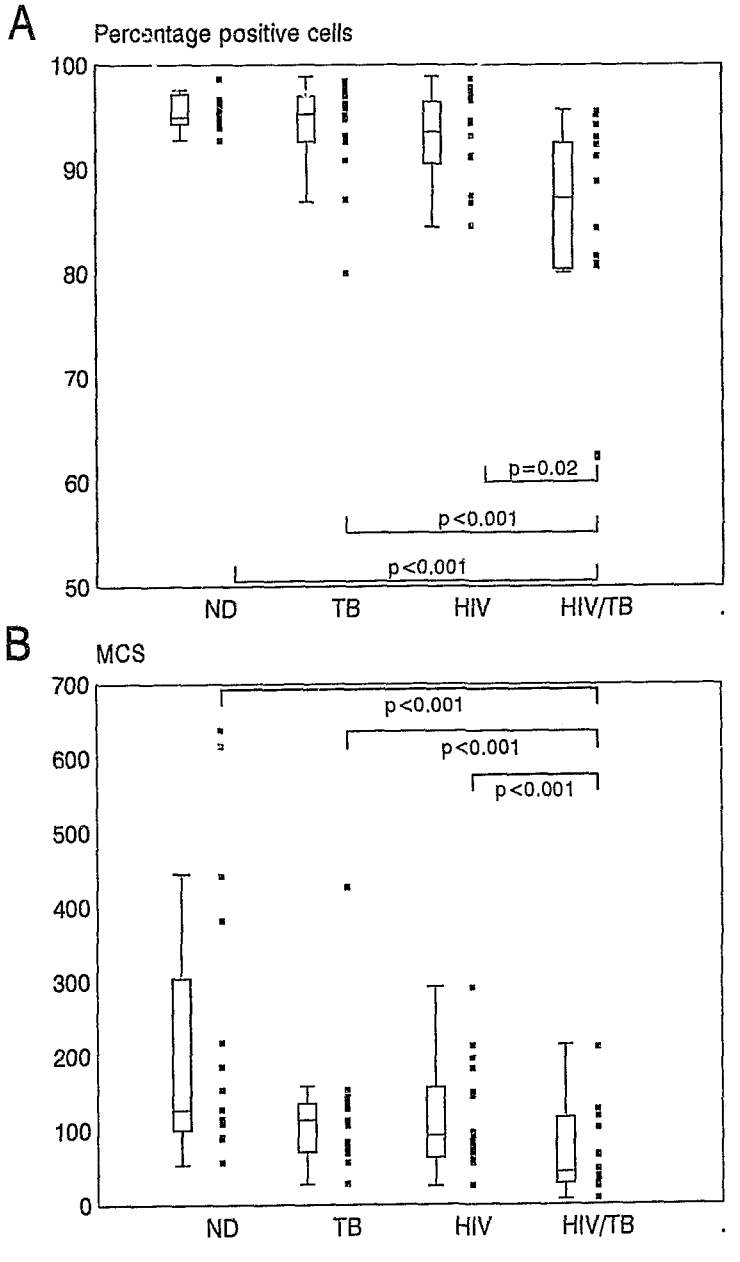

Figure 4.1 Proportion of CD16-fluorescing (A) and CD16 fluorescence intensity (B) of whole blood PMN from ND, TB, HIV, and HIV/TB study groups. Data are presented as individual values (solid squares), medians (horizontal bar), 25th and 75th percentiles (boxes) and the 10th and 90th percentiles (bars). Significant differences between groups are indicated.

4.3.2 Expression of IL-8 receptors A and B

4.3.2.1 Immunological status of study groups

Four subject groups, comprising 16 individuals in each group were recruited to analyse the expression of IL-8RA and IL-8RB on the surface of whole blood PMN. The immunological characteristics of these individuals are shown in Table 4.2.

Table 4.2 Immunological status of study groups (IL-8 receptor study)

	Study group			
	ND	TB	HIV	HIV/TB
n	16	16	16	16
White blood cells				
count : $x10^3/\mu l$	5.4 ± 0.2	10.3 ± 1.1	5.0 ± 0.3	5.7 ± 0.5
Granulocytes				
count : $x10^3/\mu l$	2.8 ± 0.2	6.7 ± 1.0	2.4 ± 0.2	3.2 ± 0.4
percentage of cells	50.6 ± 2.0	62.4 ± 3.6	47.8 ± 2.6	55.9 ± 3.4
CD4 cells				
count : cells/μl	982 ± 46	1030 ± 113	337 ± 50	296 ± 68
percentage of cells	44.8 ± 1.7	45.3 ± 2.4	14.2 ± 2.0	15.3 ± 2.1
CD8 cells				
count : cells/μl	580 ± 36	804 ± 93	1577 ± 227	1130 ± 207
percentage of cells	27.8 ± 1.7	30.7 ± 2.1	61.6 ± 3.2	60.9 ± 2.7
CD4 : CD8	1.7 ± 0.2	1.6 ± 0.2	0.3 ± 0.05	0.3 ± 0.05

Results are expressed as the mean ± SEM

Only the HIV group comprised different individuals to those recruited for the CD16 study (see Section 4.2.1). Significant differences observed comparing the leukocyte counts and percentages were however the same as those for the CD16 study, as described in Section 4.3.1.1

4.3.2.2 IL-8RA and IL-8RB expression on peripheral blood PMN

The effect of HIV-1 disease and pulmonary TB on the expression of IL-8 receptors on PMN was evaluated using whole venous blood obtained from individuals within the 4 groups described in Section 4.2.1, and indirectly staining cells with mAbs that specifically bind each of the two IL-8 receptors (Chuntharapai *et al.*, 1994). Figure 4.2A shows the proportion of PMN expressing receptor A for the different groups. The median % positive PMN was 97.2 (range 94.2 - 98.3) for healthy individuals. By comparison, there was a significant reduction in the percentages of the IL-8RA found on PMN from the TB group ($p<0.05$), the HIV group ($p<0.05$), and the HIV/TB group ($p<0.001$). The corresponding median channel shifts, expressed as relative fluorescence intensity determinations are shown in Figure 4.2B. There was a significant reduction in the relative fluorescence intensity of receptor A in the TB group, the HIV group, and the HIV/TB group when compared to the control group. IL-8RA expression in the HIV/TB group was significantly reduced relative to the HIV and TB groups.

The proportion of IL-8RB-positive PMN was also significantly reduced in each of the three infected groups ($p<0.001$) when compared to the control group of healthy individuals (Figure 4.3A), whose median was 97.7 (range 94.2 - 99.0). The same was true for comparisons of the intensity of IL-8RB-specific fluorescence (Figure 4.3B). Both TB groups showed a significantly reduced expression of IL-8RB relative to that found for the HIV group, with a greater reduction observed in HIV-1 seropositive individuals with concurrent pulmonary TB.

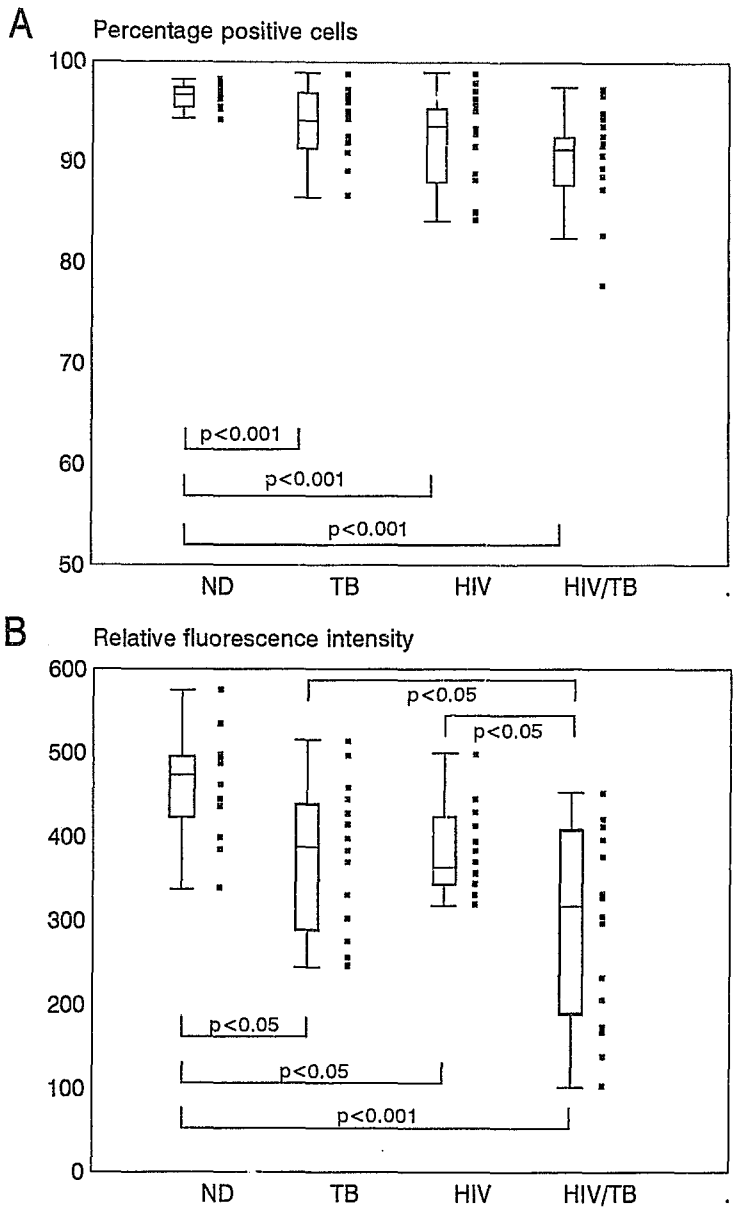

Figure 4.2 IL-8 Receptor A staining of granulocytes from ND, TB, HIV, and HIV/TB study groups. Granulocytes were gated and the percentage of positive cells (A), and the relative fluorescence intensity (B) were determined. Data are presented as individual values (solid squares), medians (horizontal bar), 25th and 75th percentiles (boxes), and the 10th and 90th percentiles (bars). Significant differences between the groups are indicated.

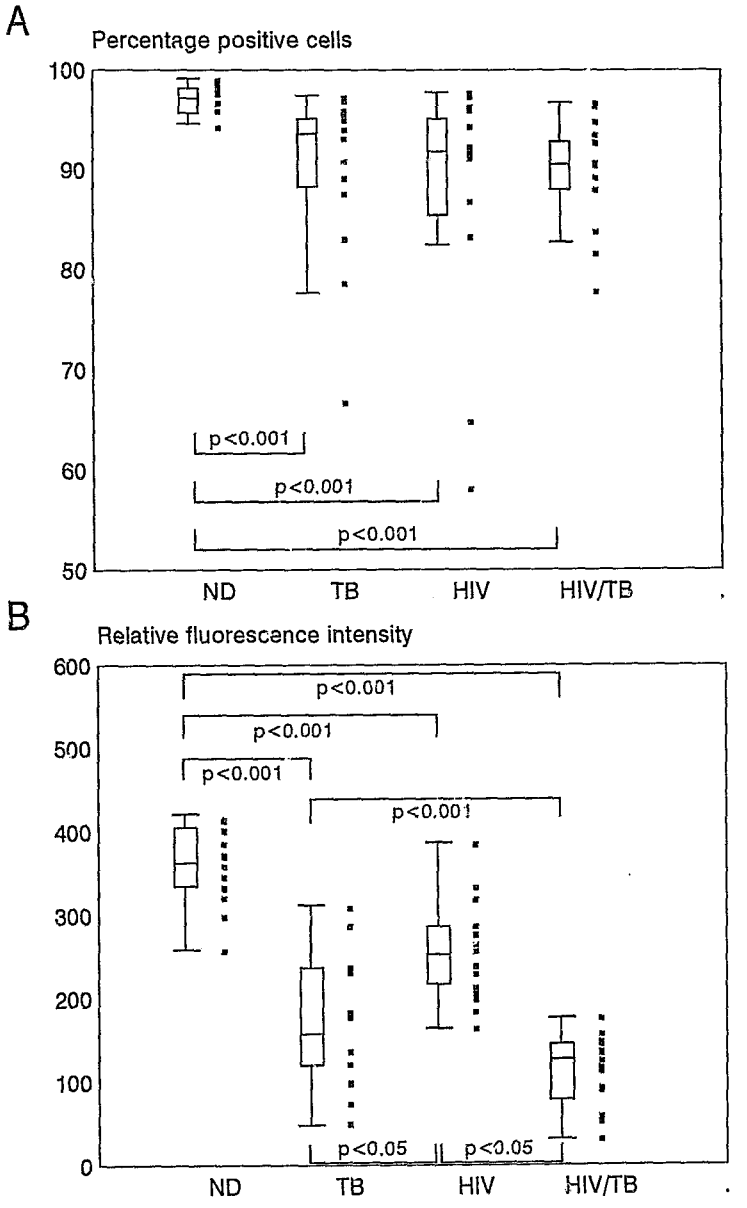

Figure 4.3 IL-8 Receptor B staining of granulocytes from ND, TB, HIV, and HIV/TB study groups. Granulocytes were gated and the percentage of positive cells (A), and the relative fluorescence intensity (B) were determined. Data are presented as individual values (solid squares), medians (horizontal bar), 25th and 75th percentiles (boxes), and the 10th and 90th percentiles (bars). Significant differences between the groups are indicated.

4.3.2.3 Relationship between IL-8 receptor expression and duration of anti-TB treatment

As a much greater degree of suppression of IL-8RB occurred on PMN of individuals in the two TB groups when compared to the HIV only group, a situation unlike that found for the IL-8RA, it was important to consider the possible role of anti-TB drug therapy in this phenomenon.

The mean duration of anti-TB treatment did not differ significantly between the TB and HIV/TB groups ($p>0.05$) and the times ranged from 1 week to a maximum of 26 weeks. When the two groups were combined there was no correlation between the expression of either IL-8 receptor on PMN and the duration of their anti-TB drug therapy ($p>0.05$). Interestingly, the TB group on its own showed a weak negative correlation between the fluorescence intensity of IL-8RA and the duration of anti-TB treatment ($r=-0.63$, $p<0.05$). When patients were stratified into two groups on the basis of time of treatment, approximately half had received treatment for <2 months and the other half for >2 months. There was no significant difference between the expression of IL-8RA (% positive) or IL-8RB receptor (% positive or MCS) between the <2 month and the >2 month treatment groups ($p>0.05$). However, the intensity of IL-8RA expression in TB patients was significantly higher in the <2 month treatment group when compared to that of the >2 month group ($p=0.01$).

4.3.2.4 Relationship between IL-8 receptor expression on PMN and lymphocytes

The percentage of lymphocytes expressing IL-8RA and IL-8RB, determined only on the basis of their FSC and SSC characteristics, are shown in Table 4.3. The purpose for monitoring each patients lymphocytes was to provide an indication as to whether there may be a relationship between down-regulation of IL-8 receptors on PMN and that on lymphocytes. The range of % IL8R-positive lymphocytes obtained in the ND group was in agreement with previously described values (Chuntharapai *et al.*, 1994). The lymphocyte compartment was largely unaltered in the TB group with respect to IL-8RA/B expression when compared with that of normal donors. Significant reductions in expression were mainly observed in the two HIV-1 infected groups for IL-8RA and only in the co-infected HIV/TB group for IL-8RB. There was no correlation between expression of either receptor on PMN (% or fluorescence intensity) and proportions of lymphocytes expressing the particular IL-8 receptor ($p>0.05$).

Table 4.3 Gross determination of percentage of lymphocytes showing positivity for IL-8RA and IL-8RB in the 4 study groups

Group	*n*	IL-8RA#	p*	IL-8RB#	p*
ND	16	16.01 (4.77 - 26.59)		17.68 (4.33 - 30.27)	
TB	16	11.64 (5.01 - 22.86)	-	14.68 (4.24 - 31.02)	-
HIV	16	7.62 (0.28 - 16.61)	0.003	13.07 (6.47 - 29.13)	-
HIV/TB	16	5.99 (2.66 - 11.33)	<0.001	7.23 (0.88 - 23.14)	<0.001

#Results are expressed as the median percentage with the percentage range in parentheses
*Significance values of infection groups each compared to normal donors

Other significant differences between groups were TB and HIV (p=0.04) and TB and HIV/TB (p=0.003) for IL-8RA; and TB and HIV/TB (p<0.001) and HIV and HIV/TB (p<0.001) for IL-8RB

4.3.3 Relationship between IL-8 receptor expression and PMN function

4.3.3.1 Relationship between IL-8 receptor expression and PMN degranulation

Having shown that the expression of both IL-8 receptors are down-regulated on PMN of HIV and HIV/TB individuals, the expression of IL-8RA and IL-8RB on PMN and their subsequent ability to degranulate in response to IL-8 was compared. Figure 4.4 shows a composite of the results obtained from a comparison of IL-8 receptor expression and subsequent degranulation capacity in response to IL-8 in a group of 6 ND and 12 HIV/TB individuals. Impaired degranulation of PMN of the HIV/TB group, measured as percentage release of β-glucuronidase at 500 ng/ml of IL-8, was associated with a significant decrease in both the proportion of IL-8RA- and IL-8RB-expressing PMN and in the intensity of IL-8RA and IL-8RB fluorescence. When further stratified into a group of HIV/TB individuals that had a capacity to release enzyme in the range that fell above the 25th percentile calculated for the ND group (n=4), and a group that fell below this (n=8), the major predictor for reduced degranulation function was a significantly reduced fluorescence intensity of IL-8RA (p<0.05).

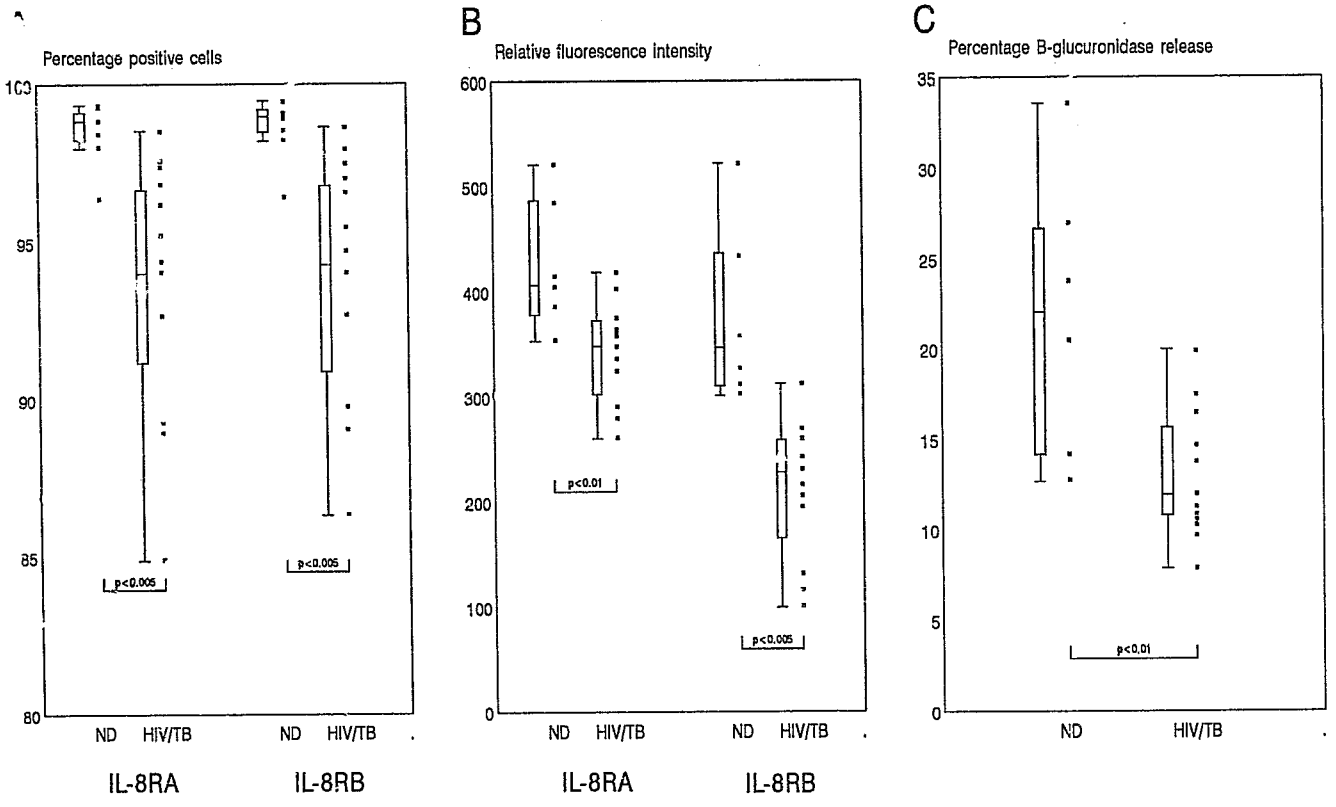

Figure 4.4 Relationship between the expression of IL-8 receptors A and B on whole blood PMN and IL-8-induced degranulation responses of isolated PMN from a group of ND (n=6) and HIV/TB (n=12) individuals. The proportion of IL-8RA- and IL-8RB-expressing PMN (A), their relative fluorescence intensities (B), and the ability of isolated PMN to degranulate in response to 500 ng/ml of IL-8 (C), are shown as individual values (solid squares); 10^{th} and 90^{th} percentiles (error bars). Boxes represent values between the 25^{th} and 75^{th} percentiles, with the median indicated. Significant differences between ND and HIV/TB groups are indicated.

4.3.3.2 Relationship between IL-8 receptor expression and PMN chemotaxis

To address the relationship between reduced IL-8 receptor expression on PMN and the chemotactic functioning of these cells, the expression of the individual IL-8 receptors on PMN from a group of 15 HIV/TB patients was compared to the ability of their PMN to migrate towards IL-8 as a chemoattractant. 6 ND were included for comparison (Table 4.4).

The HIV/TB patients were subgrouped on the basis of the magnitude of their PMN chemotactic responses as either low (CI < 4.0), intermediate (CI 4.0-6.0) or high (CI > 6.0) chemotactic indicies. There were no significant correlations between expression of either IL-8R (% or MCS) and chemotaxis. However, patients with poor chemotactic responses (low CI) had a significantly lower proportion of IL-8RA and IL-8RB-expressing PMN when compared to the intermediate and high CI groups and when compared to the ND group ($p<0.05$). The proportion of IL-8RB-expressing PMN was also significantly less in the HIV/TB intermediate and high CI groups when compared to normal control PMN ($p<0.05$), whereas the IL-8RA percentages were not different ($p>0.05$). The fluorescence intensities of both receptors in all of the HIV/TB CI subgroups were significantly reduced relative to the normal control group ($p<0.05$). The proportion of IL-8R-positive PMN was a stronger predictor for reduced function in the low CI subgroup than was receptor density. A difference as small as 2% resulted in a notable difference in chemotaxis in response to IL-8. In the intermediate and high CI subgroups, the reduced function from normal was the result of a combination of a lower proportion of IL-8RB-fluorescing PMN and reduced intensity of fluorescence of either IL-8RA or IL-8RB, various combinations in different individuals giving rise to the range of chemotactic capacities seen in these groups.

Table 4.4 Comparison of IL-8 receptor expression and capacity to migrate in response to IL-8 of PMN from the ND and HIV/TB study groups

	HIV/TB			ND
	Low (CI*<4.0)	Intermediate (CI 4.0-6.0)	High (CI>6.0)	Control (CI 7.0-12.0)
IL-8 receptors				
n	6	4	5	6
IL-8RA				
%	95.14 (92.14-96.84)	97.95 (95.45-98.77)	97.07 (96.08-98.87)	98.56 (97.60-98.92)
MCS	345.80 (263.88-509.18)	345.97 (268.48-422.22)	309.66 (142.23-412.86)	469.79 (389.96-546.14)
IL-8RB				
%	94.90 (94.30-97.54)	97.66 (96.85-98.37)	97.84 (96.36-98.82)	98.99 (97.68-99.17)
MCS	213.83 (127.86-400.32)	123.76 (64.1-165.72)	130.22 (53.08-285.08)	400.33 (246.02-579.92)

Results are expressed as medians with the range in parentheses
*CI : chemotactic index

4.4 Discussion

This section of the study involved analysing the expression of receptors belonging to two different families known to mediate PMN functions, and to determine the effect of HIV-1 infection, pulmonary TB, and co-infection with both these organisms on the expression of these receptors. The first of these, FcγRIII (CD16), interacts with the Fc portion of IgG molecules and preferentially binds immune complexes, which triggers a variety of cellular responses, including phagocytosis, secretion of inflammatory mediators, and activation of oxidative burst (Rosales and Brown, 1993). Secondly, the expression of the two IL-8 receptors A and B, both members of the superfamily of seven transmembrane G-protein coupled receptors (Taylor, 1990) were analysed. Although functionally different, IL-8RA and IL-8RB both mediate PMN functions such as chemotaxis (Loetscher *et al.*, 1994) and degranulation (Jones *et al.*, 1996).

The expression of FcγRIII (CD16) on the surface of PMN was found to be significantly reduced in HIV-1 seropositive patients with pulmonary tuberculosis when compared to those individuals with either disease alone or healthy blood donors. Results obtained with the HIV and TB groups, although not attaining significance, showed a definite trend towards decreased CD16 expression (% and MCS) relative to normal. The presence of a substantial CD16-negative neutrophil population of 25% (compared to 3% in the HIV-1 seronegative group) in the absence of any change in expression of decay accelerating factor (CD55), another GPI-anchored receptor, has been reported on PMN from HIV-1 infected individuals in another study (Boros *et al.*, 1990). This would suggest that the decreased expression is a specific defect rather than due to a general down-regulation of this family of receptors.

A number of possible explanations can be provided for reduced FcγRIII expression on neutrophils. Firstly, decreased expression of this receptor may be indicative of prior activation *in vivo*. Increased phagocytosis by PMN from HIV-1 infected individuals (Estevez *et al.*, 1986; Wenisch *et al.*, 1996) and upregulation of CD11b (Palmer and Hamblin, 1993) are consistent with cellular activation *in vivo*. It has also been reported that early in HIV-1 infection there are increased serum levels of sCD16 (Khayat *et al.*, 1987) which fall below levels obtained with healthy individuals in the presence of AIDS-defining conditions (Khayat *et al.*, 1990). Neutrophils when activated release a soluble form of the receptor (Huizinga *et al.*, 1988; Huizinga *et al.*,

1990a) and sCD16 in turn has the ability to stimulate neutrophil IL-8 production by binding to CD11b receptors (Galon *et al.*, 1996). Such events could provide an environment of consistent cellular activation in the peripheral circulation. Secondly, Dransfield *et al.* (1994) found that enhanced neutrophil apoptosis *in vitro* correlated with a reduction in CD16 expression, and based on this finding, there may be an association between reduced CD16 expression *in vivo* and neutrophil apoptosis, although this requires further investigation. Alternatively, neutrophils leaving the bone marrow may not be fully differentiated and consequently CD16 receptors, normally first expressed on neutrophils at the metamyelocyte stage (Fleit *et al.*, 1984; Terstappen *et al.*, 1990), are reduced on neutrophils prior to their entering the circulation. A further possibility that cannot be excluded is that increased levels of circulating immune complexes, if present in infected patients, may mask CD16 expression on PMN by blocking these receptors. Whatever the mechanism responsible for reduced receptor expression, it is clearly exacerbated by a combination of HIV-1 infection and pulmonary TB.

PMN from patients with HIV-1, pulmonary TB and those with dual infection have significantly diminished expression of both IL-8 receptors when compared with those of uninfected individuals. It was further apparent that these defects, common to both HIV-1 disease and TB, were present irrespective of the patient's immunological status. One difference noted in expression of the two receptors was the pronounced decrease in IL-8RB on PMN from individuals with TB, this being further aggravated by concurrent HIV-1 infection. As all TB patients were on anti-TB drug therapy the possibility that anti-TB drugs may play a role in reduced IL-8RB expression cannot be excluded and requires further evaluation. What was clear, however, was that the duration of anti-TB treatment had no apparent effect on IL-8RB expression whereas TB patients on treatment for <2 months had a higher fluorescence intensity of IL-8RA than those treated for in excess of 2 months. Future longitudinal studies of TB patients are required to clarify the possible role of anti-TB drug(s) in diminished IL-8R expression on PMN.

It was also questioned whether the down-regulation of IL-8 receptors on PMN was a result of an overall down-regulation of these receptors on all leukocytes or whether there may be some cell-type specific regulation. The presence of TB had no significant effect on the proportion of IL-8RA/B-positive lymphocytes, whereas HIV-1 markedly affected the number of cells expressing IL-8RA and IL-8RB, this being most reduced in the presence of both HIV-1 disease

and pulmonary TB. As there was no correlation between the degree of expression of either IL-8 receptor on PMN and on lymphocytes it seems likely that their regulation *in vivo* is cell-type specific, and may be altered differentially depending on the disease state.

A number of mechanisms may be responsible for down-regulation of IL-8 receptors owing to disease. Levels of IL-8 have been reported to be elevated in the serum of HIV-1 infected patients (Matsumoto *et al.*, 1993 and in the plasma of some TB patients (Friedland *et al.*, 1995; Friedland *et al.*, 1996). Results found in this study (Chapter 2) indicated significantly increased circulating levels of IL-8 in the serum and plasma of HIV-1 infected individuals, with or without concomitant pulmonary TB. On human neutrophils, IL-8 has been shown to dynamically regulate its own receptor expression. IL-8 very rapidly down-regulates its receptor expression, but the down-regulated receptor can be rapidly recycled to the surface of neutrophils (Samanta *et al.*, 1990). The down-regulation of IL-8 receptors seen here may therefore be modulated by IL-8 itself or by other cytokines whose levels may be altered. Alternatively, cells bearing IL-8 receptors may be defective in their ability to recycle these receptors, perhaps through some intracellular interference mechanism. Besides altered recycling of these receptors through extracellular or intracellular means, another related factor may be that, as mentioned before, peripheral neutrophils, at least in HIV-1 infected individuals, are in an activated state (Bandres *et al.*, 1993; Palmer and Hamblin, 1993; Pitrak *et al.*, 1996; Wenisch *et al.*, 1996).

Having found a significant reduction in the expression of both IL-8 receptors on PMN from patients with HIV-1, pulmonary TB, and those with dual infection, studies were carried out to determine if functions dependent on sufficient IL-8 receptor expression on PMN were impaired. IL-8 exerts its effects on PMN, in particular degranulation and chemotaxis, by binding to specific receptors, IL-8RA and IL-8RB. IL-8RA and IL-8RB have been shown to be functionally different and PMN responses such as the release of granule enzymes are mediated by both IL-8RA and IL-8RB (Jones *et al.*, 1996). Looking at PMN from HIV/TB patients, the group with the greatest decrease in receptor expression, it was found that the impairment of IL-8-dependent PMN functions such as degranulation and chemotaxis were associated with the reduced expression of IL-8 receptors on these cells. PMN from HIV/TB patients that had the poorest ability to degranulate at high IL-8 concentrations were also those that had the lowest density of IL-8RA on their PMN. A poor chemotactic response to IL-8 was associated with a reduction of

both IL-8 receptors on these cells.

In summary, results shown demonstrate an association between reduced IL-8 receptor expression and subsequent IL-8-induced PMN functions that are essential for the clearance of invading microbes. Altered surface receptor expression and dysfunction of PMN are consistent with clinical findings of an increased susceptibility to secondary bacterial and fungal infections, especially in patients co-infected with HIV-1 and *M. tuberculosis*.

CHAPTER FIVE

CONCLUDING REMARKS

The first section of this study was designed to look at a number of aspects involving IL-8 production, to determine the effect of infection with HIV-1, *M. tuberculosis*, and co-infection with both these organisms on the production of IL-8 *in vivo*. Results showed that whereas there is evidence of detectable levels of cell-associated IL-8 (mRNA and protein) in peripheral cells of healthy individuals, this is largely lost in the disease states studied. Coupled with this was significantly increased circulating levels of IL-8 in serum and plasma found in HIV-1 infected individuals with or without concomitant pulmonary TB. On the other hand, the capacity of peripheral mononuclear cells to produce IL-8 spontaneously *ex vivo* was enhanced in HIV-1 and TB patients and many of the HIV/TB group, but their corresponding capacities to respond to various stimuli was significantly diminished when compared to that of the normal donors. The release of IL-8 from PMN in the presence of an agonist was diminished mainly in individuals with pulmonary TB, which was further exacerbated by the presence of HIV-1 infection.

Since IL-8 is an important chemoattractant and cellular activator, a dysregulation in production is likely to play an important role in the pathogenesis of HIV-1 disease, pulmonary tuberculosis, and dual infections with both organisms. Altered production of IL-8 *in vivo* may have a number of possible consequences. Firstly, impairment of leukocyte trafficking owing to an altered concentration gradient between the periphery and the site of inflammation. As IL-8 is chemotactic for neutrophils and T lymphocytes one would expect that the cellular composition at infection sites may be altered. Altered migration of HIV-1 infected T cells may have implications for dissemination of virus to other sites of the body. IL-8 is chemotactic for approximately 10% of lymphocytes direct from peripheral blood (Larsen *et al.*, 1989) but this

proportion increases when mononuclear cells are activated with anti-CD3 antibody or PPD (Wilkinson and Newman, 1992). As these cells are anergic in HIV-1 infection it seems likely that they would be unresponsive to IL-8 as a second signal for migration. In addition, PMN functions dependent on IL-8 binding to either of its receptors are likely to be altered. As IL-8 has been shown to regulate the expression of its own receptor (Samanta *et al.*, 1990), persistently elevated levels of IL-8 could result in the down-regulation of IL-8 receptors. In addition, IL-8 shares one of its receptors (IL-8RB) with the CXC-chemokines GROα, GROβ, GROγ, NAP-2 and ENA-78, further suggesting a possible modulation of their respective functions by altered IL-8 production. At present it is not known what effects IL-8 may have on the expression of the recently identified HIV-1 co-receptors that bind members of the CC-chemokines. How IL-8 modulates these receptors may have either positive or negative implications for HIV-1 cell entry or subsequent events. Apart from the direct effects of IL-8 on cellular immune function, its role in terms of potentiation or suppression of HIV-1 needs to be questioned.

Polymorphonuclear neutrophils, which are the major cell type involved in host defence against primary or secondary bacterial or fungal infection, have a substantial dependence on IL-8 for their directed migration to sites of inflammation and, upon arrival, for a wide range of their effector antimicrobial functions. HIV-1-infected individuals have an increased incidence of bacterial infections which could be related to defective functioning of PMN. The second section of this study was aimed at detecting PMN abnormalities in patient groups by monitoring IL-8 induced β-glucuronidase release as a measure of degranulation, and by measuring PMN chemotaxis in response to IL-8. Induced release of β-glucuronidase from PMN of healthy individuals occurred in a dose-dependent manner. In contrast, PMN from HIV-1 infected individuals, with or without concurrent pulmonary tuberculosis, showed a reciprocal response in that increasing IL-8 concentrations resulted in decreased enzyme release. This reciprocal slope of the IL-8 dose-response curve was altered for almost all HIV-1 positive individuals tested, irrespective of their CD4 T-cell counts. However, individuals infected with *M. tuberculosis* alone had similar IL-8 dose-response graphs to those of normal healthy donors. In addition, PMN chemotaxis in response to IL-8 was also found to be significantly impaired in a group of HIV/TB patients, compared to a healthy ND group. These altered IL-8-mediated PMN responses may be indicative of abnormal PMN function and have important implications in the pathogenesis of HIV-1 infection in terms of their ability to clear bacterial and fungal infections.

Insight into what role IL-8 may play in pathogenesis of HIV-1 or *M. tuberculosis* infection, and co-infection with both these organisms can in part be gleaned from evaluating the distribution and expression of IL-8-specific receptors on immune cells. The third section of this study involved analysing the expression of the PMN cell surface markers, FcγRIII (CD16), and the two human IL-8 receptors A and B, and attempting to determine if the functional PMN abnormalities observed, particularly in HIV and HIV/TB patients, could be associated with an altered expression of IL-8 receptors.

Results indicated a significantly reduced expression of FcγRIII (CD16) on PMN of HIV-1 seropositive patients with pulmonary tuberculosis when compared to those individuals with either disease alone or healthy blood donors. Results obtained with the HIV and TB groups, although not attaining significance, showed a definite trend towards decreased CD16 expression relative to normal.

Neutrophil functions that may be compromised as a consequence of reduced FcγRIII expression include degranulation and clearance of circulating immune complexes. Neutrophil degranulation has been found to be triggered through FcγRIII, whereas IgG-dependent activation occurs via FcγRII (CD32) resulting in both the activation of the respiratory burst and exocytosis of granule proteins (Huizinga *et al.*, 1990b). Consistent with these delineated Fc receptor functions is the fact that PMN from HIV-1 infected individuals have enhanced phagocytosis (Estevez *et al.*, 1986; Wenisch *et al.*, 1996) but an unaltered capacity to produce reactive oxygen intermediates in response to an agonist (Wenisch *et al.*, 1996). In this latter regard, FcγRII expression has been shown to be unchanged on PMN in HIV-1 infection (Boros *et al.*, 1990). An inability to kill *Candida* sp. in the presence of enhanced phagocytosis and unimpaired oxidative burst in PMN of HIV-1 infected individuals (Wenisch *et al.*, 1996) further supports possible impairment of the non-oxidative armature of their PMN. This would be particularly relevant with an organism such as *M. tuberculosis* which is killed by neutrophils via a non-oxidative process (Jones *et al.*, 1990). Another consequence of reduced FcγRIII expression may be the inefficient removal of immune complexes from the circulation. Circulating immune complexes have been described in both HIV-1 disease (Euler *et al.*, 1985; McDougal *et al.*, 1985) and TB (Kleinhenz *et al.*, 1981). It is unlikely that cytolytic killing by neutrophils would be compromised as FcγRIIIb has been shown to be unable to mediate ADCC (Fanger *et al.*, 1989; Selvaraj *et al.*, 1989).

Perhaps the most striking clue as to the probable *in vivo* effects of reduced neutrophil FcγRIII expression is provided by an acquired abnormality in patients with paroxysmal nocturnal haemoglobinuria that results in the absence of FcγRIII expression on their neutrophils. This is thought to be responsible for the presence of circulating immune complexes and susceptibility to bacterial infections associated with this disease (Selvaraj *et al.*, 1988). Therefore, a reduction in FcγRIII on neutrophils in the presence of both HIV-1 disease and pulmonary TB, may provide an explanation for the enhanced susceptibility of these individuals to further infections with opportunistic microorganisms and may in part underlie the presence of circulating immune complexes.

A significant reduction in the percentage of PMN expressing IL-8RA and IL-8RB and in their respective fluorescence intensities was found in TB, HIV, and HIV/TB groups when compared to that obtained for the ND group. IL-8RA intensity of fluorescence was significantly decreased in the HIV/TB group when compared to the TB and HIV groups indicating a further down-regulation of IL-8RA expression owing to dual infection. On the other hand, IL-8RB fluorescence intensity was substantially reduced on PMN from patients with pulmonary TB and to a greater degree in those patients co-infected with HIV-1 and *M. tuberculosis*. In addition, the reduced expression of IL-8RA and IL-8RB on PMN from HIV/TB patients, the group with the greatest decrease in receptor expression, was found to be associated with impaired PMN function, including IL-8 induced degranulation and chemotaxis.

These results suggest that functions dependent on sufficient IL-8 receptor expression on PMN are impaired in individuals with HIV-1 disease and TB. These would include IL-8 dependent functions that are triggered through high affinity IL-8RA which specifically binds only IL-8, and functions triggered through IL-8RB which include interactions with a wider spectrum of ligands belonging to the C-X-C chemokine subfamily viz. GROα, GROβ, GROγ, NAP-2 and ENA-78 in addition to IL-8. Neutrophils are among the first cells to arrive at the site of *M.tuberculosis* infection (Montgomery and Lemon, 1993) and killing of this organism occurs via non-oxidative means (Geertsma *et al.*, 1990), a process shown to be enhanced by IL-8 (Nibbering *et al.*, 1993). Effective killing by neutrophils upon exposure to microbes is therefore reliant on a number of essential processes ranging from adhesion of peripheral blood neutrophils to endothelial cells as a first step, followed by transendothelial migration and entry into the site of infection. This

migration of neutrophils is governed by a chemokine concentration gradient established by the release of high concentrations of IL-8 mainly by monocytes/macrophages, either due to direct infection/phagocytosis or indirectly through other cytokines such as TNF-α. In order for cells in the periphery to be responsive to IL-8 they would require expression of specific IL-8 receptors on the surface of naive cells or require preactivation with the subsequent acquisition of IL-8 receptors prior to a cell migration response. The combined effect of multiple chemokines are assumed to be responsible for the cellular composition at inflammatory sites and therefore any alteration of chemokine composition or concentration would likely result in the influx of cell populations different from normal.

In addition to effects on cellular trafficking, neutrophil functions dependent on ligand-IL-8 receptor interactions important to microbial killing may be impaired. These include oxidative killing via activation of NADPH oxidase (oxidative burst) or non-oxidative killing through the release of potent antimicrobial polypeptides (degranulation). Neutrophils of HIV-1 infected individuals have an inability to kill *Candida* sp., this despite enhanced phagocytosis and unimpaired oxidative burst (Wenisch *et al.*, 1996). This suggests defective microbial killing by neutrophils via non-oxidative means. As has been demonstrated in this study, the reduced expression of both IL-8RA and IL-8RB on PMN supports a deficient degranulation response in HIV-1 infected individuals, as both receptors mediate agonist-induced granule release (Jones *et al.*, 1996). Furthermore, the reduction in IL-8RA expression on PMN from HIV-1 infected individuals obtained in our study may not be sufficient to alter functions such as oxidative burst found to be triggered only through this receptor. An altered IL-8 receptor repertoire on PMN, with resultant dysregulation of the corresponding ligands as a consequence, may be an important feature of HIV-1 and TB pathogenesis and further may have profound effects not only on normal cell function but also on cellular trafficking in response to infection.

Increased circulating levels of IL-8 in HIV-1 infection and a diminished cellular capacity to produce IL-8 as shown in this study may have important implications for antimicrobial defences and normal immune processes. An altered production of IL-8 *in vivo* is likely to play a role in the disease pathogenesis of HIV-1, *M. tuberculosis* and co-infection of both organisms. In addition, cellular responses dependent on specific receptor engagement and the subsequent translation of signal transducing events that lead to phagocyte effector functions are clearly impaired in IL-8

receptor deficient phagocytes. Abnormal PMN functioning in HIV-1 infected individuals, as shown here by defective degranulation and chemotactic responses, have important implications in the pathogenesis of HIV-1 infection in terms of their ability to clear secondary microbial infections.

Future attempts should be aimed at defining the mechanisms that brings about these changes in order to contribute to a greater understanding of the mechanisms that lead to an enhanced risk of superinfections in immunosuppressed individuals. An understanding of the effect of diseases such as HIV-1 and pulmonary TB on regulation of G-coupled protein receptors will not only contribute towards the elucidation of chemokine networks operational *in vivo* but further help in the quest to unravel their ever-increasing role in disease pathogenesis.

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APPENDIX A: Abbreviations

ADCC	antibody-dependant cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
AMV	avian myeloblastosis virus
ARC	AIDS-related complex
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
C5a	complement 5a
°C	degrees Celsius
CD	cluster designation
cDNA	complementary deoxyribonucleic acid
CI	chemotactic index
CSF	cerebrospinal fluid
Cys	cysteine
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytidine triphosphate
DEP	diethyl pyrocarbonate
dGTP	deoxy-guanidine triphosphate
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
dTTP	deoxy-thymidine triphosphate
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ENA	epithelial cell-derived neutrophil-activating peptide
FcR	receptor for the crystallizable portion of immunoglobulins
FCS	foetal calf serum
FITC	fluorescein isothiocyanate conjugate

FMLP	N-formylmethionyl-leucylphenylalanine
FSC	foward light scatter
GAP-DH	glyceraldehyde phosphate dehydrogenase
GCP	granulocyte chemotactic protein
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosyl-phosphatidylinositol
GRO	growth regulated oncogene
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
I	iodine
IAA	isoamyl alcohol
ICAM	intercellular adhesion molecule
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
KCl	potassium chloride
kDa	kiloDalton
KH_2PO_4	potassium carbonate
LiCl	lithium chloride
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
M	molar
mAb	monoclonal antibody
MDM	monocyte-derived macrophage
MDNCF	monocyte-derived neutrophil chemotactic factor
MHC	major histocompatibility antigen
MIP	macrophage inflammatory protein
ml	millilitre
mM	millimolar

mRNA	messenger RNA
N	normal
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
NAP	neutrophil activating peptide
ND	normal donor
NK	natural killer
nm	nanometre
nM	nanomolar
NP40	nonidet P40 (nonphenylpolyethylene glycol)
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAF	platelet activating factor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
PGL	persistent generalized lymphadenopothy
PHA	phytohaemagglutinin (lectin from *Phaseolus vulgaris*)
PLD	phospholipase D
PMN	polymorphonuclear neutrophils
pmol	picomole
PPD	purified protein derivative
RANTES	regulation upon activation normal T-expressed and secreted
RBC	red blood cells
RNA	ribonucleic acid
RNAsin	RNAse inhibitor
RT-PCR	reverse transcription - polymerase chain reaction
SDF	stromal cell-derived factor
SDS	sodium dodecyl sulphate

SSC	side light scatter
TB	tuberculosis
TCF	T-lymphocyte chemotactic factor
TEMED	N,N,N',N',-Tetramethylethylenediamine
TGF	transforming growth factor
Th	T-helper
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumor necrosis factor
Tris	Tris-(hydroxymethyl)-aminoethane
Triton X-100	polyethyleneglycol (9-10)-p-t-octylphenol
μg	microgram
μl	microlitre
μM	micromolar
WBC	white blood cells

APPENDIX B: Composition of buffers and media

1. Reagents for cytoplasmic RNA extraction

NP-40 lysis buffer

1M Tris (pH 7.5)	1ml
5M NaCl	3ml
1M $MgCl_2$	1.35ml
10% NP-40	6.5ml

Make up to 100ml with sterile diethyl pyrocarbonate (DEP)-treated water

SDS-Urea buffer

Urea	42g
20% SDS	5ml
5M NaCl	7ml
0.5M EDTA	2ml
1M Tris (pH 7.5)	1ml

Make up to 100ml with sterile DEP-treated water

2. Reagents for polyacrylamide electrophoresis (PAGE)

6% PAGE gel

40% acrylamide: bis-acrylamide (29:1)	6ml
TBE 10X	4ml
10% Ammonium persulphate	400μl
Distilled water	29.5ml
TEMED	20μl

1X TBE running buffer

89mM Tris-borate, 89mM boric acid, 2mM EDTA, pH 8.0.

3. Reagents for IL-8 bioassay

0.1M Sodium acetate buffer

Dissolve 0.8203g NaAc in 100ml distilled water

Adjust pH to 4.0 with HCl

Filter sterilize and store at 4°C

0.4M Glycine buffer

Dissolve 3.003g glycine in 100ml distilled water

Adjust pH to 10.0 with 10N NaOH

Filter sterilize and store at 4°C

4. Reagents for flow cytometry

Haematology diluent

Dissolve	NaCl	8.12g in 200ml distilled water
	KCl	0.28g in 100ml distilled water
	KH_2PO_4	0.26g in 100ml distilled water
	LiCl	0.43g in 200ml distilled water

Combine and make up to 1000ml with distilled water

Add EDTA (0.36g) and adjust pH to 7.2 with HCl.

Fixer

1.5% Formaldehyde PBS, 2% BSA

APPENDIX C: List of suppliers

Item	Manufacturer
Plasticware	
24-well microtitre plate	Corning Costar Corp., Cambridge, MA, USA
48-well microtitre plate	Corning Costar
96-well microtitre plate (round bottom)	Corning Costar
96-well microtitre plate (flat bottom)	Nunc A/S, Roskilde, Denmark
Transwell polycarbonate membrane filters (6.5mm)	Corning Costar
micro-centrifuge tubes, 1.5ml	Eppendorf, Hamburg, Germany
Falcon polypropylene tubes 15ml, 50ml	Becton Dickinson, San Jose, CA, USA
Antibodies	
FITC-conjugated mouse anti-human CD16	Becton Dickinson
FITC-conjugated mouse anti-human IgG1	Dako, Glostrup, Denmark
Monoclonal mouse anti-human IL-8RA and IL-8RB	Genentech Inc., San Francisco, USA
Mouse anti-human IgG1 and IgG2a	Serotec, Oxford, England
FITC-conjugated goat anti-mouse immunoglobulin	Dako

Nucleic acids

GAP-DH primers	Stratagene, La Jolla, CA, USA
Interleukin-8 primers	Stratagene
Oligo-dT primer	Boehringer Mannheim GmbH, Germany

Enzymes

DNA polymerase, Taq	Promega, Madison, WI, USA
Avian myeloblastosis virus (AMV) reverse transcriptase	Promega

Cytokines

G-CSF human recombinant (*E.coli*)	Boehringer Mannheim
GM-CSF human recombinant (*E.coli*)	Boehringer Mannheim
IFN-γ human recombinant (*E.coli*)	Boehringer Mannheim
IL-1β human recombinant (*E.coli*)	Boehringer Mannheim
IL-2 human recombinant (*E.coli*)	Boehringer Mannheim
IL-4 human recombinant (*E.coli*)	Boehringer Mannheim
IL-6 from human lymphocytes	Boehringer Mannheim
IL-8 human recombinant (*E.coli*)	Boehringer Mannheim
TNF-α human recombinant (*E.coli*)	Boehringer Mannheim
TNF-β human recombinant (*E.coli*)	Boehringer Mannheim

Reagents for electrophoresis

40% acrylamide : bis-acrylamide	Sigma Chemical Co., St Louis, MO, USA
Ammonium persulphate	Pharmacia Biotech, Uppsala, Sweden
TEMED	Sigma

Cell culture media

RPMI 1640	Sigma
Foetal calf serum	Sterilab, Kempton Park, South Africa
Streptomycin	Sigma
Penicillin G	Sigma
Hepes (2-[-4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid	Boehringer Mannheim

Organic chemicals

Chloroform	Merck, Darmstadt, Germany
Ethanol	Merck
Formaldehyde	Merck
Hydrochloric acid	Sigma
Isoamylalcohol	Merck
Phenol	Merck

General chemicals

Ammonium chloride	Sigma
Boric acid	Merck
EDTA	Boehringer Mannheim
Ethidium bromide	Boehringer Mannheim
Ficoll (Histopaque-1077)	Sigma
Glycine	Sigma
Lithium chloride	Merck
Magnesium chloride	Promega
Potassium carbonate	Sigma
Potassium chloride	Sigma
SDS (n-dodecylsulphate sodium salt)	Merck
Sodium acetate	Merck
Sodium chloride	Merck

Sodium hydroxide	BDH, Poole, England
Tris (hydroxymethyl) aminoethane	Boehringer Mannheim
Tris (hydroxymethyl) aminoethane hydrochloride	Boehringer Mannheim
Urea	Merck

Miscellaneous reagents

Amplicor HIV-1 Monitor test	Roche Diagnostic Systems, Branchburg, NJ, USA
Biotrak Interleukin-8 ELISA	Amersham, Buckinghamshire, England
p24 antigen ELISA	Coulter Corp., Hileah, FL, USA
MACS magnetic cell sorting system	Miltenyi Biotec, Bergisch Gladbach, Germany
Bovine serum albumin	Sigma
Cytochalasin B	Sigma
dNTPs	Boehringer Mannheim
Dithiothreitol	Boehringer Mannheim
FACS lysing solution	Becton Dickinson
FMLP	Sigma
Lipopolysaccharide	Sigma
Phytohaemagglutinin	Sigma
p-nitrophenyl-β-D-glucuronide	Sigma
Purified protein derivative	Sigma
Nonidet P40	Fluka, Buchs, Switzerland
Triton X-100 (polyethyleneglycol (9-10)-p-t-octylphencl)	Boehringer Mannheim
Human placental ribonuclease inhibitor (RNAsin)	Promega
AMV reverse transcriptase 5X reaction buffer	Promega
Thermo DNA polymerase 10X reaction buffer	Promega

APPENDIX D: Statistical evaluation of results

Statgraphics™ software (STSC Inc., Rockville, MA, USA) was used in performing all the statistical analysis of the results obtained.

Comparison of two samples (Mann-Whitney U-test and Wilcoxon signed ranks test)

The comparison of data between groups, eg. comparing IL-8RA fluorescence intensity for the ND group to IL-8RA fluorescence intensity for the HIV/TB group was done using the comparison of two samples procedure. In this case, the unpaired test (Mann-Whitney U-test) was used, which determines whether two medians differ. The Mann-Whitney U-test is used when data from two independent samples, possibly of different size are being compared.

Comparing data within groups, eg. comparing the spontaneous release of IL-8 from ND PBMC cultures to PHA-induced IL-8 release in ND PBMC cultures was done using the Wilcoxon signed ranks test. The ranks test, which has a relative efficiency of 0.96, is used when comparing two measures taken from the same source.

Spearman rank correlation

Rank correlation coefficients were also used to compare data within groups. The Spearman rank correlation coefficient uses the ranks of the data rather than the actual data values, with the system scaling the coefficient to fall between -1 (perfect disagreement) and +1 (perfect agreement).

APPENDIX E: Ethical clearance

This study was approved by the University of the Witwatersrand Ethical Committee, protocol number M 960719. Patients were recruited after informed consent was obtained and confidentiality of all records ensured.

APPENDIX F: Publications

Some of the findings in this thesis have been published, or submitted for publication.

Meddows-Taylor S, Martin DJ, and Tiemessen CT. (1997). Altered expression of FcγRIII (CD16) on polymorphonuclear neutrophils from individuals with human immunodeficiency virus type 1 disease and pulmonary tuberculosis. *Clin. Diagn. Lab. Immunol.* **4:** 789-791.

Meddows-Taylor S, Martin DJ, and Tiemessen CT. (1998). Reduced expression of interleukin-8 receptors A and B on polymorphonuclear neutrophils from persons with human immunodeficiency virus type 1 disease and pulmonary tuberculosis. *J. Infect. Dis.* **177:** 921-930.

Meddows-Taylor S, Martin DJ, and Tiemessen CT. (1998). Dysregulated production of interleukin-8 in individuals infected with HIV-1 and *Mycobacterium tuberculosis.* Submitted.

Meddows-Taylor S, Martin DJ, and Tiemessen CT. (1998). Impaired interleukin-8-induced degranulation of polymorphonuclear neutrophils from human immunodeficiency virus type 1-infected individuals. Submitted.