

HIV CORECEPTORS: DISTRIBUTION AND MODULATION

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ABSTRACT

The chemokine receptors, CXCR4 and CCR5, are the major coreceptors for HIV-1 entry into CD4⁺ cells. In addition, they mediate chemotaxis in response to stromal cell-derived factor (SDF-1 α) (CXCR4) and macrophage inflammatory protein (MIP)-1 α , MIP-1 β and regulated on activation, normal T cell expressed and secreted (RANTES) (CCR5). Therefore, cell surface expression of these receptors has important implications for the study of the pathogenesis of HIV disease, in terms of viral entry as well as cellular trafficking.

The first section of this study involved examining the effect of delays in sample staining on the expression of CXCR4 and CCR5 on a number of leukocyte subsets. An initial cross-sectional analysis comparing receptor expression on one cohort of HIV-1 seropositive subjects that was stained at time 0, with another matched cohort stained 24h after venesection, revealed significant differences in the expression of both receptors. These findings were then corroborated by a longitudinal study carried out on healthy donors and HIV-1 seropositive individuals. A significant increase of CXCR4 expression on all cellular subsets in the percentage of cells, as well as fluorescence intensity was observed. Conversely, CCR5 expression was reduced. However, this was significant only in terms of fluorescence intensity of the CD3⁺, CD4⁺ and CD8⁺ lymphocytes. It was then determined how quickly these changes take place, as well as whether these alterations in expression only occurred when using ethylenediaminetetra-acetic acid (EDTA) anticoagulated blood. Samples were analysed immediately, 3h, 6h, and 24h after collection, using whole blood anticoagulated with EDTA, heparin and acid citrate dextrose (ACD), using CD3 as a representative marker. Changes in receptor expression could be seen as early as 3h after venesection, with major changes occurring after 24h. Similar results were found with all three anticoagulants evaluated. These results emphasise the importance of strict control on time if correct results are to be obtained when staining for CXCR4 and/or CCR5 on whole blood samples. For this reason, all subsequent staining was carried out within 6h of collection of EDTA anticoagulated blood.

The second part of this work involved investigating age-related changes in CXCR4 and CCR5 expression. Flow cytometric analysis was performed to investigate CXCR4 and CCR5 expression on peripheral blood leukocytes in cord blood (n=27) and peripheral blood (n=34) of uninfected infants born to HIV-1 seropositive mothers and in uninfected adults (n=18). The infant samples were stratified into three groups based on age: 4.5 months, 9 months and 15 months. CXCR4 was expressed at very high levels in cord blood, decreased at 4.5 months and then increased to adult levels. In contrast, the proportion of leukocytes expressing CCR5 expression was very low in cord blood, and this subsequently increased with age. Thus, results support age-related differences in cellular expression of CXCR4 and CCR5.

We then questioned whether infection with *Mycobacterium tuberculosis* (TB group) and HIV-1 (HIV group), alone or in combination (HIV/TB group), had an effect on the expression of CXCR4 and CCR5 on different subsets of peripheral leukocytes. Overall, CXCR4 expression was significantly reduced on all cell types in all disease groups when compared to uninfected normal donors. Of significance was a notable reduction in CXCR4 expression on CD8⁺ cells, NK cells, monocytes and on granulocytes of patients within the two TB groups (TB and HIV/TB) when compared to the HIV group. The duration of anti-tuberculosis treatment was significantly negatively correlated with the expression of CXCR4 on CD4⁺ and CD8⁺ memory cells, monocytes and NK cells, viral load, and proportions of CD38-expressing CD8⁺ lymphocytes, in HIV/TB patients. By contrast, CCR5 expression was increased in all the disease groups on most cell subsets analysed, except for on monocytes in the two TB groups. There was no change in CCR5 expression on CD4⁺ cells when based on the disease groupings. However, higher proportions of CD4⁺ naive and CD8⁺ lymphocytes as well as B cells expressing CCR5 correlated with advancing HIV-1 disease as measured by percentage of CD4⁺ T cells, as did decreased proportions of CXCR4-expressing CD4⁺ naive cells.

As the expression of CXCR4 and CCR5 on leukocyte subsets was altered in HIV-1 infected individuals who were not receiving any antiretroviral therapy, we wished to evaluate the response of these two receptors to highly active antiretroviral therapy (HAART). CXCR4 expression in those patients who had been on HAART for eight weeks did not normalize to pre-therapy levels. In contrast, CCR5 expression was significantly reduced on all cell types (except on NK cells) in

the HIV-infected patients on HAART when compared with pre-therapy levels. A positive correlation was observed between the percentage of CD3⁺ cells expressing CXCR4 and the CD4⁺ T cell percentage in the patients on therapy, and a negative correlation was found between the proportion of CD3⁺ cells expressing CCR5 and the CD4⁺ T cell percentage in the patients both before starting treatment, and when on therapy. Additionally, regardless of evidence of increased immune activation in African individuals in this study, as measured by the percentage of CD8⁺CD38⁺ cells, the virological response to HAART was as effective as that reported for non-African persons. Thus, CCR5 may provide an further immunophenotypic marker of early immune reconstitution following HAART.

Recent studies have suggested the importance of measuring the density of antigen expression in addition to determining the percentage of cells expressing the antigen. Thus, the aim of this section of this study was to use quantitative flow cytometry to determine CXCR4 and CCR5 antigen density as measured in terms of antibodies bound per cell (ABC), in order to ascertain if this technique would provide greater sensitivity in the detection of small differences in coreceptor expression between different study groups. To this end, the number of CXCR4 and CCR5 ABC were measured in cord blood and adult samples. On evaluation of the number of CXCR4 ABC from the cord blood and adult groups, all cellular subsets analysed from the adult group, except for NK cells, were found to have significantly lower levels of CXCR4 ABC than the corresponding subset from the cord blood group, while significantly lower percentages of CXCR4-expressing cells were only found on the CD3⁺CD8⁺, CD19⁺ cells and on granulocytes. In addition, differences in the number of CXCR4 ABC between the groups was large, while the differences in percentage of CXCR4-expressing cells was small. Further, a significantly higher level of CCR5 ABC was observed in the adult samples when compared with the cord blood samples on the total CD3⁺ cells, CD3⁺CD4⁺, CD3⁺CD8⁺ lymphocytes, and NK cells.

CCR5 Δ 32/wt adults were found to have higher number of CXCR4 ABC on CD3⁺ cells when compared with CCR5wt/wt individuals. However, with respect to percentage of cells expressing CXCR4, no differences between the two groups were observed. However, a significant reduction in the percentage of CCR5-expressing CD3⁺ and CD4⁺ cell subsets was observed together with a reduction in the number of CCR5 ABC on CD3⁺, CD4⁺, CD8⁺, NK and CD14⁺ cells from

CCR5 Δ 32/wt individuals in comparison with CCR5wt/wt individuals. Taken together, these results indicate that more subtle changes in CXCR4 and CCR5 expression can be detected by quantitation of these coreceptors.

As the expression of CXCR4 and CCR5 may be regulated by their ligands, it was important to measure the plasma levels of these chemokines in relation to age, presence of a CCR5 Δ 32 allele, and infection with HIV-1 and *M. tuberculosis*, and to compare plasma chemokine levels with coreceptor expression. MIP-1 β was chosen as a representative for the CC chemokine. Additionally, as IL-7 and SDF-1 have functions in common, IL-7 was included in these analyses. To determine age-related differences in cytokine levels, SDF-1 α and IL-7 levels were determined in 6 cord blood and 19 adult samples, and MIP-1 β levels determined in 10 cord blood and 15 adult samples. SDF-1 α and IL-7 levels were found to be significantly lower in cord blood plasma when compared with adult plasma, while MIP-1 β levels were significantly higher in cord than adult plasma.

The relationship between the presence or absence of the CCR5 Δ 32 polymorphism in adults and circulating cytokine levels was then evaluated. SDF-1 α and IL-7 levels were analysed on plasma samples from 9 CCR5 Δ 32/wt and 10 CCR5wt/wt healthy donors, and MIP-1 β levels on plasma samples from 7 CCR5 Δ 32/wt and 8 CCR5wt/wt healthy donors. In comparison with CCR5wt/wt individuals, significantly lower SDF-1 α plasma levels were found for CCR5 Δ 32 heterozygotes, with a trend towards an increase in MIP-1 β plasma levels, while the plasma IL-7 levels of the two groups were similar. These data suggest a link between reduced CCR5 surface expression and reduced peripheral SDF-1 α levels in CCR5 Δ 32 heterozygous individuals, the mechanism of which is unknown.

The effect of infection with HIV-1 and/or *M. tuberculosis* on plasma cytokine levels was investigated. SDF-1 α , IL-7 and MIP-1 β levels were determined in healthy normal donors (ND or control group), in the HIV group, and in the TB group. Due to insufficient sample volume only SDF-1 α and IL-7 levels were determined in the HIV/TB group. Plasma levels of all three cytokines were significantly higher in the TB group than the ND group. In addition, SDF-1 α

plasma levels in the TB and HIV/TB groups were significantly higher than the HIV group, while IL-7 plasma levels in the HIV/TB group were significantly higher than in the TB group. Negative correlations were found between SDF-1 α and IL-7 levels and CXCR4 expression on all cell subsets, as well as between MIP-1 β levels and CXCR4 expression on CD8 $^+$, NK, CD14 $^+$ cells and granulocytes, while positive correlations were found between SDF-1 α plasma levels and CCR5 expression on CD3 $^+$ cells, and between IL-7 plasma levels and CCR5 expression on CD3 $^+$, CD8 $^+$ cells and on CD14 $^+$ monocytes, and a negative relationship was found between MIP-1 β and CCR5 expression on CD14 $^+$ monocytes. Moreover, a weak positive correlation was found between SDF-1 α and IL-7 levels, and between levels of SDF-1 α and MIP-1 β . Plasma levels of SDF-1 α were significantly higher in the HIV-1 infected patients with a CD4 $^+$ percentage of <14% when compared with patients with a >14% CD4 $^+$ T cell percentage, with a trend towards a reduction in MIP-1 β plasma levels in the <14% CD4 $^+$ T cell group when compared with the >14% group. IL-7 plasma levels were not significantly different between the two groups. Additionally, a negative correlation between plasma levels of SDF-1 α and percentage of CD4 $^+$ T cells, and a positive correlation between both SDF-1 α and IL-7 levels with plasma HIV-1 viral load was found. No correlation was found between MIP-1 β plasma levels and percentage of CD4 $^+$ T cells or log₁₀ HIV-1 RNA copies/ml. Collectively, these results suggest a role for SDF-1 α and IL-7 in HIV-1 disease progression.

Next, the effect of antiretroviral therapy on the plasma levels of these cytokines in HIV-1 infected adults was determined by measuring plasma cytokine levels before starting HAART, and again eight weeks later. Although the results were not significant, there was an overall reduction in the median levels of SDF-1 α and IL-7 plasma levels, and an increase in the median MIP-1 β levels during this period.

In summary, this study has shown that CXCR4 and CCR5 are reciprocally expressed, and easily modulated by both *in vivo* (age, infection, HAART and CCR5 Δ 32 polymorphism) and *ex vivo* factors such as standing time. Furthermore, small differences in receptor expression are best assessed using quantitation of surface receptor density.

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy 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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Sharon Shalekoff

15th day of July, 2003

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PRESENTATIONS AND PUBLICATIONS

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

Presentations

1. *Chemokines and Chemokine Receptors: Disease targets for therapeutic development, 1999*. November 15-16. McClean, Virginia. Distribution of CXCR4 and CCR5 on peripheral blood leukocytes of persons infected with Human Immunodeficiency Virus type 1 and *Mycobacterium tuberculosis*: Implications for pathogenesis. S Shalekoff, S Pendle, D Johnson, DJ Martin and CT Tiemessen.
2. *X111th International AIDS Conference, 2000*. Durban, South Africa. Generalised downregulation of CXC-G protein-coupled receptors and the classical chemoattractant receptor CD88 on peripheral blood leukocytes of persons infected with HIV-1 and *Mycobacterium tuberculosis*. CT Tiemessen, S Shalekoff and S Meddows-Taylor.
3. *X111th International AIDS Conference, 2000*. Durban, South Africa. CXCR4 and CCR5 expression on peripheral blood leukocytes of persons infected with Human Immunodeficiency Virus type 1 and *Mycobacterium tuberculosis*. S Shalekoff, S Pendle, D Johnson and CT Tiemessen.

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1. Shalekoff S, and Tiemessen CT (2001). Duration of sample storage dramatically alters expression of the Human Immunodeficiency Virus Coreceptors CXCR4 and CCR5. *Clin. Diagn. Lab. Immunol.* **8**: 432-436.
2. Shalekoff S, Pendle S, Johnson D, Martin DJ, and Tiemessen CT (2001). Distribution of the Human Immunodeficiency Virus coreceptors CXCR4 and CCR5 on leukocytes of persons with Human Immunodeficiency Virus type 1 infection and pulmonary tuberculosis: implications for pathogenesis. *J Clin Immunol* **21**: 390-401.
3. Shalekoff S and Tiemessen CT. Circulating levels of SDF-1 α and IL-7 in HIV-1 infection and pulmonary tuberculosis are reciprocally related to CXCR4 expression on peripheral blood leukocytes. *AIDS Res Hum Retroviruses*. In press.
4. Shalekoff S and Tiemessen CT. CCR5 Δ 32 Heterozygosity is associated with an increase in CXCR4 cell surface expression. *AIDS Res Hum Retroviruses*. In press.
5. Shalekoff S, Martin DJ, Johnson D, and Tiemessen CT. Changes in expression of CXCR4 and CCR5 on leukocytes from persons with Human Immunodeficiency Virus type 1 infection in response to highly active antiretroviral therapy. Submitted.
6. Shalekoff S, Gray GE, and Tiemessen CT. Age-related changes in the expression of CXCR4 and CCR5 on peripheral blood leukocytes from uninfected infants born to Human Immunodeficiency Virus-1 infected mothers. Submitted.

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

In order for human immunodeficiency virus (HIV) to infect cells there needs to be an interaction between the virus envelope glycoproteins (gp120/41) and at least two cellular receptors: the CD4 molecule, and a seven-transmembrane domain G-protein-coupled chemokine receptor. The chemokine receptors CXCR4 and CCR5 are the major HIV coreceptors (Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996). Macrophage-tropic (M-tropic) HIV-1 strains utilise the CCR5 receptor (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996), while T cell tropic (T-tropic) isolates utilise the CXCR4 receptor (Feng *et al.*, 1996). These HIV-1 viruses have been newly classified based on their coreceptor usage and the strains that utilise CCR5 are termed R5, while those that utilise only CXCR4 are referred to as X4 viruses. On the other hand, there are viruses that utilise both receptors with equivalent efficiency, and these are termed R5X4 (Berger *et al.*, 1998).

In late 1995 macrophage inflammatory protein (MIP)-1 α , MIP-1 β and regulated on activation, normal T cell expressed and secreted (RANTES) were shown to have an inhibitory effect on the growth of different strains of HIV-1, HIV-2 and simian immunodeficiency virus (SIV) (Cocchi *et al.*, 1995), and in 1996 it was shown that CCR5 was the natural receptor for those chemokines (Raport *et al.*, 1996; Samson *et al.*, 1996a). Stromal cell-derived factor-1 (SDF-1), the natural ligand for CXCR4 profoundly inhibits the replication of X4 HIV-1 strains (Amara *et al.*, 1997).

The importance of these chemokine receptors in HIV-1 infection was further emphasised by the finding that individuals who are homozygous for a 32 bp deletion in the CCR5 gene are highly resistant to infection with HIV-1 (Dean *et al.*, 1996; Huang *et al.*, 1996; Samson *et al.*, 1996a).

This deletion results in an unstable truncated form of the protein which is not expressed at the cell surface (Liu *et al.*, 1996a). Approximately 1% of Caucasians are homozygous for this deletion. The heterozygous form of the deletion occurs in approx 15-20% of Caucasians, and is rare in Black African and Asian populations. This mutation occurs in African American populations but at much lower frequencies (Samson *et al.*, 1996b; Zimmerman *et al.*, 1997). The heterozygous form of the deletion seems to confer moderate protection to individuals infected with HIV-1. This is shown as lower viral loads, slower decline in CD4⁺ T cells and increased time for progression to acquired immune deficiency syndrome (AIDS) (Dean *et al.*, 1996; Huang *et al.*, 1996; Zimmerman *et al.*, 1997).

The normal biological function of chemokines and their receptors is thought to involve regulation of the immune response, particularly migration during inflammatory responses, and immune development. A number of studies have addressed the distribution and expression of CXCR4 and CCR5 on various immune cells (Bleul *et al.*, 1997; Forster *et al.*, 1998; Yi *et al.*, 1998), and it has been suggested that the differential expression of these receptors has important implications for HIV-1 tropism and pathogenesis *in vivo*. Whereas most of these studies have focussed only on cell types that are permissive for HIV-1, it is important to also consider other cell types that express these receptors and address their role in immune function and pathogenesis. It is for this reason that we undertook to study these chemokine receptors by focussing more on their expression on various immune cells, as opposed to considering only aspects pertaining to viral entry, or only those cell types permissive for HIV-1 infection. This information is important in understanding HIV-1 pathogenesis and in delineating the role of the two major HIV-1 coreceptors in disease progression in both adults and in children.

1.2 Literature review

1.2.1 Chemokines

Chemokines are small (8-12 kDa), mostly basic, structurally related molecules that play a central role in host defense mechanisms. They are produced by a number of cell types in response to bacterial, viral, parasitic, and mycobacterial pathogens. They have diverse biological properties, among which the timely recruitment of immune effector cells to the sites of infection is critical

to the development of an organised host cellular response (for review see Rollins (Rollins, 1997)).

1.2.1.1 Classification

Most chemokines have four characteristic cysteines (cys) linked by disulphide bonds (Cys 1-Cys3 and Cys2- Cys4) and can be divided into 4 major families, CXC , CC, C and CX₃C, based on the number and spacing of the conserved cysteines (Rossi & Zlotnik, 2000).

1.2.1.1.1 CXC Chemokines (α -chemokines)

The CXC family of chemokines has four conserved cysteines with its first 2 terminal cysteine residues separated by a nonconserved amino acid. CXC genes exhibit 20 to 90% amino acid homology, and are clustered on chromosome 4, except for SDF-1, which is localised on chromosome 10, and is the most divergent member (Modi & Chen, 1998), and breast and kidney chemokine (BRAK) which is located on chromosome 5 (Murphy *et al.*, 2000). In general, the CXC chemokines mainly target neutrophils and T cells, but not monocytes. The CXC chemokines can be further divided into proteins containing the amino acid motif glutamic acid-leucine-arginine (ELR) present in the portion of the molecule immediately preceding Cys-1 (ELR⁺), and the few that do not (ELR⁻) (Clark-Lewis *et al.*, 1991). ELR⁺ CXC chemokines bind CXCR1 and CXCR2 (Ahuja & Murphy, 1996; Wolf *et al.*, 1998) and generally attract neutrophils. In contrast, the ELR⁻ CXC chemokines are primarily lymphocyte chemoattractants, and bind CXCR3 (Cole *et al.*, 1998; Loetscher *et al.*, 1996a), CXCR4 (Bleul *et al.*, 1996a; Oberlin *et al.*, 1996) or CXCR5 (Legler *et al.*, 1998). ELR⁺ CXC chemokines include interleukin (IL)-8, growth-related oncogene (GRO)- α , GRO- β , GRO- γ , epithelial cell-derived neutrophil-activating factor (ENA)-78, platelet basic protein and granulocyte chemoattractant protein (GCP)-2, and ELR⁻ CXC chemokines comprise platelet factor (PF)-4, monokine induced by γ -interferon (Mig), interferon- γ -inducible protein (IP)-10, interferon-inducible T cell α -chemoattractant (I-TAC), SDF-1 α/β and B cell-attracting chemokine-1/B lymphocyte chemoattractant (Zlotnik *et al.*, 1999).

1.2.1.1.2 CC Chemokines (β -chemokines)

The majority of CC chemokines have four conserved cysteines, with a small subgroup having six. The CC family has its 2 terminal cysteines without separation. Most of the CC chemokine genes are clustered on chromosome 17 (Baggiolini *et al.*, 1994). Exceptions include the genes encoding MIP-3 β and ESKine on chromosome 9, MIP-3 α /liver- and activation-regulated chemokine (LARC) on chromosome 2, eotaxin-2 and eotaxin-3 on chromosome 7, and thymus- and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) on chromosome 16 (Murphy *et al.*, 2000). The amino acid identity of the CC chemokines ranges from 25 to 70% homology (Baggiolini *et al.*, 1994). CC chemokines generally attract monocytes and T cells, and to a lesser extent eosinophils and basophils, but not neutrophils.

1.2.1.1.3 C Chemokine (γ -chemokine)

The C chemokine family, has only two cysteines in the conserved positions, corresponding to cysteines 2 and 4 in the other groups. The gene encoding the C chemokine is found on chromosome 1, a unique location for chemokines (Kelner *et al.*, 1994; Kennedy *et al.*, 1995). Lymphotactin was the original and only member of this family, and earned its name by being a potent chemoattractant for T lymphocytes (Kelner *et al.*, 1994; Kennedy *et al.*, 1995), natural killer (NK) cells (Hedrick *et al.*, 1997) and thymocytes (Kelner *et al.*, 1994). Recently, Yoshida *et al.* (Yoshida *et al.*, 1998) identified the orphan receptor G-protein-coupled receptor (GPR)5 as the specific receptor for lymphotactin. GPR5 has now been assigned the provisional name XCR1.

1.2.1.1.4 CX₃C Chemokine (δ -chemokine)

The CX₃C chemokine subfamily has four conserved cysteines with its first 2 cysteines separated by three amino acids. Fractalkine (or neurotactin) is the single known member of this family, and the gene encoding fractalkine is located on chromosome 16 (Bazan *et al.*, 1997). Unlike other previously described chemokines, fractalkine is a multimodular protein consisting of a chemokine domain, a mucin-like stalk, a transmembrane domain, and a cytoplasmic tail. This molecule can exist in either a membrane-bound form expressed on endothelial cells and neurons, or shed as a 95 kDa glycoprotein. The membrane-bound form functions as an adhesion molecule

by binding directly to CX₃CR1 (Imai *et al.*, 1997), and the soluble form induces migration of monocytes and T lymphocytes, and NK cells *in vitro* (Bazan *et al.*, 1997; Imai *et al.*, 1997).

1.2.1.2 New nomenclature for chemokines

The rapid pace of discovery of new chemokines has led to the proliferation of synonyms, with different groups reporting the same molecule under different names, resulting in confusion in the literature. For example, MIP-3 β is also known as Epstein-Barr virus-induced receptor ligand chemokine (ELC) and exodus-3. To address this problem, a new nomenclature was developed by A. Zlotnik and O. Yoshie (Zlotnik & Yoshie, 2000), based on the fact that the genes for chemokines already have a standardised designation. The CXC gene family is called SCYB (small cytokine B family), the CC gene family is called SCYA, the C gene family is called SCYC, and the CX₃C family SCYD. In this new nomenclature the SCY abbreviation is replaced with CXCL for the CXC family, CCL for the CC family, XCL for lymphotactin, and CX₃CL for fractalkine. In the CC chemokines there are some spaces reserved for ligands that have been identified in the mouse, that have not yet been identified in the human. This nomenclature offers the advantage that each ligand can be instantly identified as a member of a particular class (see Table 1.1).

1.2.1.3 Functions of chemokines

1.2.1.3.1 Role in leukocyte movement

Probably the most important biological function of chemokines, is their capacity to stimulate directed leukocyte migration or chemotaxis. Chemokines mediate the recruitment of leukocytes, firstly by activating integrins (molecules that are involved in cell adhesion), and secondly by providing directional cues to facilitate the migration of adherent leukocytes across endothelium (Butcher & Picker, 1996; Springer, 1994). Chemokines are secreted at sites of inflammation and infection, and are locally retained on matrix and cell-surface heparin sulphate proteoglycans. The main stimuli for chemokine production are early proinflammatory cytokines, such as IL-1, tumour necrosis factor (TNF)- α , bacterial products such as lipopolysaccharide (LPS), and viral infection (Baggiolini *et al.*, 1994). In chemotaxis, cells move in the direction of an increasing chemokine concentration gradient which is established surrounding the inflammatory stimulus,

Table 1.1 New nomenclature for human chemokines

Systemic name	Common Synonyms	Systemic name	Common Synonyms
<u>CXCL</u>		<u>CCL</u>	
CXCL1	Gro α ; MGSA	CCL1	I-309
CXCL2	Gro β ; MIP-2 α	CCL2	MCP-1; MCAF
CXCL3	Gro γ ; MIP-2 β	CCL3	MIP-1 α
CXCL4	PF-4	CCL4	MIP-1 β
CXCL5	ENA-78	CCL5	RANTES
CXCL6	GCP-2	(CCL6)	
CXCL7	NAP-2	CCL7	MCP-3
CXCL8	IL-8	CCL8	MCP-2
CXCL9	Mig	(CCL9/10)	
CXCL10	γ IP-10	CCL11	Eotaxin
CXCL11	I-TAC	(CCL12)	
CXCL12	SDF-1; PBSF	CCL13	MCP-4
CXCL13	BLC; BCA-1	CCL14	CC-1; HCC-1; NCC-2; CCK-1/CCCK-3; Ck β 1; MCIF
CXCL14	BRAK; bolequine	CCL15	HCC-2; leukotactin-1; MIP-5; CC-2; NCC-3; MIP-1 δ
CXCL15	Lungkine	CCL16	HCC-4, LEC; NCC-4; LMC; monotactin-1; LCC-1

Systemic name	Common Synonyms
<u>CCL</u>	
CCL17	TARC
CCL18	DC-CK1; PARC; AMAC-1; MIP-4; ck β 7
CCL19	MIP-3 β ; ELC; exodus-3; ck β 11
CCL20	MIP-3 α ; LARC; exodus-1
CCL21	6Ckine; SLC; exodus-2; TCA4;ck β 9
CCL22	MDC
CCL23	MPIF-1; MIP-3; ck β 8-1
CCL24	MPIF-2; eotaxin-2; ck β 6
CCL25	TECK; ck β 15
CCL26	Eotaxin-3; MIP-4 α

Systemic name	Common Synonyms
<u>XCL</u>	
XCL1	lymphotactin/SCM-1 α /ATAC
XCL2	SCM-1 β
<u>CX₃CL</u>	
CX ₃ CL	fractalkine/neurotactin

Reproduced and modified from Rossi and Zlotnik (Rossi & Zlotnik, 2000).

as well as on the surface of the overlying endothelium. Selectins facilitate primary adhesion or tethering, and rolling of the leukocyte on the endothelial cell surface (Lawrence & Springer, 1991). This slow velocity leads to rolling leukocytes being brought into contact with chemokines presented on the surface of endothelial cells by proteoglycans (Tanaka *et al.*, 1993b). Chemokines then bind to their receptors on the surface of the leukocytes, triggering rapid G-protein-mediated signaling, resulting in activation of integrin adhesiveness (Springer, 1994). The integrins can then bind to members of the immunoglobulin (Ig) super family, such as intercellular adhesion molecule (ICAM)-1, ICAM-2, and ICAM-3, on the endothelium, which results in firm attachment of the leukocyte to the endothelium and facilitates transendothelial migration (Rot, 1993; Springer, 1994).

1.2.1.3.2 Role in leukocyte activation

Chemokines play an important role in the regulation of leukocyte activation. For example, neutrophils activated by IL-8 have enhanced phagocytosis, superoxide generation, and granule release (Baggiolini *et al.*, 1994). Current evidence indicates that all leukocyte subsets can be activated by relevant chemokines. Monocytes are activated by monocyte chemoattractant protein (MCP)-1, MIP-1 α and RANTES (Sozzani *et al.*, 1993) and eosinophils by eotaxin, MIP-1 α and RANTES and MCP-3 (Dahinden *et al.*, 1994; Elsner *et al.*, 1996; Rot *et al.*, 1992). MIP-1 α , which activates a broad range of leukocytes, has also been shown to be a negative regulator of haemopoietic stem cells (Graham *et al.*, 1990).

Some chemokines can provide important costimulatory signals for T cell proliferation, and promote lymphocyte effector functions. The CC chemokines, RANTES and MIP-1 α enhance T cell proliferation by inducing the T cell costimulatory molecule B7-1 in antigen-presenting cells. In addition, these chemokines can augment cytotoxic T lymphocyte (CTL) and NK cell cytotoxicity (Taub *et al.*, 1996).

1.2.1.3.3 Role in disease

The generation of chemokines is an essential part of host defense against infection. Chemokines of different classes are induced as a response to various bacterial pathogens. In general, CXC

chemokines are likely to play a major role in acute bacterial infections (such as *Streptococcus pneumoniae* (Mizgerd *et al.*, 1996)), which are characterised by the predominance of neutrophils in the inflammatory reaction, while the CC or C chemokines are more likely to be associated with bacterial pathogens which cause subacute or chronic infections (such as *Borrelia burgdorferi*), which involve mononuclear phagocytes and lymphocytes. There is also evidence that chemokines have an antifungal role. A murine model to evaluate the pathogenesis of *Cryptococcus neoformans* revealed the importance of the CC chemokine, MCP-1, in the clearance of the infection. Chemokines have also been shown to have role in the pathogenesis of mycobacterial and viral diseases (Cook *et al.*, 1995; Zhang *et al.*, 1995a).

Apart from the involvement of chemokines in infectious diseases, discussed above, inappropriate activation of the chemokine network has been linked to several other diseases. These include atherosclerosis (Nelken *et al.*, 1991), autoimmune disease (Arimilli *et al.*, 2000; McManus *et al.*, 1998; Peichl *et al.*, 1991), inflammation or allergy (Goebeler *et al.*, 1998; Kulke *et al.*, 1998; Sousa *et al.*, 1994), allogeneic transplant rejection (Hancock *et al.*, 2000; Pattison *et al.*, 1994), and cancer (Youngs *et al.*, 1997).

1.2.1.3.4 Role in suppression of HIV

The CC chemokine RANTES, MIP-1 α , and MIP-1 β are major HIV-1 suppressive factors produced by CD8⁺ cells that inhibit R5-using non syncytium-inducing (NSI) HIV isolates (Cocchi *et al.*, 1995). While the CXCR4 ligand SDF-1 is constitutively expressed in most tissues, CD8⁺ cells express very low levels of SDF-1 mRNA transcripts, and the suppression of SI isolates by this α -chemokine is likely to occur via a CD8⁺ T cell-independent mechanism (Lacey *et al.*, 1997; Ohashi *et al.*, 1998). Furthermore, macrophage-derived chemokine (MDC) produced by CD8⁺ T cells was found to effectively suppress both R5 and X4 HIV isolates (Pal *et al.*, 1997). Taken together, these results indicate that CC chemokine are responsible for a large proportion of HIV-1 suppressor activity produced by CD8⁺ T cells. It has been suggested that the mechanism involved in this suppressive activity is induction of coreceptor internalization into endosomes by chemokines, which effectively prevent the formation of the gp120-CD4-coreceptor complex (Garzino-Demo *et al.*, 1998). There is also the possibility that chemokines

may target other replication steps apart from entry (Garzino-Demo *et al.*, 1998; Ward *et al.*, 1998).

1.2.1.3.5 Role in angiogenesis/angiostasis

Angiogenesis, the biological process through which blood vessels are generated, is important in physiological, as well as pathological processes. The CXC chemokines display disparate angiogenic activity depending on the presence or absence of the ELR motif. Thus, ELR⁺ CXC chemokines (IL-8, ENA-78, GRO α , β , and γ), are powerful promoters of angiogenesis, while ELR⁻ chemokines, (PF-4, IP-10, and Mig) are potent inhibitors of angiogenesis (Moore *et al.*, 1998; Strieter *et al.*, 1995). The net biological balance between angiogenic and angiostatic activity may be important in regulating overall angiogenesis during wound repair, chronic inflammation and tumourigenesis.

1.2.1.3.6 Role in development

Chemokines and their receptors regulate the movement of maturing T and B cells. Recent studies have provided evidence indicating that there are changes in chemokine receptor expression during development. As immature T and B cells go through various stages of maturation, they may express different chemokine receptors, and consequently they are responsive to different chemokines which induce their migration to new locations where their further development may take place (Bowman *et al.*, 2000; Campbell *et al.*, 1999).

SDF-1 is a chemokine which plays an essential role in development. Mice lacking in SDF-1 die perinatally with major defects in cardiac septal formation, gastrointestinal vasculogenesis, cerebellar development, myelopoiesis and B cell lymphopoiesis, but have normal T cell lymphopoiesis (Nagasawa *et al.*, 1996; Tachibana *et al.*, 1998; Zou *et al.*, 1998).

Thus, it is clear that the biological effects of chemokines are complex, involving both homeostatic activities, including recruitment of leukocytes, wound healing and B and T cell development, as well as pathological processes like inflammation, autoimmunity, and tumour

growth.

1.2.2 Chemokine receptors

1.2.2.1 Structural features

Chemokine receptors are a large family of membrane-bound G-protein-coupled molecules composed of seven-transmembrane domains (Murphy, 1994). Chemokine receptors are structurally related, and this is manifested mainly by the number of conserved structural motifs mostly found within the transmembrane domains, rather than overall sequence similarity. Their dominant characteristics include: the relatively small size in comparison with other seven-transmembrane receptors (339-373 amino acids in length), the amino-terminus is extracellular and is acidic overall; the sequence DRYLAIVHA, or a variation of it is in the second intracellular loop; the basic third intracellular loop is short; there is a cysteine in each of the four extracellular domains; the carboxy-terminus is intracellular and contains many serine and threonine residues that act as phosphorylation sites for receptor regulation. The seven-transmembrane domains pass as α -helices perpendicularly to the plasma membrane forming 3 intracellular and 3 extracellular connecting loops which are composed of hydrophilic amino acids. The highly conserved cysteines in extracellular loops 1 and 2 are linked by a disulphide bond which leads to the formation of a compact tertiary structure.

1.2.2.2 Classification

Chemokine receptors can be divided into separate groups based on their ligand specificity (Table 1.2). These include CXC receptors, CC receptors, C receptor, CX₃C receptor, viral chemokine receptors, orphan receptors and promiscuous receptors. CXCR1 to CXCR6 selectively bind CXC chemokines. CXCR6 was previously known as Bonzo, (an orphan receptor) until its ligand was identified as CXCL16, for the 16th chemokine of the CXC subfamily (Matloubian *et al.*, 2000). The CC receptor family consists of 11 receptors, CCR1 to CCR11. A further receptor, designated D6, was simultaneously cloned by two independent groups. It was initially named CCR10 (Bonini *et al.*, 1997), but because it was found to be unable to induce calcium flux on ligand binding, and appears not to be a signaling receptor (Nibbs *et al.*, 1997), the receptor has yet to be allocated a CCR number. It has recently been reported that the C chemokine,

lymphotactin, specifically binds to GPR5 (Yoshida *et al.*, 1998), which was previously termed an orphan receptor (Heiber *et al.*, 1995). GPR5 has now been designated XCR1. The CX₃C chemokine fractalkine has been shown to bind with high affinity to V28, an orphan chemokine receptor, which has been subsequently designated CX₃CR1 (Imai *et al.*, 1997). In addition, several virus-encoded chemokine receptors have been described. Human cytomegalovirus encodes a chemokine receptor, US28, which binds the CC chemokine, RANTES, MCP-1, MIP-1 α and MIP-1 β (Gao & Murphy, 1994), and when expressed at high levels *in vitro* can function as a coreceptor for several strains of HIV-1 and HIV-2 (Pleskoff *et al.*, 1997; Rucker *et al.*, 1997). The gene ECRF3, of Herpesvirus saimiri, encodes a functional receptor for the CXC chemokines IL-8, NAP-2 and GRO- α (Ahuja & Murphy, 1993; Neote *et al.*, 1993). Orphan receptors refer to those chemokine receptors whose ligands have not yet been identified. These include GPR15/BOB, APJ, GPR1 and ROC1. The Duffy blood group antigen will bind chemokine ligands of either CXC or CC groups and is therefore called a promiscuous receptor. It is divergent from the rest of the receptor family, while still belonging to the seven-transmembrane class of receptors. There is redundancy in the chemokine receptor-ligand system, as single receptors can bind multiple chemokines, and many chemokines can function with more than one receptor within a group.

1.2.2.3 Signal transduction mechanisms

Signalling by chemokine receptors occurs through interaction with pertussis toxin sensitive (Murphy, 1994; Rollins, 1997) heterotrimeric guanine triphosphate (GTP)-binding proteins (G-proteins). G-proteins are composed of α , β and γ subunits (for more detail, see Murphy (Murphy, 1996) and Bokoch (Bokoch, 1995)). The binding of a chemoattractant to its receptor induces receptor activation and conformational changes in the G-protein-interacting intracellular loops of the receptor, thus exposing the previously concealed G-protein-binding sites (Farrens *et al.*, 1996). G-proteins are inactive when guanine diphosphate (GDP) is bound, and are activated by G-protein-coupled receptors performing as guanine nucleotide exchange factors leading to GTP binding to the α subunit. GTP binding leads to the dissociation of G α GTP from G $\beta\gamma$ (Gilman, 1987). The G $\beta\gamma$ subunits bind and activate phospholipase C (PLC) β which cleaves phosphatidylinositol 4,5-bisphosphate into two second messengers, diacylglycerol (DAG) and inositol triphosphate (IP₃) in the cytoplasm. IP₃ induces mobilization of calcium from

Table 1.2 Chemokine receptors and their ligands

Receptor	Ligand
<u>CXC Chemokine Receptors</u>	
CXCR1 (IL8RA)	IL-8, GCP-2
CXCR2 (IL8RB)	IL-8, GRO- α , β , γ , ENA-78, NAP-2
CXCR3 (IP10/Mig R, GPR9)	Mig, IP-10, I-TAC
CXCR4 (LESTR, fusin)	SDF-1
CXCR5 (BLR1, MDR15)	BCA-1
CXCR6 (Bonzo, STRL33, TYMSTR)	CXCL16
<u>CC Chemokine Receptors</u>	
CCR1 (CKR1)	MIP-1 α , RANTES, MCP-2, MCP-3, MCP-4, MIP-5, Lkn-1
CCR2 (CKR2)	MCP-1, MCP-3, MCP-4
CCR3 (eotaxin receptor)	Eotaxin, eotaxin-3, RANTES, MCP-2, 3, 4, Lkn-1, MPIF-2.
CCR4 (ChemR1)	TARC, MDC
CCR5 (ChemR13)	MIP-1 α , MIP-1 β , RANTES, MCP-2
CCR6 (STRL22, DRY-6, GPR-CY4, CKR-L3)	LARC
CCR7 (EBI-1, BRL-2)	SLC, ELC
CCR8 (TER1, CKR-L1, ChemR1)	I-309
CCR9 (GPR-9-6)	TECK
CCR10 (GPR2)	ESkine
CCR11 (GPR1)	MCP-1, MCP-2, MCP-4
<u>C Receptor</u>	
XCR1 (GPR5)	Lymphotactin
<u>CX₃C Receptor</u>	
CXC ₃ CR1 (V28)	Fractalkine
<u>Viral Chemokine Receptors</u>	
US28	RANTES, MIP-1 α , MIP-1 β , MCP-1, MCP-3,
ECRF3	IL-8, NAP-2, GRO- α
KSHV GPCR	IL-8, NAP-2, PF-4, I-309, MGSA, RANTES
UL12	RANTES, MIP-1 α , MIP-1 β , MCP-1
E1	Eotaxin

Receptor	Ligand
<u>Orphan Receptors</u>	
GPR15/BOB	?
APJ	?
GPR1	?
ROC1	?
ChemR23	?
<u>Chemokine binding proteins</u>	
Duffy (DARC)	RANTES, MCP-1, IL-8, GRO- α , NAP-2
D6 (CCR9,CCR10)	MCP-1,MCP-2, MCP-4, RANTES, HCC-1

Reproduced from Murphy *et al.*(Murphy *et al.*, 2000)

intracellular stores. DAG, in conjunction with Ca²⁺ activates protein kinase C, resulting in a cascade of signal transduction events, both intracytoplasmically and in the nucleus. After activation, chemokine receptors may become partially or fully refractory to repeated stimulation with the same or other agonists (Mackay, 1996). This desensitization involves the G $\beta\gamma$ complex binding to certain G-protein-activated receptor kinases which induce phosphorylation of serine and threonine residues on the carboxy-terminal region of the receptor (Mueller *et al.*, 1994; Richardson *et al.*, 1995), preventing further coupling of the receptor and the G-protein. This process may be critical for preserving the capability of the cell to sense a chemoattractant gradient (Murphy, 1996). In addition, there is evidence to suggest that G-protein-coupled receptors signal not only through interaction with G-proteins, but also with additional cellular proteins (Hall *et al.*, 1999).

1.2.2.4 T helper cell subsets

T helper (Th) lymphocytes can be divided into Th1, Th2 and Th0 subsets on the basis of their cytokine production (Firestein *et al.*, 1989; Mosmann & Coffman, 1989). Murine Th1 cells secrete IL-2, interferon (IFN)- γ , TNF and lymphotoxin, and are important in cell-mediated responses, whereas Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and are associated with humoral-type responses (Abbas *et al.*, 1996; Mosmann & Coffman, 1989; Romagnani, 1994).

Th0 cells are precursors to the Th1 and Th2 subsets and have an unrestricted cytokine profile (Firestein *et al.*, 1989). An exact separation of Th1 and Th2 subsets comprising all cytokines, as reported in mice, is seldom found in humans, therefore a preferable definition of Th1 cells may be ability to produce IFN- γ but not IL-4, and Th2 cells secreting IL-4 but not IFN- γ (Romagnani, 1997). It has been proposed that a switch from the Th1 to Th2 cytokine profile occurs in HIV infection, and that this is an important component of the progression to AIDS (Clerici *et al.*, 1993; Clerici & Shearer, 1993; Klein *et al.*, 1997; Meyaard *et al.*, 1994). However, this remains controversial, with studies either not supporting this shift (Graziosi *et al.*, 1994), or proposing a shift from the Th1 to the Th0 phenotype (Maggi *et al.*, 1994; Romagnani *et al.*, 1994). Recently a number of studies have been carried out to evaluate chemokine receptor expression on Th1 and Th2 cells. CXCR3 and CCR5 have been shown to be associated with the Th1 phenotype (Bonecchi *et al.*, 1998), and CCR4 (Bonecchi *et al.*, 1998) and CCR8 with a Th2 phenotype (Zingoni *et al.*, 1998). Sallusto *et al.* (Sallusto *et al.*, 1997) showed that IL-4 producing cells express CCR3, suggesting that CCR3 was correlated with a Th2 phenotype. However, Bonecchi *et al.* (Bonecchi *et al.*, 1998) found extremely low levels of CCR3 expression in Th2 cells suggesting that CCR3 is only expressed on a small subset of Th2 cells. CXCR4 mRNA was found to be expressed in equal amounts in Th1 and Th2 cells (Bonecchi *et al.*, 1998).

1.2.2.5 Coreceptor function in HIV fusion/entry

Entry of HIV-1 into cells is a multistep process that is mediated by the viral envelope (Env) glycoprotein. The HIV-1 virion, in common with other retroviruses, is enveloped by a lipid membrane from which Env protrudes. Env is initially synthesised as a single gp160 polypeptide precursor. Post-translational proteolytic cleavage of the gp160 generates the heavily glycosylated surface gp120 subunit, which is derived from the amino-terminal portion of gp160, and is noncovalently attached to the transmembrane gp41 subunit, derived from the carboxy-terminal portion of the precursor (Wyatt & Sodroski, 1998). Env is a trimeric structure (Lu *et al.*, 1995) consisting of three gp120/gp41 complexes associated via noncovalent bonds within gp41. The first step required for binding of the viral particle to the target cell is the binding of gp120 to CD4. This binding then triggers conformational changes in gp120 (Lu *et al.*, 1995; Sattentau *et al.*, 1993; Trkola *et al.*, 1996), that contribute to the creation or exposure of the coreceptor-

binding determinants and subsequently allows interaction with a chemokine receptor. The gp120-coreceptor binding is speculated to be the final trigger that results in the exposure and extension the gp41 peptide (usually covered by the gp120) to form a triple-stranded coiled-coil structure, which enables the fusion peptide at its amino-terminus to insert into the plasma membrane of the target cell (Chan *et al.*, 1997; Doms & Peiper, 1997; Weissenhorn *et al.*, 1997), thus making gp41 a fundamental part of both the viral and cellular membranes.

1.2.2.6 Structural determinants of coreceptor function

1.2.2.6.1 CXCR4

CXCR4 was initially cloned by Loetscher *et al.* (Loetscher *et al.*, 1994), and was given the acronym LESTR (leukocyte-derived seven-transmembrane domain receptor). LESTR was subsequently identified as a necessary cofactor for the entry of T-tropic viruses into CD4-expressing cells (Feng *et al.*, 1996). LESTR was known as an orphan receptor until SDF-1 was identified as its ligand, and then it was renamed CXCR4 (Bleul *et al.*, 1996a; Oberlin *et al.*, 1996). CXCR4 contains 352 amino acids, has two amino-linked glycosylation sites, and has four conserved Cys residues in the ectodomain that are predicted to form 2 disulphide bonds, one connecting the amino-terminal domain with extracellular loop 3, and one connecting extracellular loops 1 and 2 (Federspiel *et al.*, 1993) (Figure 1.1A). As a result, CXCR4 and other chemokine receptors form compact structures in which all four extracellular domains are in close proximity with one another (Horuk, 1994). The first and second extracellular loops are crucial determinants for all viral strains studied to date (Brelot *et al.*, 1997; Lu *et al.*, 1997). The second extracellular loop appears to be critical for use of this receptor by both T- and dual-tropic virus strains. These loops are more acidic than the similar regions in CCR5, and CXCR4 is atypical among chemokine receptors in having this strongly negative surface charge (Loetscher *et al.*, 1994). These domains are additionally involved in SDF-1 binding (Doranz *et al.*, 1999), which may help to explain the capacity of this chemokine to block CXCR4-dependent viral infection. Dual-tropic viruses are able to use the loops of CXCR4 while maintaining the ability to use the amino-terminal of CCR5 (Lu *et al.*, 1997). Recent work by Kajumo *et al.* (Kajumo *et al.*, 2000) has shown that tyrosine (Y), aspartic acid (D), and glutamic acid (E) rich clusters in the amino-terminus and extracellular loop 2 (ECL2) are important for viral entry, with substitutions in ECL2 having a greater effect on X4 tropic strains, and substitutions in the amino-terminus having more of an effect on dual tropic strains.

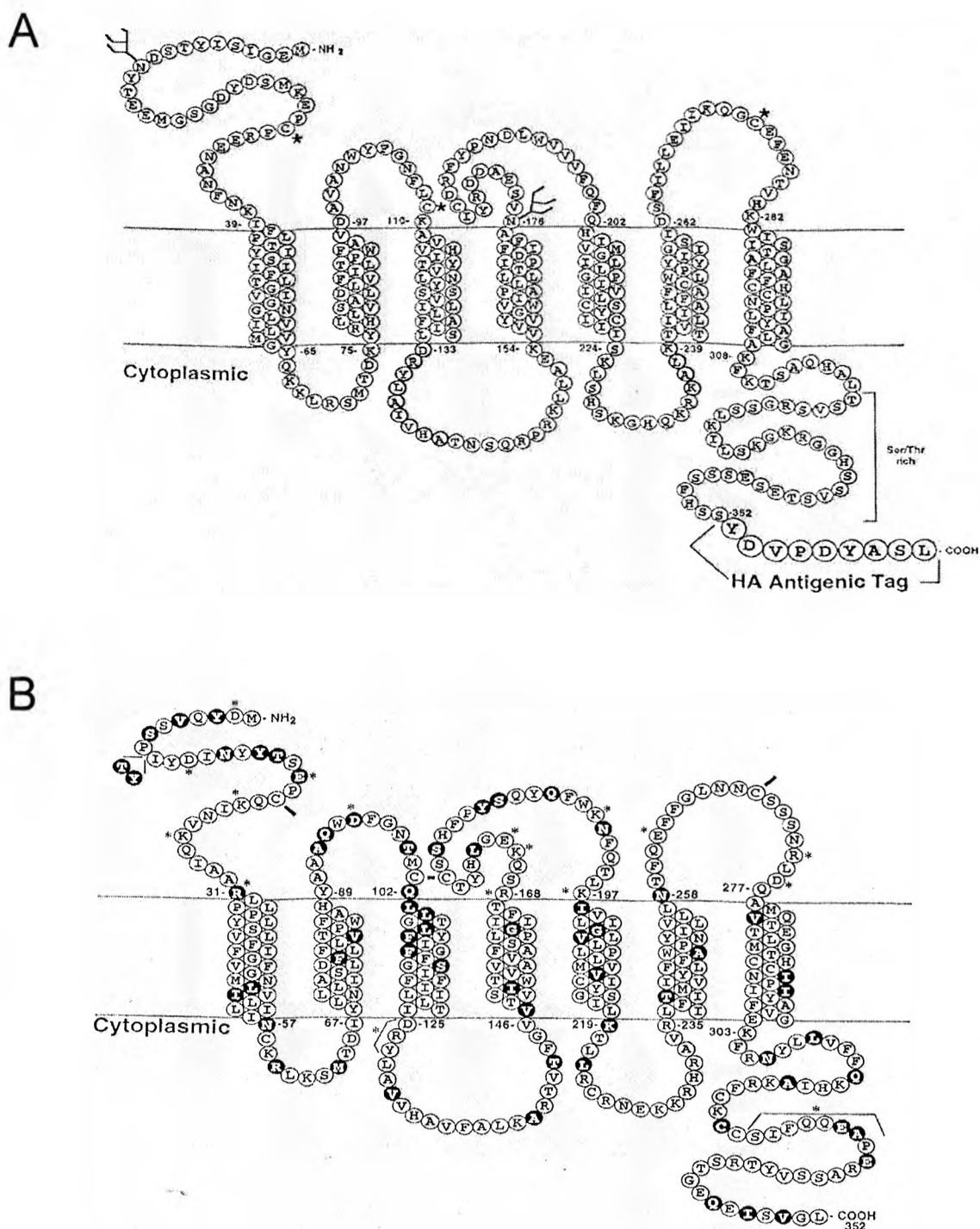


Figure 1.1 (A) CXCR4: coreceptor for X4 (T-tropic) HIV-1 strains. Asterisks indicate Cys residues in the ectodomain. Shading indicates conserved regions that are characteristic of G protein coupled receptors. Reproduced from Berson *et al.* (Berson *et al.*, 1996) (B) CCR5: coreceptor for R5 (M-tropic) HIV-1 strains. Shaded residues are those which are different in the human and the mouse homologue. Asterisks represent CCR5 mutations that were used in the study, and asterisk and bar indicates the cytoplasmic deletion in the CCR5 Δ tail. Reproduced from Doranz *et al.* (Doranz *et al.*, 1997b).

1.2.2.6.2 CCR5

CCR5 was originally cloned by Samson *et al.* (Samson *et al.*, 1996a) (Figure 1.1B) and was found to bind the CC chemokine, MIP-1 α , MIP-1 β , and RANTES (Raport *et al.*, 1996). Later, CCR5 was shown to be the major coreceptor, together with CD4, for entry of M-tropic strains (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996; Doranz *et al.*, 1996). Env-CCR5 interactions are conformationally complex and studies have indicated that residues in all four extracellular domains contribute to coreceptor function, with the amino-terminal domain and the extracellular loops comprising distinct domains (Atchison *et al.*, 1996; Bieniasz *et al.*, 1997; Doranz *et al.*, 1997b; Lu *et al.*, 1997; Picard *et al.*, 1997; Ross *et al.*, 1998; Rucker *et al.*, 1996) (reviewed by (Doms & Peiper, 1997)). Tyrosine and negatively charged amino acids, in the amino-terminal domain of CCR5 are important for binding and entry of R5 and R5X4 viruses (Doranz *et al.*, 1997b; Farzan *et al.*, 1998). The conformational integrity of the extracellular domains is also important for effective viral fusion and entry (Genoud *et al.*, 1999; Picard *et al.*, 1997; Rucker *et al.*, 1996; Wu *et al.*, 1997a). In general, M-tropic viral strains appear to be remarkably well-adapted for CCR5 use and can tolerate significant alterations in either the amino-terminal or extracellular loops, indicating that in some instances either of these structural features may be adequate for coreceptor function (Atchison *et al.*, 1996; Doranz *et al.*, 1997b; Lu *et al.*, 1997; Rucker *et al.*, 1996). Thus, at least to some degree, there are functionally redundant binding sites for Env on CCR5 (Arvanitakis *et al.*, 1997). By contrast, dual-tropic viruses are far more sensitive to changes in CCR5 structure, particularly to changes in the amino-terminal domain (Bieniasz *et al.*, 1997; Doranz *et al.*, 1997b; Picard *et al.*, 1997; Rucker *et al.*, 1996).

1.2.2.7 HIV-1 Env determinants for coreceptor use

The envelope protein (Env) is responsible for fusion of the viral and host membranes and is recognised to be the primary determinant of viral tropism. The overall positive amino acid charge of the V3 loop can be used to predict the phenotype of a viral isolate, with a higher charge associated with a SI phenotype, and utilization of the CXCR4 coreceptor (Isaka *et al.*, 1999). Other regions of Env, such as the V1 and V2 loops have been implicated as playing a role in viral tropism. Introduction of the V1/V2 regions of a dual tropic virus enabled a M-tropic virus to use CXCR4 (Cho *et al.*, 1998), and antibodies to non-V3 loop determinants inhibited the

interaction of Env with CCR5 (Verrier *et al.*, 1997). Additionally, the loss of an N-linked glycosylation site within the V3 region influenced the switch from a R5 to X4 phenotype, and occurred in association with an increase in V3 charge (Pollakis *et al.*, 2001). These results suggest that other regions of Env are involved in determining coreceptor usage.

1.2.3 Other chemokine receptors and HIV

Although CCR5 and CXCR4 are the most widely used HIV-1 coreceptors, several other chemokine receptors have been shown, *in vitro*, to have coreceptor activity to a greater or lesser degree (Table 1.3). Not all chemokine receptors can act as HIV-1 coreceptors, and no coreceptor activity was found in most studies for CCR1, CCR4, and CCR6, and for CXC chemokine receptors except CXCR4 and CXCR6, and for several orphan receptors. In addition, HIV-1 coreceptor activity was detected for US28 (Pleskoff *et al.*, 1997), a chemokine receptor homologue encoded by human cytomegalovirus.

Table 1.3 Summary of minor HIV-1 coreceptors

Chemokine Receptor	Reference
CXCR6/Bonzo/STRL33/TYMSTR	Alkhatib <i>et al.</i> , 1997b; Deng <i>et al.</i> , 1997; Edinger <i>et al.</i> , 1998a; Liao <i>et al.</i> , 1997; Loetscher <i>et al.</i> , 1997
CCR2b	Doranz <i>et al.</i> , 1996
CCR3	Alkhatib <i>et al.</i> , 1997a; Bazan <i>et al.</i> , 1998; Choe <i>et al.</i> , 1996; He <i>et al.</i> , 1997; Ross & Cullen, 1998
CCR8	Horuk <i>et al.</i> , 1998; Rucker <i>et al.</i> , 1997
CCR9	Choe <i>et al.</i> , 1998
CX ₃ CR1	Combadiere <i>et al.</i> , 1998
BOB/GPR15	Deng <i>et al.</i> , 1997; Edinger <i>et al.</i> , 1998a; Farzan <i>et al.</i> , 1997
GPR1	Edinger <i>et al.</i> , 1998a; Farzan <i>et al.</i> , 1997
ChemR23	Samson <i>et al.</i> , 1998
APJ	Choe <i>et al.</i> , 1998
US28	Pleskoff <i>et al.</i> , 1997

1.2.4 Polymorphisms in chemokines and chemokine receptors

The length of time from infection with HIV to the development of AIDS, is known to vary considerably between individuals. Additionally, there are small group of individuals who remain uninfected, despite repeated exposure to HIV (Paxton *et al.*, 1996). The exact mechanisms for this are still not completely understood, but host genetic factors are partly responsible. Over the past few years polymorphisms in some chemokines and chemokine receptors have been identified that may impact on HIV-1 transmission and rate of disease progression (O'Brien & Dean, 1997).

1.2.4.1 CCR5 Δ 32

Two groups simultaneously identified a 32 bp deletion in the CCR5 gene in sequences encoding the second extracellular loop that resulted in premature truncation of the polypeptide (Liu *et al.*, 1996a; Samson *et al.*, 1996b), which is not expressed at the cell surface (Liu *et al.*, 1996a; Rana *et al.*, 1997) and lacks coreceptor activity (Liu *et al.*, 1996a; Samson *et al.*, 1996b). The allele frequency of CCR5 Δ 32 in the Caucasian population is approximately 10%, with 1% of Caucasians being homozygous and approximately 15-20% being heterozygous (Dean *et al.*, 1996; Samson *et al.*, 1996b). Individuals who are homozygous for the CCR5 Δ 32 allele are highly resistant, but not immune (Dean *et al.*, 1996; O'Brien *et al.*, 1997; Theodorou *et al.*, 1997) to virus infection, while heterozygotes progress to AIDS more slowly (Dean *et al.*, 1996; Huang *et al.*, 1996; Liu *et al.*, 1996a; Michael *et al.*, 1997a; Paxton *et al.*, 1998; Samson *et al.*, 1996b). Although cells from homozygous individuals are resistant to infection by CCR5-restricted viruses, they are readily infectable *in vitro* by viruses that use other coreceptors such as CXCR4 (Connor *et al.*, 1996; Liu *et al.*, 1996a; Rana *et al.*, 1997; Samson *et al.*, 1996b). The CCR5 Δ 32 allele is most prevalent in northern European populations (16%), less common in southern European populations (4%), and is extremely rare in African populations (Martinson *et al.*, 1997; Samson *et al.*, 1996b).

1.2.4.2 CCR5-m303

The CCR5-m303 mutation is a single nucleotide polymorphism (T→A substitution at position 303) referred to as m303 or CCR5-m303 (Quillent *et al.*, 1998). CCR5-m303 leads to the

introduction of a premature stop codon in the open reading frame of the CCR5 gene. This prevents the expression of functional CCR5 protein with HIV-1 coreceptor function. This mutation has been found to occur in three of 209 European blood donors (1%), which is 10-20% lower than the frequency than the $\Delta 32$ allele.

1.2.4.3 CCR5 59029-G/A

A 59029-G/A CCR5-promoter region polymorphism has been linked to altered rates of HIV-1 disease progression (McDermott *et al.*, 1998). HIV-1 positive individuals who were homozygous for the 59029-G allele had slower disease progression compared with 59029-A homozygotes (median time to AIDS 10.4 years versus 6.6 years). Despite the fact that CCR5 $\Delta 32$ and CCR2-64I have been shown to be in strong linkage disequilibrium with the CCR5 59029-A allele, the protective effect of 59029-G in relation to 59029-A was demonstrated to be independent of the protective effects of CCR5 $\Delta 32$ and CCR2-64I. This polymorphism, however, has no effect on HIV-1 transmission. As 59029-G has a 45% lower promoter activity than 59029-A, this suggests that the mechanism of action of this polymorphism is differential regulation of CCR5-1 gene transcription. Allele frequencies of both 59029-G, and 59029-A were high in all racial groups tested, thus this polymorphism may affect HIV pathogenesis globally.

1.2.4.4 CCR2-64I

The CCR2-64I mutation, a single amino acid change (G→A substitution) at position 190, results in a valine→isoleucine substitution at position 64 in the CCR2b protein. This mutation has been found at an allelic frequency of 9.8% in Caucasians compared to 15.1% in African Americans (Smith *et al.*, 1997). The CCR2-64I allele, in both heterozygotes and homozygotes, is associated with a 2-3 year delay in progression to AIDS, but has no impact on HIV-1 transmission (Kostrikis *et al.*, 1998). The protective effect of CCR2-64I was initially questioned (Michael *et al.*, 1997b), but it is now clear that the effect is masked unless seroincident (rather than seroprevalent) cohorts are studied (Kostrikis *et al.*, 1998; Smith *et al.*, 1997). As CCR2b is used as a coreceptor by relatively few viral strains it is unlikely that the protective effect associated with this mutation is due to a direct effect on viral infection. As valine and isoleucine have the same chemical and functional characteristics, this mutation should supposedly not modify the

structure of the receptor. However, a recent report by Mellado *et al.* (Mellado *et al.*, 1999) showed that CXCR4 can heterodimerize with CCR2-64I, but not with wild type CCR2. Moreover, the authors have previously suggested that chemokine heterodimerization may help to prevent HIV-1 infection (Rodriguez-Frade *et al.*, 1999). Taken together, these results may help to explain why the CCR2-64I mutation is protective.

1.2.4.5 RANTES-403

The two most common RANTES promoter compound genotypes, G1 (-403G/G, -28C/C) and G4 (-403G/A, -28C/C) have been found in 67% and 23% of Caucasians, respectively. The G4 compound genotype occurred more frequently in HIV-positive individuals (30%) than in exposed uninfected individuals (21.1%), while the G1 compound genotype was more common in exposed uninfected individuals (72.4%) than in HIV-positive individuals (59.9%). In addition, seroconverters who lacked CCR5 Δ 32, and possessed the G4 compound genotype had increased time for progression to AIDS than those who had the G1 compound phenotype (McDermott *et al.*, 2000). Thus, the G4 genotype is associated with increased risk of HIV-1 acquisition, and paradoxically, slower progression to AIDS than the G1 phenotype. The -403A construct has previously been shown to increase activity of the RANTES promoter versus the -403G construct (Nickel *et al.*, 2000), consequently the CD4⁺ cells of G4-positive individuals may secrete more RANTES. This may enhance cellular inflammation thus facilitating viral replication or transfer, however, HIV-1 must then compete with RANTES for binding to CCR5 resulting in slower viral replication.

1.2.4.6 SDF-1 3' A

The SDF-1 3' A mutation (also designated as SDF-3'UTR-801G-A) is a single nucleotide polymorphism (G→A substitution at position 801) found in a conserved part of 3'-untranslated region of the SDF-1 gene that encodes the ligand for CXCR4 (Winkler *et al.*, 1998). The polymorphism occurs at an allelic frequency of 21.1% in Caucasians compared to 5.7% in African Americans (Winkler *et al.*, 1998). Winkler *et al.* (Winkler *et al.*, 1998) reported that the SDF-1 3' A mutation, in the homozygous form, delays the onset of AIDS. The authors postulated that the mutation could increase the amount of SDF-1 available to bind to CXCR4, consequently

preventing the emergence of late stage X4 viruses. However, these results were contradicted in other studies (Easterbrook *et al.*, 1999; Mummidi *et al.*, 1998). Additional studies are required to clarify the impact of this mutation on disease progression.

1.2.5 Evolution of coreceptor use by HIV-1 *in vivo*

R5-tropic viruses are the most commonly transmitted viruses and are the predominant virus type during the early stages of infection (Connor *et al.*, 1993; Roos *et al.*, 1992; Schuitemaker *et al.*, 1992; Zhu *et al.*, 1993). X4 viral strains emerge in about 50% of patients with time, commonly after years of HIV-1 infection (Tersmette *et al.*, 1988; Tersmette *et al.*, 1989). This switch in viral phenotype is linked to accelerated immune system failure and advancement to AIDS (Connor *et al.*, 1993; Schuitemaker *et al.*, 1992; Tersmette *et al.*, 1988; Tersmette *et al.*, 1989). Dual-tropic, R5X4, viruses may represent an important evolutionary intermediate (Collman *et al.*, 1992), and although it is not clear if transition from M- to T-tropism must necessarily progress through this intermediate phenotype, it has been suggested that dual-tropic viruses evolve from M-tropic viruses by gaining the ability to use CXCR4 while retaining the capacity to use CCR5. Changes in coreceptor use are largely as a result of mutations in the Env protein, and these in turn correlate with changes in viral tropism (Bieniasz *et al.*, 1997). Shifts in tropism frequently include changes in the V3 loop of the gp120 subunit, often leading to an increase in basic charge in one or both ends of the loop (de Jong *et al.*, 1992; Fouchier *et al.*, 1992; Hwang *et al.*, 1991; Liu *et al.*, 1990). There are non-V3 loop determinants, however, that also play a role in viral tropism (Kim *et al.*, 1995; O'Brien *et al.*, 1990; Shioda *et al.*, 1991; Westervelt *et al.*, 1992).

1.2.6 Coreceptor based therapeutic strategies

Although current antiretroviral therapies have resulted in dramatic reductions in mortality and morbidity, they are not without their problems: they do not clear HIV-1 from infected persons (Finzi *et al.*, 1999); drug resistant viral strains are emerging that are resistant to both reverse transcriptase and protease inhibitors (Flexner, 1998; Perrin & Telenti, 1998; Yerly *et al.*, 1999); and side effects such as lipodystrophy, hyperlipidaemia and protease-related diabetes have been observed (Carr *et al.*, 1998; Flexner, 1998; Lucas *et al.*, 1999). The finding that chemokine

receptors are coreceptors for HIV-1, and the fact that individuals who are homozygous for the CCR5 32 bp deletion are relatively resistant to HIV infection, has initiated the investigation for novel antiviral approaches which prevent or reduce CCR5 and/or CXCR4 expression. It is known that CCR5 is not essential for normal cellular and physiologic function, as it is absent in some individuals (Liu *et al.*, 1996a; Samson *et al.*, 1996b). However, CXCR4 together with its ligand SDF-1, are physiologically indispensable (Hargreaves *et al.*, 2001; Moser & Loetscher, 2001; Nagasawa *et al.*, 1996; Nanki & Lipsky, 2000; Onai *et al.*, 2000; Tachibana *et al.*, 1998; Zou *et al.*, 1998). The interaction between SDF-1 and CXCR4 is necessary for physiologic trafficking and endothelial transmigration of lymphocytes, myelopoiesis and lymphopoiesis of B cells, costimulation of T cells and plasma cell migration to the bone marrow. Thus, there are some concerns about the use of CXCR4 agonists as therapeutic agents against HIV. Inhibition via CCR5 is a particularly appealing option as the viruses establishing a new infection are generally CCR5-tropic. These approaches have distinct advantages over strategies that are primarily targeted to viral components: they are specifically aimed at the coreceptor, and therefore, HIV may not be able to evade this strategy; and they are directed at inhibition of viral entry rather than limiting virus production in infected cells. These therapeutic strategies may be used to augment current antiretroviral drug treatments, to allow lower doses of highly active antiretroviral therapy (HAART) and to permit structured treatment interruptions. They are unlikely to be immunogenic, consequently they would make good candidates for regimens that require continued maintenance. Finally, these approaches could be modified to target both CCR5 and CXCR4, thus making cells resistant to R5 as well as X4 viruses.

1.2.6.1 Immune restoration

Studies have shown that when human CD4⁺ T cells were cultured *in vitro* with anti-CD3/CD28 conjugated beads, they became resistant to infection (Levine *et al.*, 1996). This antiviral effect was shown to be due to high levels of CC chemokine (MIP-1 α , MIP-1 β and RANTES) and the downmodulation of CCR5 (Carroll *et al.*, 1997; Riley *et al.*, 1997). A recent study has shown that TNF- α provides an essential costimulatory signal for the synthesis of the CC chemokine in activated CD4⁺ T cells (Brice *et al.*, 2000). These findings show that CCR5 can be manipulated, and have led to two techniques that capitalize on these observations. One strategy aims at providing immune restoration by autologous transfusion of virus-resistant CD4⁺ T cells, which

have been stimulated in culture with anti-CD3/CD28 monoclonal antibodies, into HIV infected individuals. This procedure, however, also increases replication of X4 viruses (Brice *et al.*, 1998), and therefore should only be used early on in the course of infection when the M-tropic strains are prevalent. Phase 1 human trials are in progress, but wide clinical utilization of this therapy is unlikely because of its cost and cumbersome nature of the treatment.

A second possibility is to provide HIV infected individuals, who are infected with a M-tropic strain, with stem cells or cord blood from individuals who are homozygous for the $\Delta 32$ deletion. The recipient, however, would have to undergo relatively unpleasant treatments to prevent graft rejection and graft-versus-host disease. This, together with the fact that there is limited availability of stem cells, makes this approach unlikely for large-scale use.

1.2.6.2 Gene therapy

1.2.6.2.1 Ribozymes

Ribozymes are enzymatic RNA molecules that can be designed to specifically bind and cleave target RNAs, and by so doing prevent or reduce translation of proteins encoded by the targeted sequence. In the first of such approaches to target HIV, ribozymes were designed against HIV *gag* sequences (Sarver *et al.*, 1990) and HIV-1 5' leader sequences (Yu *et al.*, 1993). Recently, a number of groups synthesised ribozymes targeted to the CCR5 receptor (Cagnon & Rossi, 2000; Goila & Banerjea, 1998; Gonzalez *et al.*, 1998) which were able to specifically reduce CCR5 mRNA, resulting in decreased CCR5 surface expression (Cagnon & Rossi, 2000; Feng *et al.*, 2000; Gonzalez *et al.*, 1998). As a result, the cells were resistant to infection by R5 HIV-1 viruses, but not X4 HIV-1 strains (Cagnon & Rossi, 2000; Feng *et al.*, 2000). Furthermore, anti-CCR5 ribozyme did not have any deleterious effects on subsequent differentiation and maturation, when introduced into haematopoietic stem cells (Bai *et al.*, 2000). Therefore, anti-CCR5-ribozyme gene therapy could be used as an adjunct to ribozymes that directly target HIV RNA, or in combination with current antiretroviral treatments to prevent or retard disease progression.

1.2.6.2.2 Intrakines

CCR5 and CXCR4 are synthesised in the endoplasmic reticulum (ER) and are then transported to the cell surface. This approach aims to decrease the surface expression of the HIV-1 coreceptors, CCR5 and CXCR4, by trapping them in the ER. To this end, MIP-1 α , RANTES (Yang *et al.*, 1997) and SDF-1 α genes (Chen *et al.*, 1997a) were linked to the tetrapeptide sequence KDEL (an ER retention sequence) (Munro & Pelham, 1987) and cloned into expression vectors. These modified chemokines were termed intracellular cytokines or intrakines. Stimulated peripheral blood leukocytes (PBLs) transduced with the recombinant vector were viable and had normal cell proliferation and DNA synthesis in response to IL-2, and anti-CD3. Intrakines are constitutively impeded from leaving the ER by the tetrapeptide which acts as a molecular leash and so prevents newly synthesised CCR5 or CXCR4 from reaching the surface, probably by forming intracellular complexes in the ER. MIP-1 α - and RANTES-intrakine decreased CCR5 surface expression on the transduced lymphocytes and protected them from entry by M-tropic viruses. Similarly SDF-1 α -intrakine decreased CXCR4 expression and prevented infection by T-tropic HIV-1. CD3 and CD4 surface expression was not affected in either case. Strategies to co-inactivate CCR5 and CXCR4 are being evaluated, and results indicate that lymphocytes with the phenotypic knock-out of CCR5 and CXCR4 can resist infection by M-tropic, T-tropic and dual-tropic HIV-1 viruses. Additionally, the transduced lymphocytes were able to maintain normal cell features, that included responsiveness to both mitogen and recall antigen. This approach has therapeutic applications in that genetically modified lymphocytes or stem cells could be infused back into patients to retard or avert disease progression. Since intrakines have a half-life of > 4h (in contrast to chemokines which have a half-life of < 10 min *in vivo*) and as genetically modified lymphocytes or stem cells can live for months, intrakine therapy should have a lasting anti-HIV effect. The disadvantages of this approach includes lack of specificity, as chemokines typically interact with multiple receptors. Although CCR5 is thought to be nonessential, it is expected that the RANTES intrakine strategy would also affect the other RANTES receptors, CCR1 and CCR3 (Jung & Littman, 1999). Important technical issues which remain to be resolved include the specificity and efficiency of cellular transduction, and resultant expression and persistence of the therapeutic genes.

1.2.6.2.3 Intrabodies

Intrabodies are intracellularly expressed antibodies that can be designed to bind and prevent expression and function of their target molecules. A CCR5-specific single-chain antibody, ST6, has recently been developed (Steinberger *et al.*, 2000). ST6 was retained in the endoplasmic reticulum by coupling the ER retention sequence, KDEL, to the carboxy-terminus of the protein. The intrabody efficiently blocked both human and rhesus CCR5 surface expression, and therefore the cells containing the intrabody were resistant to infection with R5 HIV-1 and SIV viruses. This approach is likely to be more specific than the RANTES intrakine approach, as ST6 reacts with an epitope in the first extracellular domain of CCR5, which is conserved in human and non-human primates and is not found in any other protein. The authors suggest that introducing ST6 into haematopoietic stem cells in infected individuals may generate a pool of cells that are protected from R5 HIV-1 viruses. As with other CCR5-targeted strategies however, there is the risk that they may encourage a switch to the more virulent X4 viruses.

1.2.6.3 Monoclonal antibodies

A further strategy to prevent HIV interacting with its coreceptor is to develop monoclonal antibodies (mAbs) that target chemokine receptors. Endres *et al.* (Endres *et al.*, 1996) developed a murine mAb against CXCR4, designated 12G5, which binds the second extracellular loop of CXCR4. Inhibition of viral entry by 12G5 has been shown to be both cell type and virus isolate dependent (McKnight *et al.*, 1997; Strizki *et al.*, 1997). A number of anti-CCR5 mAbs have been generated. Of these, 2D7 and PRO 140 have been shown to be the most potent inhibitors of HIV-1 entry (Olson *et al.*, 1999). 2D7 recognizes the second extracellular loop of CCR5, which is a significant domain for gp120 as well as chemokine binding (Wu *et al.*, 1997a). 2D7 efficiently inhibits the calcium flux mediated by RANTES, MIP-1 α and MIP-1 β and effectively blocks the entry of several R5 and R5X4 viruses *in vitro*. PRO 140 (previously called PA14) is an anti-CCR5 monoclonal antibody that recognizes an epitope that includes residues in the amino-terminus, as well as in the second extracellular loop of CCR5 (Olson *et al.*, 1999). The concentration of PRO 140 needed to effectively prevent HIV-1 entry, does not affect the chemokine receptor activity of CCR5. Trkola *et al.* (Trkola *et al.*, 2001) have recently shown that PRO 140 exhibits cross-subtype anti-HIV-1 activity, and thus can inhibit entry of a broad spectrum of R5 isolates. The potential benefits of antibody-based inhibitors include multivalent

binding potential, and a predicted long half-life which would allow an extended dosing schedule. There are, however, still a number of obstacles which may hamper the development of mAbs for therapeutic use, including high production costs, accessibility of the targeted cellular population, the requirement for injection as well as potential immunogenicity of the mAb. These products, however, may cause fewer side effects than small molecule inhibitors.

1.2.6.4 Genetically engineered enveloped virus particles

Recent work has shown that when the normal envelope protein of Rhabdoviruses rabies (Mebatsion *et al.*, 1997) and vesicular stomatitis virus (Schnell *et al.*, 1997) was replaced with CD4 and CXCR4 the virus could bind and infect only those cells that express HIV gp120/gp41. Those cells that are about to release new viral particles will by necessity express gp120/41, and will therefore bind the vector, thus allowing the virus to enter the cell where it can replicate and destroy only that particular cell. There are, however, a number of concerns over using a novel replicating virus therapeutically. These include capacity of the vector to exclusively target productively infected cells, the transmissibility of the vector from individual to individual, or from mother to infant, and the potential detrimental sequelae of administering a live replicating vector to immunocompromised recipients.

1.2.6.5 Chemokines

The CCR5 ligands, MIP-1 α , MIP-1 β and RANTES, and the CXCR4 ligand, SDF-1 have been shown to block infection of M-tropic and T-tropic HIV-1 strains, respectively (Amara *et al.*, 1997; Cocchi *et al.*, 1995). Thus, the administration of chemokines in their natural form is a natural approach to chemokine-based anti-HIV therapy. However, as they have inflammatory side effects and a very short plasma half-life, mutated or chemically modified chemokine analogues of RANTES and SDF-1 β , with reduced pro-inflammatory activity are being developed.

1.2.6.5.1 RANTES analogues

A number of amino-terminal modified analogues of RANTES have been described. These

analogues all have antagonistic properties, are unable to mobilize calcium and induce chemotaxis, and are inhibitory to M-tropic HIV-1 strains, and have no effect on T cell line adapted HIV-1 strains and are more effective inhibitors of HIV-1 infection than the natural chemokines as they reduce the rate of receptor recycling, thus efficiently sequestering CCR5 inside the cell. These include derivatives truncated of seven, eight, or nine amino acids at the amino-terminus, RANTES (8-68) (Ylisastigui *et al.*, 1998), RANTES (9-68) (Arenzana-Seisdedos *et al.*, 1996), and RANTES (10-68) (Ylisastigui *et al.*, 1998) respectively; Met-RANTES (Proudfoot *et al.*, 1996) which has as an additional methionine at the amino-terminus; and the two chemically modified RANTES produced by total synthesis, amino-oxypentane (AOP)-RANTES (2-68) (Simmons *et al.*, 1997) and amino-nonanoyl (NNY)-RANTES (2-68) (Mosier *et al.*, 1999). Compared to RANTES or Met-RANTES, AOP-RANTES was more potent in blocking infection of peripheral blood mononuclear cells (PBMCs) by primary NSI, M-tropic strains that use CCR5 as a coreceptor (Simmons *et al.*, 1997). AOP-RANTES can also inhibit viral entry via CCR3, although inefficiently compared to its effect on CCR5-mediated entry (Elsner *et al.*, 2000). NNY-RANTES was more effective in preventing HIV-1 infection in the human peripheral blood lymphocyte-SCID mouse model, however, a subset of mice rapidly developed resistant viruses (Mosier *et al.*, 1999).

1.2.6.5.2 SDF-1 analogue

Met-SDF-1 β was developed through the addition of an amino-terminal methionine to SDF-1 β . It inhibits T-tropic HIV-1 replication more efficiently than does wild-type SDF-1 β and has no effect on M-tropic HIV-1 replication. The almost complete inhibition of T-tropic HIV-1 replication caused by Met-SDF-1 β appears to be related to prolonged downregulation of CXCR4 (Yang *et al.*, 1999). In addition, when used in combination with antiretroviral drugs, no toxicity was observed (Rusconi *et al.*, 2000b).

1.2.6.6 Small molecule antagonists

1.2.6.6.1 CXCR4 antagonists

1.2.6.6.1.1 AMD3100

AMD3100 is a member of novel class of low molecular weight compounds called biocyclams.

Biocyclams were first reported in 1992 to be a potent inhibitors of HIV-1 and HIV-2 replication (De Clercq *et al.*, 1992; De Clercq *et al.*, 1994). However, it was only recently that they were shown to act through binding specifically to CXCR4, and so preventing the interaction between the envelope glycoprotein of X4 viruses and CXCR4 (Donzella *et al.*, 1998; Schols *et al.*, 1997). AMD3100 is the most specific and most potent CXCR4 antagonist that has been reported. AMD3100 competes with SDF-1 for binding to CXCR4, blocks the calcium flux and chemotactic responses induced by SDF-1 (Schols *et al.*, 1997), and does not initiate an intracellular signal on its own. It has been recently demonstrated to interact with aspartic acids of CXCR4 extracellular loop 2 (Labrosse *et al.*, 1998). Blanco *et al.* (Blanco *et al.*, 2000) have shown that AMD3100 is able to inhibit both syncytium formation and apoptosis induction when PBMCs or CD4⁺ cell lines were cocultured with chronically infected H9/IIIB cells. It is able to inhibit the replication of X4 HIV-1 strains at nanomolar concentrations and is non-toxic up to 500 μ M concentrations. It is not active against M-tropic strains of HIV-1 (Schols *et al.*, 1997). More recently, Zhang and Moore (Zhang & Moore, 1999) reported that a dual-tropic virus (R5X4) was inhibited, although only partially, by AMD3100. The extracellular domain of CXCR4, specifically the anionic residues in extracellular loop 2, is important for AMD3100 binding (Labrosse *et al.*, 1998). Results from Este *et al.* (Este *et al.*, 1999) indicate that by using AMD3100 to selectively block CXCR4, it is possible to alter the phenotype of already existing more pathogenic X4 viral strains to less pathogenic R5 strains, and to prevent the shift from R5 HIV strains to the X4 strains, and by so doing extend the asymptomatic phase of the infection. However, resistance to AMD3100 has been reported (de Vreese *et al.*, 1996), and the resistant virus is not able to switch coreceptor use to the CCR5 coreceptor (Schols *et al.*, 1998). The oral bioavailability of AMD3100 is low, and thus has to be administered parenterally. Initial phase I clinical trials were completed in 1999 (Hendrix *et al.*, 2000), which then proceeded to Phase II trials. However, significant cardiac side effects were observed, thus leading to the subsequent withdrawal of AMD3100 from further development (Scozzafava *et al.*, 2002).

1.2.6.6.1.2 ALX40-4C

ALX40-4C is a highly cationic oligopeptide containing 9 arginine residues that was originally developed as an inhibitor of the interaction of the HIV Tat protein with its RNA target TAR (Sumner-Smith *et al.*, 1995). Subsequent to the discovery of the HIV coreceptors in 1996 it was

determined that ALX40-4C can inhibit fusion and entry of X4, but not R5 virus strains (O'Brien *et al.*, 1996), and that the true target of ALX40-4C is CXCR4 (Doranz *et al.*, 1997a). ALX40-4C interferes with SDF-1-induced calcium mobilization and prevents binding of the 12G5 monoclonal antibody to CXCR4-expressing cells. ALX40-4C interacts with the negatively charged surface of the first and second extracellular loop of CXCR4, and therefore its mechanism of action is through direct blocking of Env interactions, instead of downregulation of the receptor (Doranz *et al.*, 2001). A recent phase I/II clinical trial showed no significant or consistent reductions in HIV viral load. However, only 12 of the patients enrolled in this study were infected with X4 viral strains (Doranz *et al.*, 2001). ALX40-4C was well tolerated in this study, and therefore these results suggest that CXCR4 antagonists can be used safely. ALX40-4C cannot be considered as absolutely specific for CXCR4 because through its highly cationic character, it also interferes with the entry of some herpes viruses (Sumner-Smith *et al.*, 1995).

1.2.6.6.1.3 T22

T22 ([Tyr5, 12, Lys7]-polyhemusin II), a synthesised 18 amino acid cationic peptide (Nakashima *et al.*, 1992) derived from blood cells of American horseshoe crabs, is a small molecule CXCR4 antagonist (Murakami *et al.*, 1997). T22 specifically inhibits infection by X4 viruses, but not R5 viruses, as well as chemotaxis in response to SDF-1. Recently, viral determinants for T22 susceptibility were mapped to the V3 loop of gp120. T22 binds to the amino-terminus and two of the extracellular loops of CXCR4, and consequently interferes with SDF-1 α binding (Murakami *et al.*, 1999). There is *in vitro* data to suggest the concentrations of T22 that are necessary to obtain significant antiviral activity, only partially inhibit the responses to SDF-1 (Murakami *et al.*, 1997).

1.2.6.6.1.4 T134

T134 is a small (14 amino acid residues) analogue of T22 (18 amino acid peptide), with reduced positive charge, that prevents HIV entry by binding to CXCR4, and has no effect on CCR5. It has highly potent antiviral activity and is considerably less toxic than T22. Moreover, T134 is effective against X4 strains that are resistant to AMD3100 (Arakaki *et al.*, 1999).

1.2.6.6.2 CCR5 antagonists

1.2.6.6.2.1 TAK-779

TAK-779, a quaternary ammonium derivative, is the first small molecule found to target replication of M-tropic R5 HIV-1 strains (Baba *et al.*, 1999). TAK-779 inhibits binding of RANTES to CCR5, and prevents CCR5-mediated calcium mobilization at nanomolar concentrations. It antagonizes CCR2b to a lesser extent than CCR5, but has no effect on CCR1 or CCR3. TAK-779 blocks replication of R5 clinical isolates as well as laboratory strains, but is totally inactive against X4 HIV-1 (Baba *et al.*, 1999). Recently, it was demonstrated that TAK-779 prevents the viral surface glycoprotein gp120 from binding to CCR5, and thus it blocks HIV-1 replication at the membrane fusion stage. The binding site for TAK-779 is positioned near the extracellular surface of CCR5, inside a cavity developed between transmembrane helices 1, 2, 3, and 7 (Dragic *et al.*, 2000). Unlike RANTES, it does not induce internalization of CCR5, nor does it increase entry of X4 viruses.

1.2.6.6.2.2 SCH-C

SCH-(351125) (SCH-C) is a small molecule antagonist of CCR5. It is an oxime-piperidine compound, that unlike TAK-779, has no cross-reactivity with CCR2, and is orally bioavailable (Strizki *et al.*, 2001). Animal pharmacokinetic data indicate that this compound may be used in a once- or twice daily dosing regimen. SCH-C entered phase 1 studies in the US in March 2001. Studies, however, were suspended in April 2001, due partly to prolongation of the QTc interval (signal conduction in the heart) at the highest dose. A second generation CCR5 receptor antagonist SCH-350634, whose antiviral potency is superior to that of SCH-C is in preclinical development (Este, 2002).

As HIV-1 often evolves from using CCR5 to CXCR4 *in vivo*, there are concerns that blocking CCR5 could result in the emergence of CXCR4-using variants, and conceivably accelerate disease progression. So far, selection for HIV-1 variants that were resistant to AD101, a CCR5-specific small molecule inhibitor, caused the emergence of variants that were also resistant to SCH-C. However, these viruses were not able to use CXCR4, but continued to be dependent on CCR5 (Trkola *et al.*, 2002).

1.2.6.7 Synergy between coreceptor based therapies and other antiretroviral agents

As with other classes of antiretroviral therapies, it is unlikely that coreceptor based entry inhibitors will be used as monotherapies due to insufficient potency, as well as because of the risk of developing resistant viruses. Synergy has been observed between AMD3100 and the fusion inhibitor T20 (Tremblay *et al.*, 2000). SCH-C demonstrates synergy with nucleoside reverse transcriptase inhibitors (zidovudine and lamivudine), non-nucleoside reverse transcriptase inhibitors (efavirenz) and protease inhibitors (indinavir), as well as with T20 (Tremblay *et al.*, 2002). In addition, combining AOP-RANTES with Met-SDF-1 β resulted in 95-99% inhibition of infection with R5 and X4 viruses, compared with 32-61% with the drugs individually (Rusconi *et al.*, 2000a).

1.2.7 Expression of CXCR4 and CCR5 on leukocyte subsets

Although both CXCR4 and CCR5 are expressed on peripheral blood leukocytes (Bleul *et al.*, 1997; Loetscher *et al.*, 1994; Wu *et al.*, 1997b), their expression on T cell subsets is relatively reciprocal. CXCR4 is mainly expressed on T cells with a naive, unactivated phenotype (CD26^{low}, CD45RA⁺, CD45RO⁻), while CCR5 is predominantly expressed on T cells with an activated, memory phenotype (CD26^{high}, CD45RA⁻, CD45RO⁺) (Bleul *et al.*, 1997; Ostrowski *et al.*, 1998; Wu *et al.*, 1997b).

1.2.8 Modulation of CXCR4 and CCR5 receptor expression

A number of studies have addressed the role of cytokines on CXCR4 and CCR5 receptor expression. In general, CXCR4 expression on T cells is increased by IL-2 (Bleul *et al.*, 1997; Jourdan *et al.*, 2000), IL-4 (Jinquan *et al.*, 2000; Jourdan *et al.*, 1998; Valentin *et al.*, 1998; Wang *et al.*, 1998a), IL-7, and IL-15 (Jourdan *et al.*, 2000), and decreased by IL-10 (Jinquan *et al.*, 2000), while IL-13 (Bailer *et al.*, 2000) and granulocyte macrophage-colony stimulating factor (GM-CSF) (Di Marzio *et al.*, 1998) decreased CXCR4 expression on macrophages. CCR5 expression is upregulated on T cells by IL-2 (Bleul *et al.*, 1997; Weissman *et al.*, 2000; Zou *et al.*, 1999), and on monocytes by IL-10 (Houle *et al.*, 1999; Sozzani *et al.*, 1998). In contrast, IL-2 was found to decrease CCR5 expression on macrophages (Kutza *et al.*, 1998), and IL-10 to decrease CCR5 expression on T cells (Patterson *et al.*, 1999). IL-13 has recently been found to

downmodulate CCR5 expression on primary macrophages (Bailer *et al.*, 2000). The effect of IFN- γ on the modulation of both CXCR4 (Penton-Rol *et al.*, 1998) (Biolchini *et al.*, 2000; Lee *et al.*, 1999a; Shirazi & Pitha, 1998) and CCR5 (Hariharan *et al.*, 1999; Penton-Rol *et al.*, 1998; Zaitseva *et al.*, 2000; Zella *et al.*, 1998) is controversial.

The use of oral contraceptives has been shown to increase the number of CD4⁺ T cells in the cervical epithelium that express CCR5, but has no influence over the expression of CXCR4 (Prakash *et al.*, 2002). As thalidomide inhibits the synthesis of TNF- α , and has been used successfully to enhance weight gain in patients with HIV-1 and mycobacterial infections (Klausner *et al.*, 1996), its effect on the HIV-1 coreceptors was studied. Oral thalidomide administered to six men reduced the upregulation of CXCR4 and CCR5 induced by *ex vivo* simulation of blood with LPS, staphylococcal or mycobacterial antigens (Juffermans *et al.*, 2000b).

Concurrent infections in patients infected with HIV-1 may alter the susceptibility of target cells to HIV-1 infection, and could thus potentially accelerate the pathogenesis of the disease. Consequently, the effect of bacterial and viral infections on the expression of CXCR4 and CCR5 has been addressed in a number of studies. *Treponema pallidum* (Sellati *et al.*, 2000) as well as *Mycobacterium avium* complex (Wahl *et al.*, 1998) increased CCR5 expression on CD4⁺ monocytes, but had no effect on CXCR4 expression. In addition, one study by Juffermans *et al.* (Juffermans *et al.*, 2000a) found that lipoarabinomannan (LAM) (a cell wall component of *M. tuberculosis*), lipoteichoic acid (LTA) (a cell wall component of *Staphylococcus aureus*) and staphylococcal enterotoxin B (a superantigen produced by *S. aureus*) induced an upregulation of both CXCR4 and CCR5 on CD4⁺ T cells, while another study by Juffermans *et al.* (Juffermans *et al.*, 2002) found that LPS, LAM and LTA induced a reduction in CXCR4 and CCR5 expression on monocytes. In addition, patients with active tuberculosis were shown to have increased expression of CXCR4 and CCR5 on CD4⁺ T cells (Juffermans *et al.*, 2001). Thus, effect of bacterial infections on the expression of CXCR4 and CCR5 is controversial, and may depend in part on cell type and assay conditions. Additionally, influenza has been shown to upregulate CXCR4 cell surface expression on CD4⁺ cells (Puri *et al.*, 2000).

A number of studies have addressed the effect of HIV on the expression of CXCR4 and CCR5, and while the upregulation of CCR5 expression has been consistently demonstrated in HIV-1 infected individuals in comparison with healthy controls (Giovannetti *et al.*, 1999; Nicholson *et al.*, 2001; Ostrowski *et al.*, 1998; Pierdominici *et al.*, 2002), some studies have found that the expression of CXCR4 is upregulated (Pierdominici *et al.*, 2002), while others have found that CXCR4 expression is reduced relative to healthy individuals (Forster *et al.*, 1998; Giovannetti *et al.*, 1999; Nicholson *et al.*, 2001; Ostrowski *et al.*, 1998).

1.2.9 Coreceptors for SIV and HIV-2

HIV-2 and SIV strains have also been shown to utilize chemokine receptors as coreceptors for entry. It was previously thought that all SIV isolates, including both T-tropic and M-tropic strains, could use CCR5, but not CXCR4 for entry (Chen *et al.*, 1997b; Edinger *et al.*, 1997). However, recent studies have demonstrated that diverse SIV isolates can use CXCR4 for viral entry (Owen *et al.*, 2000). Chen *et al.* (Chen *et al.*, 1997b) have also shown that replication can occur efficiently in human peripheral blood mononuclear cells from individuals who are homozygous for the CCR5 Δ 32 deletion, as well as in the CEMx174 cell line which lacks CCR5. STRL33 (Alkhatib *et al.*, 1997b), GPR1, and GPR15 (Farzan *et al.*, 1997), CCR8 (Rucker *et al.*, 1997), ChemR23 (Samson *et al.*, 1998) and APJ (Edinger *et al.*, 1998b) have also been shown to function as coreceptors for a number of SIV strains, however their utilization does not appear to correlate strictly with cell tropism (Deng *et al.*, 1997; Farzan *et al.*, 1997). Zhang *et al.* (Zhang *et al.*, 1998) and Pohlman *et al.* (Pohlmann *et al.*, 1999) found that primary SIV isolates could replicate efficiently in cells expressing STRL33/CXCR6, while HIV-1 isolates could not. This observation underscores the possibility that STRL33/CXCR6 is more likely to be physiologically relevant for SIV strains than for HIV-1 strains.

Several studies have shown that HIV-2 isolates can also use CCR5, CXCR4, or both, as coreceptors for entry (McKnight *et al.*, 1998; Rucker *et al.*, 1997; Sol *et al.*, 1997). Moreover, many HIV-2 isolates are promiscuous in their coreceptor usage, in that they are able to use, apart from CCR5 and CXCR4, one or more of CCR1, CCR2b, CCR3, CCR4 and BOB as coreceptors (Guillon *et al.*, 1998; McKnight *et al.*, 1998; Morner *et al.*, 1999). In addition, a recent report by

Kanbe *et al.* (Kanbe *et al.*, 1999) demonstrated that CXCR5/BLR1 is a novel coreceptor for HIV-2, but not for HIV-1 or SIV.

1.3 Study objectives

The overall aim of this study was to assess *ex vivo* and *in vivo* factors that may modulate CXCR4 and CCR5 expression, on peripheral blood leukocytes, that include technical factors like standing time prior to staining, the anticoagulant used, age, the presence of disease, (HIV-1 or *M.tuberculosis*, co-infection with HIV-1 and *M.tuberculosis*), treatment with HAART, and peripheral levels of cytokines. Two and three-colour flow cytometry was carried out throughout the study, as the facility for four-colour staining only became available towards the end of the study.

The specific objectives of this study were thus:

1. To determine important technical issues such as the stability of CXCR4 and CCR5 expression in whole blood, and to assess whether this would be affected in any way by the choice of anticoagulant.
2. To examine the expression of CXCR4 and CCR5 on various leukocyte subsets from healthy adults, as a baseline from which to compare all further cohorts.
3. To assess CXCR4 and CCR5 expression on cord blood and infant samples and to compare these to adults in order to study age-related changes in receptor expression.
4. To determine whether the presence of HIV-1 infection or pulmonary tuberculosis, occurring either singly or as a co-infection would modulate coreceptor expression.
5. To question whether highly active antiretroviral therapy of short duration administered to HIV-1 infected adults normalizes CXCR4 or CCR5 expression.

6. To evaluate a sensitive flow cytometric assay to quantitate the number of CXCR4 and CCR5 antibodies bound per cell (a measure of receptor density).

7. To correlate the expression of CXCR4 and CCR5 with the plasma levels of their ligands, SDF-1 α and MIP-1 α , MIP-1 β and RANTES, respectively. IL-7 was included for comparison as it shares certain functions with SDF-1 α .

CHAPTER TWO

***EX VIVO* MODULATION OF CXCR4 AND CCR5 EXPRESSION IN WHOLE BLOOD**

2.1 Introduction

Flow cytometric analysis of the chemokine receptors, CXCR4 and CCR5, is a valuable tool particularly in HIV-1 disease, and has the potential for future use for predictive purposes, and for monitoring the success of various therapies.

As it is often very difficult to obtain specimens on the day that they were collected, it was considered important to determine whether CXCR4 and CCR5 were stable markers and could be analysed up to 24h after collection. In this study, the expression of CXCR4 and CCR5 was investigated on whole blood samples taken from healthy individuals, and from individuals infected with HIV-1 in order to identify whether there are any changes in receptor expression due to time. In addition, results obtained from blood collected into EDTA, heparin and acid citrate dextrose (ACD) vacutainers were compared.

2.2 Materials and methods

2.2.1 Study participants

Cross-sectional analysis was performed on whole blood of a total of 20 HIV-1 seropositive individuals. Of these samples, 11 were stained within 6h of venesection (time 0) (t0) and 9 were stained 24h after sample collection (overnight) (ON). All samples were left at room temperature prior to staining. None of these patients were receiving antiretroviral therapy. The characteristics of the study subjects are presented in Table. 2.1. The groups were matched with respect to age, sex, race, CD4⁺ T cell count and viral load. Blood was collected by venipuncture into Vacutainer tubes containing EDTA (Becton Dickinson, San Jose, CA).

Longitudinal analysis was performed on whole blood samples obtained from 6 healthy individuals and from 7 HIV-1 infected individuals. These samples were stained within 6h (t0) after blood was drawn, and again after being left at room temperature ON.

In addition, samples from 2 healthy individuals were collected in heparin, EDTA and ACD (Becton Dickinson, San Jose, CA) vacutainer tubes. Samples were stained immediately (t0), 3h (t1), 6h (t2) and 24h (t3) after sample collection.

Table 2.1 Immunological status of individuals included in the cross-sectional analysis*

	Study group	
	t0	ON
No. of subjects	11	9
Age (years)	38 (25-56)	32.5 (30-39)
Sex (male/female)	6/5	6/3
Race (black/white)	6/5	6/3
CD4 ⁺ T cell count (x10 ³ /μl)	454 (57-657)	326 (21-709)
Viral load (log HIV-1 RNA copies/ml)	4.7 (2.9-5.2)	4.2 (2.4-5.4)

*Results are expressed as medians, with the range in parentheses. Study groups: t0, samples stained within 6h; ON, samples stained 24h after collection.

2.2.2 Monoclonal antibodies

Using two- and three-colour staining, different subsets of mononuclear cells expressing either CXCR4 or CCR5 (CXCR4-phycoerythrin (PE) and CCR5-PE, Pharmingen, San Diego, Calif.) were identified with markers that distinguish T cells (CD3-peridinin chlorophyll protein (PerCP), CD4⁺ and CD8⁺ lymphocytes (CD4-PerCP, CD8-PerCP, CD3-fluorescein isothiocyanate (FITC)), B cells (CD19-FITC Becton Dickinson, San Jose, CA), monocytes (CD14-FITC, Coulter, Hialeah, FL), and CD16⁺CD56⁺CD3⁻ NK cells (CD16-FITC, Becton Dickinson and CD56-FITC, Serotec). In order to control for nonspecific staining, quadrants were set using isotype-matched controls (IgG1-PerCP, IgG2a-PE, Becton Dickinson and IgG1-FITC (DAKO)

and IgG2a-FITC (Serotec) (Appendix B Panel 1).

2.2.3 Immunofluorescent staining

All samples were stained by adding 5 μ l of each monoclonal antibody (mAb) as a cocktail to 50 μ l of whole blood. Samples were incubated at room temperature for 10 minutes. Erythrocytes were lysed with FACS lysing solution (Becton Dickinson), washed with PBS, and resuspended in 250 μ l of cell fixative: 1.5% (vol/vol) formaldehyde (Merck, Darmstadt, Germany) with 2% (wt/vol) bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.).

2.2.4 Flow cytometry

A FACSort flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser was used for all analyses. Ten thousand cells were acquired per sample, and the data analysed using Cellquest software (Becton Dickinson), and expressed as the percentage of cells expressing the antigens of interest, as well as their mean fluorescence intensity (MFI) values. Calibrite beads (Becton Dickinson) were run on a weekly basis to ensure the stability of the flow cytometer. B cells and NK cells were analysed after gating for lymphocytes based on CD45 staining and forward scatter (FSC) and side scatter (SSC) properties. CD4⁺ and CD8⁺ T cells were identified by initial gating on total CD3⁺ cells and SSC. Monocytes and granulocytes were identified using their FSC and SSC properties, and the presence or absence of CD14 staining, respectively.

2.2.5 Statistical analysis

Data in the cross-sectional study were analysed using the Mann-Whitney U test, and data from the longitudinal study analysed using the Wilcoxon test for matched samples. SPSS^R software (SPSS Inc., Chicago, Illinois) was used for all analyses. Values of $P < 0.05$ were considered to be statistically significant.

2.3 Results

2.3.1 Cross-sectional analysis

As part of a study to investigate the effect that infection with HIV-1 has on CXCR4 and CCR5 receptor expression, EDTA anticoagulated blood samples from a cohort of HIV-1 infected patients (n=9) were stained. These samples, however, had been delayed due to transportation difficulties. To control for possible effects due to standing time, these results were compared with those obtained from a patient cohort (comparable with respect to age, sex, race, CD4⁺ T cell count, and viral load) where samples were stained within 6h (n=11) (Table 2.1). CXCR4 and CCR5 surface expression on leukocyte subsets in whole venous blood from 11 HIV-1 infected individuals stained within 6h, and 9 HIV-1 infected individuals stained after 24h were measured using flow cytometry. When CXCR4 staining of whole blood within 6h (t₀) was compared with staining after overnight (ON) incubation, there were significant increases in CXCR4 expression on all cell subsets, as reflected in both the percentage of fluorescing cells (except for on polymorphonuclear neutrophils) (Figure 2.1A), and in the mean fluorescence intensity (MFI). In contrast, there was a trend towards a decrease in CCR5 expression in the samples that were stained after ON incubation compared with those stained within 6h. However, this was significant only with respect to the fluorescence intensity of the CD3⁺ and CD4⁺ cells (Figure 2.1B).

2.3.2 Longitudinal analysis

In order to confirm these observations, longitudinal analysis was performed on whole blood samples obtained from 6 healthy individuals (normal donor group) and from 7 HIV-1 infected individuals (HIV group). These samples were stained within 6h (t₀) after blood was drawn, and again after being left at room temperature ON. As shown in Figure 2.2A, delaying the sample staining resulted in a significant upregulation of CXCR4 expression on all cell types, as shown in both proportions of cells expressing CXCR4 as in the MFI. Exceptions were the proportions of CXCR4-expressing polymorphonuclear neutrophils and MFI of CXCR4 on NK cells in the HIV group. Conversely, CCR5 expression tended to be reduced within all cell subsets evaluated except for CD14⁺ monocytes (MFI) in the HIV group (Figure 2.2B). However, significance was only attained for intensity of CCR5 fluorescence on CD3⁺, CD4⁺ and CD8⁺ lymphocytes in both

groups; proportions and MFI of CCR5-expressing CD14⁺ monocytes in the normal donor group; and the proportions of CCR5-expressing CD3⁺ lymphocytes in the disease group. Figure 2.3 shows representative dotplots of data (CXCR4 and CCR5 expression on CD3⁺ cells) from a normal donor.

A. CXCR4

B. CCR5

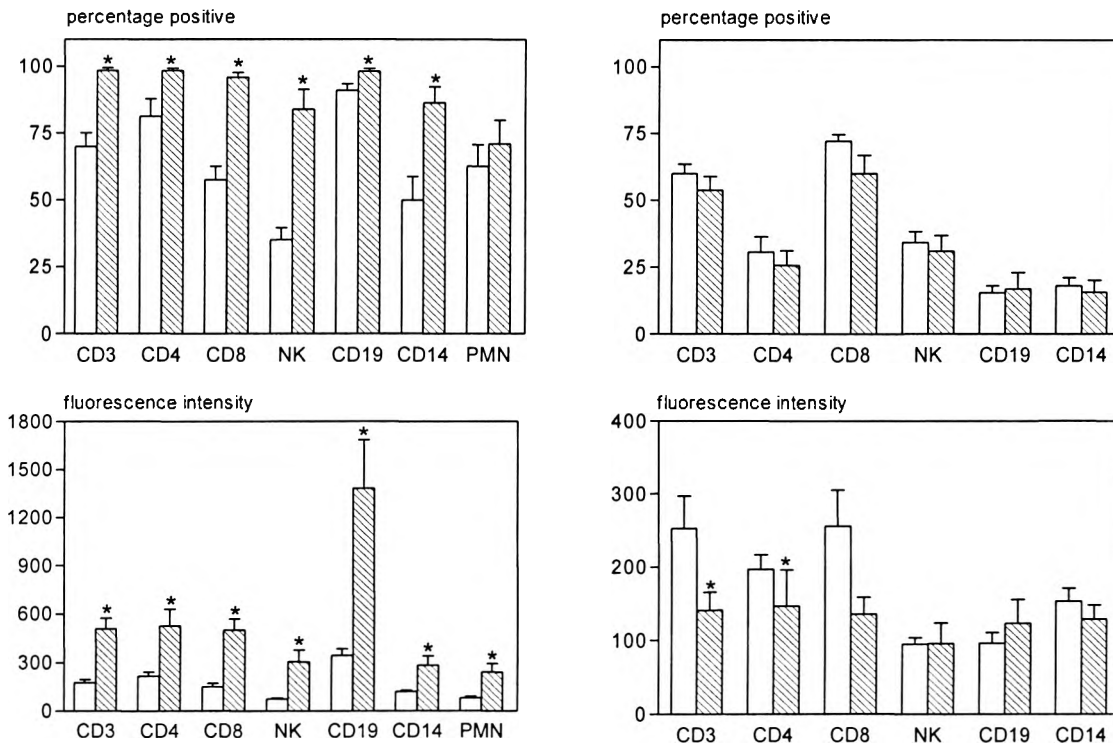
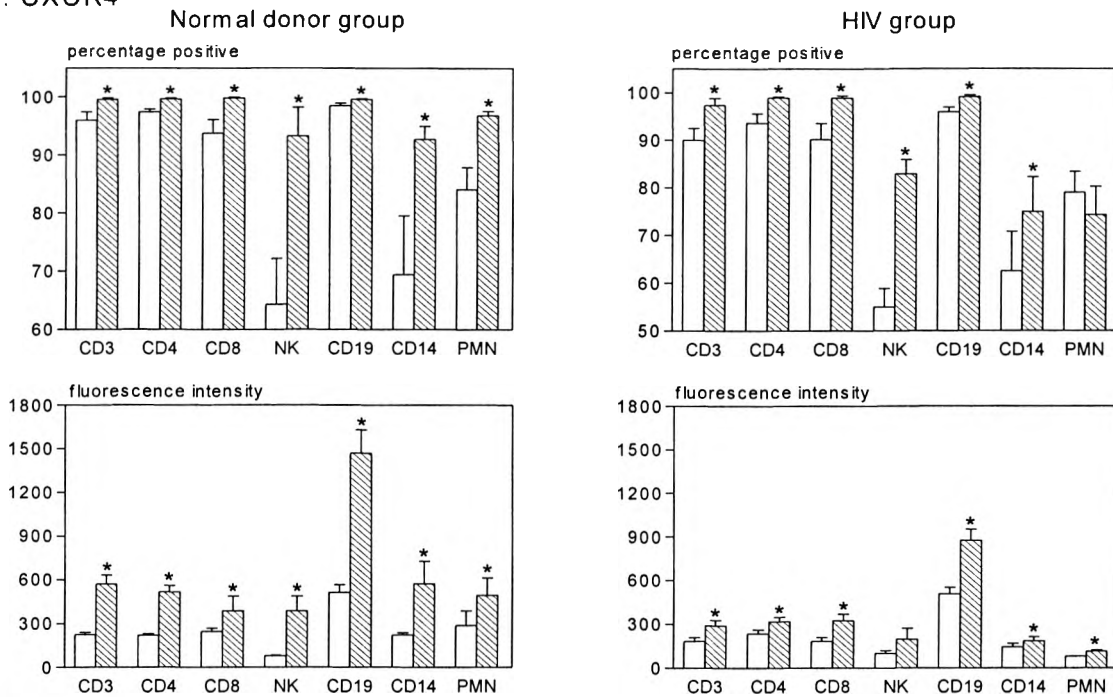


Figure 2.1 Cross-sectional analysis of CXCR4 (A) and CCR5 (B) receptor expression on cellular subsets from HIV-1-infected individuals (HIV group) stained within 6h (t0) (open bars) or left ON (hatched bars). The results are expressed as the percentage of positive cells as well as the MFI (fluorescence intensity). The values represent the mean \pm the standard error of the mean. Significant differences between t0 and ON samples are indicated by *, $P < 0.05$.

These findings raised the question as to whether the use of anticoagulants other than EDTA would give similar results. CD3⁺ cells were used as the representative subset for the comparisons since these cells showed significant alterations in both CXCR4 and CCR5 expression in the cross-sectional as well as the longitudinal analyses. As shown in Figure 2.4A, CXCR4 upregulation occurred over the times indicated with all three anticoagulants evaluated, with the greatest increase in fluorescence intensity occurring after overnight incubation with all three anticoagulants. However, the most dramatic increase was observed with heparin after standing overnight. In contrast to the upregulation of CXCR4 expression, CCR5 expression was

A. CXCR4



B. CCR5

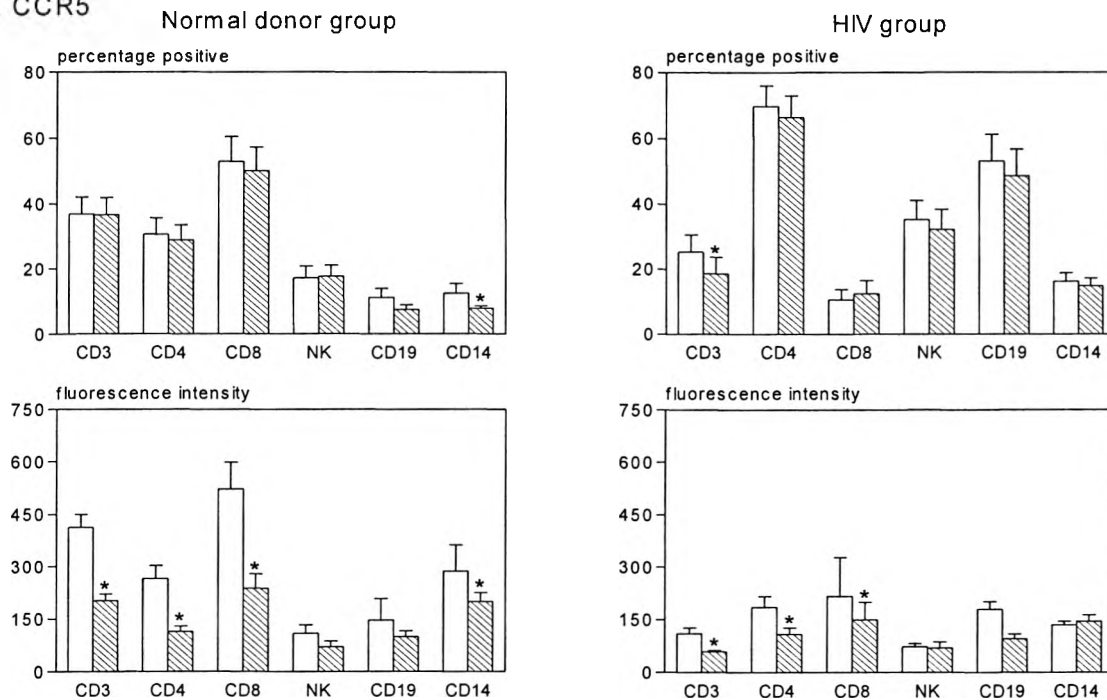


Figure 2.2 Longitudinal analysis of CXCR4 (A) and CCR5 (B) receptor expression on cellular subsets from healthy individuals (normal donor group) and HIV-1 infected individuals (HIV group) stained within 6h (t0) (open bars) or left ON (hatched bars). The results are expressed as the percentage of positive cells as well as the MFI (fluorescence intensity). The values represent the mean \pm the standard error of the mean. Significant differences between t0 and ON samples are indicated by *, $P < 0.05$.

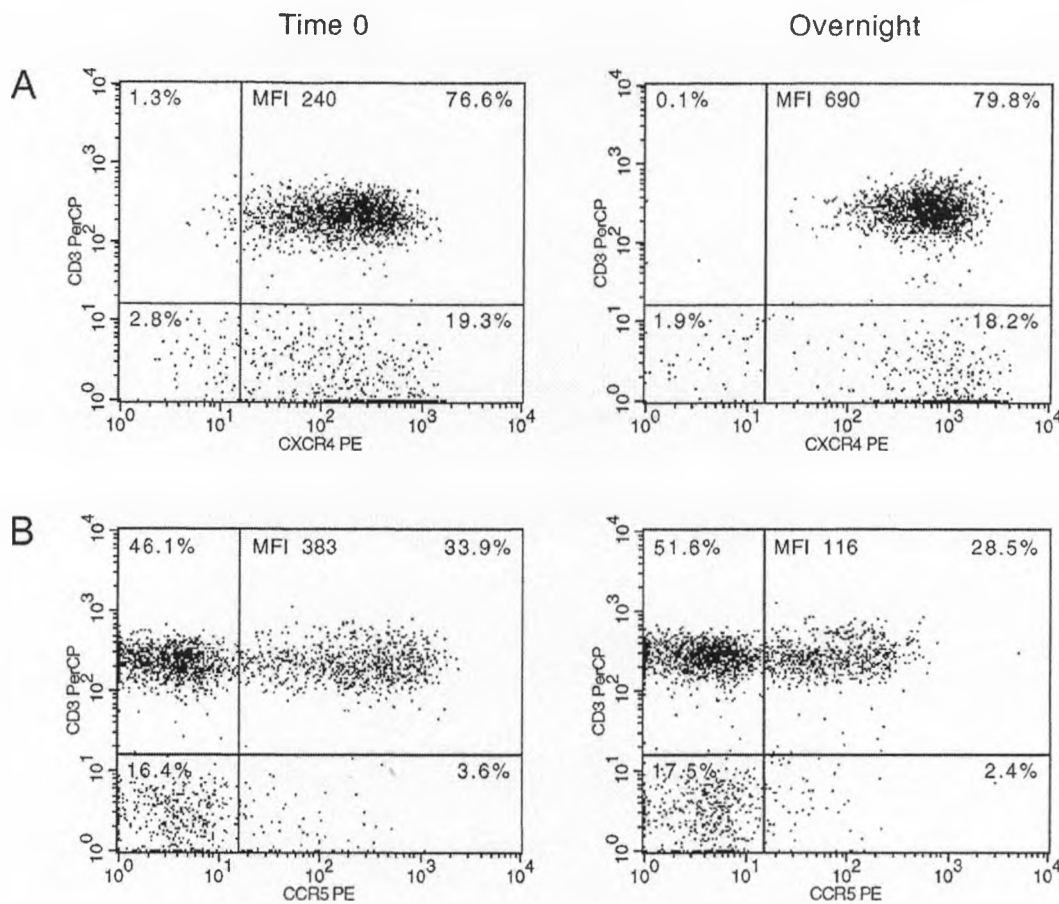


Figure 2.3 Representative dotplots of normal donor CD3⁺ lymphocytes expressing CXCR4 (A) and CCR5 (B) from time zero (left) and ON samples (right). The percentage of cells in each quadrant, as well as the MFI, of the CD3⁺ CXCR4⁺ and CD3⁺ CCR5⁺ cells are indicated.

downregulated with respect to the proportions of CD3⁺ cells expressing CCR5 as well as the MFI of the cells (Figure 2.4B). Interestingly, a greater downregulation in the proportions of CCR5-expressing CD3⁺ cells after ON incubation was observed with ACD in comparison with EDTA and heparin.

2.4 Discussion

Previous experience evaluating the modulation of markers of immune activation (Shalekoff *et al.*, 1998a) demonstrated the need to evaluate receptors under study for their stability particularly with respect to factors such as standing time, and anticoagulant prior to embarking on extended

A. CXCR4

B. CCR5

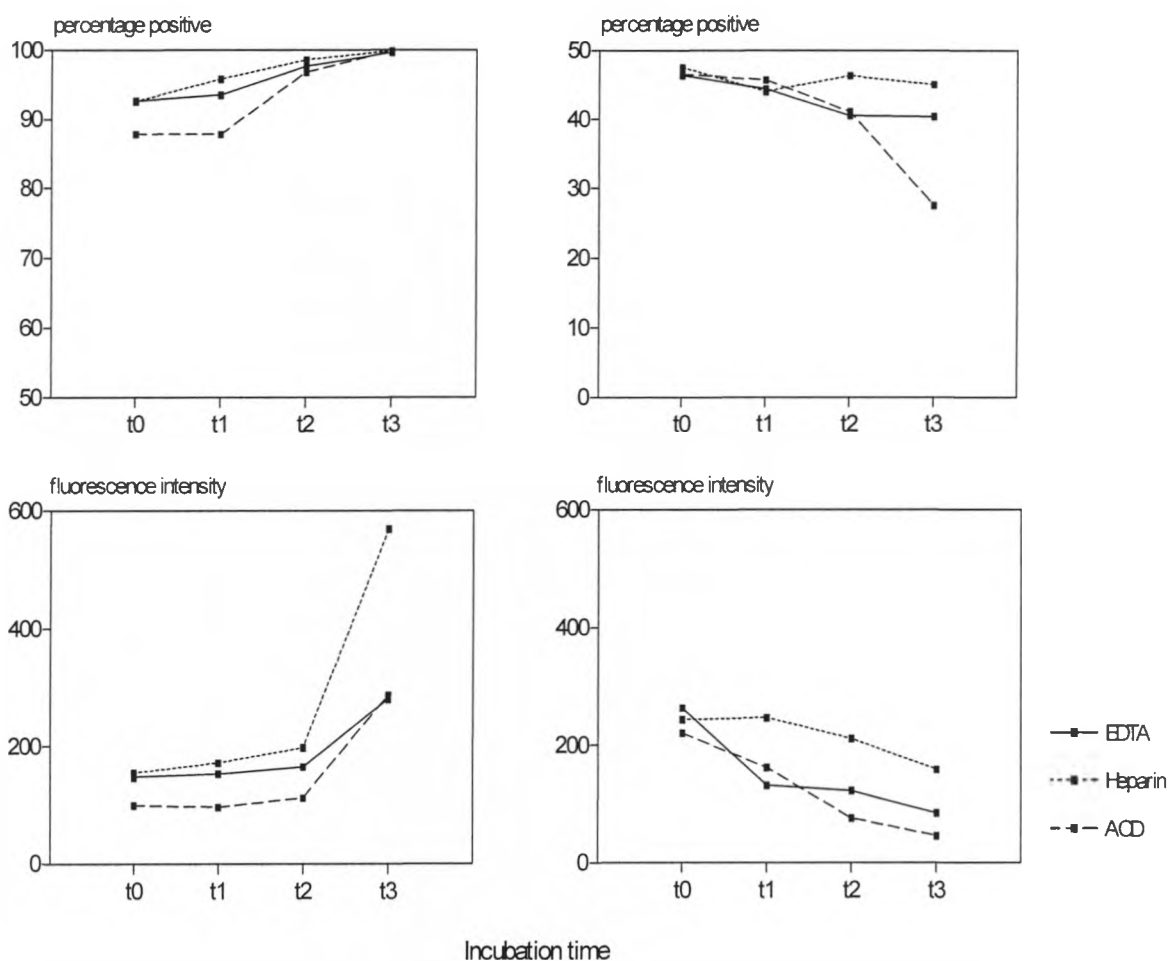


Figure 2.4 Longitudinal analysis of CXCR4 (A) and CCR5 (B) expression on CD3⁺ cells. Whole blood samples anticoagulated with EDTA, heparin, and ACD were stained immediately on being drawn (t0), at 3h (t1), at 6h (t2), and after standing ON (t3). The data are representative of experiments using two healthy donors.

studies. The extent of the modulation of CXCR4 and CCR5 expression in this study with time was surprising. The rapid upregulation of CXCR4 expression may be due to the fact that various leukocytes have been shown to contain large intracellular stores of this receptor (Forster *et al.*, 1998). CXCR4 is localised in endosomal compartments from where it can recycle to the cell surface (Signoret *et al.*, 1997), and CXCR4 has been shown to be increased on the surface of lymphocytes after only a few hours of culture (Bermejo *et al.*, 1998). Studies on the effect of activation of peripheral blood leukocytes on CXCR4 expression have been contradictory, with phytohaemagglutinin being shown by some groups to decrease CXCR4 expression (Bermejo *et al.*, 1998; Forster *et al.*, 1998; Signoret *et al.*, 1997), while others have reported a rapid

upregulation of expression (Bleul *et al.*, 1997).

In summary, our results have clearly demonstrated that CXCR4 and CCR5 expression was reciprocally altered with sample standing time. This has been confirmed in another study (Hultin *et al.*, 1999). What was also apparent was that CXCR4 was more easily modulated, with significant alterations being found for most cell subsets, while CCR5 expression was most significantly altered in the fluorescence intensity of CD3⁺, CD4⁺ and CD8⁺ subsets. Furthermore, CXCR4 and CCR5 were modulated similarly in both the normal donor and HIV cohorts. Moreover, these alterations in receptor expression occurred with all three anticoagulants examined. Since it is often impossible to stain patient samples immediately due to logistical constraints, 6h was used as a cutoff time in which samples should be processed for all further staining in this study. Furthermore, EDTA was selected as the anticoagulant of choice as overall changes in receptor expression were less with standing time. In conclusion, the data presented here demonstrate that, in order to avoid compromising the accuracy of results due to *ex vivo* effects, careful consideration of time of venesection should be taken, particularly in the case of longitudinal samples from the same patient that are to be compared. It should be further emphasised that awareness in this regard would preclude inconsistencies in findings of receptor expression that are likely to occur within and between different laboratories.

CHAPTER THREE

AGE-RELATED CHANGES IN THE EXPRESSION OF CXCR4 AND CCR5 ON PERIPHERAL BLOOD LEUKOCYTES FROM UNINFECTED INFANTS BORN TO HUMAN IMMUNODEFICIENCY VIRUS-1 INFECTED MOTHERS

3.1 Introduction

The immune system of the infant is functionally less mature at birth and undergoes a process of sequential maturation and development that is reflected in qualitative and quantitative changes in a number of leukocyte subsets. Infants have increased numbers of lymphocytes compared with adults, as well as different distributions of the major lymphocyte subsets (Denny *et al.*, 1992; McKinney & Wilfert, 1992; Shahabuddin *et al.*, 1998a; Shahabuddin *et al.*, 1998b). T cells from infants are functionally different from those of adults, they secrete less IL-2, IFN- γ and IL-4 in response to a variety of stimuli, and newborn CD4⁺CD45RA⁺ naive T cell subsets are known to transform more rapidly to CD4⁺CD45RO⁺ memory T cells (Early & Reen, 1999). In addition, studies investigating neonatal T cell-B cell collaboration found both cell types to be functionally deficient (Splawski *et al.*, 1991).

While a number of studies have evaluated the expression of CXCR4 and CCR5 in adult populations, focussing primarily on cells that are permissive for HIV-1 entry, very little is known about the expression of these receptors on peripheral blood leukocytes of infants. As a greater knowledge of the developing immune system of the infant will provide valuable understanding with respect to the pathogenesis of neonatal disease, especially with respect to perinatal HIV-1 infection, it is important to determine the expression of these chemokine receptors on leukocytes of infants, and to assess whether age-related changes in their expression exist, and to question if these could account for differing disease susceptibility and progression found between adults and children. Consequently, the aim of this study was to characterize CXCR4 and CCR5 expression

patterns on peripheral blood leukocytes in children of different ages, and to compare these with values obtained for healthy adults.

3.2 Materials and methods

3.2.1 Study participants

Cross-sectional analysis was performed on a total of 79 subjects, 61 infants and 18 adults. A total of 29 cord blood samples were stained for flow cytometry, and of these 2 were determined to be infected with HIV-1 *in utero* (HIV DNA PCR positive at <48h) and were excluded from all cross-sectional analyses. The remaining 27 infants were confirmed HIV negative at three months by DNA PCR, and were included in the age-related studies. Thirty-four samples were collected from uninfected infants born to HIV-1 infected mothers enrolled within the Petra study (The Petra Study Team, 2002). These 34 infants were stratified into 3 groups based on age: 4.5 months (n=9), 9 months (n=16), 15 months (n=9). All 61 infants were determined HIV-1 seronegative by ELISA (Abbott HIV-1 IMX) performed at 18 months of age. The 18 adult volunteers (mean age 37.8 years; range 26-57) were recruited from among laboratory workers, and they had no known risk factors for HIV-1 infection.

3.2.2 Monoclonal antibodies

Two- and three-colour staining was performed using the following monoclonal antibodies (mAbs): CD45-fluorescein isothiocyanate (FITC)/CD14-phycoerythrin (PE), CD3-peridinin chlorophyll (PerCP), CD4-PerCP, CD8-PerCP, CD19-FITC, CD45RA-FITC, CD16-FITC purchased from Becton Dickinson (San Jose, CA); CXCR4-PE and CCR5-PE obtained from PharMingen (San Diego, CA); CD38-FITC and CD45RO-FITC obtained from DAKO (Glostrup, Denmark); CD56-FITC obtained from Serotec (Oxford, UK); CD14-FITC from Coulter (Hialeah, FL). The appropriate isotypic control antibodies used were IgG1-PerCP, IgG2a-PE (Becton Dickinson); IgG1-FITC (DAKO); IgG2a-FITC (Serotec).

3.2.3 Flow cytometry

EDTA anticoagulated whole blood was stained within 6h of collection, by adding 5 µl of each

mAb as a cocktail, to 50 μ l of blood, and incubated for 10 minutes at room temperature. After labelling, samples were lysed with FACS lysing solution (Becton Dickinson), washed with PBS, and then resuspended in 250 μ l of cell fixative (1.5% (vol/vol) formaldehyde with 2% (wt/vol) BSA). Mononuclear cell subsets expressing either CXCR4 or CCR5 were identified using markers to distinguish T cells (CD3), B cells (CD19), and monocytes (CD14). NK cells were identified as CD16⁺CD56⁺CD3⁻ cells. The naive/memory phenotype of the CD4 and CD8 subsets was studied together with coreceptor expression using the CD45RA (naive) and CD45RO (memory) mAbs. In addition, CD8 cells were separately stained for the CD38 marker of cellular activation. Details of mAb panels are shown in Appendix B Panel 2.

Samples were analysed on a FACSort flow cytometer using Cellquest version 3.1 software. Ten thousand cells were acquired per sample. Lymphocyte and granulocyte populations were identified and gated based on their CD45 staining and their forward (FSC) and side (SSC) scatter properties, and monocytes based on their staining with CD14 antibody, and the data presented as the percentage of cells expressing the particular antigens of interest.

3.2.4 Statistical Analysis

Comparison of CXCR4 and CCR5 expression on different leukocyte subsets between the different study groups was done by use of the Mann-Whitney U test. The Spearman correlation coefficient was calculated to analyse correlations between age and CXCR4 and CCR5 expression.

3.3 Results

3.3.1 Percentages of lymphocyte subpopulations

Results for percentages of lymphocyte subsets of HIV-1 exposed uninfected infants and uninfected adults are shown in Table 3.1. To investigate changes related to age, cord blood was compared to 4.5 month, 4.5 month to 9 month, 9 month to 15 month, and 15 month to adult. There was no significant change in the percentage of CD3⁺ lymphocytes from cord blood through to 15 months, however there was then a significant increase in these cells in adults ($P < 0.01$). When the T cells were divided into CD4 and CD8 subsets, no significant changes were observed

within the CD4⁺ cell subset, while there was a significant reduction in the CD8 subset from cord blood to 4.5 months ($P<0.01$), and an increase in the adult group when compared to the 15 month group ($P<0.01$). The CD4/CD8 ratio increased from cord blood to 4.5 month and then decreased, with a significantly lower ratio in the adult group when compared to the 15 month group ($P<0.05$). The percentage of NK cells showed a decline from 14% in cord blood to 8% at 4.5 months ($p=0.05$), followed by a slow increase to a median value of 15% in the adult group. The percentage of CD19⁺ B lymphocytes increased almost twofold during the first 4.5 months of life from a median value of 15% in the cord blood samples to a median of 28% ($P=0.001$), then remained stable throughout the infant samples, followed by a decrease to 11% in the adult group ($P<0.001$).

3.3.2 T cell differentiation and activation

The percentage of CD4⁺ lymphocytes expressing the naive marker, CD45RA, decreased gradually with age from a median of 89% of cord blood CD4⁺ cells, and at 9 months was significantly lower than at 4.5 months (80% vs 85%, respectively) ($P<0.05$), did not change significantly at 15 months, and decreased further to 51% in the adults ($P=0.001$) (Table 3.1). In the CD8 cell subset, 98% of cells in the cord blood group were also CD45RA⁺, and this decreased significantly at 4.5 months ($P<0.001$). Thereafter, there was a trend towards a reduction in CD45RA⁺ cells, but this was not significant. In contrast, the memory marker, CD45RO was expressed on a low number of CD4⁺ (9%) and CD8⁺ (4%) cells and increased significantly from cord blood to 4.5 months ($P=0.001$), 4.5 to 9 months ($P<0.05$) and 15 months to adult ($P<0.001$, CD4 and $P<0.01$, CD8). A median of 95% of cord blood lymphocytes expressed CD38, which decreased significantly to 91% at 4.5 months ($P<0.05$), remained stable, then declined further. This was significant from 9 months to 15 months ($P<0.05$), and from 15 months to adult ($P<0.001$).

3.3.3 Expression of CXCR4 and CCR5 on leukocyte subsets

3.3.3.1 *In utero* HIV-1 infection and CXCR4/CCR5 expression

As staining for CXCR4 and CCR5 expression must be done within six hours of sample collection, all cord blood samples were stained and analysed prior to infection status of the infant being determined. In total, 29 cord blood samples were analysed, and of these two were found to

Table 3.1 Percentages of lymphocyte subsets for study subjects

Cell type or ratio	Median % total lymphocytes (range) for group:				
	cord blood (n=27)	4.5 months (n=9)	9 months (n=16)	15 months (n=9)	adult (n=18)
CD3 ⁺ T cells	62 (48-73)	59 (49-66)	57 (39-71)	61 (59-68)	71* (57-77)
CD4 ⁺ cells	41 (24-59)	41 (28-51)	35 (20-54)	41 (30-48)	40 (33-54)
CD8 ⁺ cells	27 (11-37)	20* (11-29)	20 (9-38)	18 (9-35)	28* (18-39)
CD4/CD8 ratio	1.6 (0.7-5.7)	2.1 (1.1-4.4)	1.9 (0.8-5.6)	2.2 (1.0-5.0)	1.5* (0.9-3.0)
CD16 ⁺ /CD56 ⁺ CD3 ⁻ NKcells	14 (5-36)	8* (3-13)	8 (2-25)	7 (6-15)	15* (4-22)
CD19 ⁺ B cells	15 (6-24)	28* (20-39)	30 (14-48)	25 (17-84)	11* (5-17)
CD45RA ⁺ lymphocytes	89 (80-99)	90 (84-92)	86* (65-93)	84 (83-87)	65* (43-94)
CD45RA ⁺ CD4 cells	89 (55-98)	85 (80-89)	80* (57-89)	79 (72-87)	51* (27-93)
CD45RA ⁺ CD8 cells	98 (92-100)	93* (70-96)	85 (62-98)	83 (66-94)	75 (44-99)
CD45RO ⁺ lymphocytes	8 (4-25)	12* (7-31)	16* (6-32)	16 (11-19)	38* (27-62)
CD45RO ⁺ CD4 cells	9 (4-23)	15* (9-34)	23* (13-45)	21 (12-25)	55* (37-78)
CD45RO ⁺ CD8 cells	4 (2-9)	8* (3-45)	20* (5-55)	21 (11-34)	34* (10-61)
CD38 ⁺ lymphocytes	95 (85-97)	91* (82-95)	91 (73-95)	86* (77-92)	59* (42-77)
CD38 ⁺ CD8 cells	97 (87-100)	95 (86-98)	95 (83-98)	93 (73-97)	48* (22-75)

*Differences were determined by the Mann-Whitney U test and are indicated compared to the previous age group ($P < 0.05$).

have significantly higher expression of CCR5, which was particularly apparent on CD8⁺ cells, when compared with other cord blood samples. Figure 3.1 shows a representative example of CCR5-expressing CD8⁺ cells from a cord blood sample with low CCR5-expressing CD8⁺ cells (1.3%/37.6%=3.5%) (A), and from a cord blood sample with high CCR5-expressing CD8⁺ cells (7.9%/27.1%=29.2%) (B). The median value of CCR5-expressing CD8⁺ cells from the low expressing group was 2.4% (range 0.27%-5.23%), while the percentage of CCR5-expressing CD8⁺ cells from the two samples with high expression was 29.2% and 41.52%. However, no obvious difference in CXCR4 expression were observed on these cord blood samples in comparison with the samples having low levels of CCR5 expression. These two infants were subsequently demonstrated to be HIV DNA PCR positive. For one infant the PCR was performed on a <48h specimen, while the second infant was lost to follow-up and the only specimen available on which to base the diagnosis was the cord blood specimen which was determined HIV DNA PCR positive. Only HIV negative infants were included in all age-related comparisons that follow.

3.3.3.2 Age-related differences in expression of CXCR4 and CCR5 expression

Whole blood samples from the five study groups were analysed for the expression of CXCR4 and CCR5. The proportion of cells expressing CXCR4 and CCR5 on various leukocyte subsets are shown in Figures 3.2 and 3.3, respectively. All cell types expressed both receptors except for granulocytes, which did not express CCR5. In general, greater proportions of cells expressed CXCR4 than CCR5 in all age groups studied. The proportion of cells expressing CXCR4 was highest in cord blood (Figure 3.2). Thereafter, proportions of CXCR4-expressing cells declined at 4.5 months and then increased to adult levels. For example, a median 98.9% of CD3⁺ lymphocytes expressed CXCR4 in cord blood, this decreased to 90.5% in the 4.5 month group, and then gradually increased to 94% at 15 months and to 96.1% in the adult group (Figure 3.2A). Similar patterns were evident in the other cell subsets with the most dramatic reduction from cord blood to 4.5 months occurring in the NK cells and CD14⁺ monocytes (Figure 3.2D and F, respectively).

In contrast to the high expression of CXCR4 on cord blood and the subsequent decrease at 4.5 months, the proportions of cells expressing CCR5 were initially very low and gradually increased

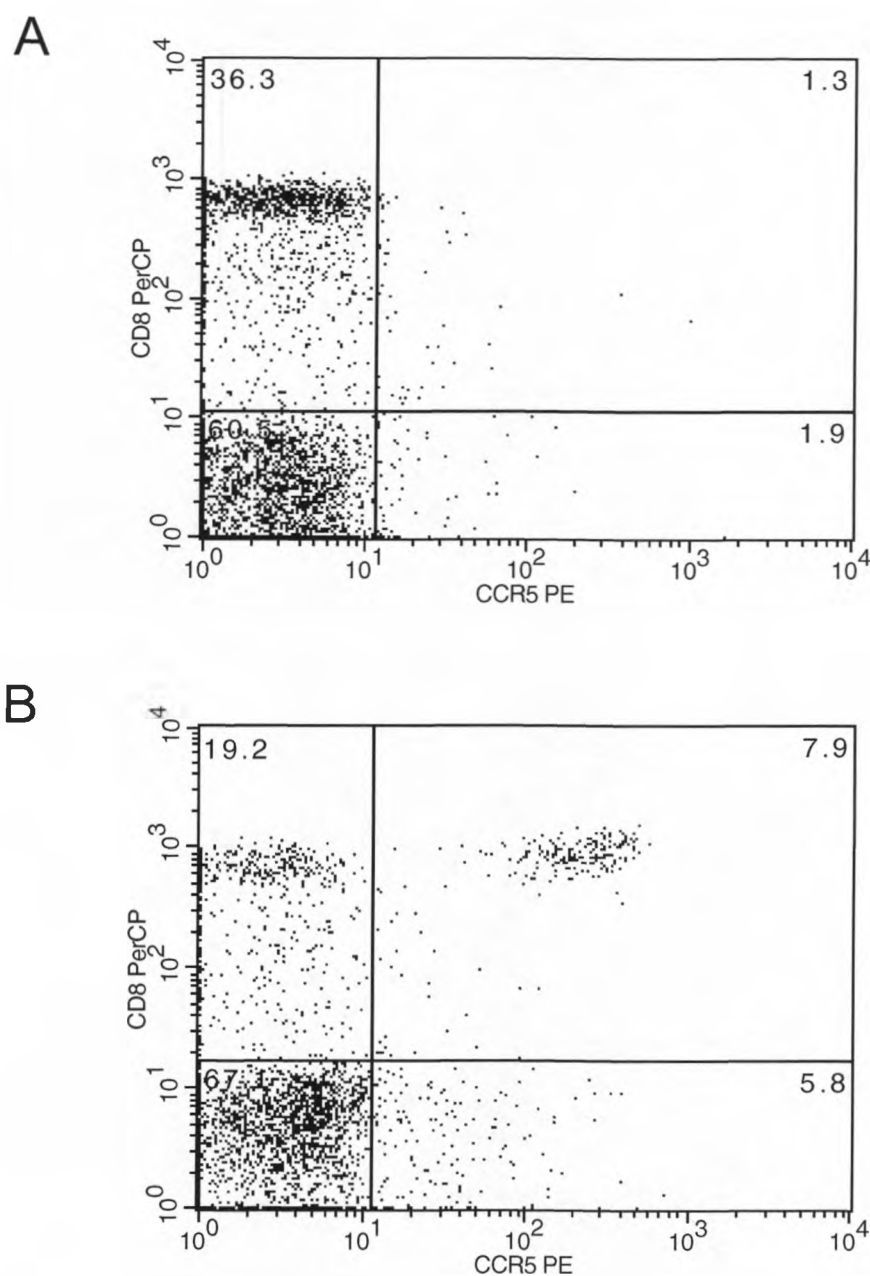


Figure 3.1 CCR5 expression on CD8⁺ lymphocytes. Representative dotplots of cord blood samples from a HIV-1 negative (A) and an intrauterine HIV-1 infected (B) infant. The percentage of cells in each quadrant is indicated.

with age (Figure 3.3). For example, the percentage of CD3⁺ lymphocytes that expressed CCR5 gradually increased from a median of 1.3% in cord blood, to 10% at 4.5 months, to 16% at 9 months, to 17% at 15 months, and to 35.5 % in adults (Figure 3.3A). Similar patterns were observed on the other cell types except for CD19⁺ lymphocytes, where there was no significant age-related increase in CCR5 expression (Figure 3.3E), and the CD14⁺ monocytes where there

was only a significant increase in expression in the 9 month ($P<0.05$) and adult group ($P<0.01$) when compared with the cord blood samples (Figure 3.3F).

3.3.4 CD45RA and CD45RO

T lymphocytes can be divided into functional subsets, the so-called naive and memory subsets, identified by cell surface markers CD45RA and CD45RO, respectively. Although recent work has shown that CD45RA is not able to completely discriminate naive from memory cells (De Rosa *et al.*, 2001), we were only able to perform three-colour analysis, and therefore used CD45RA as a crude marker for naive cells for work in this Chapter, as well as for all further analyses in this thesis.

3.3.4.1 CXCR4 and CCR5 expression on CD45RA and CD45RO subsets

To evaluate whether the age-related changes observed in coreceptor expression, described above, were due to the alterations in CXCR4 and CCR5 expression on CD45RA and/or CD45RO subsets, CD4⁺ and CD8⁺ cells expressing the aforementioned markers were gated and the proportions of cells expressing either CXCR4 or CCR5 was determined. Figure 3.4 shows the proportion of CD4⁺CD45RA⁺ (A) and CD4⁺CD45RO⁺ cells (E) and CD8⁺CD45RA⁺ (B) and CD8⁺CD45RO⁺ cells (F) that also express CXCR4. CXCR4 was expressed almost ubiquitously on CD4⁺CD45RA⁺ cells and to a lesser extent on CD4⁺CD45RO⁺ cells, while higher proportions of CD8⁺CD45RO⁺ cells than CD8⁺CD45RA⁺ expressed CXCR4 in the cord blood, 15 month and adult groups, and at 4.5 and 9 months higher proportions of CD8⁺CD45RA⁺ than CD8⁺CD45RO⁺ cells expressed CXCR4. The decrease in CXCR4 expression on total CD4⁺ and CD8⁺ cells at 4.5 months compared to cord blood (Figure 3.2B and C) was due to a reduction in CXCR4 expression on both naive and memory subsets, with a greater reduction evident on the CD45RO subsets (Figure 3.4E and F). Thereafter, there was a trend towards an increase in CXCR4. In addition, CXCR4 was expressed on a higher proportion of CD4⁺CD45RA⁺ cells, than on CD8⁺CD45RA⁺ (Figure 3.4A and B). This was significant in all groups.

Patterns of CCR5 expression were different from those of CXCR4 expression. CCR5 expression on both CD4⁺ and CD8⁺ cells was strongly restricted to the memory subsets (CD45RO). The

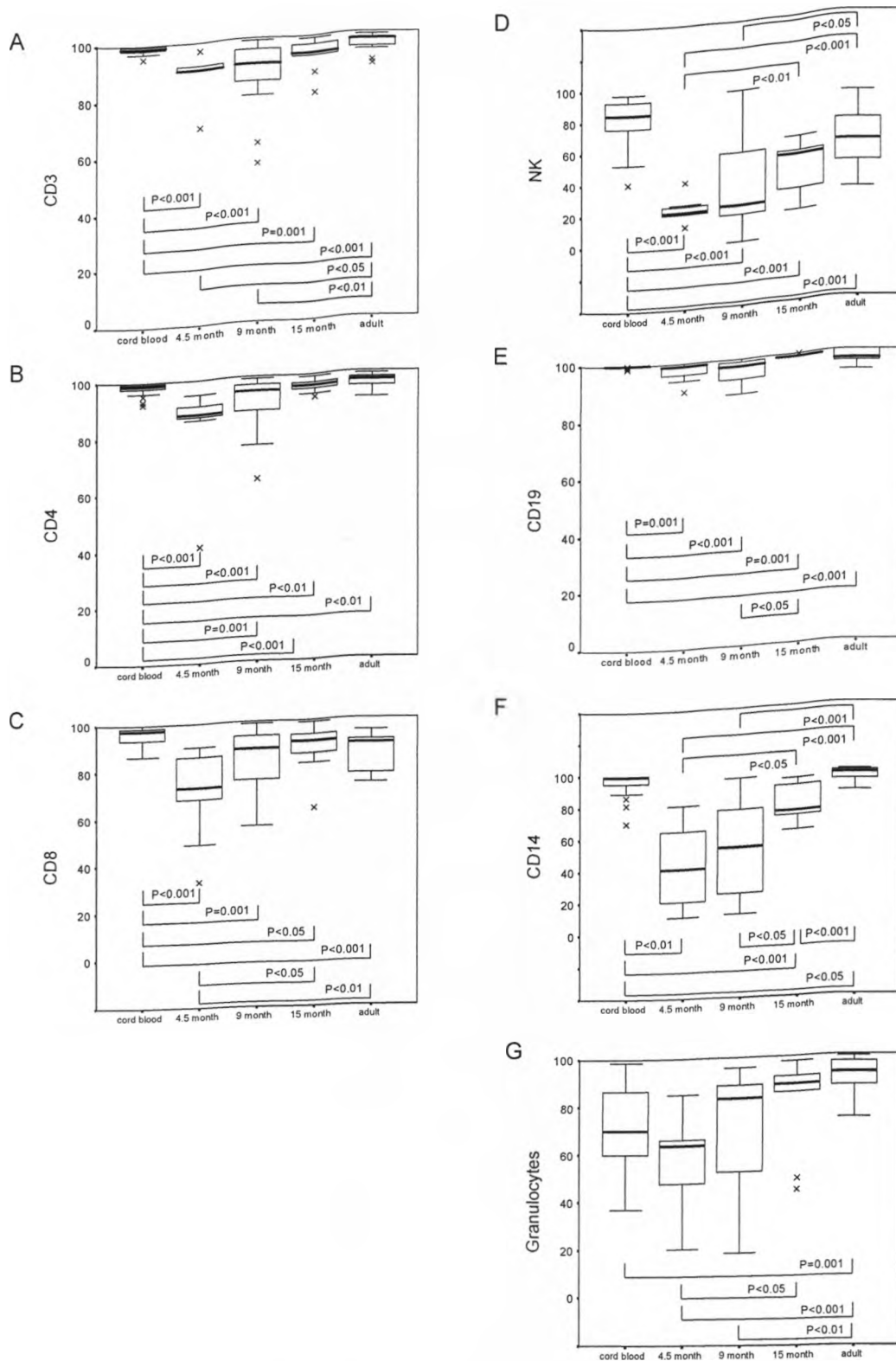


Figure 3.2 Expression of CXCR4 on whole blood leukocytes from subjects within each of the five different age groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), CD14 cells (F) and granulocytes (G). Results are shown as percentages of cells expressing CXCR4. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

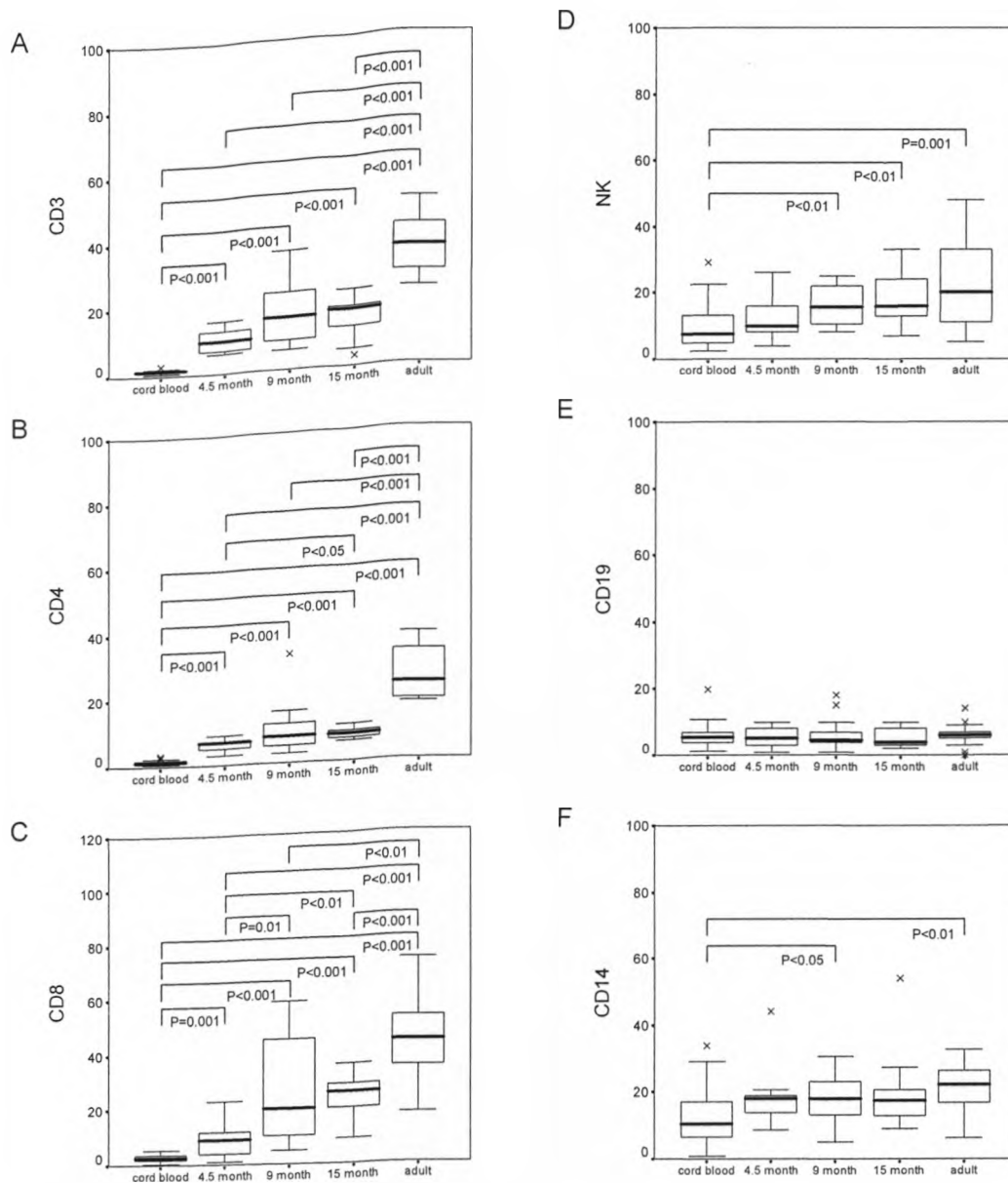


Figure 3.3 Expression of CCR5 on whole blood leukocytes from subjects within each of the five different age groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 cells (F). Results are shown as percentages of cells expressing CCR5. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

age-related increase in the proportions of CD4⁺ and CD8⁺ cells that express CCR5 (Figure 3.3B and C) was due to increased proportions of both naive and memory subsets that express CCR5 (Figure 3.4C, D, G and H). This increase was significant for both CD4⁺CD45RA⁺ and CD4⁺CD45RO⁺ ($P < 0.001$) and CD8⁺CD45RA⁺ ($P < 0.05$) and CD8⁺CD45RO⁺ ($P < 0.001$) cells compared to cord blood. Thereafter, there was a trend towards an increase in CCR5 expression on the memory and naive CD4⁺ cells, and there was a significant increase in CCR5 expression on both naive and memory CD8⁺ cells from 4.5 months to 9 months ($P < 0.05$ and $P = 0.01$, respectively) and on naive CD8⁺ cells from 15 months to adult ($P < 0.001$). Moreover, CCR5 was expressed on a higher proportion of CD8⁺ than CD4⁺ cells in all groups except the cord blood group.

3.3.4.2 Expression of CD45RA and CD45RO on CXCR4- and CCR5-expressing lymphocytes

Because T lymphocytes can be divided into naive and memory subsets which have been shown to have a tendency for particular routes of recirculation *in vivo*, and to demonstrate different chemotactic properties *in vitro*, cells were further examined for the proportions of CXCR4- and CCR5-expressing CD4⁺ and CD8⁺ cells that co-expressed CD45RA or CD45RO. Analysis revealed that CXCR4 expression on lymphocytes from cord blood was associated with a predominantly naive phenotype. Very high proportions of CD4⁺CXCR4⁺ cells (Figure 3.5A) co-expressed CD45RA (90.3%), as did CD8⁺CXCR4⁺ cells (Figure 3.5B) (96.7%). With increasing age there was a reduction in the proportion of CD4⁺CXCR4⁺ cells and CD8⁺CXCR4⁺ co-expressing CD45RA, and an increase in the proportion of these cells expressing CD45RO, such that in the adult group, CD4⁺CXCR4⁺ cells co-expressed essentially equal proportions of CD45RA and CD45RO (56.4% vs 53.5%, respectively). Analysis of the adult CD8⁺CXCR4⁺ lymphocytes, however, reveals that the majority of CD8⁺CXCR4⁺ lymphocytes are also CD45RA⁺ (73.5%). Examination of cord blood CCR5-expressing CD4⁺ lymphocytes revealed similar proportions of cells co-expressing CD45RA and CD45RO (Figure 3.5C and G). However, significantly higher proportions of CCR5-expressing CD8⁺ cells co-expressed CD45RA (Figure 3.5D) than expressed CD45RO (Figure 3.5H). Both CD4⁺ and CD8⁺ cells then demonstrated age-related reductions in CD45RA and increases in CD45RO, and in the adult group much higher proportions of CD4⁺CCR5⁺ cells co-expressed of CD45RO (93%) than CD45RA (13%) with

similar proportions of CD8⁺CCR5⁺ (59.5% vs 61%) cells expressing these markers.

3.3.5 Expression of CD38 on CXCR4- and CCR5-expressing lymphocytes

CD38 is an important immunoregulatory molecule (Cockayne *et al.*, 1998) and is also a marker of the cell maturation phenotype (Reinherz *et al.*, 1980), therefore the levels of its expression on CXCR4- and CCR5-expressing lymphocytes were examined. Figure 3.6 shows a representative example of the proportion of CXCR4- and CCR5-expressing lymphocytes in cord blood, 15 month and adult samples. CD38 expression was high on both CXCR4- and CCR5-expressing lymphocytes in cord blood. CD38 expression on CXCR4-expressing lymphocytes was maintained in the infant groups until 9 months, with median values of 95%, 94% and 94% in the cord blood, 4.5 month and 9 month groups respectively, and then decreased slightly at 15 months (90%) ($P<0.05$), and then declined significantly to adult levels (57%) ($P<0.001$). CD38 expression on CCR5-expressing lymphocytes decreased significantly at 4.5 months (85%) compared to cord blood levels (94%), did not change significantly at 9 months (82.5%), and 15 months (81%), and then decreased substantially in the adult group (39%) ($P<0.001$). In all groups studied (except cord blood) significantly higher proportions of CXCR4⁺ lymphocytes expressed CD38 than did CCR5⁺ lymphocytes ($P<0.05$).

3.3.6 Relationship between CXCR4 and CCR5 expression and age

Whether the expression of either CXCR4 or CCR5 on various leukocyte subsets correlates with age was evaluated. As CXCR4 expression was high on cord blood and then decreased at 4.5 months and then increased again, the cord blood samples were excluded from the analysis. Positive correlations between CXCR4 expression and age were found for the following cell subsets, CD3⁺ cells ($r = 0.457$, $P=0.001$), CD4⁺ cells ($r = 0.519$, $P<0.001$), NK cells ($r = 0.570$, $P<0.001$), CD14⁺ monocytes ($r = 0.760$, $P<0.001$), and granulocytes ($r = 0.621$, $P<0.001$). There was no correlation between age of study subjects and CXCR4 expression on CD8⁺ cells or on CD19⁺ B cells. There was a positive correlation between age and proportions of cells expressing CCR5 on CD3⁺ cells ($r = 0.893$, $P<0.001$), CD4⁺ cells ($r = 0.908$, $P<0.001$), CD8⁺ cells ($r = 0.862$, $P<0.001$), and NK cells ($r = 0.454$, $P<0.001$) and CD14⁺ monocytes ($r = 0.402$, $P<0.001$). There was no correlation between age of study subjects and CCR5 expression on CD19⁺ B cells.

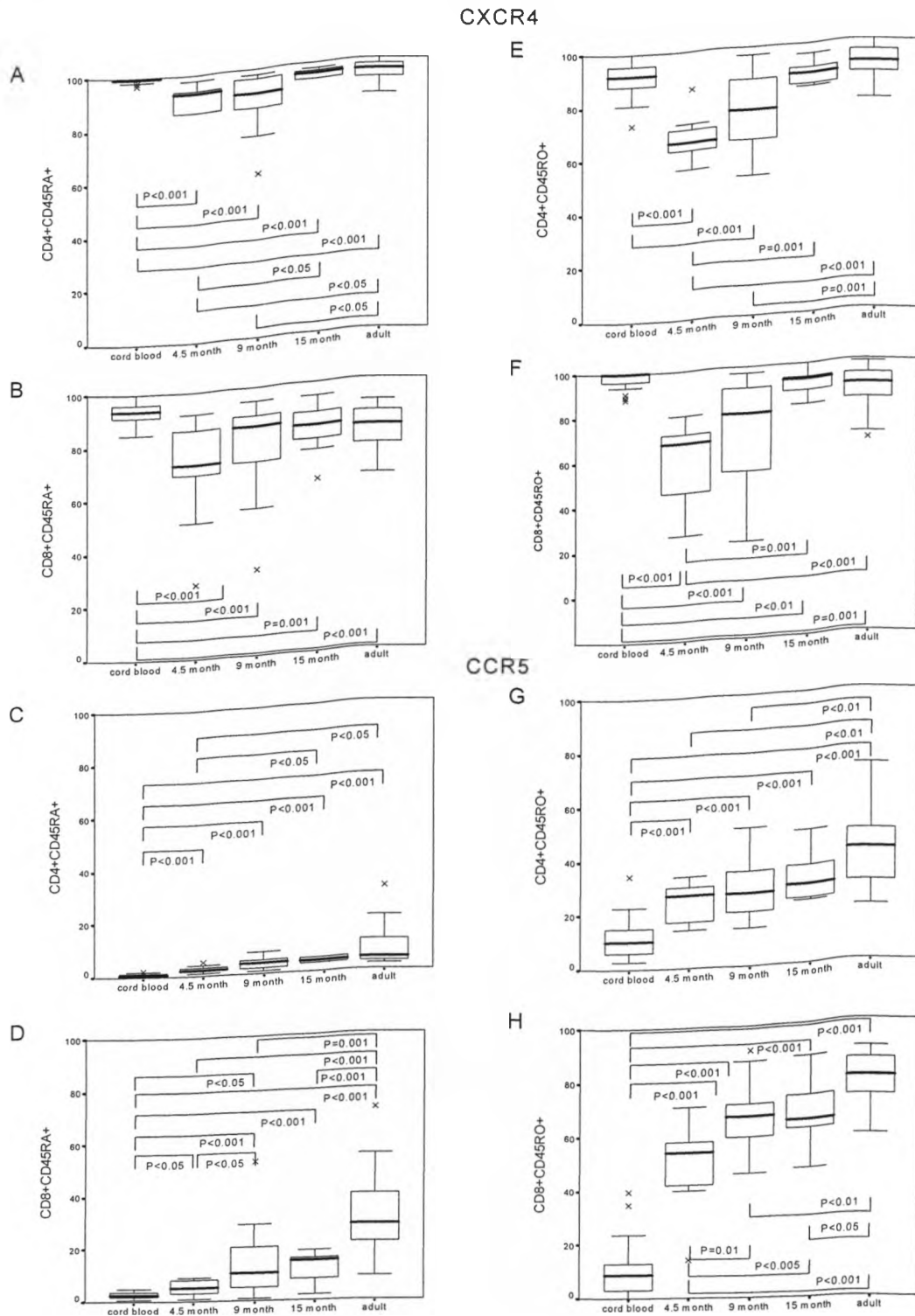


Figure 3.4 Expression of CXCR4 and CCR5 on naive (CD45RA) and memory (CD45RO) CD4⁺ and CD8⁺ lymphocytes from subjects within each of the five different age groups. (A) CXCR4-expressing CD4⁺CD45RA⁺ cells, (B) CXCR4-expressing CD8⁺CD45RA⁺ cells, (C) CCR5-expressing CD4⁺CD45RA⁺ cells, (D) CCR5-expressing CD8⁺CD45RA⁺ cells, (E) CXCR4-expressing CD4⁺CD45RO⁺ cells, (F) CXCR4-expressing CD8⁺CD45RO⁺ cells, (G) CCR5-expressing CD4⁺CD45RO⁺ cells, and (H) CCR5-expressing CD8⁺CD45RO⁺ cells. Results are shown as percentages of cells. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

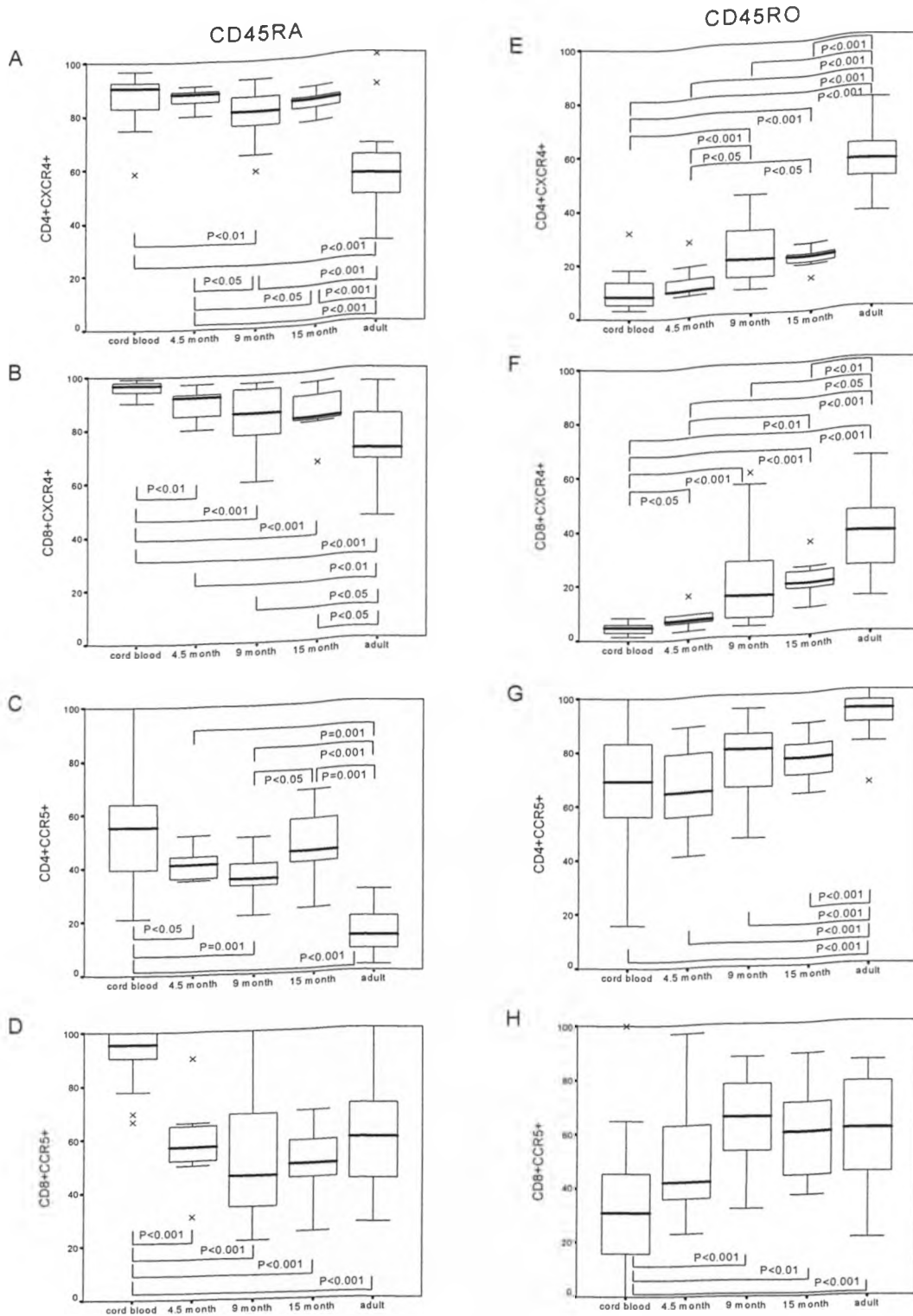


Figure 3.5 Expression of CD45RA and CD45RO on CXCR4- and CCR5-expressing CD4⁺ and CD8⁺ lymphocytes from subjects within each of the five different age groups. (A) CD45RA expressing CD4⁺CXCR4⁺ cells, (B) CD45RA expressing CD8⁺CXCR4⁺ cells, (C) CD45RA expressing CD4⁺CCR5⁺ cells, (D) CD45RA expressing CD8⁺CCR5⁺ cells, (E) CD45RO expressing CD4⁺CXCR4⁺ cells, (F) CD45RO expressing CD8⁺CXCR4⁺ cells, (G) CD45RO expressing CD4⁺CCR5⁺ cells, and (H) CD45RO expressing CD8⁺CCR5⁺ cells. Results are shown as percentages of cells. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

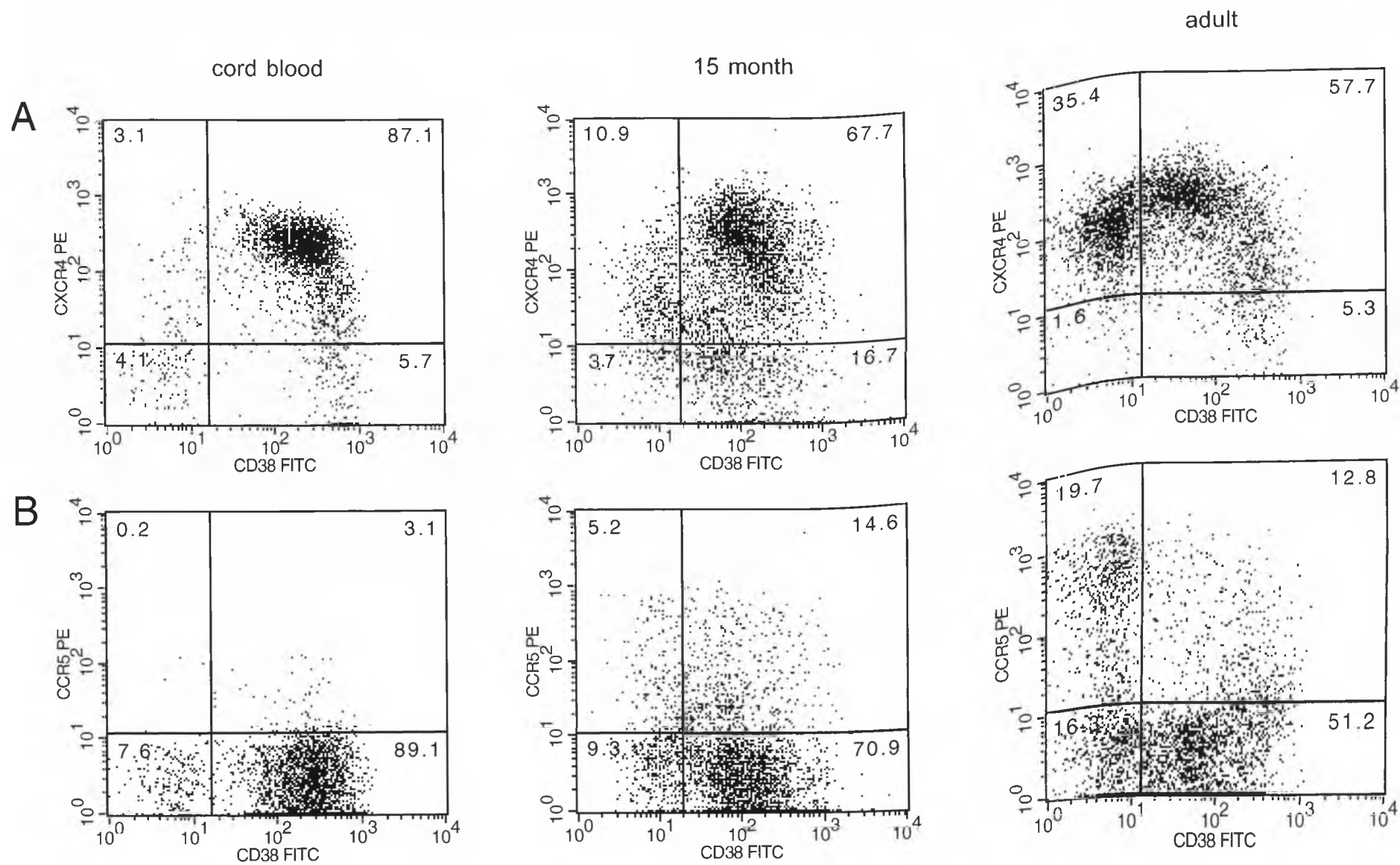


Figure 3.6 CD38 expression on (A) CXCR4- and (B) CCR5-expressing lymphocytes. Whole blood samples were stained with CXCR4 PE or CCR5 PE and CD38 FITC and the results were analysed by gating on the lymphocyte population according to forward and side scatter. Representative dotplots from cord blood (left), 15 month (middle), and adult (right) groups. The percentage of cells in each quadrant is indicated.

3.4 Discussion

To date not much is known about how the extent of cellular expression of the two major HIV-1 coreceptors might impact upon transmission of HIV-1 from mother to infant, or on subsequent disease progression in the child once HIV-1 infection has been acquired. This study has identified significant differences in the expression of CXCR4 and CCR5 on peripheral blood leukocytes in relation to age (analysed in cord blood, 4.5 month, 9 month and 15 month infants, and adults). CXCR4 was highly expressed on cord blood samples, thereafter decreased significantly in all cell subsets, and then increased gradually to adult levels. Although CXCR4 expression was high on both cord blood and adult cell subsets, CXCR4 expression was still significantly higher in cord blood than in adult blood. Unlike CXCR4, CCR5 was expressed at low levels in cord blood and increased gradually to adult levels, in all subsets except for CD19⁺ B cells where cord blood levels were not significantly lower than adult levels. Therefore, the data show that CXCR4 and CCR5 expression in infants is distinctly different from expression in healthy adults. Results confirm those from other studies (Auewarakul *et al.*, 2000; Mo *et al.*, 1998) in that the percentage of CD4⁺ cells that expressed CCR5 was lower in the cord blood than in adult blood (1.4% vs 23%, respectively). Mo *et al.* (Mo *et al.*, 1998) found cord blood monocytes expressed only slightly less CCR5 than did adult cells (62% versus 78%), and although in our study a higher proportion of cord blood monocytes (11%) expressed CCR5 than did cord blood CD4⁺ cells, this was still significantly less than adult monocytes (22%). The reason for these differences may be that isolated PBMC were used for their experiments (Mo *et al.*, 1998), whereas whole blood was used in this study.

Mother-to-child transmission of HIV-1 can occur during the intrauterine period, the intrapartum period, or postnatally through breast-feeding. Early positive HIV cultures or PCR assays (<48h) are indicative of intrauterine infection. The percentage of HLA-DR-expressing CD8⁺ lymphocytes has been postulated to be a possible marker of *in utero* HIV infection (Rich *et al.*, 1997). Uninfected infants, or those with late positive HIV cultures were likely to have 2% of their CD8⁺ cells expressing HLA-DR⁺ lymphocytes, in comparison with 5% CD8⁺ cells expressing HLA-DR⁺ in those infants thought to be infected *in utero*. Here, substantial differences between CCR5-expressing CD8⁺ lymphocytes between uninfected and infected infants (2.4% vs. 29.2% and 41.52%) were observed. Whether this difference would be reduced with increased sample

size remains to be determined. However, it would appear that CCR5 may be a more sensitive marker than HLA-DR. CCR5 is unlikely to be a specific marker of HIV infection, but is more likely to be elevated due to generalised immune activation associated with HIV infection. Additionally, CCR5 has been shown to be elevated in adult HIV-1 infection (Giovannetti *et al.*, 1999; Ostrowski *et al.*, 1998). Furthermore, flow cytometric phenotyping of cord blood CD8⁺ cells is a non-invasive and rapid technique that may assist in the early diagnosis of HIV infection. Thus, these results deserve further study and may contribute to understanding the pathogenesis of vertical HIV transmission.

On evaluation of coreceptor expression on CD4⁺ and CD8⁺ naive and memory cells, it was observed that CXCR4 was expressed on a higher proportion of CD4⁺ naive cells than CD8⁺ naive cells, while CCR5 was expressed on a higher proportion of CD8⁺ naive than CD4⁺ naive cells, and a higher proportion of CD8⁺ memory than CD4⁺ memory cells.

Virus populations in the infected infant have been shown to be more homogeneous than those of their mothers (Jansson *et al.*, 1997; Scarlatti *et al.*, 1993; Wolinsky *et al.*, 1992), which suggests that either a single genotype is transmitted, or is initially replicating in the child. Whether such a restriction in the virus transmitted is in any way related to the coreceptor expression on permissive cells remains largely unknown. However, HIV-1 has been shown to primarily infect CXCR4-expressing cells in placentae from nontransmitting HIV-1 infected mothers, whereas infection of predominantly CCR5-expressing cells was demonstrated in placentae from transmitting women (Behbahani *et al.*, 2000). Macrophage-tropism of mother's HIV-1 isolates have been shown to be associated with an increased risk of transmission of HIV-1 to the infant (De Rossi *et al.*, 1997). Taken together, these data are consistent with transmission of a homogeneous population of NSI isolates that use CCR5 as coreceptor.

Persistent use of the CCR5 receptor has been associated with slow progression of HIV-1 disease (Xiao *et al.*, 1998), while the use of the CXCR4 receptor is associated with rapid progression (Schuitemaker *et al.*, 1992). Perinatally infected children experience more rapid progression to disease than do HIV-1 infected adults (Rogers *et al.*, 1987; Scott *et al.*, 1989), and thus the

question of an association between CXCR4 and CCR5 receptor expression and clinical disease is important. However, infants are more likely to be infected with NSI strains of HIV-1 and progress to symptomatic disease rapidly with no evidence of syncytium-inducing (SI) isolates (Spencer *et al.*, 1994). SI isolates are however found in older HIV-1 infected children (Spencer *et al.*, 1994).

Individuals homozygous for a 32bp deletion in the CCR5 gene (which results in an unstable truncated form of the protein which is not expressed at the cell surface) (Liu *et al.*, 1996a), are highly resistant to infection with HIV-1 (Dean *et al.*, 1996; Huang *et al.*, 1996; Samson *et al.*, 1996b), while individuals heterozygous for the deletion have increased time for progression to AIDS (Dean *et al.*, 1996). Similarly, the homozygous $\Delta 32$ genotype, among children of HIV-1 infected mothers, may confer resistance to infection from mother-to-child transmission of HIV-1 (Mandl *et al.*, 1998; Philpott *et al.*, 1999). It has not been conclusively demonstrated whether CCR5 $\Delta 32$ heterozygous children are protected from infection. A significant reduction in heterozygosity among vertically infected children was found in one study (Mandl *et al.*, 1998), while another showed that transmission to CCR5 $\Delta 32$ heterozygotes only occurred at viral loads greater than 4 log₁₀ copies/ml (Ometto *et al.*, 2000). Conversely, no association between infant CCR5 $\Delta 32$ /wt genotype and reduction in risk of mother-to-child transmission of HIV-1 was found in other studies (Edelstein *et al.*, 1997; Misrahi *et al.*, 1998). However, both adults (Dean *et al.*, 1996; Michael *et al.*, 1997a) and children (Misrahi *et al.*, 1998; Romiti *et al.*, 2000; Sei *et al.*, 2001) heterozygous for the deletion have increased time for progression to AIDS.

That expression of CCR5 is so low on cord blood cells and those of infants, raises the question of susceptibility to infection by CCR5-utilizing strains of HIV-1. It is interesting that CCR5-utilizing HIV-1 strains are very readily transmitted from mother to child (De Rossi *et al.*, 1997), and not restricted to CXCR4-utilizing viruses, as might be thought if receptor expression were a restricting factor. An explanation for this may be that CCR5 can either be upregulated by cellular activation as occurs *in vitro* through the addition of mitogen, and thereby allowing for increased viral entry, or because entry of even small amounts of virus can lead to active virus turnover through some post-entry enhancement of replication promoted through cellular activation. *In vitro* experiments by others have further shown that the threshold of CCR5

expression required for HIV-1 replication in thymocytes is very low (Pedroza-Martins *et al.*, 1998). Although the percentage of CD14⁺ monocytes expressing CCR5 was lower in cord blood than in adult blood, cord blood has a greater absolute number of monocytes than does adult blood (De Paoli *et al.*, 1988; Denny *et al.*, 1992). Peripheral monocytes could harbour HIV-1 that upon later differentiation into macrophages would become activated. Further support favouring the lack of restriction afforded by low CCR5 expression in cord blood cells comes from a study showing unstimulated cord blood mononuclear cells are preferentially infected by M-tropic NSI HIV-1 isolates, whereas adult PBMC are more susceptible to T cell-tropic SI viral strains (Reinhardt *et al.*, 1995).

It must be kept in mind that the uninfected infants studied were born to HIV-1 seropositive mothers, and so are defined as exposed-uninfected infants. T-lymphocyte maturation abnormalities have been detected in uninfected HIV-exposed infants (Clerici *et al.*, 2000b), and so results here must not be seen as necessarily representative of uninfected infants that are not exposed to HIV-1. Prior exposure to HIV-1 may have consequences for cellular maturation and differentiation that may distinguish these two uninfected groups. Delineation of these differences and the possible consequences thereof awaits further studies. The adult group used here was uninfected with no likelihood of HIV-1 exposure, but since differences in receptor expression seen here on the basis of age are profound, it was felt justified to use this adult group as an appropriate control for the purpose of demonstrating age-related differences.

In summary, results have shown that CXCR4 and CCR5 are differentially expressed on infant and adult peripheral leukocytes. These differences are likely to relate to differences in cell functions attributed to these receptors. Data from clinical studies show that low CCR5 expression on infant cells does not restrict the ability of HIV-1 to establish a productive infection *in vitro*, or restrict HIV-1 transmission from mother-to-infant. Likewise, high CXCR4 expression does not associate with preferential transmission of SI viral variants. Therefore, CXCR4 or CCR5 expression *per se* is not predictive of the viral variant that ultimately establishes infection in the child. These findings have implications for cellular trafficking *in vivo* and suggest that capabilities of peripheral cells to move in response to a chemotactic stimulus would be expected to become more efficient with increasing age. A greater understanding of the differences in

disease pathogenesis between infants and adults, and the role of the major HIV-1 coreceptors is important in the design of novel approaches for the prevention of HIV-1 transmission and for attenuation of disease progression in HIV-1 infected individuals.

CHAPTER FOUR

DISTRIBUTION OF THE HUMAN IMMUNODEFICIENCY VIRUS CORECEPTORS CXCR4 AND CCR5 ON LEUKOCYTES OF PERSONS WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION AND PULMONARY TUBERCULOSIS: IMPLICATIONS FOR PATHOGENESIS

4.1 Introduction

Tuberculosis (TB) is a major cause of morbidity and mortality among HIV-1 infected individuals worldwide, with countries in the developing world and especially in sub-Saharan Africa the most affected by the tuberculosis epidemic. Data presented at the 2001 Organization of African Unity Summit on HIV/AIDS, TB and Other Infectious Diseases, in Abuja, Nigeria showed that TB cases are increasing by 10% per year in Africa because of the HIV epidemic. There were nearly two million new cases of TB identified in Africa in 1999, with approximately two-thirds of individuals with TB also infected with HIV-1.

As HIV infection induces a decline in cell-mediated immunity, HIV-1 infected individuals are at an increased risk of developing tuberculosis, either from a reactivation of latent infection (Braun *et al.*, 1991; Selwyn *et al.*, 1989), exogenous reinfection (Small *et al.*, 1993), or a rapid progression of a recent infection (Daley *et al.*, 1992). Tuberculosis usually occurs early in the course of HIV-1 infection, and may be the first opportunistic infection encountered by HIV-1 infected individuals (Theuer *et al.*, 1990). This is presumably because *M. tuberculosis* is more virulent than other HIV-1 associated pathogens, such as *Pneumocystis carinii* and *M. avium complex* (Hopewell, 1989).

Studies have shown that patients with TB have an accelerated course of HIV-1 infection. HIV-1 infected patients with tuberculosis have shorter survival than HIV-1 infected patients without

tuberculosis (Leroy *et al.*, 1997; Whalen *et al.*, 1995; Whalen *et al.*, 1997; Whalen *et al.*, 2000). *In vitro* data has shown that active tuberculosis leads to increased HIV-1 replication. HIV-1 infected mononuclear phagocytes had a threefold increase in p24 production when co-infected with *M. tuberculosis* (Zhang *et al.*, 1995b). In addition, a number of clinical studies have shown that patients with active tuberculosis have an increased HIV-1 viral load compared with HIV-1 infected persons without TB. Goletti *et al.* (Goletti *et al.*, 1996) measured HIV-1 plasma viral load before, during, and after the development of *M. tuberculosis* disease and found increased levels of virus replication during acute TB, which returned to a baseline level after treatment (Goletti *et al.*, 1996). Zhang *et al.* (Zhang *et al.*, 1995b) found a striking increase in p24 levels in bronchoalveolar lavage fluid from sites of *M. tuberculosis* infection compared with sites with no *M. tuberculosis* infection in HIV-1 infected patients.

The distribution and expression of CXCR4 and CCR5 on various immune cells has been addressed in a number of studies (Bleul *et al.*, 1997; Forster *et al.*, 1998; Yi *et al.*, 1998), and it has been suggested that the differential expression of these receptors has important implications for HIV-1 tropism and pathogenesis *in vivo*. Although most of these studies have focussed only on cell types that are permissive for HIV-1, it is important to also consider other cell types that express these receptors and address their role in immune function and pathogenesis, as the normal biological function of chemokines and their receptors is thought to involve regulation of the immune response, particularly migration during inflammatory responses, and immune development. Therefore, the aim of this Chapter was to determine the effect that infection with HIV-1, *M. tuberculosis* and co-infection with both of these organisms has on CXCR4 and CCR5 expression *in vivo* and to question their role in the pathogenesis of these two important diseases.

4.2 Materials and methods

4.2.1 Study participants

Cross-sectional analysis was performed on a total of 54 individuals, comprising 14 healthy normal donors (ND or control group), 12 HIV-1 seropositive patients without active TB (HIV group), 13 patients with pulmonary TB (TB group), and 15 patients co-infected with *M. tuberculosis* and HIV-1 (HIV/TB group). The healthy volunteers were recruited from among

laboratory workers, and they had no known risk factors for HIV-1 infection. None of the patients in the HIV and HIV/TB groups had received any antiretroviral treatment. All but one of the patients with pulmonary TB were on standard anti-TB therapy (rifampin, isoniazid, pyrazinamide, and ethambutol for 2 months, and then excluding ethambutol for the remaining period), one HIV patient was on fluconazole and co-trimoxazole, and another HIV-1 patient on isoniazid and co-trimoxazole, at the time of sample collection.

4.2.2 Monoclonal antibodies

Two- and three-colour staining was performed as described in Section 3.2.2. (Appendix B panel 2).

4.2.3 Flow cytometry

EDTA anticoagulated whole blood was stained within 6h of collection and samples analysed on a FACSort flow cytometer using Cellquest version 3.1 software as in Section 3.2.3.

4.2.4 Viral load quantitation

Plasma HIV-1 RNA levels were determined using the Quantiplex™ HIV RNA bDNA system (Chiron Diagnostics, East Walpole, MA) according to the manufacturer's instructions at the central NHLS laboratory.

4.2.5 Statistical analysis

Comparison of CXCR4 and CCR5 expression on different leukocyte subsets between the different study groups was done by use of the Mann-Whitney U test. The Spearman correlation coefficient was calculated to determine correlations between various immune and viral parameters and expression of either chemokine receptor.

4.3 Results

4.3.1 Immune characteristics of patients

The immunological characteristics of patients within the different study groups are shown in

Table 4.1. Significant differences were observed between the total white blood cell counts (WCC) of the TB group and those of the HIV and HIV/TB groups ($p < 0.05$). The increase in WCC in TB patients is directly due to an increase in absolute polymorphonuclear leukocyte count (Meddows-Taylor *et al.*, 1998; Meddows-Taylor *et al.*, 1999a). Absolute CD4⁺ T cell counts in TB patients were significantly higher than those for either HIV group ($P < 0.05$), and no patients showed a tuberculosis-induced suppression of CD4 counts (< 300 cells/ μ l). CD4⁺ T cell counts did not differ between the HIV and HIV/TB groups ($P > 0.05$), but viral loads were significantly higher in the HIV/TB group ($P = 0.001$). Proportions of CD38-expressing CD8⁺ lymphocytes (CD8⁺CD38⁺) were significantly elevated in the HIV and HIV/TB groups relative to that found in normal donor and TB groups ($P < 0.05$). Although small increases in these proportions of cells were seen in TB compared to ND, and HIV/TB compared to HIV groups, these differences were not significant ($P > 0.05$).

4.3.2 CXCR4 and CCR5 expression on leukocyte subsets

The expression of CXCR4 and CCR5 on various leukocyte subsets was evaluated by staining whole venous blood from individuals in the four study groups with fluorescent-conjugated mAbs that specifically bind either of the two chemokine receptors together with cell-type specific markers. The distribution and proportional representation of both CXCR4 and CCR5 on various leukocyte subsets from healthy blood donors is shown in Table 4.2. Both receptors were expressed on all peripheral blood cell types, except for granulocytes which did not express CCR5. Greater proportions of cells expressed CXCR4 as opposed to CCR5. For example, 97% of CD3⁺ leukocytes expressed CXCR4, whereas only 34.5% of these cells expressed CCR5. Also evident was that CXCR4 was expressed on a greater percentage of CD4⁺ (96.5%) than on CD8⁺ cells (87%), while CCR5 was expressed on a greater percentage of CD8⁺ (44.5%) than CD4⁺ cells (23%).

It was next questioned if the presence of HIV-1 infection, pulmonary tuberculosis or co-infection with HIV-1 and *M. tuberculosis* could modulate the expression of either of the two receptors *in vivo*. Figure 4.1 shows the percentage of cells that express CXCR4 within the different leukocyte subsets defined by specific markers. By comparison with healthy subjects, there was a significant

Table 4.1 Immunological status of individuals within the study groups*

	Study group			
	ND	TB	HIV	HIV/TB
No. of subjects	14	13	12	13
Age (years)	37.5 (27-58)	45 (22-57)	38 (25-56)	33 (19-48)
White blood cell count (x10 ³ /μL)	nd	8.1 (3.6-13.8)	4.7 (2.1-7.7)	6.9 (1.7-10.5)
CD4 ⁺ T cells (x10 ³ /μL)	nd	922 (343-1794)	439 (25-657)	171 (10-906)
(percentage)		44 (21-69)	21 (3-38)	9 (2-22)
CD8 ⁺ T cells (x10 ³ /μL)	nd	628 (77-1621)	959 (235-1535)	1179 (336-4003)
(percentage)		37 (10-48)	51 (33-69)	66 (36-78)
CD4:CD8 ratio	nd	1.2 (0.6-6.5)	0.3 (0.05-0.9)	0.17 (0.03-0.51)
Viral load (log HIV-1 RNA copies/ml)			4.66 (2.95-5.16)	5.45 (4.00-6.47)
CD8 ⁺ CD38 ⁺ cells	48.5 (22-75)	65 (19-97)	79 (60-99)	89 (58-99)

*Results are expressed as medians, with the range in parentheses.

Study groups: ND, normal donors; TB, tuberculosis patients; HIV, HIV-1 infected patients without active tuberculosis; HIV/TB, co-infected patients

nd: not determined.

Percentile P25 and P75 values for healthy adults (as absolute counts and percentages, respectively) are as follows: for leukocytes, 3.6 to 10.0; for CD4 cells, 700 to 1,100 and 38 to 46; for CD8 cells, 500 to 900 and 31 to 40; for CD4:CD8, 1.0 to 1.5.

reduction in CXCR4-expressing leukocytes, on all cell subsets, and in all disease groups evaluated ($P \leq 0.05$). In addition, there was a significant reduction in CXCR4-expressing CD8⁺ ($P < 0.05$) and NK cells ($P = 0.05$) in the TB group when compared to that found for the HIV group. CD14⁺ monocytes and granulocytes from both TB groups had reduced expression of CXCR4 when compared with the HIV-only group. This was significant for both TB groups for the CD14⁺ monocytes ($P \leq 0.001$), and for the TB-only group for the granulocytes ($P < 0.01$).

In contrast to that of CXCR4, CCR5 expression was increased in the TB, HIV, and HIV/TB groups within most cell subsets when compared with the normal donor group (Figure 4.2). Exceptions included the CD14⁺ monocytes where only the HIV group had a significantly increased expression above that of the normal donor group ($P < 0.05$), and the CD4⁺ cell subset which showed no disease-associated modulation of CCR5 expression. Leukocyte subsets that showed a significantly increased expression of CCR5 ($P < 0.05$) relative to that found for the TB-only group were CD3⁺ (HIV/TB group) and CD19⁺ (HIV and HIV/TB groups) cells.

Table 4.2 Proportions of specific leukocytes from 14 healthy blood donors that express CXCR4 and CCR5*

Leukocyte subset	CXCR4	CCR5
CD3	97 (89-99)	34.5 (23-50)
CD4	96.5 (91-99)	23 (17-38)
CD8	87 (73-95)	44.5 (18-74)
NK	55.5 (28-89)	20.5 (5-48)
CD19	97.5 (93-100)	6 (0-14)
CD14	96.1 (86-98)	21.5 (11-33)
Granulocytes	93.2 (74-99)	0

*Results are expressed as medians, with the range in parentheses, of 14 healthy individuals

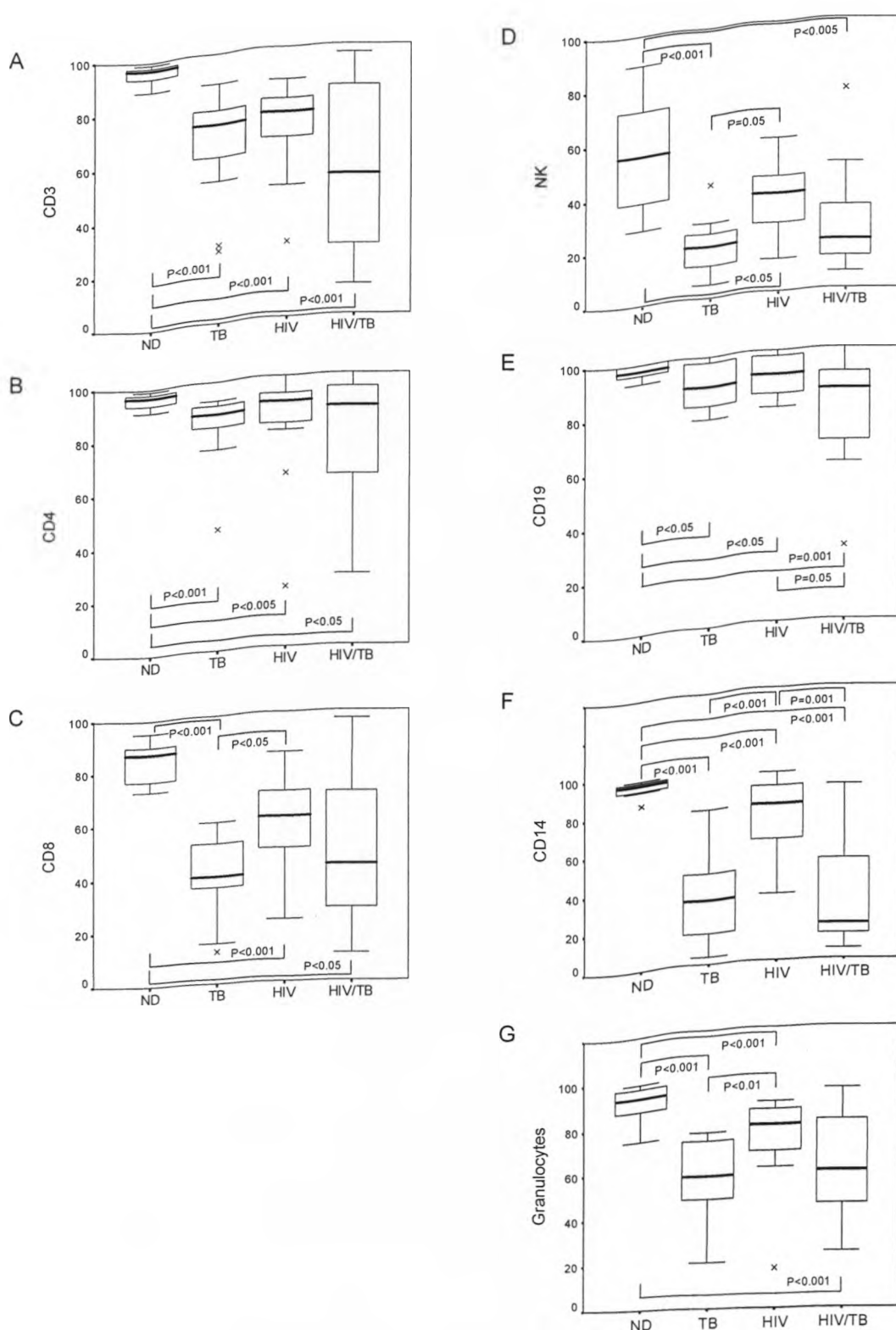


Figure 4.1 Expression of CXCR4 on whole blood leukocytes from subjects in the ND, TB, HIV and HIV/TB study groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells and granulocytes (G). Results are shown as percentages of cells expressing CXCR4. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

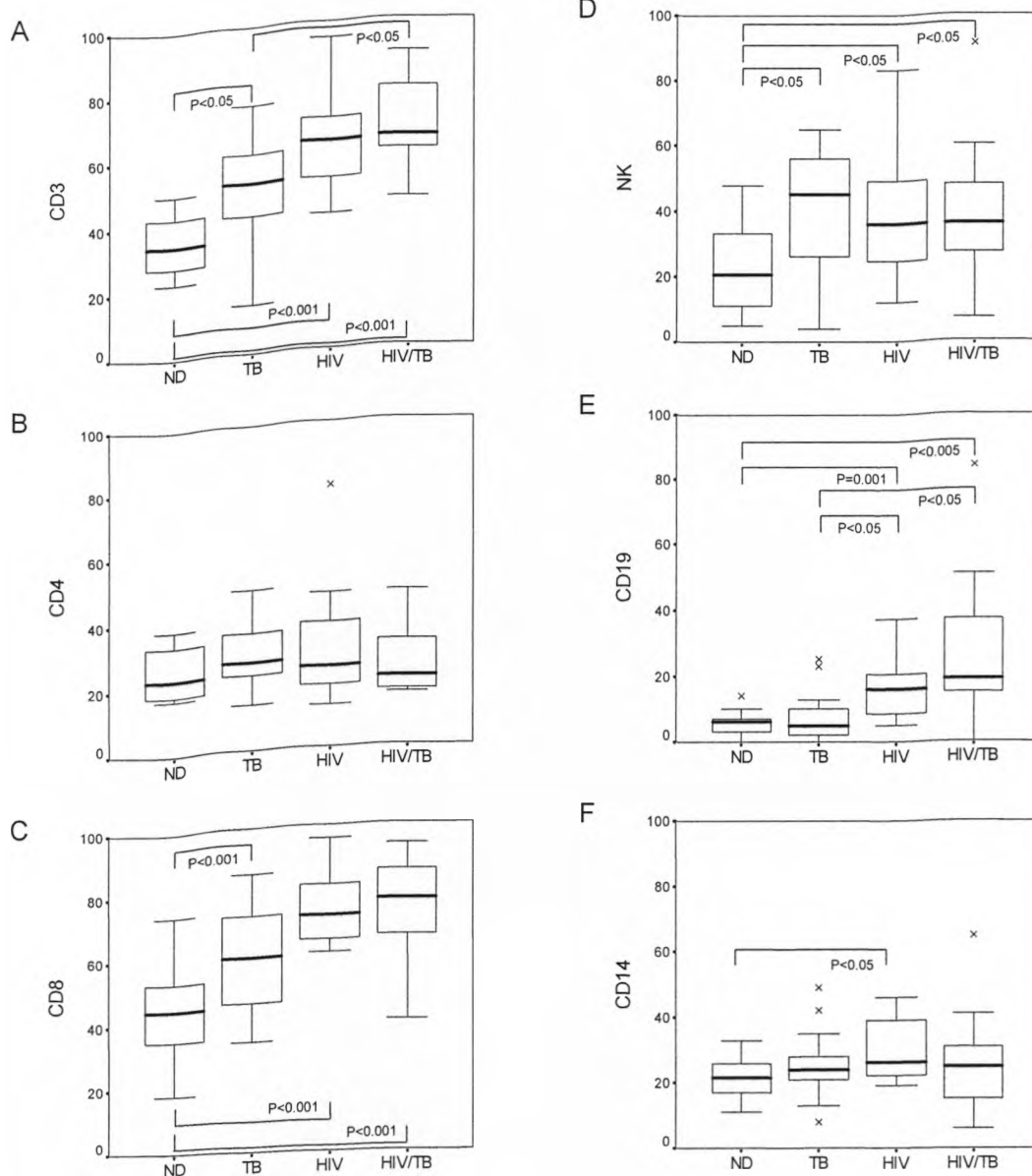


Figure 4.2 Expression of CCR5 on whole blood leukocytes from subjects in the ND, TB, HIV and HIV/TB study groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells. Results are shown as percentages of cells expressing CCR5. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

4.3.3 Expression of CD45RA and CD45RO

The extent of CD45RA and CD45RO expression on cells of the different study groups was analysed in two ways: (i) by gating CD4⁺ and CD8⁺ cells expressing CD45RA or CD45RO and determining the proportions expressing either CXCR4 or CCR5 (Figure 4.3), and (ii) by gating CD4⁺ and CD8⁺ cells expressing either of the chemokine receptors and then determining the proportions of these cells expressing CD45RA or CD45RO (Figure 4.4). As can be seen in Figure 4.1B, there was a slight reduction in the proportions of CD4⁺ cells that co-expressed CXCR4 in all disease groups compared to the control group, with no distinct differences noted between disease groups. However, when the CD4⁺ cell subset was further divided into naive and memory phenotypes, no change from normal controls was observed in CXCR4 expression on CD4⁺CD45RA⁺ cells in any of the groups (Figure 4.3A), whereas a significant reduction in CXCR4 expression was evident within the CD45RO compartment (Figure 4.3E). This latter result very clearly showed the strong TB influence in reducing CXCR4 relative to that which occurs in presence of HIV-1 infection. This effect was again seen in the CD8⁺ cell subset with the effect of TB on the reduction of CXCR4 expression, evident in both naive and memory cell compartments (Figure 4.3B and F). While no differences were observed on the basis of study group in proportions of CD4⁺ cells that expressed CCR5 (Figure 4.2B), there were trends towards an increase in its expression on naive, but a decrease on memory CD4⁺ cells in the HIV/TB group (Figure 4.3C and G). The increase in proportions of CD8⁺ cells expressing CCR5 (Figure 4.2C) was due to an increase in its expression within the CD45RA compartment in the TB group, but was the result of a combination of both increased proportions of naive and memory CD8⁺ cells in the HIV and HIV/TB groups (Figure 4.3D and H).

The second way in which the results were analysed determined what proportions of total CXCR4- and CCR5-expressing CD4⁺ and CD8⁺ cells co-expressed CD45RA or CD45RO. Table 4.3 shows that the distribution of CXCR4 expression on lymphocytes of healthy control persons was associated with a predominantly naive phenotype, with 62.5% of total lymphocytes expressing CD45RA compared with 40% expressing CD45RO. The reciprocal was true for CCR5 expression, with higher proportions of lymphocytes expressing CD45RO (76%) than CD45RA (38.5%). On further analysis, CD4⁺CXCR4⁺ cells had equal proportions of naive (55%) and memory cells (51.5%), as did CD8⁺CCR5⁺ cells. On the other hand, CD4⁺CCR5⁺ cells consisted

of much higher proportions of memory than naive cells, with the opposite true for CD8⁺CXCR4⁺ cells.

The effect of HIV-1 disease and pulmonary tuberculosis on the expression of CD45RA and CD45RO on CXCR4- and CCR5-expressing CD4⁺ and CD8⁺ subsets was then evaluated. In both the HIV and HIV/TB groups a trend existed towards a reduction in the percentage of CXCR4⁺CD4⁺ cells that co-expressed CD45RA, and an increase in the same cells that co-expressed CD45RO, relative to that found in the uninfected control group. The opposite trends were seen in the expression of CD45RA and CD45RO on CCR5-expressing CD4⁺ cells with an increase in CD45RA expression in the HIV/TB group and a decrease in CD45RO expression in the same group (Figure 4.4). In contrast, significant changes in both CD45RA and CD45RO expression on CXCR4 and CCR5-bearing CD8⁺ cells were evident, particularly in the presence of HIV-1 infection (HIV and HIV/TB groups). CD45RA expression was decreased (Figure 4.4B and D) whereas that of CD45RO was increased (Figure 4.4F and H). In the presence of TB however, the tendency was the opposite to that of HIV-1 infection, in that a marginal increase in CD45RA was evident with a concomitant decrease in CD45RO.

4.3.4 Expression of CD38 on CXCR4- and CCR5-expressing lymphocytes

CD38 is widely used as a marker of T and B lymphocyte activation, and so the levels of its expression on CXCR4- and CCR5-expressing lymphocytes were examined. In the normal donor group, significantly higher proportions of CXCR4⁺ lymphocytes expressed CD38 (median 59% range: 40 - 78%) than did CCR5⁺ lymphocytes (median 39.5% range: 22 - 65%) (P<0.001). On examination of CXCR4- and CCR5-expressing lymphocytes, proportions of cells expressing CD38 in both the HIV groups were found to be significantly higher than in the normal donor and TB groups (Figure 4.5A). Moreover, the TB group also showed significantly greater numbers of CD38-expressing CCR5⁺ lymphocytes (but not CXCR4⁺ lymphocytes) than the normal donor group. A representative example showing the results obtained for a healthy donor and for an HIV/TB patient is shown in Figure 4.5B. In the healthy individual (top panel) 58% of CXCR4⁺ lymphocytes were also CD38⁺ (54.6%/93.5%), whereas only 32.5% of CCR5⁺ lymphocytes expressed CD38 (11%/33.8%). However, in the HIV/TB individual (bottom panel), 96% of

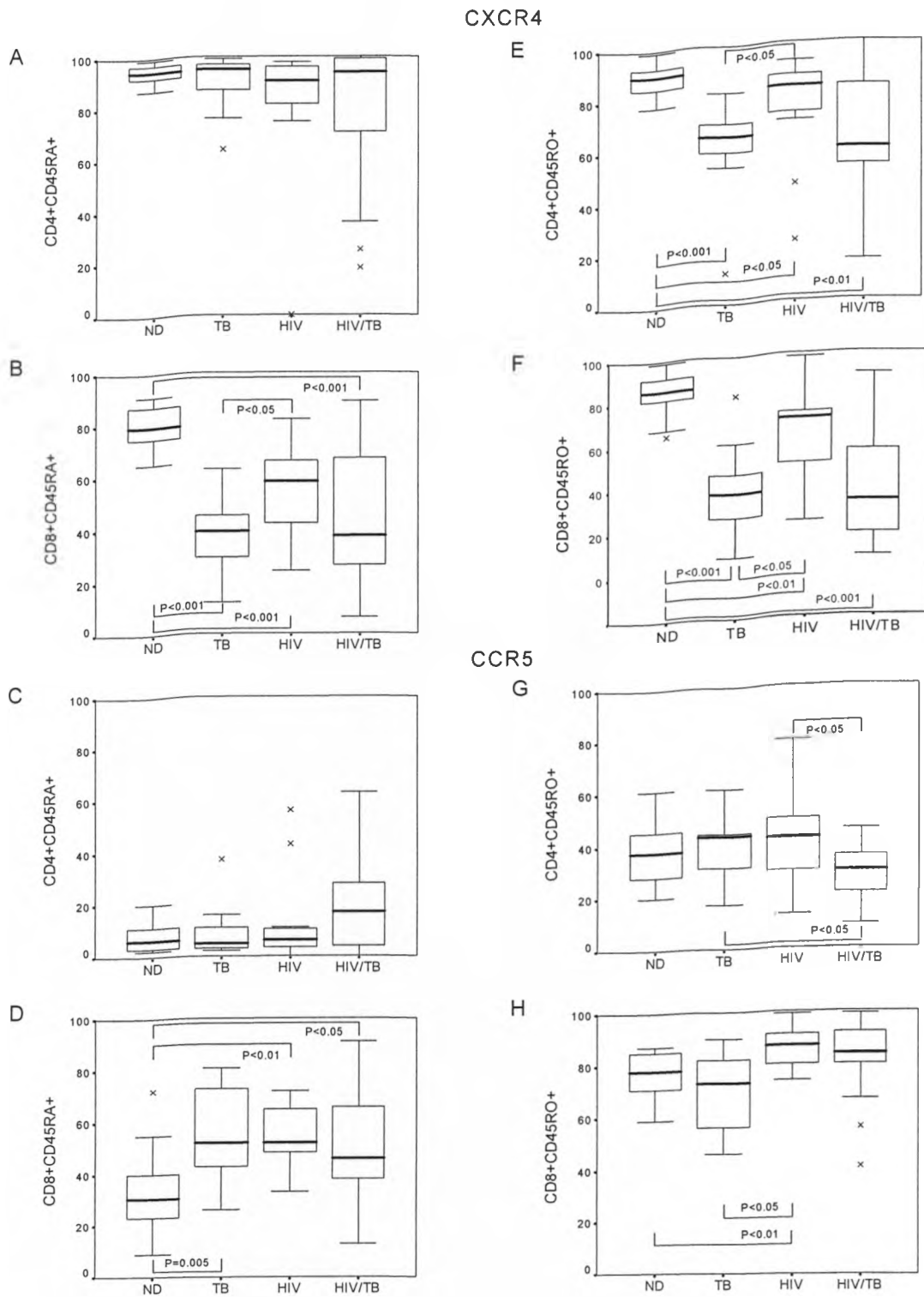


Figure 4.3 Expression of CXCR4 and CCR5 on naive (CD45RA) and memory (CD45RO) CD4⁺ and CD8⁺ lymphocytes. (A) CXCR4-expressing CD4⁺CD45RA⁺ cells, (B) CXCR4-expressing CD8⁺CD45RA⁺ cells, (C) CCR5-expressing CD4⁺CD45RA⁺ cells, (D) CCR5-expressing CD8⁺CD45RA⁺ cells, (E) CXCR4-expressing CD4⁺CD45RO⁺ cells, (F) CXCR4-expressing CD8⁺CD45RO⁺ cells, (G) CCR5-expressing CD4⁺CD45RO⁺ cells, and (H) CCR5-expressing CD8⁺CD45RO⁺ cells. Results are shown as percentages of cells. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

Table 4.3 Proportions of CD45RA and CD45RO on CD4⁺ and CD8⁺ cells that coexpress CXCR4 and CCR5*

Leukocyte subset	CD45RA	CD45RO
Total lymphocytes		
CXCR4 ⁺	62.5 (41-74)	40 (32-51)
CCR5 ⁺	38.5 (20-99)	76 (42-89)
CD4		
CXCR4 ⁺	55 (34-64)	51.5 (35-77)
CCR5 ⁺	15 (6-30)	89.5 (66-100)
CD8		
CXCR4 ⁺	70 (45-94)	37.5 (13-64)
CCR5 ⁺	59.5 (28-100)	61 (21-86)

*Results are expressed as medians, with the range in parentheses, of 14 healthy individuals.

CXCR4⁺ lymphocytes (77.8%/81.4%) and 97% of CCR5⁺ lymphocytes (71.9%/73.8%) co-expressed CD38.

4.3.5 Relationship between CXCR4 and CCR5 expression and HIV-1 stage of disease

To determine whether expression of either CXCR4 or CCR5 on various leukocyte subsets is associated with stage of HIV-1 disease, the expression of CXCR4 and CCR5 on the various cell subsets in relation to the percentage of CD4⁺ T cells was compared. HIV and HIV/TB groups were combined and stratified according to measures of >14% and <14% CD4⁺ T cells. Although there was a trend towards a decrease in CXCR4 expression on all mononuclear cell subsets in the <14% CD4⁺ T cell group when compared with the >14% group, the results were not significant (data not shown). However, there was a significant reduction in the proportion of granulocytes that express CXCR4 in the <14% CD4⁺ T cell group when compared to the >14% CD4⁺ T cell group ($P < 0.05$). The proportions of CD3⁺, CD8⁺ and CD19⁺ cells that co-expressed CCR5, on the other hand, were significantly higher in the patients that had CD4⁺ T cell counts of <14%, with a trend to this effect in CD4⁺ cells (Figure 4.6). Higher proportions of CD4⁺ and CD8⁺

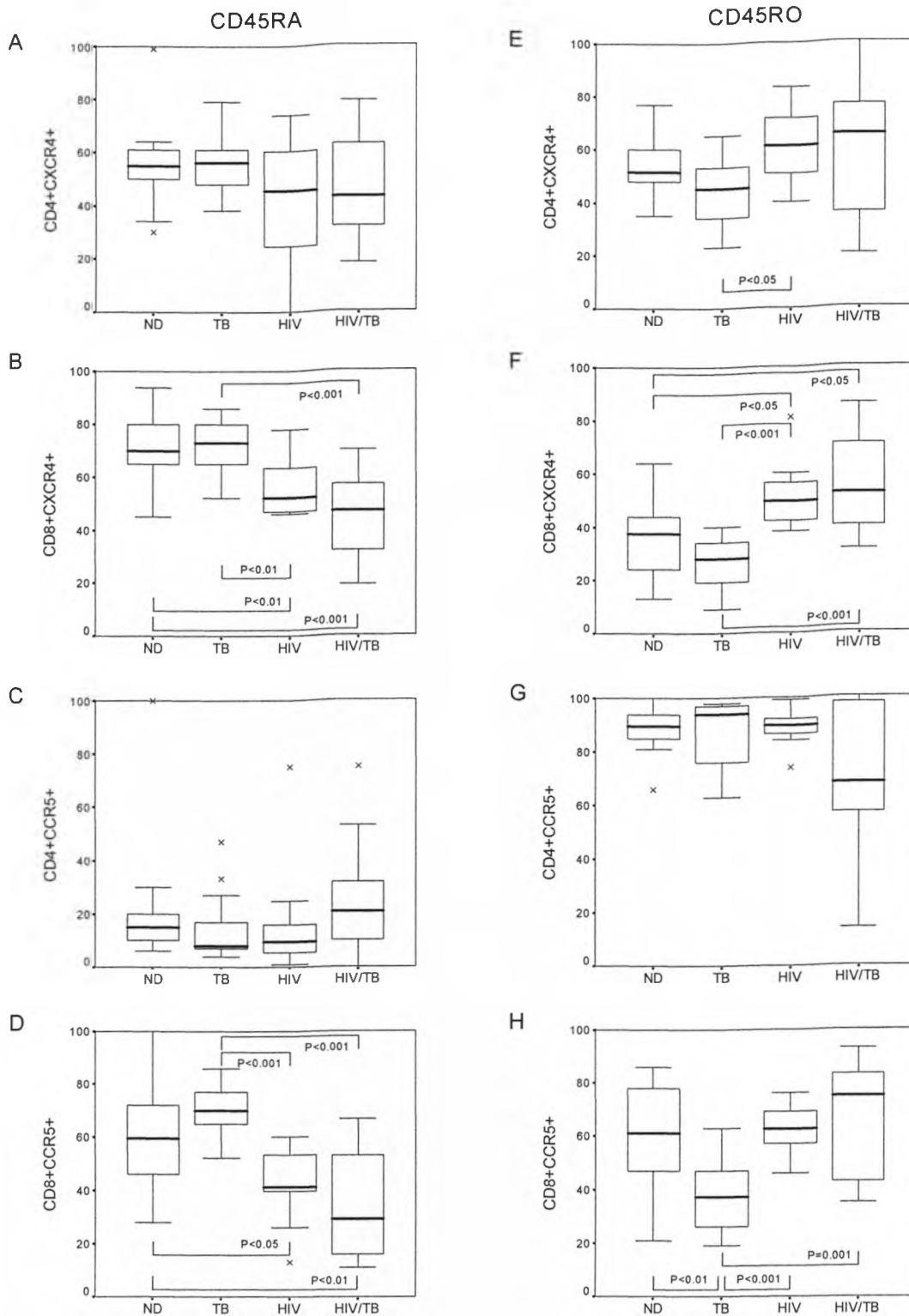


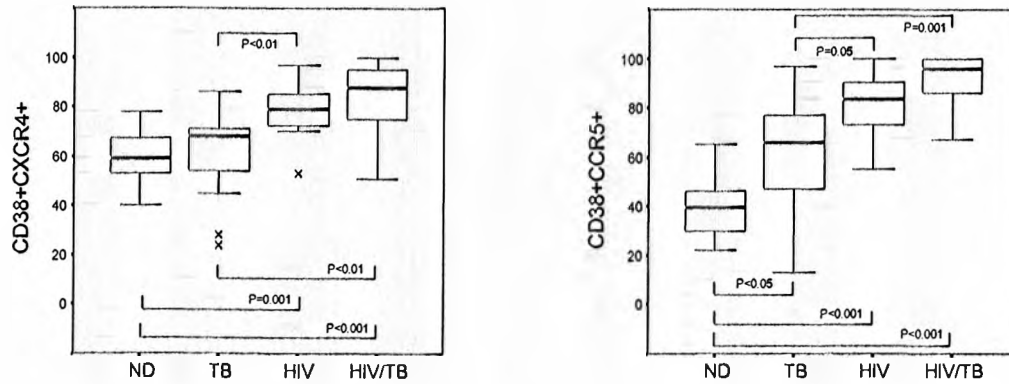
Figure 4.4 Expression of CD45RA and CD45RO on CXCR4- and CCR5-expressing CD4⁺ and CD8⁺ lymphocytes. (A) CD45RA expressing CD4⁺CXCR4⁺ cells, (B) CD45RA expressing CD8⁺CXCR4⁺ cells, (C) CD45RA expressing CD4⁺CCR5⁺ cells, and (D) CD45RA expressing CD8⁺CCR5⁺ cells. (E) CD45RO expressing CD4⁺CXCR4⁺ cells, (F) CD45RO expressing CD8⁺CXCR4⁺ cells, (G) CD45RO expressing CD4⁺CCR5⁺ cells, and (H) CD45RO expressing CD8⁺CCR5⁺ cells. Results are shown as percentages of cells. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

lymphocytes and B cells expressing CCR5 were significantly negatively correlated with advancing HIV-1 disease as measured by percentage of CD4⁺ T cells ($r = -0.428$, $P < 0.05$; $r = -0.547$, $P = 0.005$; $r = -0.653$, $P < 0.001$, respectively). Proportions of CD8⁺CD38⁺ cells were significantly negatively correlated with CD4⁺ T cell percentage ($r = -0.632$, $P = 0.001$), as was viral load ($r = -0.511$, $P = 0.011$). It was further evident that within the CD4⁺ cell subset, it was the expression of CXCR4 and CCR5 on naive cells ($r = 0.406$, $p < 0.05$ and $r = -0.692$, $P < 0.05$, respectively), and not the memory cells that correlated with CD4⁺ T cell percentage, that is, there was an increase in CCR5-expressing naive CD4⁺ cells, but a decrease in CXCR4-expressing naive CD4⁺ cells with advancing disease.

4.3.6 Relationship between CXCR4 and CCR5 expression and duration of anti-tuberculosis treatment

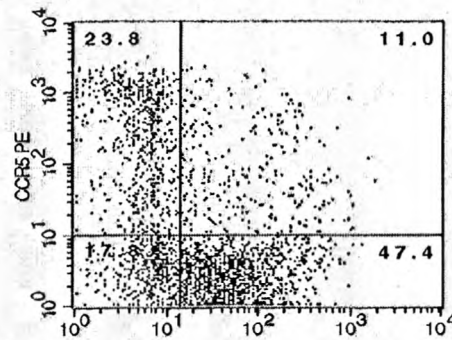
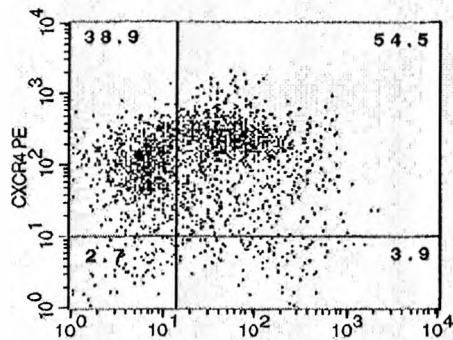
As there was a significant reduction of CXCR4 expression on CD8⁺, NK cells and granulocytes in the TB group, and monocytes in the TB and HIV/TB groups when compared with the HIV-only patients, it was important to assess the possibility that anti-tuberculosis therapy may be implicated in reduced expression of this particular receptor. The mean duration of anti-tuberculosis treatment did not differ significantly between the TB and HIV/TB groups (mean 8.3, range 3-20 weeks, and mean 7.5, range 0-26 weeks, respectively; $P > 0.05$). In the TB group there was no significant correlation between the expression of CXCR4 and the duration of anti-tuberculosis treatment. However, the HIV/TB group showed a negative correlation between the expression of CXCR4 on CD3⁺ ($r = -0.596$, $P < 0.05$), CD4⁺ ($r = -0.611$, $P < 0.05$), CD14⁺ ($r = -0.653$, $P < 0.05$), and on NK cells ($r = -0.685$, $P < 0.05$) and the duration of treatment. Further analysis showed that it was on the memory, and not the naive CD4⁺ cells that CXCR4 was reduced with time of treatment ($r = -0.707$, $P = 0.01$). The same was true for CXCR4 expression on CD8⁺ memory cells ($r = -0.637$, $P < 0.05$), even though a significant correlation was not noted when total CD8⁺ cells were analysed. Correlations also existed between duration of treatment and various other immunological parameters. In the HIV/TB group, proportions of CD8⁺CD38⁺ cells were negatively correlated with duration of anti-tuberculosis treatment ($r = -0.606$, $P < 0.05$), as was viral load ($r = -0.634$, $P < 0.05$). Positive correlations were found in this same group between total WCC ($r = 0.607$, $P < 0.05$), absolute lymphocyte count ($r = 0.685$, $P < 0.05$), absolute CD4⁺ T cell count ($r = 0.685$, $P < 0.05$), absolute CD8⁺ T cell count ($r = 0.683$, $P < 0.05$), absolute monocyte count ($r = 0.833$,

A



B

Uninfected individual



HIV/TB patient

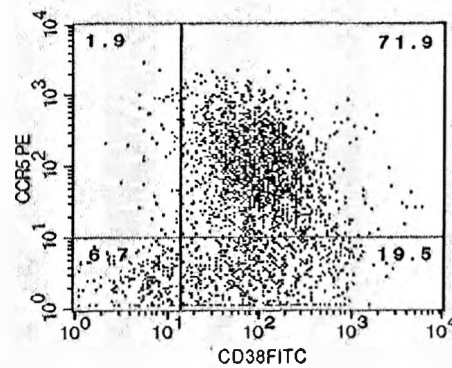
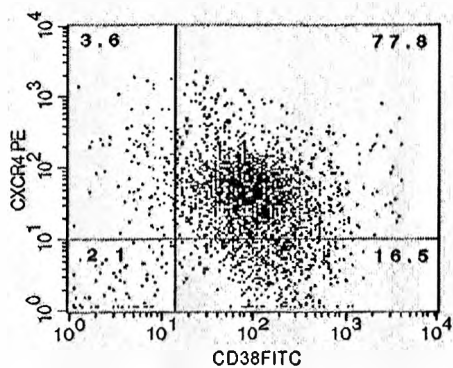


Figure 4.5 CD38 expression on CXCR4- and CCR5-expressing lymphocytes. (A) ND, TB, HIV, and HIV/TB study groups. Boxes represent values between the 25th and 75th percentiles, with the median indicated. Significant differences between groups are indicated. (B) Representative dot-plots showing CD38⁺CXCR4⁺ and CD38⁺CCR5⁺ lymphocytes from an uninfected individual and a HIV/TB patient. Percentages of cells in each quadrant are indicated.

P=0.001) and the duration of anti-TB drug treatment. In contrast to the cellular expression of CXCR4, there were no significant correlations between CCR5 expression on any cell type and the duration of anti-tuberculosis therapy in either the TB or the HIV/TB groups.

4.4 Discussion

The effects of HIV-1 and *M. tuberculosis* infection on each other are considered bidirectional (Del Amo *et al.*, 1999), with co-infection associated with higher morbidity and mortality, and an increased risk of acquisition of new secondary infections (Whalen *et al.*, 1995). As there is an ever-increasing burden in Africa of both HIV-1 disease and tuberculosis, alone or in combination, an understanding of the interactions of the respective infecting organisms and the immune consequences to the host are imperative for constructive intervention. This study was undertaken to evaluate the distribution of HIV-1 coreceptors CXCR4 and CCR5 on peripheral blood leukocytes of healthy individuals, HIV-1 infected patients, individuals with pulmonary tuberculosis and those with both HIV-1 disease and pulmonary tuberculosis. Unlike other studies which have focussed on coreceptor expression on CD4-expressing cells, the major targets for HIV-infection, the levels of coreceptor expression on all peripheral blood cell types were evaluated in this study, as alterations in levels of expression may have important implications for host immune function.

Comparison of CXCR4 and CCR5 expression on different cell types identified by specific cellular markers showed that CXCR4 expression predominated, and was further associated with a naive phenotype, while CCR5 expression was associated with a memory phenotype, as has been previously reported (Bleul *et al.*, 1997; Forster *et al.*, 1998). Unlike Bleul *et al.*, (Bleul *et al.*, 1997) who found that CXCR4 expression was restricted to mononuclear cells, a large proportion of granulocytes (93%) expressed CXCR4 in this study.

This study has shown that a significant reduction in the proportion of leukocytes expressing CXCR4 occurs in patients infected with HIV-1, those with pulmonary tuberculosis, and those with dual infection, while the proportions of CCR5-bearing leukocytes were significantly increased from that found for control persons. Furthermore, different cellular subsets are

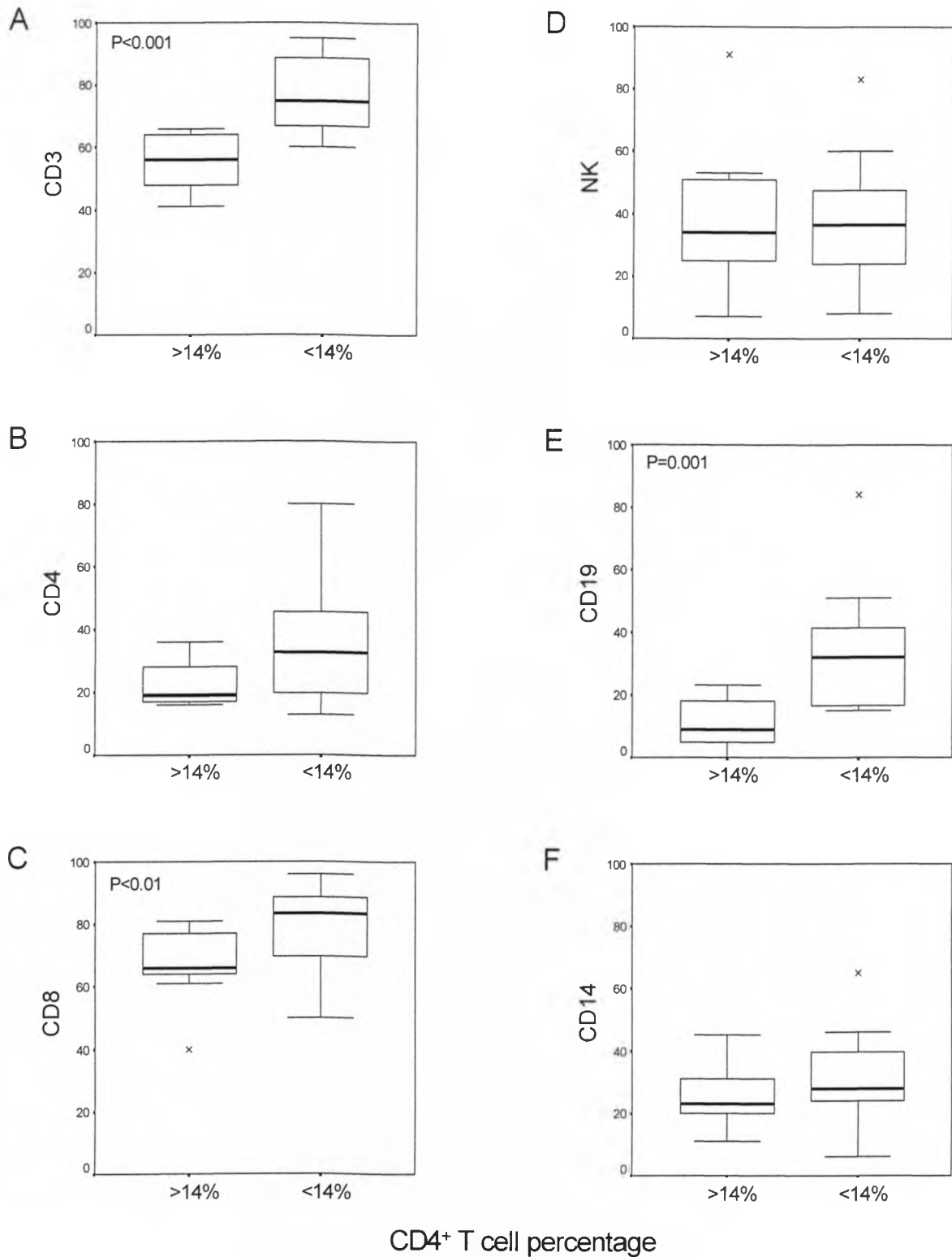


Figure 4.6 Relationship between CCR5 expression within various leukocyte subsets and the stage of HIV-1 disease as described by a CD4⁺ T cell percentage of >14% and <14% (horizontal axis). Proportions of CCR5-expressing CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E) and CD14 (F) cells are shown. Significant differences are indicated.

modulated in a similar way dependent on the disease state, suggesting that common mechanisms may underlie modulation of these receptors on different cell types. It was also apparent from results that, in general, the CXCR4 receptor was modulated to a greater extent by the presence of pulmonary TB or its treatment, whereas CCR5 was modulated to a greater extent by the presence of HIV-1 infection.

Recently, a number of studies have shown that CXCR4 is downregulated on CD4⁺ and CD8⁺ cells of HIV-1 infected persons (Forster *et al.*, 1998; Giovannetti *et al.*, 1999; Ostrowski *et al.*, 1998). These findings are corroborated in the present study where CXCR4 expression was significantly reduced in these cells in all the disease groups studied. In contrast to Forster *et al.* (Forster *et al.*, 1998) who found that when evaluating CXCR4⁺ T cells in HIV-1 seropositive individuals, both CD4⁺ and CD8⁺ cells were equally downregulated, this study found a much more profound downregulation of this receptor on CD8⁺ cells. A substantial downregulation of CXCR4 expression on CD8⁺ cells, NK cells, CD14⁺ monocytes and granulocytes was apparent in the two TB groups when compared to the HIV-only group or controls, was noted in the TB and HIV/TB groups, demonstrating the aforementioned distinct effect of pulmonary TB on CXCR4 expression. The pattern of downregulation of CXCR4 on granulocytes, owing to the presence of TB, is reminiscent of some previously reported results obtained when monitoring related receptors, in particular CXCR2, one of the two receptors that specifically bind IL-8 (Meddows-Taylor *et al.*, 1998). Reduced expression of the IL-8 receptors (CXCR1 and CXCR2) was also associated with a reduced ability of neutrophils to respond to the appropriate ligand, namely IL-8, by directed migration, calcium flux (Meddows-Taylor *et al.*, 1998) and degranulation (Meddows-Taylor *et al.*, 1999b). These similarities in the patterns of reduced receptor expression raise the question of a common mechanism/s underlying disease-related downregulation of these G-protein-coupled receptors.

Results obtained for expression of CXCR4 in the two TB groups contrasted with those of Juffermans and coworkers who found that the *in vitro* addition of lipoarabinomannan, a cell wall component of *M. tuberculosis*, to whole blood of healthy donors resulted in an upregulation of both CXCR4 and CCR5 on CD4⁺ T cells (Juffermans *et al.*, 2000a), and that patients with active TB also presented with increased expression of both HIV-1 coreceptors (Juffermans *et al.*, 2001).

Patient numbers in the latter study were small (n=8), and half had pulmonary TB, the other half had extrapulmonary TB, and some of these patients were HIV-1 infected. It cannot be assumed that immune responses in pulmonary TB are the same as found in extrapulmonary TB, and so comparison with this study is difficult. Furthermore, all their patients were newly diagnosed, whereas all but one of our patients were on anti-TB therapy for some duration.

As there was a greater modulation of CXCR4 expression in the two TB groups than the HIV-only group, and as the majority of the patients infected with *M. tuberculosis* were receiving anti-tuberculosis drug therapy, it was important to determine the possible role of administration of these drugs on receptor expression. The HIV/TB group showed a significant negative correlation between the expression of CXCR4 on CD3⁺, CD4⁺ and CD8⁺ memory cells, CD14⁺ and NK cells and the duration of treatment. There were no correlations between CCR5 expression and duration of treatment. In this same group, a positive correlation between length of anti-tuberculosis drug therapy and improvements in peripheral absolute cell counts, and a negative correlation between viral load and cellular activation as measured by the proportions of CD8⁺CD38⁺ cells was observed. There are other studies that suggest that anti-TB treatment may play a role in diminished chemokine receptor expression (Meddows-Taylor *et al.*, 1998), and diminished cell function (Shalekoff *et al.*, 1998b).

By contrast to CXCR4, the proportions of different leukocytes expressing CCR5 were increased in the TB, HIV and HIV/TB groups, except for CCR5-expressing monocytes which were significantly increased only in the HIV group. Interestingly, the CCR5-expressing CD4⁺ cells were not elevated in any of the disease groups. Our results contrast with those of Ostrowski *et al.* (Ostrowski *et al.*, 1998) who demonstrated that CCR5 expression was upregulated only on CD4⁺ T cells, and not on CD8⁺ T cells or on monocytes, of HIV-1 infected individuals when compared to uninfected controls. Another group (Giovannetti *et al.*, 1999) found the expression of CCR5 in HIV-1 infected patients to be significantly increased on both CD4⁺ and CD8⁺ T cells.

Greater proportions of CCR5-bearing CD4⁺ and CD8⁺ lymphocytes, as well B cells, CD8⁺CD38⁺ cells and increased viral loads, correlated with advancing disease as measured by

percentage of CD4⁺ T cells in HIV-1 infected persons with pulmonary tuberculosis. There was a trend towards a reduction in CXCR4 with disease stage, but this was not significant. Of interest was the fact that the increase in CCR5-expressing CD4⁺ cells, and the decrease in CXCR4-expressing CD4⁺ cells with disease progression was a result of these changes in the proportions of naive, and not memory cells.

Many studies have shown that increased coreceptor expression *in vitro* leads to increased viral entry (Sozzani *et al.*, 1998; Wang *et al.*, 1998b). Increased CCR5 receptor expression could lead to an increase in entry of NSI or R5 viruses. An increase in CCR5 expression on CD14⁺ cells in HIV-1 seropositive individuals was observed, and this could therefore be one cell type where there could be increased viral entry in HIV-1 positive individuals. Monocytes also express CXCR4 which would suggest a permissiveness for entry of X4 viruses. Entry of cell-free HIV-1 into monocytes in the peripheral circulation could facilitate the spread of HIV-1 to different tissues where monocytes differentiate into macrophages, and may then support active HIV-1 replication. It is widely recognised that in approximately 50% of HIV-1 infected individuals (much lower in subtype C infections), the phenotype of HIV-1 changes from NSI to SI with progression of disease (Connor *et al.*, 1997; Richman & Bozzette, 1994). Results here clearly show that the distribution of receptors, in terms of the proportions of cells that express either receptor, would not facilitate or reflect such a change in virus phenotype. If the switch to SI were driven by a larger availability of CXCR4-expressing cells at late stage disease, results obtained argue against such a notion. The same is true for CCR5, which increases with disease progression. That the specific phenotype of the virus may in turn drive the expression of the coreceptors directly seems unlikely.

A more likely explanation for altered coreceptor expression may be the state of immune activation, which is affected by both stage of HIV-1 disease as well as the presence of opportunistic or secondary infections. Ostrowski *et al.* (Ostrowski *et al.*, 1998) studied patients with other forms of chronic immune activation, but did not detect a reduction in the expression of CXCR4 on CD4⁺ T cells, and therefore suggested that the reduction in CXCR4 expression may be relatively specific to HIV-1 infection. Recent studies have demonstrated that CXCR4 expression was upregulated as a result of influenza virus infection (Puri *et al.*, 2000), and CCR5

expression on CD14⁺ monocytes was induced by *Treponema pallidum* (Sellati *et al.*, 2000). Taken together with the finding that infection with *M. tuberculosis* results in a downregulation of CXCR4 expression, it is unlikely that changes in coreceptor expression are specific to HIV-1 as an infecting agent, and are more likely to be a consequence of generalised immune activation or, as mentioned before, anti-TB therapy. It has been shown that monocytes isolated from TB patients are more permissive for HIV-1 replication when infected with HIV-1 *in vitro* (Toossi *et al.*, 1993). This was suggested to be due to a greater number of activated monocytes that occur in these patients. As the presence of TB, with or without concomitant HIV-1 infection, did not result in upregulation of CCR5, it is unlikely that increased viral entry could be a factor in the enhanced HIV-1 replication shown *in vitro* (Toossi *et al.*, 1993), or *in vivo* as demonstrated by the fact that HIV/TB patients had higher viral loads than HIV-only individuals. One study found that in healthy individuals the activation marker HLA-DR was expressed to a much greater degree on CCR5⁺CD4⁺ cells than on CXCR4⁺CD4⁺ cells (Ostrowski *et al.*, 1998), whereas in this study, significantly higher proportions of CXCR4⁺ lymphocytes expressed CD38 than did CCR5⁺ lymphocytes. Moreover, in comparison with healthy individuals, CD38 expression was increased in all disease groups on CCR5⁺ lymphocytes, and in the HIV groups for CXCR4⁺ lymphocytes.

Very little has been published on the possible immunological consequences of altered CXCR4 and CCR5 expression in the context of HIV-1 disease, and even less so of tuberculosis. Cell signalling and subsequent trafficking of specific cell subsets are the most likely of functions to be affected through altered expression of either of the chemokine receptors. Reduced expression of CXCR4 on mononuclear cells might be expected to result in an impaired migration response to SDF-1 α . Likewise, an increased CCR5 expression may, in the absence of any other cellular defects or anergy, give rise to a hyper-reponse to RANTES, MIP-1 α and MIP-1 β . Reduced CXCR4 on neutrophils is more likely to affect SDF-1 α -induced exocytosis of granules, or some as yet unknown function, as this receptor does not function to mediate chemotaxis in these cells (C.Tiemessen, unpublished data). Changes in chemotactic responsiveness to the appropriate chemokines, on the basis of the degree of receptor expression, would be expected to be more profound in CD8⁺ cells, monocytes and NK cells, and to a lesser degree in CD4⁺ cells and B cells.

In summary, results show an altered CXCR4 and CCR5 repertoire on peripheral leukocytes of

persons with pulmonary TB and HIV-1 infection, and suggest that an altered ability of X4 and R5 viruses to enter CD4-bearing cells, and dysregulation of cellular trafficking, may be important features of HIV-1 and tuberculosis pathogenesis. A further understanding of what modulates expression of the HIV-1 coreceptors on various cell types *in vivo*, may contribute to delineating those immune factors that are mere markers of an infectious event from those that play a significant role in disease pathogenesis.

CHAPTER FIVE

CHANGES IN EXPRESSION OF CXCR4 AND CCR5 ON LEUKOCYTES FROM PERSONS WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION IN RESPONSE TO HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

5.1 Introduction

Results from Chapter Four confirmed findings of reduced CXCR4 (Forster *et al.*, 1998; Giovannetti *et al.*, 1999) and elevated CCR5 expression (Giovannetti *et al.*, 1999) in HIV-1 infected individuals when compared with healthy individuals, findings which may have implications for HIV-1 entry and pathogenesis. HAART has been shown to be effective in increasing numbers of CD4⁺ T cells, and in reducing HIV-1 viral load, both in the periphery (Gulick *et al.*, 1997) and in lymphoid tissues (Cavert *et al.*, 1997). Although a number of studies have addressed the effect of HAART on chemokine receptor expression, the results have not been consistent (Giovannetti *et al.*, 1999; Giovannetti *et al.*, 2001; Nicholson *et al.*, 2001; Pierdominici *et al.*, 2002).

HIV-1 infected and uninfected individuals living in Africa, have been shown to have increased immune activation, when compared with individuals living in Europe (Rizzardini *et al.*, 1996; Rizzardini *et al.*, 1998). Additionally, African individuals have been shown to have an increased percentage of CCR5- (Clerici *et al.*, 2000a; Kalinkovich *et al.*, 2001; Kalinkovich *et al.*, 1999) and CXCR4-expressing cells (Kalinkovich *et al.*, 2001). As other studies evaluating the effect of HAART on CXCR4 and CCR5 expression, were performed on European individuals, it was important to determine whether African individuals would respond in a similar way in light of this heightened immune activation. Consequently, this study was undertaken to determine whether the reduction in immune activation known to occur following the use of HAART, would result in a concomitant increase in CXCR4 and reduction in CCR5 expression. Immune activation

was evaluated in this study using CD38 as a marker of activated CD8⁺ cells. Furthermore, as alterations in CXCR4 and CCR5 expression on a number of leukocyte subsets in relation to HIV-1 disease or co-infection with *M. tuberculosis* were observed in Chapter Four, it was important to determine whether HAART would reverse the effects of HIV-1 infection on coreceptors expression within the various leukocyte subsets.

5.2 Materials and methods

5.2.1 Study participants

Eight asymptomatic antiretroviral drug-naive HIV-1 infected patients were enrolled for this study (Table 5.1). CD4⁺ T cell counts ranged between 173 and 657/ μ l, CD4⁺ T cell percentages between 6.5 and 36, and viral loads between 3.42 and 4.84 log₁₀ RNA copies/ml. Of the eight patients, one was male and seven were female. The median age was 41 (range 26-68 years). Blood samples were taken at baseline (BL) and after eight weeks of HAART for evaluation of coreceptor expression. Additional specimens were obtained from patient six at a further two time points, each eight weeks apart, viz, 16 and 32 weeks after initiation of therapy, from patient seven at 32 weeks, and from patient eight at 16 weeks after the onset of HAART. Five patients received one protease inhibitor and two nucleoside reverse transcriptase inhibitors (NRTI), and three patients received one non-nucleoside reverse transcriptase inhibitor (NNRTI) and two NRTIs. Eighteen healthy individuals (five were male and thirteen were female) were studied in parallel as controls (ND group), and blood taken on one occasion. Of these, 14 were white, two were coloured and two were black, and their median age was 36.5 (range 26-57 years).

5.2.2 Monoclonal antibodies

Two- and three-colour staining was performed as described in Section 3.2.2.

5.2.3 Flow cytometry

EDTA anticoagulated whole blood was stained within 6h of collection and samples analysed on a FACSort flow cytometer using Cellquest version 3.1 software as in Section 3.2.3.

5.2.4 Viral load quantitation

Plasma HIV-1 RNA levels were determined as in Section 4.2.4.

5.2.5 Statistical analysis

Comparison of CXCR4 and CCR5 expression on different leukocyte subsets between HIV-1 infected patients at baseline (BL), and eight weeks after HAART was done using the Wilcoxon signed rank test (paired samples), and between the HIV-1 infected patients (at BL and HAART), and the ND group using the Mann-Whitney U test (unpaired samples). The Spearman correlation coefficient was calculated to analyse correlations for paired samples (BL vs HAART).

Table 5.1 Baseline immunological and virological status of HIV-1 infected individuals

Patients	Age (years)	Race	Sex	CD4 ⁺ T cells		Viral load (log HIV-1 RNA copies/ml)	CD8 ⁺ CD38 ⁺ cells (percentage)
				x10 ³ /μl	percentage		
1	44	W	F	456	23.8	4.63	75.08
2	28	B	F	657	32.8	4.01	77.12
3	60	W	F	378	17.8	3.84	79.66
4	68	W	M	286	19.6	3.69	64.57
5	26	B	F	490	36	4.27	88.95
6	38	B	F	353	17.2	3.42	63.33
7	42	B	F	201	14.3	4.84	72.42
8	40	B	F	173	6.5	4.62	78.78

W: White; B: Black; F: female; M: male

5.3 Results

5.3.1 Virological and immunological response to HAART

All patients had a good virological and immunological response after eight weeks of HAART, with reductions in viral load of at least 1.8 log and an increase in percentage of CD4⁺ T cells, and

in most subjects, an increase in absolute CD4⁺ T cell counts. In addition, the percentage of CD8⁺CD38⁺ cells was significantly lower in the patients on HAART when compared with pre-therapy levels (P<0.05). These however, were still significantly higher than ND levels (P<0.05).

5.3.2 Comparison of CD38 expression on CD8⁺ cells from South African individuals with those from European and American individuals

As individuals living in Africa have been found to have increased immune activation, in comparison with European individuals (Rizzardini *et al.*, 1996; Rizzardini *et al.*, 1998), it was thought important to compare the percentage of CD8⁺CD38⁺ cells as a measure of this activation. CD8⁺CD38⁺ data from South African individuals from this study, and published results from American and European studies are shown in Table 5.2. The percentage of CD8⁺CD38⁺ cells in uninfected and HIV-1 infected individuals living in South African was substantially higher than CD8⁺CD38⁺ cells from uninfected and HIV-1 infected individuals from other published studies.

Table 5.2 Comparison of percentages of CD8⁺CD38⁺ lymphocytes from different studies

Uninfected	HIV-1 infected (asymptomatic)	HIV-1 infected (AIDS)	Reference
48 (22-75)¹	76 (63-89)¹		Current study
22 (16-36) ¹	58 (44-91) ¹	80 (42-91) ¹ ARC 96 (91-99) ¹ AIDS	Kestens <i>et al.</i> , 1992*
20.3 ± 14.4 ²	40.1 ± 15.4 ²	52.4 ± 19.4 ²	Giorgi <i>et al.</i> , 1993
18 ³	47 ³		Landay <i>et al.</i> , 1995**
5.2 ± 0.74 ⁴	25.8 ± 1.01 ⁴	33.6 ± 2.1 ⁴	Bofill <i>et al.</i> , 1996
14 (5.3-38.1) ⁵			Taylor <i>et al.</i> , 1996
8 (4-17) ⁵	36 (14-82) ⁵	69 (31-97) ⁵	Liu <i>et al.</i> , 1997

Results are expressed as median (range)¹, mean ± SD², median³, mean ± SEM⁴, median (5-95th percentile)⁵

*Asymptomatic and lymphadenopathy groups combined; AIDS Related Complex (ARC) and AIDS grouped separately

**HIV-1 infected patients had a median CD4% of 21%

5.3.3 Alterations in expression of CXCR4 and CCR5 on leukocyte subsets

The expression of CXCR4 and CCR5 on leukocyte subsets from eight HIV-1 infected patients was analysed at BL and eight weeks after the initiation of HAART, and compared with 18 healthy individuals (ND). Before the initiation of HAART, the percentage of CD3⁺ (P<0.001), CD4⁺ (P<0.01), CD8⁺ (P<0.05), CD19⁺ (P<0.05) and CD14⁺ (P<0.001) cells expressing CXCR4 were significantly reduced when compared to the ND group (Figure 5.1). After eight weeks of treatment, CXCR4 expression on CD3⁺, CD8⁺, NK and CD14⁺ cells, was still significantly reduced when compared to the ND group, and in none of the subsets was there a significant increase in CXCR4 expression in the patients after therapy when compared to BL data. The granulocytes showed no significant disease- or HAART-associated alterations in CXCR4 expression (P>0.05). In addition, it was found that the expression of CXCR4 on CD3⁺ cells was positively correlated to the percentage of CD4⁺ T cells ($r = 0.994$, P<0.001) in the patients on HAART.

On examination of CXCR4 expression on leukocyte subsets for each patient individually, it was observed that CXCR4 expression was increased on CD3⁺, CD4⁺, and CD8⁺ cells in patients one, two, three and four, decreased on patients six, seven and eight, and remained largely unchanged on patient five when compared with their pre-treatment levels (Figure 5.2).

In contrast to results found for CXCR4 expression, the percentage of cells expressing CCR5 was significantly elevated in the HIV-1 infected patients before therapy within the CD3⁺ (P<0.001), CD8⁺ (P<0.001), NK (P<0.05) and CD19⁺ (P<0.05) subsets when compared with healthy individuals, with a trend to this effect in the CD4⁺ cells and CD14⁺ monocytes (Figure 5.3). Although there was a significant post-therapy reduction (P<0.05) in all subsets except for the NK cells (where there was an increase in percentage of cells expressing CCR5), the levels of CCR5 expression on the CD3⁺ and CD8⁺ cells were still significantly elevated when compared to ND levels. Furthermore, a negative correlation between percentage of CD4⁺ T cells and the expression of CCR5 on CD3⁺ cells was observed in the HIV-1 positive patients both at BL ($r = -0.905$, P<0.01) and when on HAART ($r = -0.838$, P<0.01).

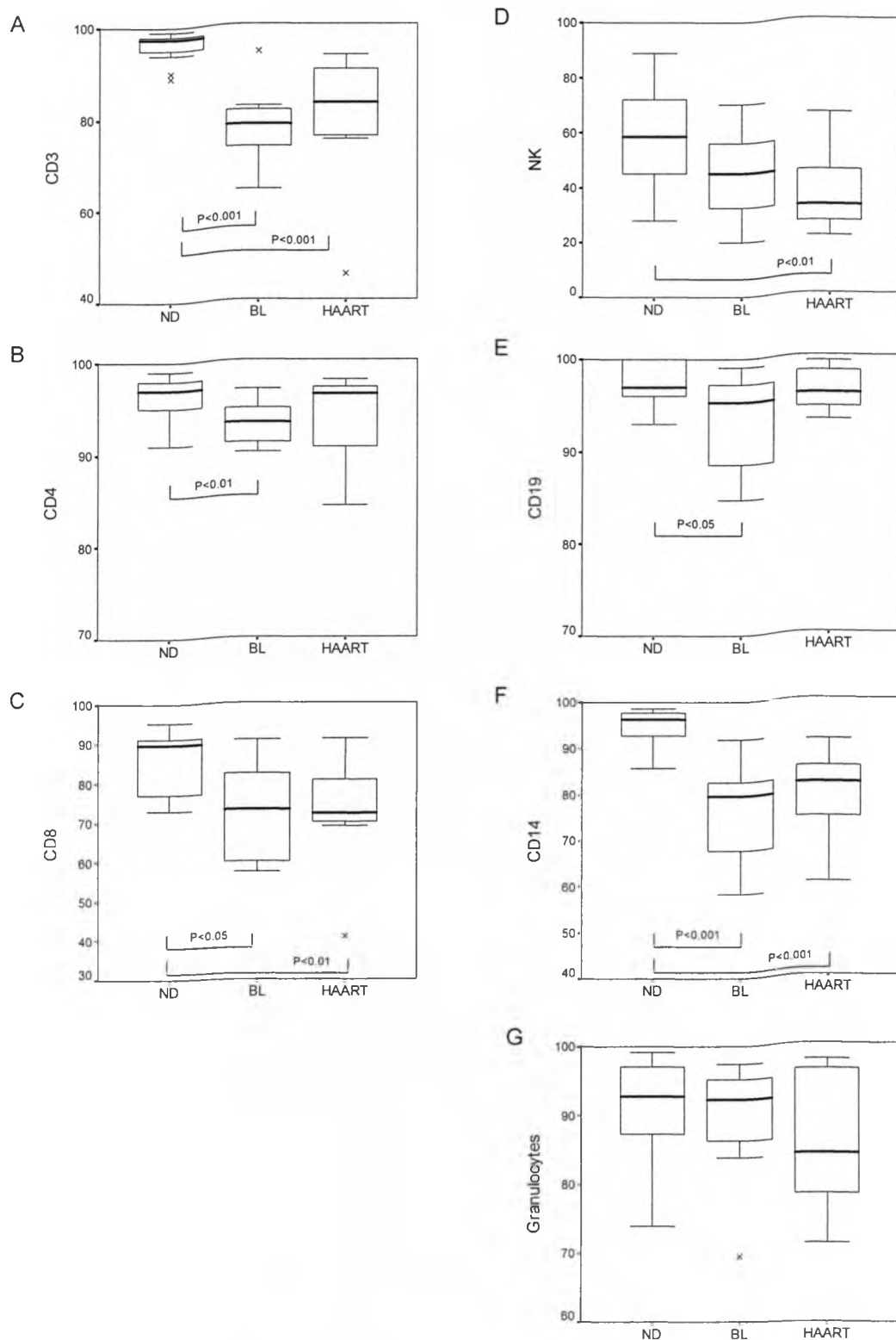


Figure 5.1 Expression of CXCR4 on whole blood leukocytes from subjects in the ND, BL, HAART study groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells and granulocytes (G). Results are shown as percentages of cells expressing CXCR4. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

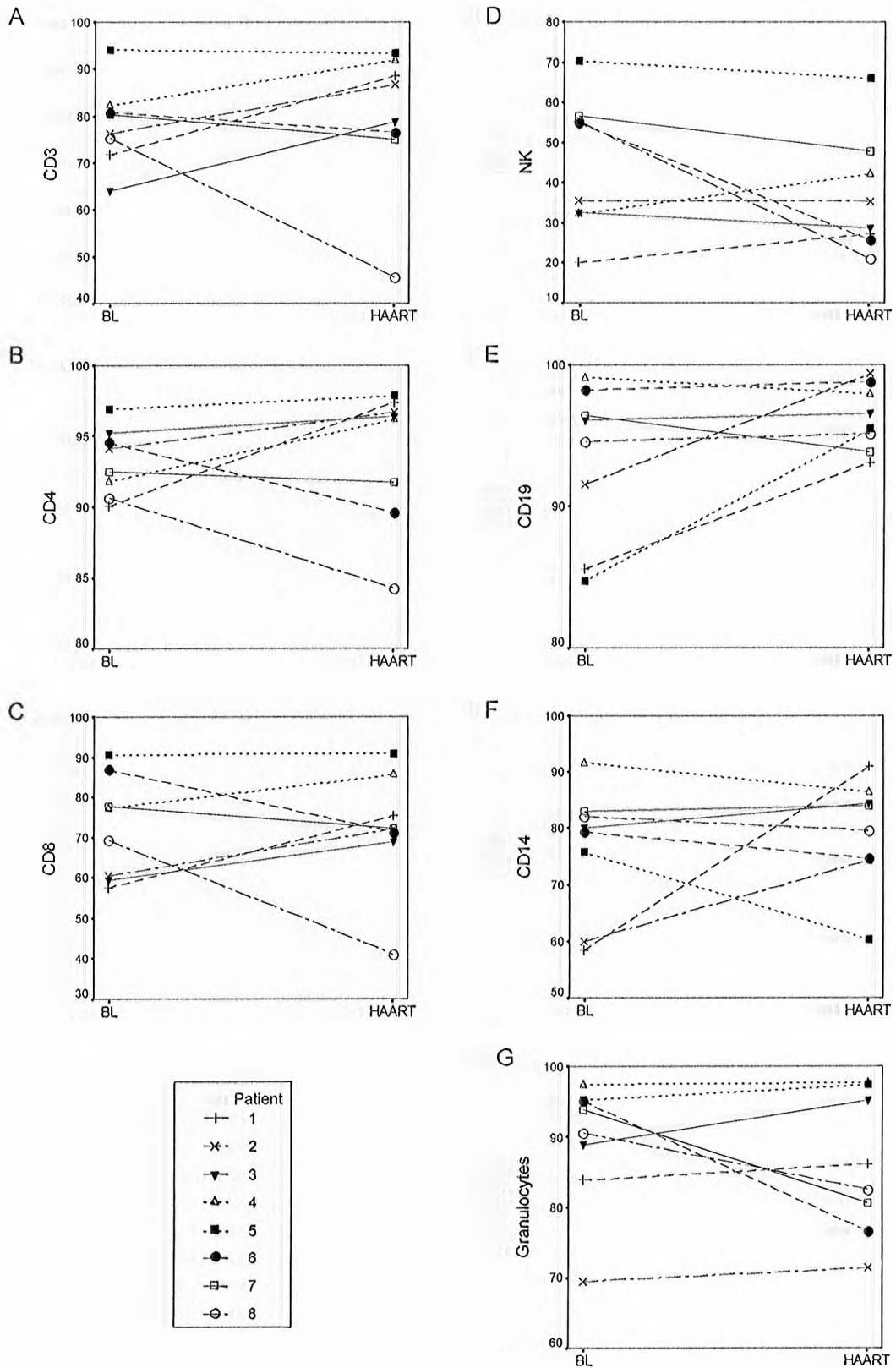


Figure 5.2 Expression of CXCR4 on whole blood leukocytes from HIV-1 infected subjects at BL, and after eight weeks of HAART: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells and granulocytes (G). Results are shown as percentages of cells expressing CXCR4 from individual patients.

On an individual patient basis, there was a trend towards a reduction in CCR5 expression in all patients within the CD3⁺, CD4⁺ and CD8⁺ cell compartments, and in seven of eight patients on the CD19⁺ and CD14⁺ cells (Figure 5.4). Large reductions in CCR5 expression (10-20%) were observed in some patients, while in others expression was only reduced by between 3-5%. Interestingly, the increase in CCR5 expression on CD19⁺ and CD14⁺ cells occurred in the same patient (patient three). In contrast to the reduction in CCR5 expression observed on other cell subsets, there was an increase in CCR5 expression on NK cells in five of the seven patients.

It was next questioned whether following the three patients who had reductions in CXCR4 expression on most subsets, for longer time periods would allow the CXCR4 expression to increase, and to determine whether further reduction in CCR5 expression would occur. Specimens were obtained from patient six at a further two time points, each eight weeks apart, viz, 16 and 32 weeks after initiation of therapy. It was only possible, however, to obtain specimens from patient seven at 32 weeks, and from patient eight at 16 weeks after the onset of HAART.

Overall, CXCR4 expression, after a decrease at eight weeks, returned to levels that were similar to BL values, which therefore remained lower than the levels found in the healthy controls. In addition, CCR5 expression, over the extended time period did not show further reductions in expression (data not shown). The viral load for patient six remained undetectable up to 32 weeks, while that for patient seven increased at 16 weeks and 32 weeks, while still remaining 1 log lower than BL levels. The viral load for patient 8 also increased at 16 weeks, but remained 2 log lower than at BL.

5.3.4 Expression of CD45RA and CD45RO

An initial evaluation of total CD4 and CD8 cells that were CD45RA⁺ or CD45RO⁺ revealed a reduction in expression of CD45RA and an increase of CD45RO expression in HIV-1 infected individuals when compared to the normal donors. However, this was significant only for CD8⁺ cells (data not shown).

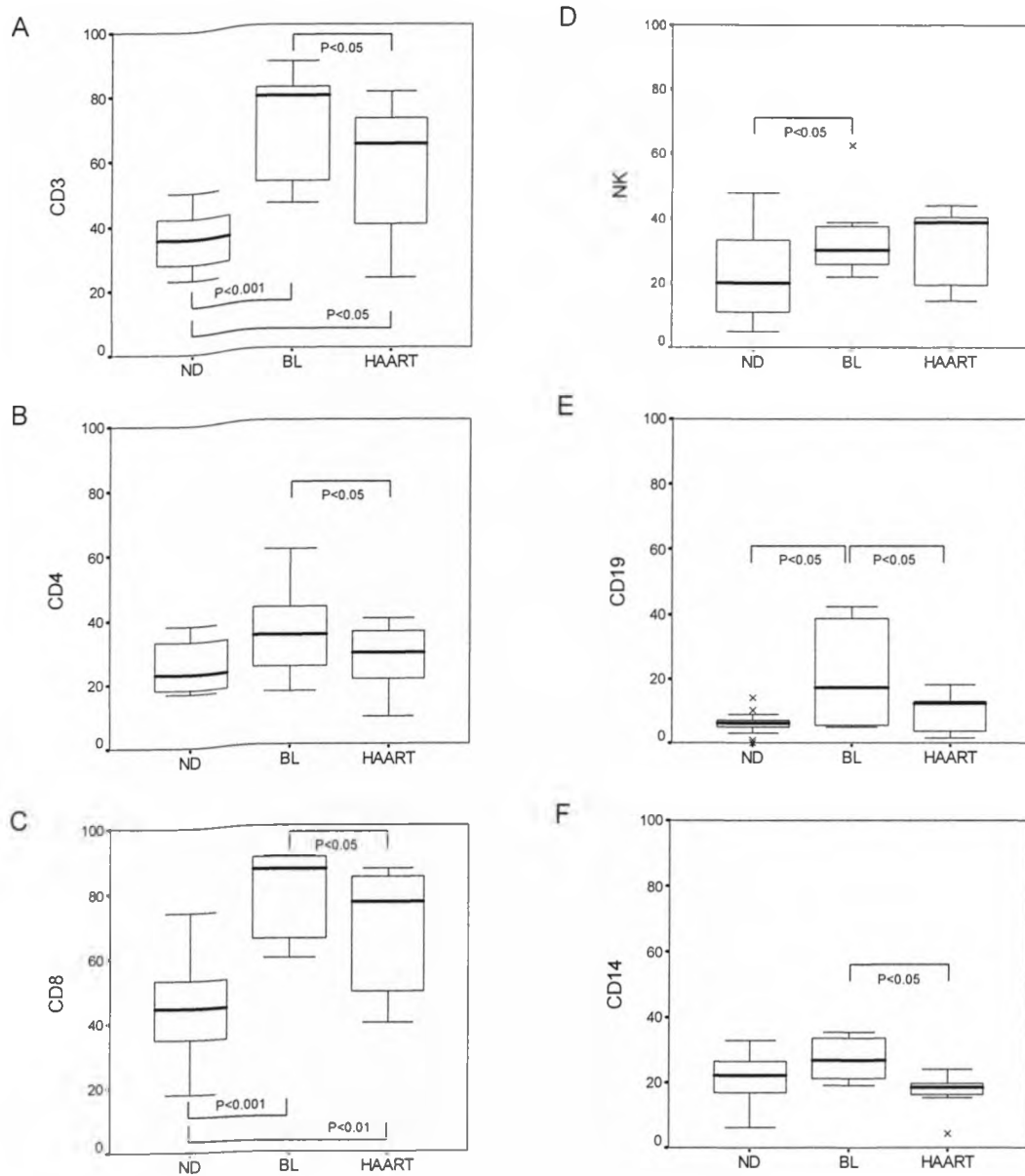


Figure 5.3 Expression of CCR5 on whole blood leukocytes from subjects in the ND, BL, HAART study groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells. Results are shown as percentages of cells expressing CCR5. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

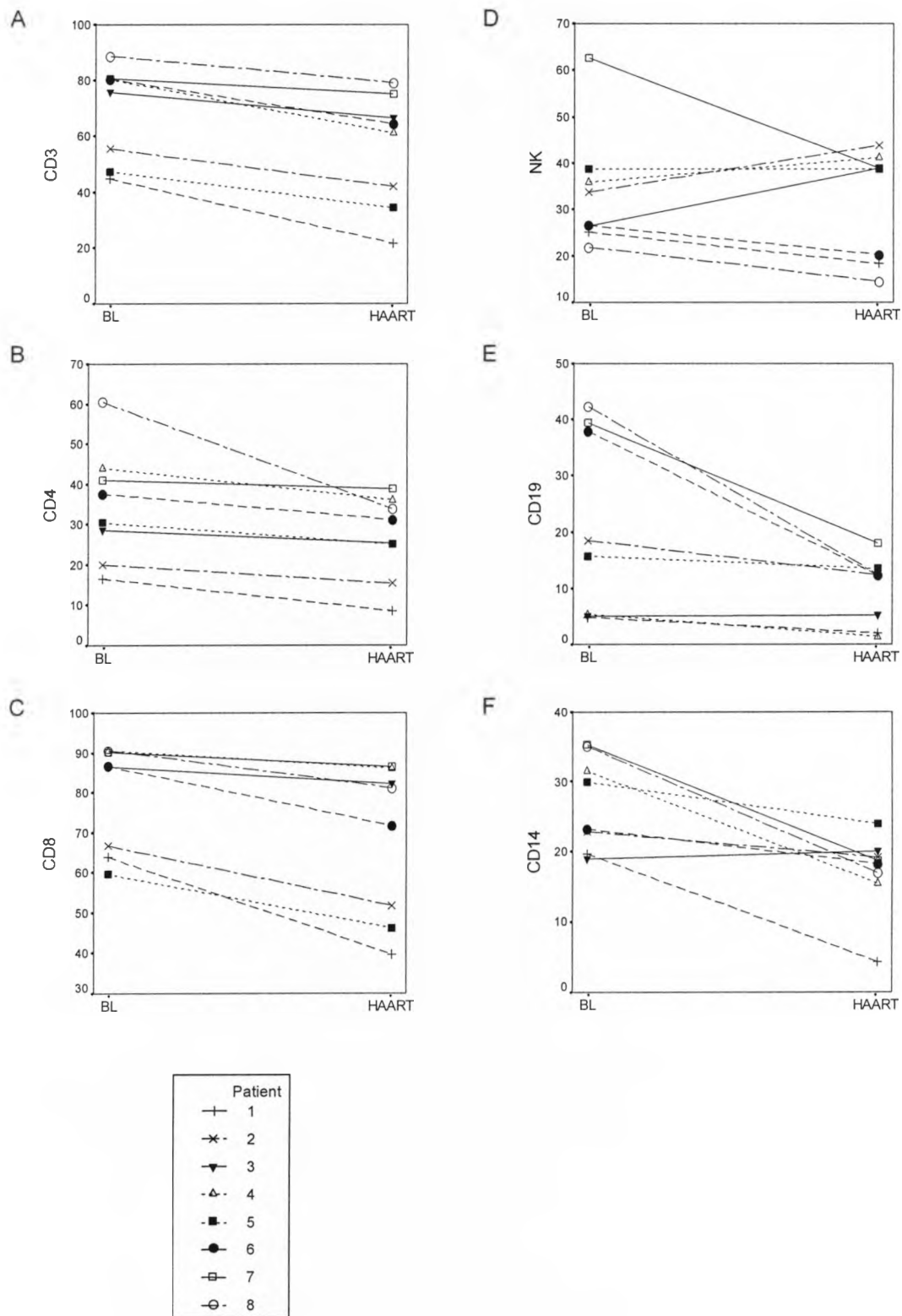


Figure 5.4 Expression of CCR5 on whole blood leukocytes from HIV-1 infected subjects at BL, and after eight weeks of HAART: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells. Results are shown as percentages of cells expressing CCR5 from individual patients.

The expression of CCR5 on CD4⁺ cells and CD8⁺ cells was inversely related to levels of both naive CD45RA⁺ CD4 cells (for CD4: $r = -0.765$, $P=0.001$; for CD8: $r = -0.756$, $P=0.001$) and naive CD45RA⁺ CD8 cells (for CD4: $r = -0.585$, $P=0.017$; for CD8: $r = -0.694$, $P=0.003$). In addition, a positive correlation was observed between CCR5 expression on CD4⁺ cells and CD8⁺ cells and memory CD45RO⁺ CD4 cells (for CD4: $r = 0.859$, $P<0.001$; for CD8: $r = 0.841$, $P<0.001$) and CCR5 expression on CD8⁺ cells and memory CD45RO⁺ CD8 cells ($r = 0.547$, $P=0.028$) in HIV-1 infected patients either at BL or on HAART. In contrast, only a weak negative correlation was found for the expression of CXCR4 on CD4⁺ cells and memory CD45RO⁺ CD4 cells ($r = -0.521$, $P=0.039$).

5.3.5 Is alteration in expression of CXCR4 and CCR5 related to shifts in CD45RA and CD45RO or up/downregulation of the coreceptors?

The alterations in receptor expression may be due to alterations in the percentages of naive or memory cells expressing the coreceptor, or to changes in proportions of naive and memory cells, or both. Therefore, the coreceptor expression was analysed in two ways: (i) by gating CD4⁺ and CD8⁺ cells expressing CD45RA or CD45RO and determining the proportions expressing either CXCR4 or CCR5, and (ii) by gating CD4⁺ and CD8⁺ cells expressing either of the chemokine receptors, and then determining the proportions of these cells expressing CD45RA or CD45RO.

Figure 5.5 shows the proportions of CD45RA- and CD45RO-expressing CD4⁺ and CD8⁺ cells co-expressing CXCR4 or CCR5. CXCR4 receptor expression on CD8⁺CD45RA⁺ and CD8⁺CD45RO⁺ cells ($P<0.05$) was significantly lower in the HIV-1 infected patients at BL than in the healthy controls (Figure 5.5B and F), but was not significantly different on CD4⁺CD45RA⁺ and CD4⁺CD45RO⁺ cells (Figure 5.5A and E). These results are very similar to those obtained in Chapter Four, with the exception of the significant reduction in CXCR4 expression on CD4⁺CD45RO⁺ (Figure 4.3E), which is probably as a result of the larger number of patients in the HIV group in that Chapter. There were no significant changes in CXCR4 expression after HAART (compared with BL) on either the CD45RA⁺ and CD45RO⁺ CD4 and CD8 cells. In contrast, CCR5 expression was significantly elevated on the CD4⁺CD45RA⁺ ($P<0.05$), CD8⁺CD45RA⁺ ($P=0.001$) and CD8⁺CD45RO⁺ ($P<0.001$), (with a trend to that effect on CD4⁺CD45RO⁺ cells) (Figure 5.5C, D, G and H). Again, results here are similar to Chapter Four,

with the exception of no increase in CCR5 expression on CD4⁺CD45RA⁺ cells (Figure 4.3C). Although CCR5 expression was significantly reduced after HAART when compared with BL in CD4⁺CD45RA⁺ ($P<0.05$), CD8⁺CD45RA⁺ ($P<0.05$) and CD8⁺CD45RO⁺ ($P<0.05$) subsets, the percentage of cells expressing CCR5 remained elevated above the ND levels in both CD8⁺ cell subsets.

The second way in which the results were analysed determined CD45RA and CD45RO expression on CXCR4-expressing and CCR5-expressing CD4⁺ and CD8⁺ cells (Figure 5.6). Evaluation of CXCR4-expressing cells in HIV-1 infected persons at BL revealed comparable results to those found for CD45RA and CD45RO expression on total CD4 and CD8 cells described above, i.e. there was a reduction in CD45RA expression (Figure 5.6A and B) and an increase in CD45RO expression (Figure 5.6E and F) in comparison with healthy individuals. On analysis of CCR5-expressing CD4⁺ and CD8⁺ cells no significant changes in CD45RA and CD45RO expression on the CCR5⁺ CD4⁺ subset were found (Figure 5.6C and G). In the CCR5⁺ CD8⁺ cells, however, similar trends were evident to that found for CXCR4⁺ cells (Figure 5.6D and H). Results obtained here were similar to results from Chapter Four (Figure 4.4). After treatment, there was a significant increase in CD45RA expression on the CXCR4⁺ CD8⁺ cells and on the CCR5⁺ CD8⁺ cells ($P<0.05$), and a significant reduction in CD45RO expression on both CXCR4⁺ CD4⁺ and CXCR4⁺ CD8⁺ ($P<0.05$) cells.

5.3.6 Expression of CD38 on CXCR4-expressing and CCR5-expressing lymphocytes

The proportion of both CXCR4- and CCR5- expressing lymphocytes that expressed the activation marker CD38 was assessed. The HIV-1 infected patients at BL showed significantly larger numbers of both CD38-expressing CXCR4⁺ (median 69.3%) ($P<0.05$) and CCR5⁺ lymphocytes (median 78.1%) ($P<0.001$) when compared with the normal donors (CXCR4: median 59%; CCR5: median 40.5%). After HAART the expression of CD38 on both CXCR4⁺ (median 60.5%) and CCR5⁺ (median 54%) lymphocytes was significantly reduced ($P<0.05$) compared to BL. While the expression of CD38 after HAART was not significantly different from the normal donors on CXCR4⁺ lymphocytes, it was still significantly elevated when compared to normal donors levels on the CCR5⁺ lymphocytes ($P<0.05$).

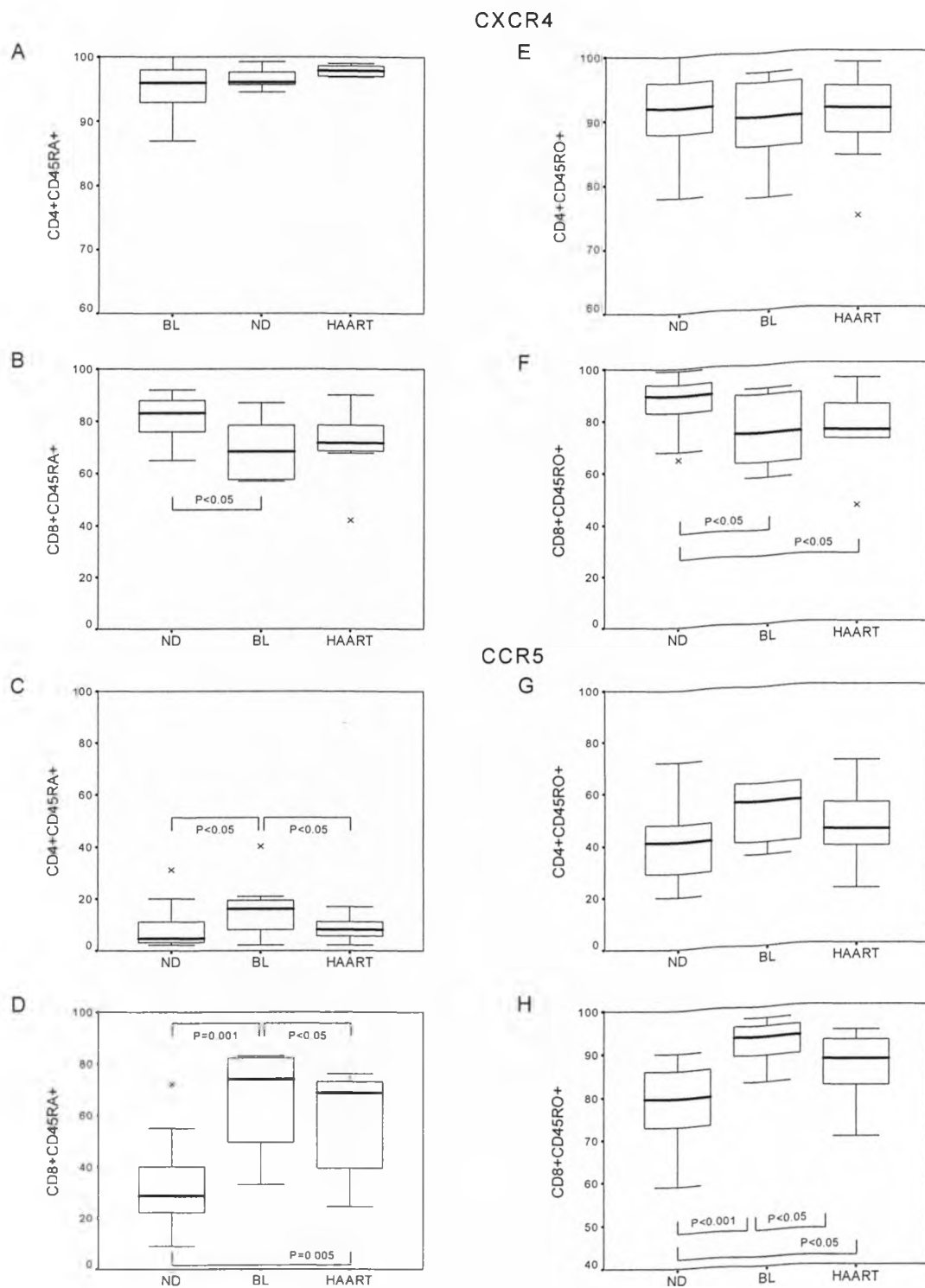


Figure 5.5 Expression of CXCR4 and CCR5 on naive (CD45RA) and memory (CD45RO) CD4⁺ and CD8⁺ lymphocytes. (A) CXCR4-expressing CD4⁺CD45RA⁺ cells, (B) CXCR4-expressing CD8⁺CD45RA⁺ cells, (C) CCR5-expressing CD4⁺CD45RA⁺ cells, (D) CCR5-expressing CD8⁺CD45RA⁺ cells, (E) CXCR4-expressing CD4⁺CD45RO⁺ cells, (F) CXCR4-expressing CD8⁺CD45RO⁺ cells, (G) CCR5-expressing CD4⁺CD45RO⁺ cells, and (H) CCR5-expressing CD8⁺CD45RO⁺ cells. Results are shown as percentages of cells. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

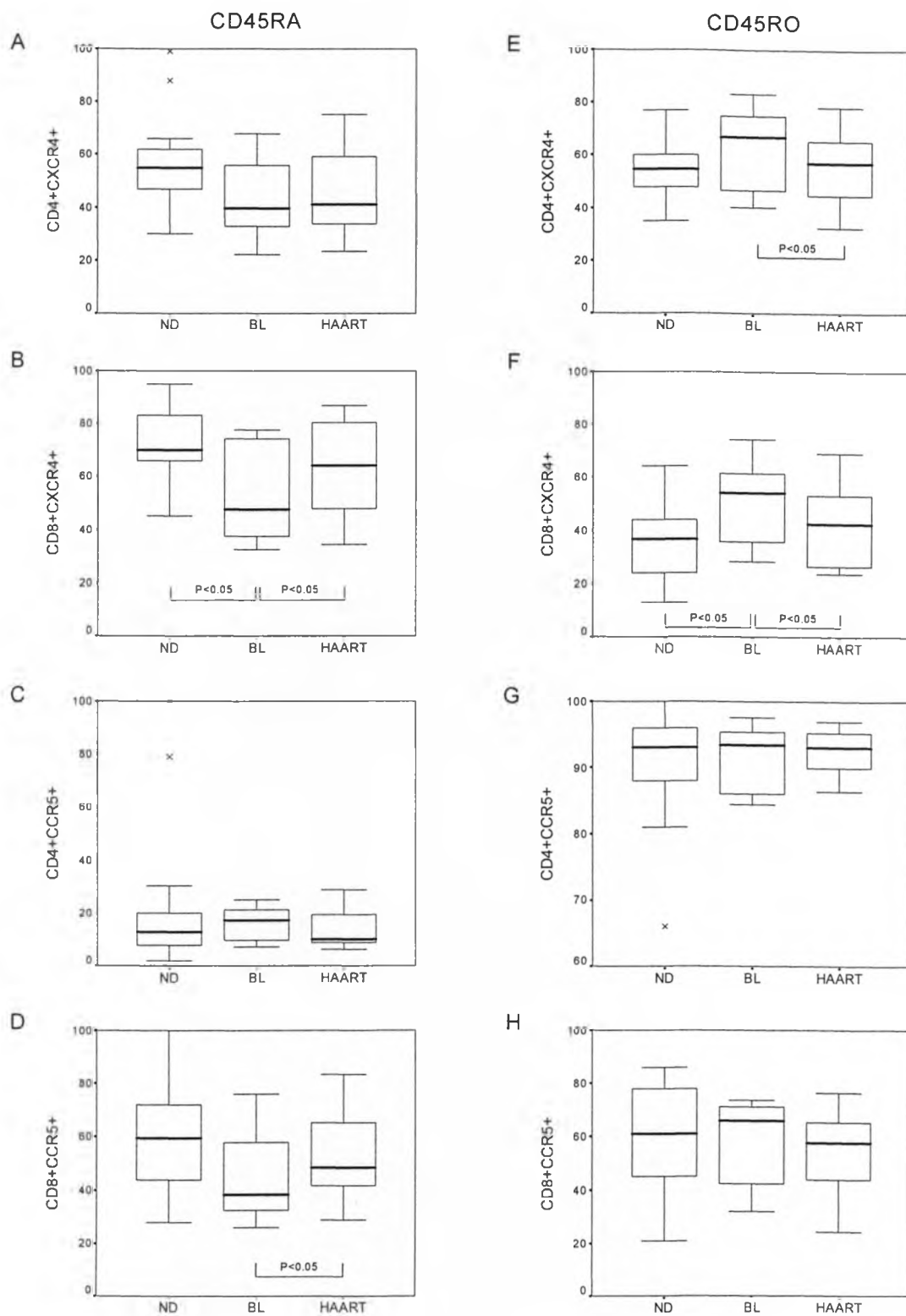


Figure 5.6 Expression of CD45RA and CD45RO on CXCR4- and CCR5-expressing CD4⁺ and CD8⁺ lymphocytes. (A) CD45RA expressing CD4⁺CXCR4⁺ cells, (B) CD45RA expressing CD8⁺CXCR4⁺ cells, (C) CD45RA expressing CD4⁺CCR5⁺ cells, (D) CD45RA expressing CD8⁺CCR5⁺ cells, (E) CD45RO expressing CD4⁺CXCR4⁺ cells, (F) CD45RO expressing CD8⁺CXCR4⁺ cells, (G) CD45RO expressing CD4⁺CCR5⁺ cells, and (H) CD45RO expressing CD8⁺CCR5⁺ cells. Results are shown as percentages of cells. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated.

5.4 Discussion

Chemokine receptors can be modulated by a number of infectious diseases, including infection with HIV (Forster *et al.*, 1998; Giovannetti *et al.*, 1999; Ostrowski *et al.*, 1998). Given that HAART is important in initiating the restoration of immune function, and results in a generalised inhibition of systemic immune activation (Andersson *et al.*, 1998), it was considered important to question whether HAART could play a role in restoring levels of CXCR4 and CCR5 expression, and to identify if CXCR4 and/or CCR5 could be used as markers of immune reconstitution in response to HAART in conjunction with CD4⁺ T cell counts and viral load. Our justification for evaluating response to HAART at a relatively early timepoint (8 weeks) was that HAART reduces viral load within one month (Holodniy *et al.*, 1993; Holodniy *et al.*, 1991), and thus may have a concomitant early effect on coreceptor expression. Nicholson *et al.* (Nicholson *et al.*, 2001) demonstrated normalization of CCR5 expression (but not CXCR4) at three to four weeks after initiation of therapy, while in other studies the first post-therapy evaluation of coreceptor expression was carried out after three months of therapy (Giovannetti *et al.*, 1999; Giovannetti *et al.*, 2001; Pierdominici *et al.*, 2002). This study therefore evaluated the early effects of HAART on the expression of CXCR4 and CCR5 on leukocyte subsets in asymptomatic antiretroviral-naive South African HIV infected individuals.

Results obtained in this study confirmed that antiretroviral-naive HIV-1 infected individuals have reduced CXCR4 and elevated CCR5 expression on most leukocyte subsets when compared to ND, as was previously demonstrated in Chapter Four. When the CD4⁺ and CD8⁺ subsets were analysed further, reduced CXCR4 expression on both CD8⁺CD45RA⁺ and CD8⁺CD45RO⁺ subsets, and elevated CCR5 expression on CD4⁺CD45RA⁺ and both CD8⁺CD45RA⁺ and CD8⁺CD45RO⁺ subsets, with a trend to this effect on the CD4⁺CD45RO⁺ subsets was found. It is noteworthy that expression of both CXCR4 and CCR5 was more profoundly regulated on CD8⁺ cells than on CD4⁺ cells, as has been previously observed in some studies (Giovannetti *et al.*, 1999), but not in others (Nicholson *et al.*, 2001; Ostrowski *et al.*, 1998). In addition, here higher proportions of CXCR4- and CCR5-expressing cells have been found than in other studies. This may, in addition to other factors (including differences in sample preparation), be environmentally driven, as individuals living in Africa have higher levels of immune activation (Rizzardini *et al.*, 1998). A study by Clerici *et al.* (Clerici *et al.*, 2000a) showed an increased expression of CCR5

in African compared with Italian residents, while Kalinkovich *et al.* (Kalinkovich *et al.*, 2001) found CXCR4 and CCR5 expression was elevated in Ethiopians evaluated shortly after their arrival in Israel, as well as in those Ethiopians living in Israel for longer periods. In addition, these new arrivals had significantly higher percentages of CD8⁺CD38⁺ cells (mean of 53.4%) in comparison with Ethiopians who had been living in Israel for more than seven years (mean of 42.5%) and non-Ethiopians (mean of 34.5%). Of significance in this present study was the substantially higher percentage of CD8⁺CD38⁺ cells in uninfected and HIV-1 infected individuals living in South Africa in comparison with percentage of CD8⁺CD38⁺ cells from European and North American studies, thus confirming the heightened immune activation in this population. Interestingly, the percentage of CD8⁺CD38⁺ cells found for the new Ethiopians in Israel was very similar to that found in this study for the healthy donors, thus suggesting levels of immune activation in both African groups.

No significant increase in CXCR4 expression was evident on any of the leukocyte subsets from HIV-1 infected patients following treatment. In contrast, there was a significant reduction in the proportions of CD3⁺, CD4⁺, CD8⁺, CD19⁺ and CD14⁺ cells that expressed CCR5. In addition, when comparing the levels of CCR5 expression after HAART to that of the ND group, only the CD3⁺CCR5⁺ and the CD8⁺CCR5⁺ remained significantly different, suggesting overall that HAART played a role in the normalization of CCR5 expression. Additionally, these results were paralleled by increases in the expression of CD45RA on CD8⁺CXCR4⁺ and CD8⁺CCR5⁺ cells, as well as decreases in CD45RO expression on both CD4⁺CXCR4⁺ and CD8⁺CXCR4⁺ cells. Furthermore, a reduction in the levels of CD38 expression was observed on both CXCR4- and CCR5-expressing lymphocytes, suggesting decreased immune activation. Thus, the reduction in CCR5 expression is accompanied by an increase in CD45RA expression, and a decrease in CD45RO and in CD38 expression.

A number of studies have evaluated CXCR4 and CCR5 expression on CD4⁺ and CD8⁺ cells in the context of HAART, and while reduction of CCR5 expression has been consistently demonstrated (Giovannetti *et al.*, 1999; Giovannetti *et al.*, 2001; Nicholson *et al.*, 2001; Pierdominici *et al.*, 2002; Smith *et al.*, 2002), reports of CXCR4 are not as consistent. Some studies have demonstrated an increase of CXCR4 expression on CD4⁺ T cells (Giovannetti *et al.*,

1999), others a reduction in its expression (Giovannetti *et al.*, 2001), while further studies observed no significant alteration in CXCR4 expression in response to therapy (Nicholson *et al.*, 2001; Pierdominici *et al.*, 2002; Smith *et al.*, 2002). The explanation for these inconsistencies is currently unknown. In our study, CXCR4 expression increased in four patients, remained largely unchanged in one patient, and decreased in three patients eight weeks after starting treatment. A possible explanation for the discrepancy in response to therapy, may be the significantly lower absolute CD4⁺ T cell count and percentage of CD4⁺ T cells at BL, in the patients that had a reduction in CXCR4 expression, when compared to the patients where the CXCR4 expression increased or was unchanged. In addition, the patients with the decrease in CXCR4 expression had a lower proportion of naive CD45RA⁺ CD4⁺ cells and a higher proportion of memory CD45RO⁺ CD4⁺ cells (data not shown). Further, patient seven was subsequently shown to have virus resistant to one NRTI and two NNRTIs. CXCR4 is very easily modulated, both *ex vivo* (Chapter Two) and *in vivo* (Chapter Four). In the latter study the presence of tuberculosis (TB) and/or anti-TB therapy had a profound effect on reducing CXCR4 on all cell subsets analysed. It was therefore ensured that no patients included in this study had received any antibiotic therapy, as effects on coreceptor expression are unknown. Additionally, it is not known if different antiretroviral regimens might affect the expression of CXCR4 (or CCR5) differently.

A reduction in CD45RA⁺ cells and concomitant increase in CD45RO⁺ cells has been frequently observed in HIV-1 infected individuals (Benito *et al.*, 1997; Mahalingam *et al.*, 1996; Roederer *et al.*, 1995). The introduction of HAART in patients with relatively advanced disease results in an initial rise in both naive and memory CD4⁺ and CD8⁺ cells, followed by an increase in naive CD4⁺ and CD8⁺ cells, accompanied by a decrease in circulating memory CD8⁺ cells (Autran *et al.*, 1997; Pakker *et al.*, 1998). As expression of CXCR4 and CCR5 has previously been shown to be associated with a naive and memory phenotype, respectively (Bleul *et al.*, 1997; Forster *et al.*, 1998), it was important to determine whether the alterations in coreceptor expression observed in HIV-1 infection were due to the underlying change in CD45RA/RO proportions (Giovannetti *et al.*, 1999) rather than, or in addition to, shifts in expression within the subsets (Blanco *et al.*, 1999; Nicholson *et al.*, 2001). With respect to CXCR4 expression on naive and memory CD4⁺ cells, there were no significant alterations compared with healthy individuals, suggesting that shifts in CD45RA and CD45RO proportions, rather than downregulation of

CXCR4 itself were responsible for the observed reduction in this coreceptor on CD4⁺ cells. In contrast, no change in the expression of CD45RA and CD45RO on CCR5-expressing CD4 cells was found, while increased CCR5 expression on both CD45RA⁺ CD4⁺ and CD45RO⁺ CD4⁺ subsets was observed, suggesting that the increased CCR5 expression on CD4⁺ cells was not due to alterations in proportions of CD45RA and CD45RO cells, but rather due to upregulation of the CCR5 coreceptor. With respect to the CD8⁺ subsets, downmodulation of CXCR4 on both naive and memory CD8⁺ cells, and upregulation of CCR5 on both naive and memory and CD8⁺ cells occurred, suggesting that changes in coreceptor expression were caused by shifts in CD45RA/RO expression, in addition to changes in expression within subsets.

Although no significant correlation was found between activation markers (CD38 expression on CXCR4- and CCR5-expressing lymphocytes and on CD8⁺ cells) and CCR5 and CXCR4 expression on various cell subsets, the reduction in immune activation paralleled the reduction in CCR5 expression, and is likely to play a role in the normalization of CCR5 expression as suggested previously (Giovannetti *et al.*, 1999; Nicholson *et al.*, 2001; Pierdominici *et al.*, 2002).

Immunological functions that may be affected by altered CXCR4 and CCR5 expression includes cell signaling and subsequent trafficking of specific leukocyte subsets. As no significant improvement in CXCR4 expression with HAART was found, there may remain an impaired response to SDF-1. CCR5 expression, although decreased by treatment, still remained significantly higher than normal donor levels in CD3⁺ and CD8⁺ subsets, indicating that these cells in particular may be hyper-responsive to MIP-1 α , MIP-1 β and RANTES. However, restoration of immune function in all other immune cell subsets analysed would be expected.

In summary, results show that CXCR4 and CCR5 expression is altered due to HAART, which to some extent normalised the expression of CCR5. It was also apparent that different cell types respond differently, and that immune restoration likely occurs in an orchestrated manner over time of treatment. Perhaps, the most striking finding was the generalised heightened immune activation in South Africans (both in healthy controls and in HIV-1 infected patients) that is probably environmentally driven. This study therefore provides the first documented report on

changes in coreceptor expression in response to HAART in a highly activated population. Furthermore, despite evidence of heightened immune activation in African individuals, the virological response to HAART in this study was as efficient as reported in non-African persons. Given these findings, CCR5 may provide an additional immunophenotypic marker that indicates early immune reconstitution following HAART.

CHAPTER SIX

QUANTITATION OF CXCR4 AND CCR5 EXPRESSION ON PERIPHERAL BLOOD LEUKOCYTES

6.1 Introduction

Results from previous Chapters have yielded information with regard to the percentage of cells expressing CXCR4 and CCR5. However, recent work has indicated the importance of measuring the amount or density of antigen expression in addition to the frequency of cells expressing the antigen. Knowledge of the level of expression of an antigen on the surface of a cell is becoming progressively more important in furthering the understanding of cell function and disease progression. For example, the intensity of CD38 expression on CD8⁺ lymphocytes from HIV-1 infected individuals is a useful prognostic tool for the development of AIDS (Liu *et al.*, 1997; Liu *et al.*, 1996b). Of importance with respect to quantitation of the HIV-1 coreceptors is that CCR5 surface density on CD4⁺ T cells has been demonstrated to correlate with HIV-1 viral load (Reynes *et al.*, 2000), and rate of decline of CD4⁺ T cells (Reynes *et al.*, 2001).

In Chapter Three, the proportion of cells expressing CXCR4, and CCR5 in different age groups was evaluated. The proportions of CXCR4-expressing cells was highest in cord blood (Figure 3.1), and thereafter declined at 4.5 months and then increased to adult levels. For example, a median of 98.9% of CD3⁺ lymphocytes expressed CXCR4 in cord blood and this decreased to 96.1% in the adult group. While the reduction in percentage of CXCR4-expression from cord blood levels to adult levels was significant, the differences were very small. In contrast, the proportions of cells expressing CCR5 are very low in cord blood and gradually increase with age (Figure 3.2). The percentage of CD3⁺ lymphocytes that express CCR5 gradually increased from 1.3% in cord blood to 35.5 % in adults (Figure 3.2A). Thus, there was a relatively large increase in percentage of CCR5-expressing cells from cord blood levels to adult levels.

Individuals homozygous for a 32 bp deletion within the CCR5 gene are highly resistant to infection with HIV-1 (Dean *et al.*, 1996; Huang *et al.*, 1996; Samson *et al.*, 1996a). This deletion results in an unstable truncated form of the protein which is not expressed at the cell surface (Liu *et al.*, 1996a). Those HIV-1 infected individuals heterozygous for the 32 bp deletion, present with a slower decline in CD4⁺ T cell counts, lower viral loads, and increased time for progression to AIDS (Dean *et al.*, 1996; Huang *et al.*, 1996; Zimmerman *et al.*, 1997). In addition, previous work has shown that individuals heterozygous for the 32 bp deletion have decreased percentages of CCR5-expressing CD4⁺ T cells as compared with individuals without the mutation (de Roda Husman *et al.*, 1999; Wu *et al.*, 1997b).

The main objective of this study was to establish the methodology for quantitative flow cytometry as a more sensitive technique to analyse the expression of CXCR4 and CCR5. In order to demonstrate the additional value of this methodology, it was firstly questioned whether the results obtained for percentages of cord blood and adult cells expressing either of the coreceptors would be further reflected in terms of the number of antibodies bound per cell (ABC), a measure of receptor density. Secondly, as a reciprocal relationship between CXCR4 and CCR5 expression was generally observed in previous Chapters, it was hypothesised that lower CCR5 surface expression in CCR5 Δ 32/wt individuals relative to CCR5wt/wt individuals would result in a reciprocal increase in the number of CXCR4 ABC in these individuals.

6.2 Materials and methods

6.2.1 Study participants

To analyse whether differences in the number of CXCR4 and CCR5 ABC exist between cord blood and adult leukocytes, a cross-sectional analysis was performed on a total of 30 subjects, comprising whole blood samples from cord blood of ten infants born to HIV-1 seronegative mothers, and from 20 healthy adults, with a median age of 38.5 (range 25-62 years).

To question whether differences in the number of CXCR4 and CCR5 ABC exist between individuals heterozygous for the CCR5 Δ 32 deletion (CCR5 Δ 32/wt) and individuals without the mutation (CCR5wt/wt), a cross-sectional study was performed on a total of 20 healthy individuals

with no known disorders that might affect chemokine receptor expression, comprising 9 CCR5 Δ 32/wt individuals and 11 CCR5wt/wt individuals, with a median age of 38 (range 26-62 years), and 39 (range 25-57), respectively.

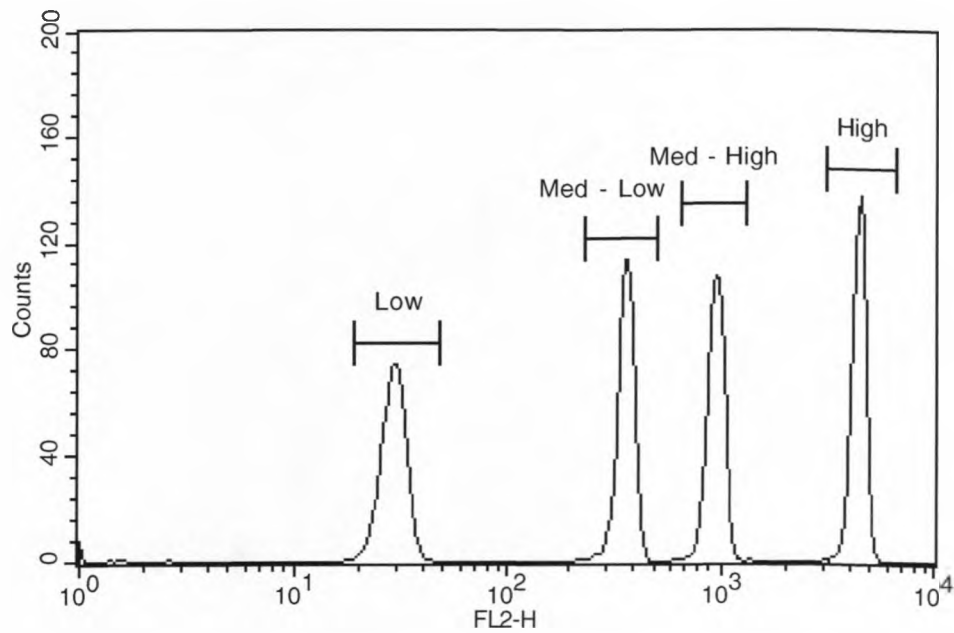
6.2.2 Quantitation

Quantitation was carried out using the commercially available QuantiBRITE system (Becton Dickinson, San Jose, Calif.). This system consists of a set of four beads that have different levels of PE covalently attached. These beads are used to calibrate the fluorescence 2 (FL2) axis in terms of numbers of PE molecules. The number of PE molecules/bead varies between lots, and lot-specific information is included in each kit. A single lot of QuantiBRITE beads, carrying 1400, 14000, 36600, and 182000 PE molecules, was used for all experiments. When used together with PE conjugates with a known ratio of PE to antibody, PE molecules can be converted to the number of ABC. Whole blood samples were stained within 6h of collection using saturating concentrations of highly purified PE-labelled antibodies to CXCR4 and CCR5 with a PE molecule-to-antibody ratio of 1:1. Either CXCR4 or CCR5 was added together with antibodies to identify CD3⁺CD4⁺ and CD3⁺CD8⁺ lymphocytes, NK cells, CD19⁺ B cells, and CD14⁺ monocytes. Granulocytes were identified based on their forward and side scatter characteristics and lack of CD14 staining. (Appendix B Panel1). Quantitation was performed gating on coreceptor-expressing cells.

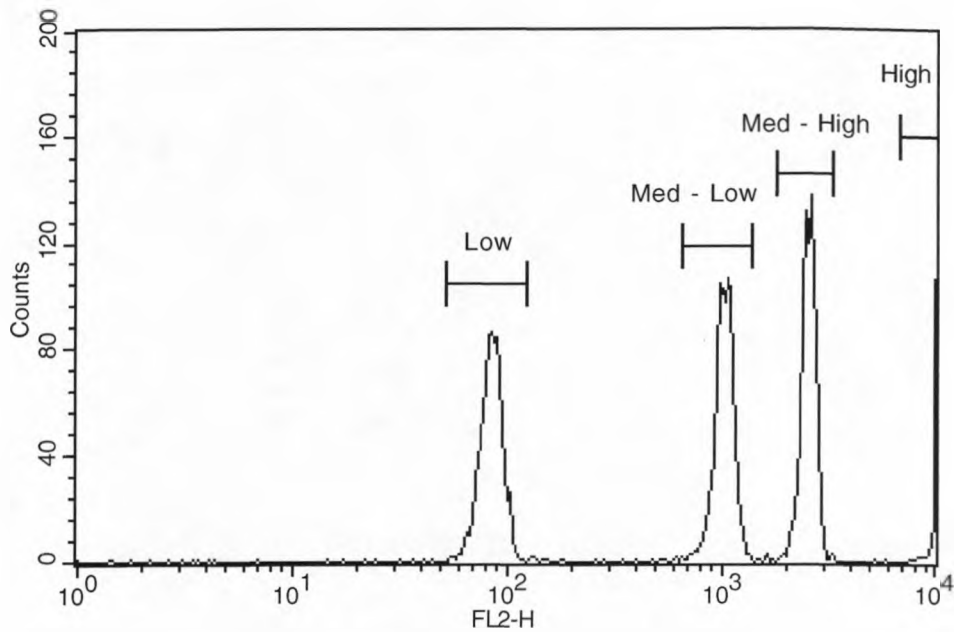
6.2.2.1 Instrument setup

The QuantiBRITE system has been specifically adapted for use with the lyse-no wash staining technique. As all of our previous studies were carried out using a lyse-wash procedure, we continued with this technique for all quantitation analyses. However, the photomultiplier tube voltages and compensation settings that were necessary to visualise all bead populations on the histogram, as shown in Figure 6.1A for the lyse-no wash method, were not optimal for samples stained using the lyse-wash technique. Thus, the beads were acquired on the flow cytometer using settings optimised for the lyse-wash technique, and the 4th bead population was then not completely on scale (Figure 6.1B). All samples were acquired on a FACSort flow cytometer using the same instrument settings as those used for the bead acquisition.

A



B



C

Marker	Geo Mean
All	1157.34
Low	82.71
Med - Low	1001.45
Med - High	2460.81
High	9878.65

Figure 6.1 Histogram of QuantiBRITE beads using instrument settings optimised for (A) lyse-no wash and (B) lyse-wash sample acquisition. Geometric means for histogram B are shown (C). Low:1400, Med-Low:14000, Med-High:36600, High:182000 PE molecules/bead.

6.2.2.2 Conversion of CXCR4 and CCR5 MFI to the number of antibodies bound per cell

In order to convert the MFI of CXCR4 and CCR5 to the number of ABC, a linear regression equation was calculated using the FL2 geometric means and the PE molecules per bead from the QuantiBRITE system. This equation was then used to convert the geometric mean of the cell population of interest into the number of ABC. As the 4th bead population was not completely on scale, it was excluded from the analysis in order to avoid inaccuracies in calculation of the regression curve, therefore only the first three bead populations were used.

6.2.2.3 Example of calculation

The geometric means of the bead population from the histogram in Figure 6.1B are shown in Figure 6.1C. \log_{10} for the geometric means and for the PE molecules per bead from the first three bead populations was calculated (Table 6.1), and a linear regression of \log_{10} FL2 against \log_{10} PE molecules per bead plotted using the following equation: $y = mx + c$

where $y = \log_{10}$ FL2 and $x = \log_{10}$ PE molecules per bead (Figure 6.2).

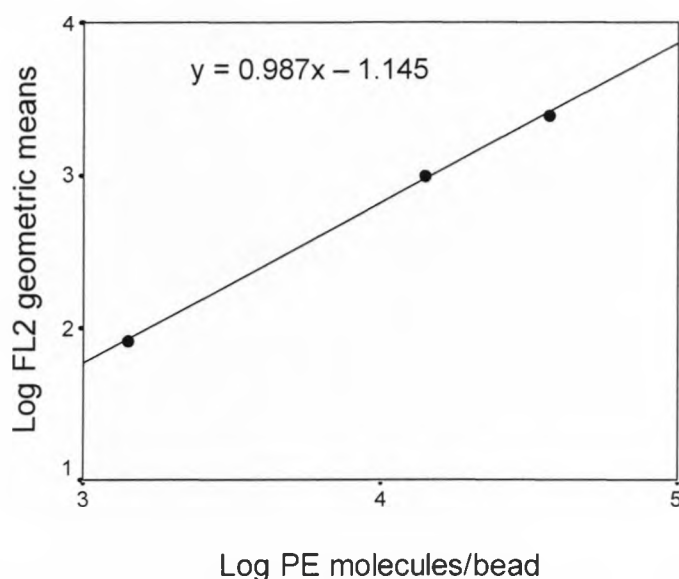


Figure 6.2 Linear regression of \log_{10} PE molecules per bead against \log_{10} FL2 using the following equation: $y = mx + c$.

Table 6.1 Calculation of \log_{10} for the FL2 geometric means and for the PE molecules per bead

FL2 Geometric Means	Log FL2	PE Molecules/Bead	Log PE Molecules/Bead
82.71	1.918	1400	3.146
1001.45	3.001	14000	4.146
2460.81	3.391	36600	4.563

The regression curve for this calculation was: $y = 0.987x - 1.145$

Next, the ABC for the different cellular subsets was determined by substituting \log_{10} FL2 geometric means into the equation and solving for \log_{10} ABC. The anti-log was then determined.

As an example, the geometric mean for CXCR4-expressing CD3⁺ cells was 435.69

First $\log_{10} 435.69 = 2.639$

From $y = 0.987x - 1.145$, therefore solve for x:

$$2.639 = 0.987x - 1.145$$

$$x = \frac{2.639 + 1.145}{0.987}$$

$$0.987$$

$$x = 3.8338 \log_{10} \text{ PE molecules per cell}$$

The number of PE molecules per cell = 6820

As the PE:mAb ratio in this study was 1:1, then the ABC is 6820.

6.2.3 Statistical analysis

Comparison of CCR5 and CXCR4 ABC between different study groups was done using the Mann-Whitney U test.

6.3 Results

In this study it was important to measure the density of CCR5 and CXCR4 expression, in terms of number of ABC, in addition to the frequency of cells expressing these antigens in order to compare results obtained with the two different methods. Results were therefore expressed as both the percentage of cells expressing the antigen of interest, as well as the number of ABC. The cell subsets evaluated were CD3⁺, CD4⁺, CD8⁺, CD19⁺, CD14⁺ and NK cells for both CCR5 and CXCR4 expression. Granulocytes were analysed for CXCR4 expression only.

6.3.1 CXCR4 and CCR5 ABC on leukocyte subsets from adult samples

Although CXCR4 was expressed on a higher percentage of all of the subsets evaluated (as demonstrated previously in Chapter Three), the number of CCR5 ABC was higher than the number of CXCR4 ABCs on the total CD3⁺ cells (median 2839 ABC vs. 2329 ABC), CD3⁺CD8⁺ cells (median 2947 ABC vs. 2192 ABC) and NK cells (1515 ABC vs. 1282 ABC). (Table 6.2 and Table 6.3). The number of CXCR4 ABC varied substantially among various subsets examined. CD19⁺ B cells (median 4402 ABC) expressed the highest amount of CXCR4, followed by the CD3⁺CD4⁺ lymphocytes and CD3⁺ CD8⁺ lymphocytes, with NK cells expressing the smallest amount (1282 ABC). The converse was true for the number of CCR5 ABC on B cells, where these cells expressed the lowest levels (901 ABC) with CD3⁺CD8⁺ cells expressing the highest levels (2947 ABC).

6.3.2 CXCR4 and CCR5 ABC on leukocyte subsets from cord blood samples

CXCR4 was expressed on a higher percentage of cord blood leukocyte subsets than was CCR5 (as demonstrated previously in Chapter Three), and the number of ABC for CXCR4 was also higher than for CCR5 (Table 6.2 and Table 6.3). Similarly to the adult subsets, CD19⁺ B cells (median 7737 ABC) expressed the highest amount of CXCR4, with NK cells expressing the smallest amount (1591 ABC). CD19⁺ B cells expressed the lowest levels (906 ABC), with CD14⁺ monocytes expressing the highest levels (median 1773 ABC) of CCR5 ABC.

6.3.3 Comparison of CXCR4 and CCR5 expression on leukocytes from cord blood and adult samples

Data were first analysed according to percentage of cells expressing either CXCR4 or CCR5. CXCR4 expression was only found to be significantly lower in the adult group in comparison with the cord blood group on the CD3⁺CD8⁺, CD19⁺ cells and on granulocytes (Table 6.2). The percentage of CCR5-expressing cells was significantly higher in the adult group when compared to the cord blood group on all cell subsets, except for CD19⁺ B cells and CD14⁺ monocytes (Table 6.3). Results from Chapter Three demonstrated that CXCR4 expression was significantly lower in the adult group on all subsets in comparison with the cord blood group, and CCR5 expression was significantly higher on all cell subsets with the exception of CD19⁺ B cells. The reason for this discrepancy in results is likely due to sample size, as 27 cord blood samples were evaluated in Chapter Three, and only 10 samples were analysed in this quantitation study. On evaluation of the number of CXCR4 ABC (Figure 6.3 and Table 6.2), all cellular subsets analysed from the adult group, except NK cells, were found to have significantly lower levels of CXCR4 ABC than the corresponding subset from the cord blood group. In addition, while differences in percentages of CXCR4-expressing cells between cord and adult samples were small, for example a median of 99.1% of cord blood CD3⁺ lymphocytes expressed CXCR4 compared with a median of 97.8% of adult CD3⁺ lymphocytes, differences in the number of CXCR4 ABC were much larger (5566 ABC for cord blood versus 2329 ABC for adult CD3⁺ lymphocytes) (Table 6.2). Also evident, was the substantial variation in levels of CXCR4 expression on the cord blood samples in comparison with the adult samples. The comparison of the number of CCR5 ABC between cord blood and adult samples is shown in Figure 6.4 and Table 6.3. A significantly higher level of CCR5 ABC was observed in the adult samples when compared with the cord blood samples on the total CD3⁺ cells, CD3⁺CD4⁺, CD3⁺CD8⁺ lymphocytes, and NK cells. Additionally, differences in CCR5 expression between cord and adult blood were large with respect to both percentage of cells (2.7% vs. 36.7%) and number of ABC (1122 ABC vs. 2838.6 ABC).

6.3.4 Comparison of CXCR4 and CCR5 expression on leukocytes from CCR5 Δ 32/wt and CCR5wt/wt individuals

When data were analysed according to percentage of cells expressing either CXCR4 or CCR5,

no difference in CXCR4 expression between the CCR5 Δ 32/wt and wt/wt individuals was observed. However, a significant reduction in CCR5 expression on CD3⁺ (P<0.05) and CD4⁺ (P<0.01) cell subsets from CCR5 Δ 32/wt individuals in comparison with CCR5wt/wt individuals was found, with a trend to this effect on the other subsets (data not shown). On analysis of the number of ABC, an increase in CXCR4 ABC on CD3⁺ cells (P<0.05) was observed from CCR5 Δ 32/wt individuals when compared with CCR5wt/wt individuals, with a trend to that effect on CD4⁺, CD8⁺, NK and CD19⁺ cells (Figure 6.5). Conversely, the number of CCR5 ABC was reduced on CD3⁺ (P<0.001), CD4⁺ (P=0.001), CD8⁺ (P=0.001), NK (P<0.05) and CD14⁺ (P<0.05) cells from the Δ 32/wt individuals when compared with wt/wt individuals, with a trend to that effect on CD19⁺ cells (Figure 6.6). Interestingly, there was a negative correlation between

Table 6.2 Comparison of the percentage CXCR4 positive cells and the number of CXCR4 ABC between cord blood and adult samples^a

Cell subset	CXCR4 percentage cord blood	CXCR4 percentage adult	CXCR4 ABC cord blood	CXCR4 ABC adult
Total CD3 ⁺	99.1 (96.0-99.9)	97.8 (92.6-99.6)	5566.2 (2138.2-10387.1)	2328.7* (1840.5-2914.4)
CD3 ⁺ CD4 ⁺	98.9 (94.2-99.8)	99.0 (97.2-99.8)	4827.4 (1894.5-8821.3)	2591.0* (2052.9-3244.2)
CD3 ⁺ CD8 ⁺	99.9 (91.7-100)	97.2* (86.6-99.4)	7373.7 (3412.9-1153.6)	2192.3* (1457.3-3030.3)
NK	69.1 (51.1-87.1)	75.1 (62.9-92.6)	1590.6 (694.7-3209.9)	1263.2 (746.5-1871.9)
CD19 ⁺	100 (98.8-100)	99.0* (94.0-100)	7737.4 (4492.6-12270.4)	4401.6* (2750.3-6417.2)
CD14 ⁺	95.0 (91.0-98.7)	95.4 (78.3-98.2)	2530.7 (1908.3-4571.8)	1882.9* (1623.8-2399.1)
Granulocytes	57.8 (29.1-82.2)	95.4* (30.6-98.6)	1897.2 (1483.1-2414.0)	1554.9* (1269.9-2469.8)

^aResults are expressed as medians with the range in parentheses

*Significant differences between cord blood and adult samples (P<0.05) are indicated.

the number of CCR5 ABC on CD3⁺ cells ($r = -0.748$, $P < 0.05$) and NK cells ($r = -0.841$, $P < 0.05$) and a positive correlation between the number of CXCR4 ABC on CD4⁺ cells ($r = 0.672$, $P < 0.05$) and the age of the CCR5 Δ 32/wt individuals. However, these correlations were not evident for the CCR5wt/wt individuals.

Table 6.3 Comparison of the percentage CCR5 positive cells and the number of CCR5 ABC between cord blood and adult samples^a

Cell subset	CCR5 percentage cord blood	CCR5 percentage adult	CCR5 ABC cord blood	CCR5 ABC adult
Total CD3 ⁺	2.7 (1.1-9.3)	36.7* (25.5-57.3)	1122.4 (641.9-1875.2)	2838.6* (1603.6-5206.8)
CD3 ⁺ CD4 ⁺	2.1 (0.8-5.7)	29.6* (17.6-39.4)	1011.7 (553.5-4725.0)	1649.1* (1062.4-4474.0)
CD3 ⁺ CD8 ⁺	2.2 (1.2-21.9)	54.1* (34.1-76.3)	1130.8 (307.7-2073.0)	2946.6* (1636.6-8605.2)
NK	10.34 (3.4-27.6)	35.1* (21.8-71.3)	968.2 (517.9-1472.0)	1515.4* (913.9-3127.4)
CD19 ⁺	8.1 (2.6-15.9)	7.5 (3.7-17.1)	905.9 (676.9-2905.1)	901.4 (450.0-1598.5)
CD14 ⁺	11.8 (2.6-15.9)	22.1 (5.5-86.6)	1773.3 (1367-2520.2)	1761.8 (1396.2-3122.3)

^aResults are expressed as medians with the range in parentheses

*Significant difference in between cord blood and adult samples ($P < 0.05$) are indicated.

6.4 Discussion

The quantitation of antigens by flow cytometry has a number of sources of variation that can be due to variability in both instrument and reagents. These include differences in fluorochrome/protein ratios between mAbs batches. Data presented as MFI or as the percentage of positive cells are always relative to controls that are specific for any given experiment. Thus the primary aim of this Chapter was to evaluate a more sophisticated method for measuring coreceptor levels. The use of beads with known numbers of PE molecules per bead, in conjunction with well-characterised PE-conjugated antibodies provides a simple technique to

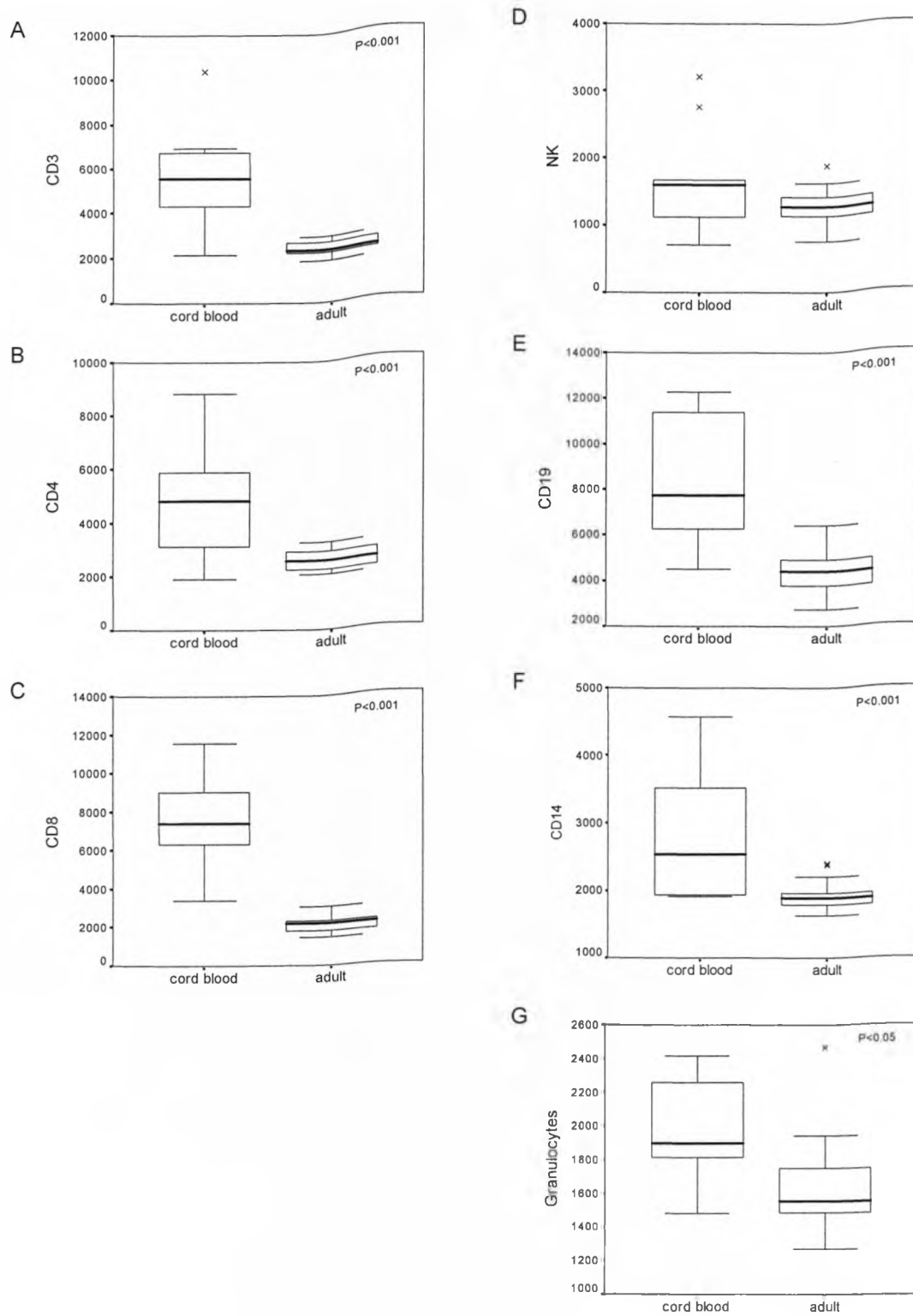


Figure 6.3 Expression of the number of CXC4 ABC on whole blood leukocytes from cord blood and adult samples: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells and granulocytes (G). Results are shown as the number of CXC4 ABC. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between the groups are indicated.

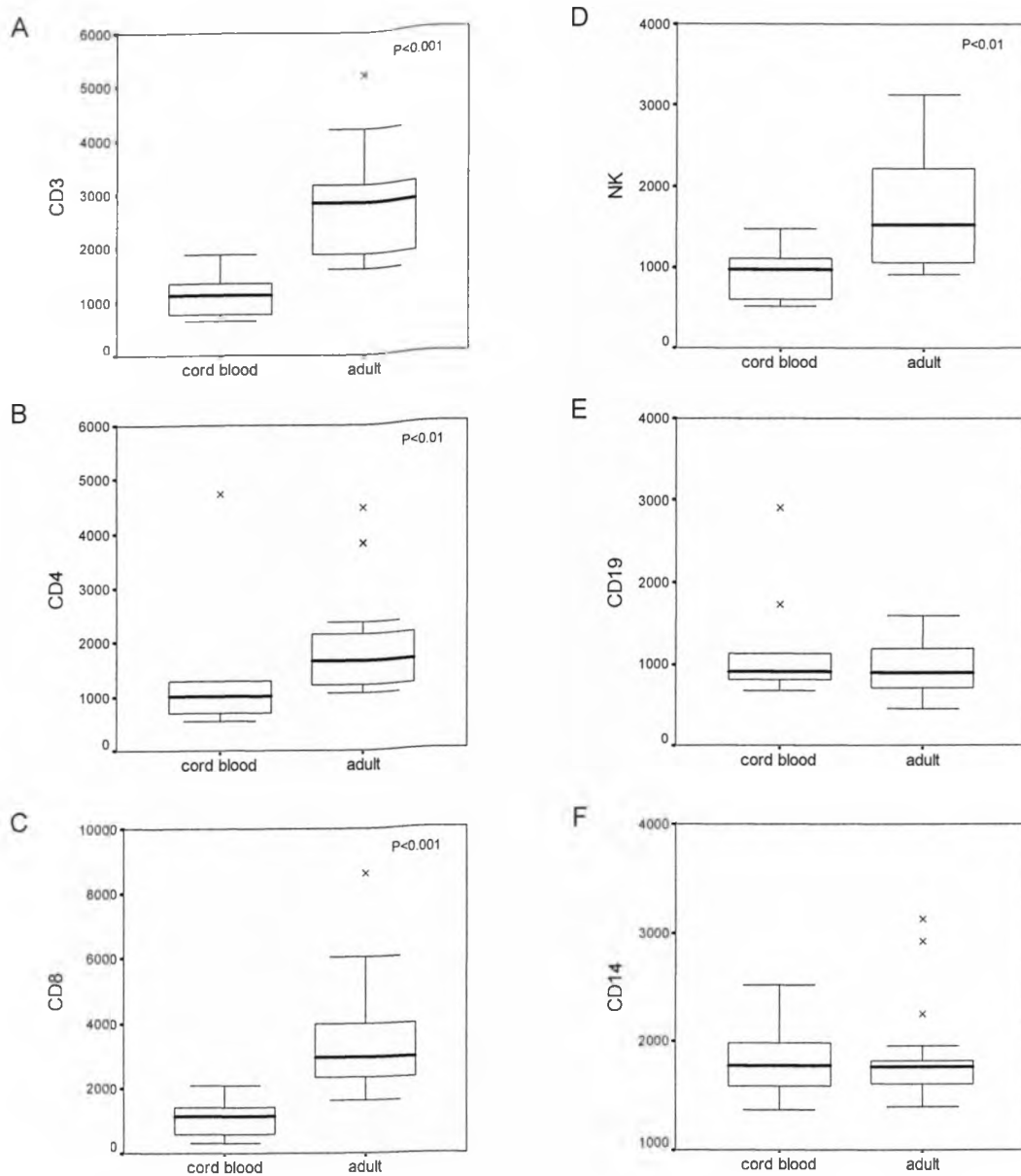


Figure 6.4 Expression of the number of CCR5 ABC on whole blood leukocytes from cord blood and adult samples: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells. Results are shown as the number of CCR5 ABC. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between the groups are indicated.

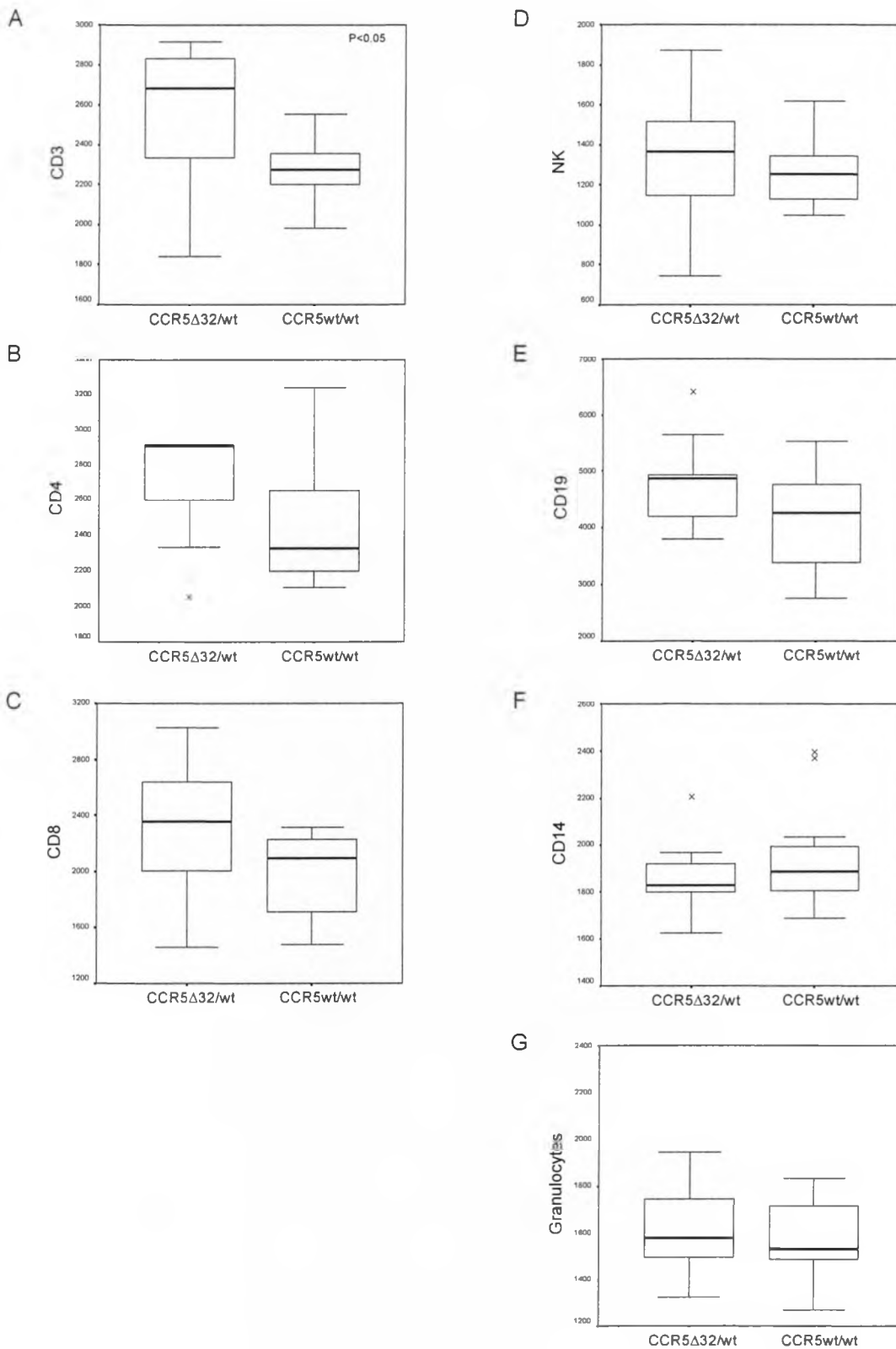


Figure 6.5 Expression of the number of CXCR4 ABC on whole blood leukocytes from CCR5 Δ 32/wt individuals and CCR5wt/wt individuals: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells and granulocytes (G). Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between the groups are indicated.

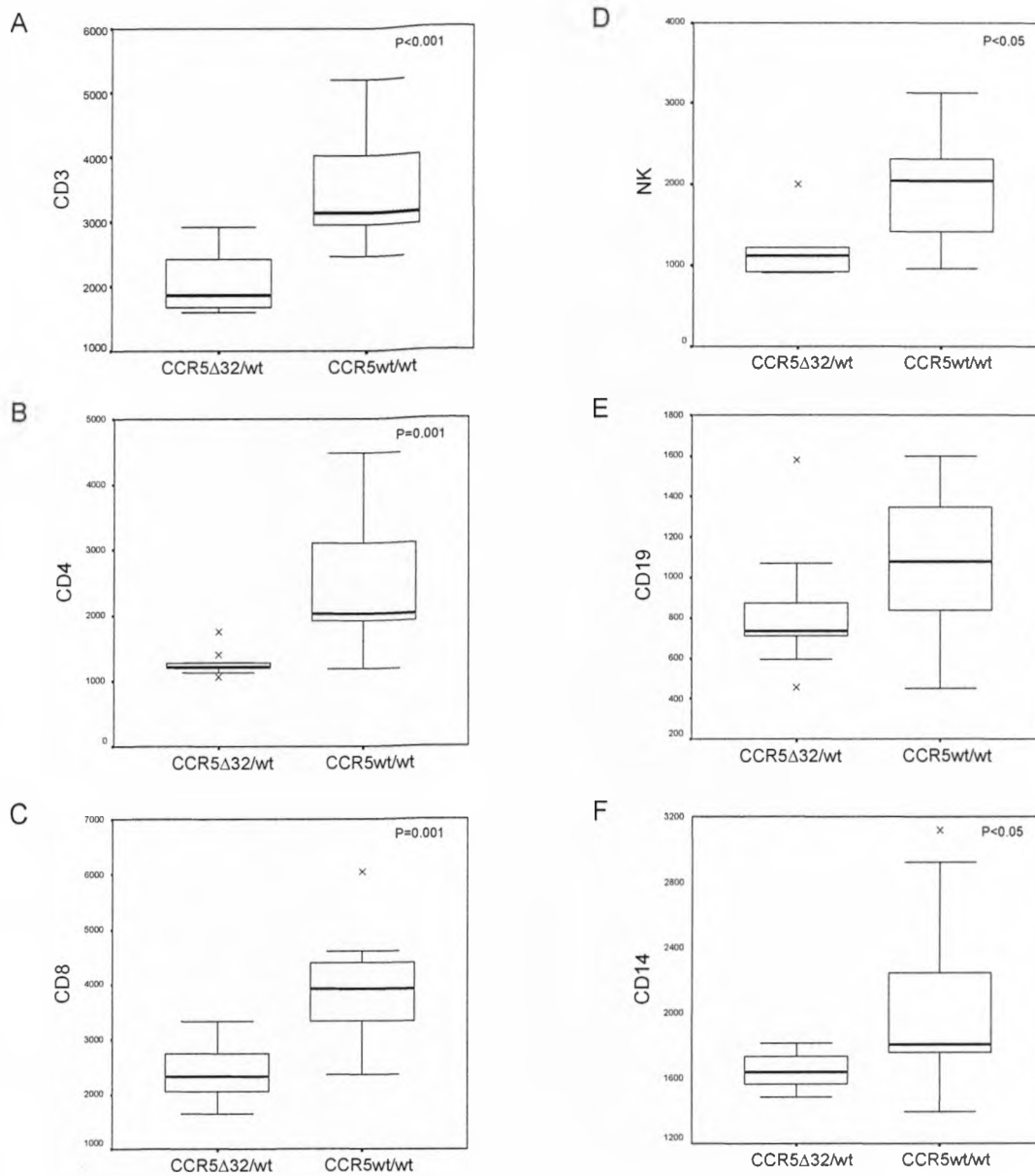


Figure 6.6 Expression of the number of CCR5 ABC on whole blood leukocytes from CCR5 Δ 32/wt individuals and CCR5wt/wt individuals: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), and CD14 (F) cells. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between the groups are indicated.

quantify CXCR4 and CCR5 levels using flow cytometry. Thus, the Quantibrite bead system, that is independent of the limitations described above, was used to determine the number of coreceptor ABC for both adult and cord blood samples. An objective of the present study was to quantitate CXCR4 and CCR5 on peripheral blood leukocyte subsets and to compare these results with the percentages of cells expressing the coreceptors. In this study it was observed that although a larger proportion of all leukocyte subsets evaluated expressed CXCR4 than CCR5, this was not always reflected in terms of the number of ABC. CD3⁺ cells, CD3⁺CD8⁺ cells and NK cells expressed higher levels of CCR5 ABC than CXCR4 ABC. In agreement with the findings of Lee *et al.* (Lee *et al.*, 1999b), CD19⁺ B cells expressed the highest number of CXCR4 ABC, with NK cells expressing the smallest number, while the highest number of CCR5 ABC was expressed on CD3⁺CD8⁺ cells, and the lowest number on CD19⁺ B cells.

The results from our work revealed differential expression of CXCR4 and CCR5 between cord and adult blood. All cord blood cell subsets evaluated in this study, except NK cells, were found to have significantly higher numbers of CXCR4 ABC in comparison with adult subsets. In contrast to the small differences in the percentage of cells positive for CXCR4 between cord blood and adult samples, large differences in the number of ABC between cord and adult blood were observed. This is in contrast with a previous study where MFI was similar on cord blood and adult T cells (Mo *et al.*, 1998). In addition, higher variability in the numbers of CXCR4 ABC in the cord blood group than the adult group was found. In contrast to the higher CXCR4 ABC in the cord blood group, these samples had significantly lower numbers of CCR5 ABC when compared with adult samples, and differences in CCR5 expression, between the cord blood and adult groups were large with respect to both percentage and number of CCR5 ABC. Of interest, was the finding that no significant difference in the number of CCR5 ABC on CD14⁺ monocytes was observed between cord blood and adult blood. Moreover, cord blood has a greater absolute number of monocytes than does adult blood (Yanase *et al.*, 1986). This has implications for HIV-1 transmission as these cells may be preferentially targeted for infection with CCR5-tropic HIV-1 strains. In addition, the substantially higher number of CXCR4 ABC in the cord blood group in comparison with the adult group may result in faster disease progression in the former group if X4 viral strains are acquired.

As expected CCR5 Δ 32/wt individuals had lower CCR5 expression with respect to both percentage of cells expressing CCR5 as well as the number of ABC than CCR5wt/wt individuals. In addition, there was an increase in the level of CXCR4 ABC on CD3⁺ cells in these individuals, however, this was not reflected in the percentage of cells expressing CXCR4. It is anticipated that the compensatory increase in CXCR4 would be greater in individuals homozygous for the Δ 32 mutation than that found for CCR5 Δ 32/wt individuals. The mechanism underlying this reciprocal increase in CXCR4 expression associated with a lower CCR5 expression is currently unknown. An important function of the chemokine/chemokine receptor system is to control leukocyte migration. SDF-1, as well as MIP-1 α , MIP1- β and RANTES induce chemotaxis of T cells. Thus, individuals heterozygous for the CCR5 Δ 32, may have increased CXCR4 expression on T cells to compensate for the reduction in CCR5 expression on these cells. Although, the CCR5 Δ 32 gene is associated with delayed disease progression, this protective effect is lost when these individuals are infected with SI CXCR4-utilizing HIV-1 strains (Michael *et al.*, 1997a), and the greater CXCR4 receptor density in these individuals begs the question as to whether more rapid disease progression is likely to occur than in CCR5wt/wt individuals, when infected with SI viruses. Why age-related changes were found with respect to CXCR4 and CCR5 ABC in the CCR5 Δ 32/wt heterozygotes and not the CCR5wt/wt individuals is currently not known. This work therefore provides preliminary evidence for a compensatory increase in CXCR4 expression in individuals heterozygous for the CCR5 Δ 32 mutation, identifying the existence of a common axis directly linking the regulation of cell surface expression of CCR5 and CXCR4. This may further help explain why HIV pirates the use of these two particular receptors as the major coreceptors for facilitating its entry into permissive cells.

In summary, quantitation of the HIV coreceptors by measure of ABC, is a more sensitive tool than the determination of percentages of cells expressing the receptors and is therefore particularly valuable for determining minor differences in receptor expression between different study cohorts. Moreover, the use of this quantitative flow cytometric technique may be useful as a monitoring tool for the efficacy of drugs designed to reduce CCR5 expression. Additionally, monitoring the number of CXCR4 and CCR5 ABC may provide information that may be useful as adjunct to CD4⁺ T cell counts and HIV-1 viral load for monitoring the efficacy of standard antiretroviral therapy.

CHAPTER SEVEN

RELATIONSHIP BETWEEN SDF-1 α , IL-7 AND MIP-1 β LEVELS AND EXPRESSION OF CXCR4 AND CCR5

7.1 Introduction

In previous chapters an extensive evaluation of CXCR4 and CCR5 expression has been carried out using a number of different study cohorts. As SDF-1 and the CC chemokine (RANTES, MIP-1 α , and MIP-1 β) are the ligands for CXCR4 and CCR5, respectively, it was important to examine the plasma levels of these chemokines in relation to age, presence of a CCR5 Δ 32 allele, and infection with HIV-1 and *M. tuberculosis*, and to compare plasma chemokine levels with coreceptor expression. The measurement of RANTES in plasma is problematic as it is stored in platelets, and may be released even under meticulous conditions of sample preparation (Klinger *et al.*, 1995; Malnati *et al.*, 1997), obscuring circulating chemokine levels. MIP-1 α binds to both CCR1 and CCR5, and while MIP-1 β was initially shown to bind to CCR8 (Napolitano & Santoni, 1999), this was later disproved (Garlisi *et al.*, 1999). Thus, MIP-1 β is specific for CCR5. Further, *in vitro* studies have shown that MIP-1 β inhibits M-tropic primary HIV-1 isolates more potently, and downregulates CCR5 expression to a greater extent than does MIP-1 α (Trkola *et al.*, 1998). For these reasons, MIP-1 β was chosen as a representative for the CC chemokine. In addition, as IL-7 and SDF-1 have functions in common, IL-7 was included in these analyses.

SDF-1 is a CXC chemokine that was first cloned from a bone marrow stromal cell line (Tashiro *et al.*, 1993), and identified as a pre-B cell growth stimulating factor (Nagasawa *et al.*, 1994). SDF-1, unlike most chemokines, is constitutively expressed in a broad range of tissues (Jiang *et al.*, 1994; Tashiro *et al.*, 1993), and is a potent chemoattractant for early-stage B cell precursors (D'Apuzzo *et al.*, 1997), monocytes and lymphocytes (Bleul *et al.*, 1996b). In addition, SDF-1 is a chemokine which plays a vital role in development, and mice deficient in this chemokine die perinatally with major defects in cardiac septal formation, gastrointestinal vasculogenesis,

cerebellar development, myelopoiesis and B cell lymphopoiesis (Nagasawa *et al.*, 1996; Tachibana *et al.*, 1998; Zou *et al.*, 1998). SDF-1 is the natural ligand for the seven-transmembrane G-protein-coupled receptor, CXCR4 (Bleul *et al.*, 1996a; Oberlin *et al.*, 1996), which also serves as the major HIV-1 coreceptor utilised by T cell tropic, SI HIV-1 strains (Feng *et al.*, 1996). SDF-1, by binding to CXCR4 has been shown to inhibit virus entry and replication by SI HIV-1 strains (Bleul *et al.*, 1996a; Oberlin *et al.*, 1996) that are usually isolated at late, symptomatic stages of HIV-1 infection.

IL-7, a cytokine produced predominantly by the stromal cells of the bone marrow (Namen *et al.*, 1988) and thymus (Fernandez *et al.*, 1994; Wolf & Cohen, 1992), is essential for the development of the immune system. IL-7 was initially described as a growth factor for precursor B cells (Namen *et al.*, 1988), and was later demonstrated to play a key role in modulating T cell development (Conlon *et al.*, 1989), and in enhancing the function of mature T cells (Gringhuis *et al.*, 1997).

MIP-1, a low m.w. heparin-binding protein doublet consisting of two peptides, MIP-1 α and MIP-1 β , has a number of inflammatory and immunoregulatory functions. Although, the MIP proteins were originally purified from lipopolysaccharide-treated murine monocytic cell lines (Sherry *et al.*, 1988; Wolpe *et al.*, 1988), and were identified as “macrophage inflammatory proteins,” they are produced by a number of cell types including T cells (Brown *et al.*, 1989) and B cells (Krzywiec *et al.*, 1999). At the protein level, mature MIP-1 α and MIP-1 β are 69 amino acids in length, and have a sequence identity of 60% (Sherry *et al.*, 1988; Wolpe & Cerami, 1989). MIP-1 α and MIP-1 β induce chemotaxis of monocytes (Ugucioni *et al.*, 1995), T cells (Schall *et al.*, 1993; Taub *et al.*, 1993), NK cells (Loetscher *et al.*, 1996b; Taub *et al.*, 1995), and dendritic cells (Sozzani *et al.*, 1997; Xu *et al.*, 1996). The two chemokines also have the ability to enhance NK- (Taub *et al.*, 1996; Taub *et al.*, 1995) and CTL-mediated cytotoxicity (Taub *et al.*, 1996). In addition, MIP-1 β can induce adhesion of T cells to endothelial cells (Gilat *et al.*, 1994; Tanaka *et al.*, 1993a).

The role of SDF-1 in HIV-1 disease progression has not been well established. A single

nucleotide polymorphism, found in a conserved part of 3'-untranslated region of the SDF-1 gene that encodes the ligand for CXCR4, had been reported to delay the onset of AIDS (Winkler *et al.*, 1998). The authors postulated that the mutation could increase the amount of SDF-1 available to bind to CXCR4, consequently preventing the emergence of late stage X4 viruses. However, these results were contradicted in other studies (Easterbrook *et al.*, 1999; Mummidi *et al.*, 1998). A positive correlation between SDF-1 levels and CD4⁺ T cell count was found in HIV-1 infected patients. In addition, these patients had lower levels of SDF-1 than uninfected controls (Derdeyn *et al.*, 1999). Contrary to this report, another study found higher plasma SDF-1 levels in HIV-1 infected individuals than in uninfected controls, and an inverse correlation with CD4⁺ T cell count (Ikegawa *et al.*, 2001), while another found no significant differences in SDF-1 levels between HIV-1 seropositive patients and healthy controls (Llano *et al.*, 2001a).

IL-7 is able to upregulate HIV-1 replication *in vitro* (Pedroza-Martins *et al.*, 2002; Smithgall *et al.*, 1996). Moreover, significantly higher plasma IL-7 levels have been found in HIV-1 infected individuals when compared with healthy individuals (Llano *et al.*, 2001b). In addition, recent studies have demonstrated an inverse correlation between circulating IL-7 levels and numbers of CD4⁺ T cells in HIV-1 infected individuals (Fry *et al.*, 2001; Llano *et al.*, 2001b; Napolitano *et al.*, 2001), and a positive correlation with HIV-1 viral load (Napolitano *et al.*, 2001).

The finding that CC chemokine inhibit HIV-1 infection *in vitro* (Cocchi *et al.*, 1995), raised the question that these molecules are involved in protective immunity against HIV-1 *in vivo*, and although much work has been carried out in this regard, the specific role of β -chemokine levels in disease progression is still unclear. One study found significantly lower plasma concentrations of CC chemokine in HIV-1infected individuals than in healthy uninfected control subjects (Kakkanaiah *et al.*, 1998), while another showed that the HIV-1infected patients had higher serum levels of RANTES than healthy controls (Aukrust *et al.*, 1998). Further, β -chemokine levels in HIV-1 infected progressors have been found alternately to be increased (Zanussi *et al.*, 1996), decreased (Aukrust *et al.*, 1998), or not significantly different when compared to non-progressing HIV-1infected individuals (McKenzie *et al.*, 1996).

In vitro experiments have shown that CXCR4 expression can be downregulated by SDF-1 (Amara *et al.*, 1997; Signoret *et al.*, 1997) and upregulated by IL-7 (Jourdan *et al.*, 2000; Llano *et al.*, 2001b; Pedroza-Martins *et al.*, 2002), and that CCR5 expression can be downmodulated by MIP-1 β (Trkola *et al.*, 1998). As clear-cut differences in surface expression of these receptors were evident in earlier Chapters with respect to age (Chapter Three), CCR5 Δ 32 polymorphism (Chapter Six) and infection with HIV-1 and *M.tuberculosis* (Chapter Four), it was hypothesised that the ligands for these receptors might show corresponding changes in their peripheral levels. IL-7 was included in these analyses because of its known effects on the developing immune system, as well as for its role in HIV-1 disease progression.

Therefore, this study was undertaken to determine the levels of SDF-1 α , IL-7 and MIP -1 β from plasma of study participants, and importantly to investigate the relationship between CXCR4 and CCR5 expression on different leukocyte subsets (CD3 $^+$, CD4 $^+$, CD8 $^+$, NK, CD19 $^+$, CD14 $^+$ cells for CXCR4 and CCR5, and granulocytes only for CXCR4) and the corresponding circulating cytokine levels.

7.2 Materials and methods

7.2.1 Study participants

7.2.1.1 Cord blood samples

Cord blood was collected from infants born to HIV-1 seronegative mothers for comparison with control healthy adult samples.

7.2.1.2 Adult samples

Adult samples were collected from 19 healthy individuals with a median age of 38 (range 25-62 years). Of these, four were male and fifteen were female. Nine of these individuals were known to be CCR5 Δ 32/wt and 10 CCR5wt/wt.

7.2.1.3 HIV, TB and HIV/TB samples

The HIV group comprised of 16 HIV-1 seropositive patients without active TB, the TB group

consisted of 13 patients with pulmonary TB, and the HIV/TB group included 11 patients co-infected with *M. tuberculosis* and HIV-1. All but one of the patients with pulmonary TB were on standard anti-TB therapy. None of the patients in the HIV and HIV/TB groups had received any antiretroviral treatment. In addition, a longitudinal study of HAART administration was performed on a subset of eight HIV-1 seropositive patients (Table 5.1). The TB patients in this study are the same cohort as in Chapter Four. Two of the HIV, and two of the HIV/TB patients from the cohort in Chapter Four could not be included in this study due to insufficient sample, and an additional six patients were included in the HIV group. The immunological characteristics of patients within the different study groups used for cytokine analysis are shown in Table 7.1.

Table 7.1 Immunological status of individuals within the HIV, TB and HIV/TB study groups

	Study group*		
	TB	HIV	HIV/TB
No. of subjects	13	16	11
Age (years)	45 (22-57)	39 (25-68)	33 (19-48)
CD4			
(cells/ μ l)	922 (343-1794)	365.5 (57-657)	61 (10-906)
(percentage)	44 (21-69)	19.2 (3-38)	13 (2-22)
Viral load		4.3 (3.42-5.16)	5.4 (4-6.5)
(log ₁₀ HIV-1 RNA copies/ml)			

*Results are expressed as medians with the range in parentheses

7.2.2 Determination of cytokine levels

Plasma SDF-1 α , IL-7 and MIP-1 β levels were determined using the Quantikine human enzyme-linked immunosorbent assay (ELISA) systems (R & D Systems, Inc., Minneapolis, United States of America) according to the manufacturer's instructions.

7.2.2.1 SDF-1 α ELISA

Briefly, 100 μ l of standard or sample was added to the wells of a microtitre plate coated with antibody to SDF-1 α . Following a 2h incubation on a horizontal shaker, the wells were washed,

and 200 μ l of horseradish peroxidase (conjugated with polyclonal antibody against SDF-1 α) was added. After a second 2h incubation on the shaker, the plate was again washed, and 200 μ l of substrate was added per well. After a final 30 min incubation, colour development was stopped by the addition of 2N sulfuric acid. The optical density of each well was determined using a microplate reader at 450 nm. The minimal detectable amount of SDF-1 α that can be determined using this assay ranged from 1.0 pg/ml to 47 pg/ml (mean 18 pg/ml).

7.2.2.2 IL-7 ELISA

Briefly, 200 μ l of standard or sample was added to the wells of a microtitre plate coated with antibody to IL-7. Following a 16h incubation, the wells were washed, and 200 μ l of horseradish peroxidase (conjugated with polyclonal antibody against IL-7) was added. After a 2h incubation, the plate was again washed, and 50 μ l of substrate was added per well. After a final 30 min incubation, colour development was stopped by the addition of 2N sulfuric acid. The optical density of each well was determined using a microplate reader at 490 nm. The minimal detectable amount of IL-7 that can be determined using this assay is less than 0.1 pg/ml.

7.2.2.3 MIP-1 β ELISA

Briefly, 150 μ l of standard or sample was added to the wells of a microtitre plate coated with antibody to MIP-1 β . Following a 2h incubation, the wells were washed, and 200 μ l of horseradish peroxidase (conjugated with polyclonal antibody against MIP-1 β) was added. After a further 2h incubation, the plate was again washed, and 200 μ l of substrate was added per well. After a final 20 min incubation, colour development was stopped by the addition of 2N sulfuric acid. The optical density of each well was determined using a microplate reader at 450 nm. The minimal detectable amount of MIP-1 β that can be determined using this assay is less than 11.0 pg/ml.

7.2.3 Statistical analysis

Comparison of SDF-1 α , IL-7 and MIP-1 β levels between different study groups was performed using the Mann-Whitney U test (unpaired samples) and comparison of these chemokine levels from HIV-1 infected patients at baseline and after HAART was done using the Wilcoxon signed rank test (paired samples). The Spearman correlation coefficient was calculated to analyse

correlations.

7.3 Results

7.3.1 Comparison of cytokine levels between cord and adult blood

To determine differences in cytokine levels between cord blood and adult blood, SDF-1 α and IL-7 levels were determined in 6 cord blood and 19 adult plasma samples, and MIP-1 β levels determined in 10 cord blood and 15 adult samples. SDF-1 α levels (Figure 7.1A) were significantly lower in cord blood plasma when compared with adult plasma ($P=0.001$) (median 701.73 and 1440.03, respectively) as were IL-7 levels (Figure 7.1B) ($P<0.001$) (median 0.81 and 2.0, respectively). In contrast MIP-1 β levels were significantly higher in cord than adult plasma ($P<0.001$) (median 108.89 and 63.61, respectively). Thus, these results are the opposite to that found for CXCR4 and CCR5 expression, where CXCR4 expression was higher and CCR5 expression lower on cord blood leukocyte subsets in comparison with adult leukocyte subsets.

7.3.2 Relationship between the CCR5 Δ 32 polymorphism and circulating cytokine levels

In order to determine the relationship between the presence or absence of the CCR5 Δ 32 polymorphism in adults and circulating cytokine levels, SDF-1 α and IL-7 levels were evaluated on plasma samples from 9 CCR5 Δ 32/wt and 10 CCR5wt/wt healthy donors, and MIP-1 β levels on plasma samples from 7 CCR5 Δ 32/wt and 8 CCR5wt/wt healthy donors. Significantly lower SDF-1 α plasma levels were found in the CCR5 Δ 32 heterozygotes (median 1037.32 pg/ml) in comparison with CCR5wt/wt individuals (median 1641.39 pg/ml) ($P<0.05$) (Figure 7.2A). Thus these results were the opposite to those found for CXCR4 ABCs on CD3 $^+$ cells (Chapter Six, paragraph 6.3.3) where increased levels of CXCR4 ABC were found on the CCR5 Δ 32 heterozygotes in comparison with CCR5wt/wt individuals. Although no difference in IL-7 levels between the two groups was detected (Figure 7.2B), there was a trend towards an increase in MIP-1 β levels in the heterozygotes (median 77.54 pg/ml) when compared with CCR5wt/wt individuals (median 61 pg/ml) (Figure 7.2C). This result is the opposite to that found for CCR5 expression where CCR5 Δ 32 heterozygotes express lower levels of CCR5 in comparison with CCR5wt/wt individuals (Chapter Six).

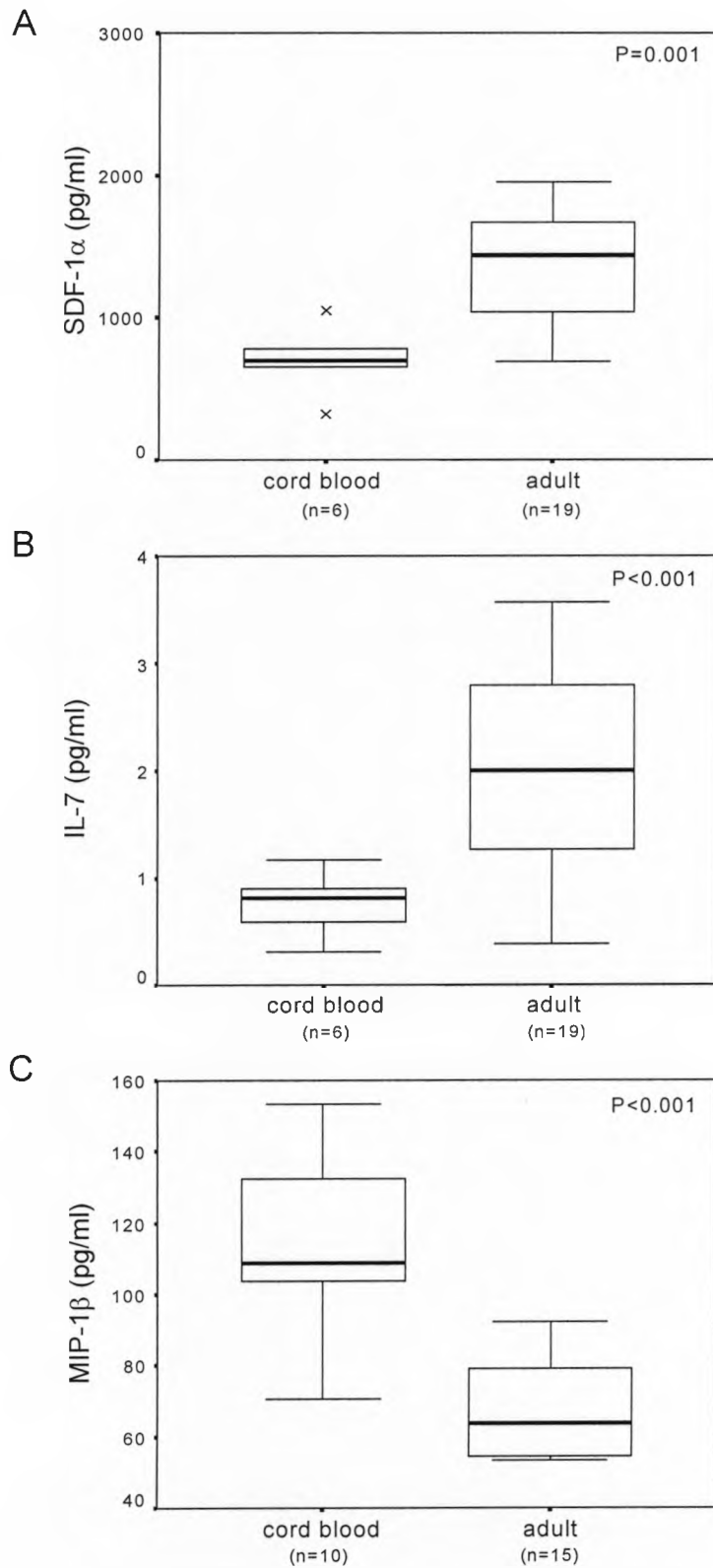


Figure 7.1 Comparison of plasma levels of SDF-1 α (A) and IL-7 (B) and MIP-1 β (C) from cord blood and adult plasma samples. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Sample numbers and significant differences between groups are indicated.

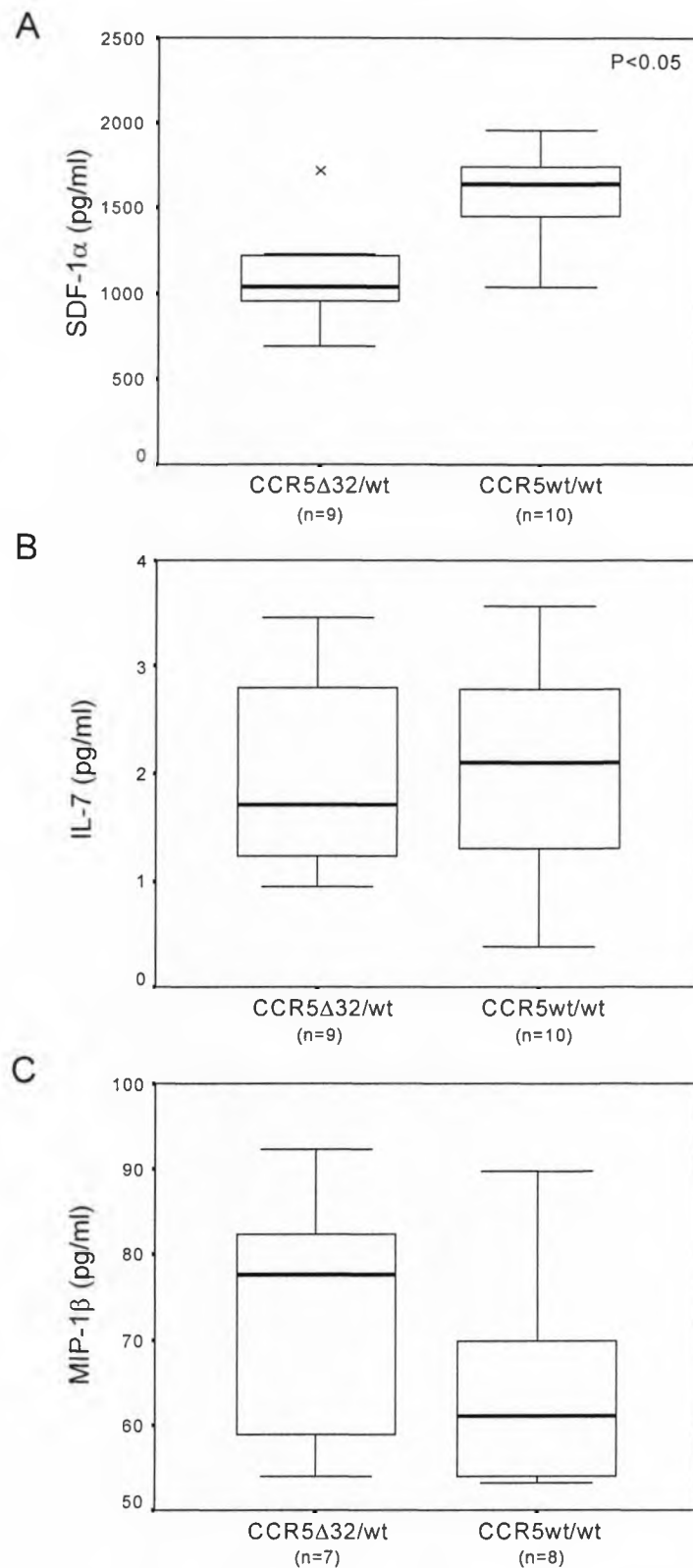


Figure 7.2 Comparison of plasma levels of SDF-1 α (A), IL-7 (B) and MIP-1 β (C) from CCR5 Δ 32/wt individuals and CCR5wt/wt individuals. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Sample numbers and significant differences between the groups are indicated.

7.3.3 Effect of infection with HIV-1 and/or *M.tuberculosis* on circulating cytokine levels

Next, the effect of infection with HIV-1 and/or *M.tuberculosis* on plasma cytokine levels was determined. SDF-1 α and IL-7 levels were investigated in patients from the HIV group, the TB group, and the HIV/TB group. SDF-1 α levels were measured for all study participants. However, due to insufficient sample IL-7 levels were determined in 12 patients from the TB group, and six patients from the HIV/TB group, and all the individuals from the ND and HIV groups. On the other hand, MIP-1 β was only determined in 15 individuals from the ND group, five patients from the TB group, and 14 HIV-1 seropositive patients, and was not measured in any of the patients in the HIV/TB group due to insufficient sample volume. The immunological characteristics of individuals within the different study groups are shown in Table 7.1. In comparison with healthy individuals, there was a significant increase in SDF-1 α levels in all disease groups ($P < 0.001$) (Figure 7.3A). In addition, SDF-1 α levels in both the TB ($P < 0.001$) and HIV/TB groups ($P = 0.001$) were significantly elevated when compared to levels in the HIV group. IL-7 levels in all disease groups were significantly higher than those found for the ND group ($P < 0.001$) (Figure 7.3B). Furthermore, IL-7 levels in the HIV/TB group were significantly higher than the TB group ($P = 0.01$). Additionally, MIP-1 β levels were significantly higher in the TB group than both the ND and HIV groups ($P < 0.001$) (Figure 7.3C).

The relationship between plasma SDF-1 α , IL-7 and MIP-1 β levels and CXCR4 expression on cellular subsets (CD3 $^+$, CD4 $^+$, CD8 $^+$, NK, CD19 $^+$, CD14 $^+$ cells and granulocytes) was investigated by combining the ND, TB, HIV and HIV/TB groups (ND, TB, HIV only for MIP-1 β) in order to establish if, in general, increased cytokine levels would be associated with decreased proportions of CXCR4-expressing cells irrespective of the disease state. Strong negative correlations were found between SDF-1 α levels and CXCR4 on all cell subsets ($P < 0.001$) (Figure 7.4). Negative correlations were also evident for IL-7 plasma levels and CXCR4 on all cell subsets (Figure 7.5), and for MIP-1 β and CXCR4 on CD8 $^+$ ($r = -0.343$, $P < 0.05$), NK ($r = -0.345$, $P < 0.05$), CD14 $^+$ cells ($r = -0.437$, $P = 0.001$) and granulocytes ($r = -0.412$, $P < 0.05$) (Figure 7.6). Exclusion of the ND group from the analysis resulted in significance attained for all cell comparisons for SDF-1 α and CXCR4, and for MIP-1 β and CXCR4 on CD14 $^+$ cells and granulocytes, but not for IL-7 and CXCR4, most likely because of insufficient sample numbers in this latter comparison (data not shown). Although significance of correlations was obtained for some cell types within each of

the independent study groups, larger sample numbers would be required to adequately show the relationships within each disease group for each cell type. However, overall the data suggests that where circulating levels of either SDF-1 α or IL-7 are high, the proportions of different CXCR4-expressing leukocyte subsets are correspondingly low. A recent study supports our hypothesis that the reciprocal relationship between plasma SDF-1 α levels and CXCR4 expression is likely a generalised phenomenon and not specific to a particular disease state in that high plasma SDF-1 levels were associated with low CXCR4 expression on CD4⁺ and CD8⁺ T cells in HIV-1 exposed-uninfected sex workers, and lower plasma SDF-1 levels were associated with higher CXCR4 expression in HIV-1 exposed-uninfected haemophiliacs (Soriano *et al.*, 2002).

Evaluation of the relationship between SDF-1 α , IL-7 and MIP-1 β plasma levels and CCR5 expression revealed a positive correlation between SDF-1 α plasma levels and CCR5 expression on CD3⁺ cells ($r = 0.398$, $P < 0.01$) and on CD8⁺ cells ($r = 0.272$, $P < 0.05$), and between IL-7 plasma levels and CCR5 expression on CD3⁺ ($r = 0.283$, $P < 0.05$) and CD8⁺ cells ($r = 0.422$, $P < 0.01$), as well as on CD14⁺ monocytes ($r = 0.313$, $P < 0.05$). In addition, a negative relationship was found between MIP-1 β and CCR5 expression on CD14⁺ monocytes ($r = -0.448$, $P < 0.01$) (data not shown). Moreover, a weak positive correlation was found between SDF-1 α and IL-7 levels ($r = 0.468$, $P < 0.001$), and between levels of SDF-1 α and MIP-1 β ($r = 0.353$, $P < 0.05$).

7.3.4 Relationship between plasma cytokine levels and HIV-1 disease progression

To determine whether plasma levels of SDF-1 α , IL-7 and MIP-1 β are associated with stage of HIV-1 disease, the HIV and HIV/TB groups (HIV only for MIP-1 β) were combined and stratified into two groups according to the percentage of CD4⁺ T cells, viz >14% and <14% CD4⁺ T cells. Plasma levels of SDF-1 α were significantly higher in the <14% CD4 group when compared with the >14% group ($P = 0.005$) (Figure 7.7A). Furthermore, lower levels of SDF-1 α significantly correlated with a higher percentage of CD4⁺ T cells ($r = -0.638$, $P < 0.001$) (Figure 7.7B). IL-7 plasma levels were not significantly different between the <14% CD4 group and the >14% group (Figure 7.7C), and no correlation was found between IL-7 plasma levels and percentage of CD4 T cells (Figure 7.7D). A smaller sample size used for this comparison most likely precluded the ability to show significance, but would need to be tested. In contrast, for MIP-1 β plasma levels there was a trend towards a slight reduction in the <14% CD4⁺ T cell group when compared with

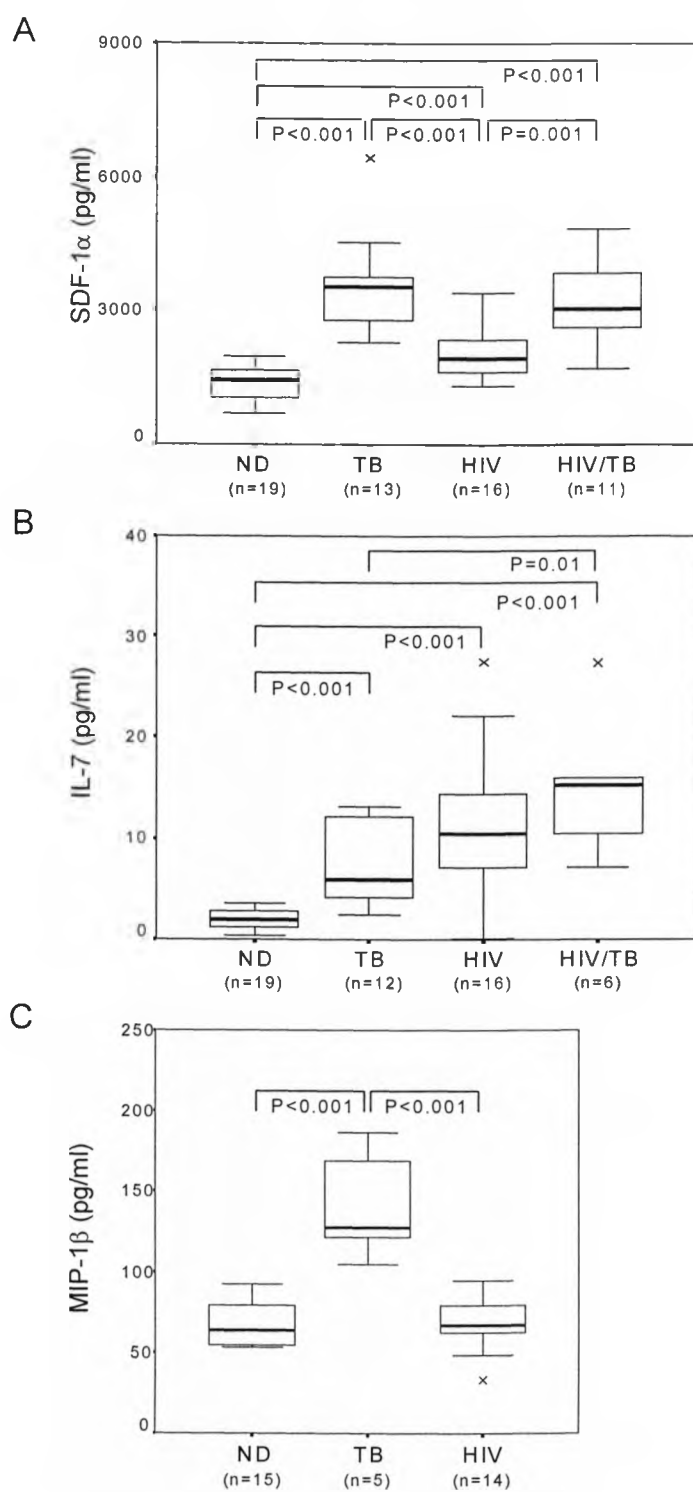


Figure 7.3 Comparison of plasma levels of SDF-1 α (A) and IL-7 (B) from subjects in the ND, TB, HIV and HIV/TB study groups and comparison of plasma levels of MIP-1 β (C) from subjects in the ND, TB and HIV study groups. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Sample numbers and significant differences between groups are indicated.

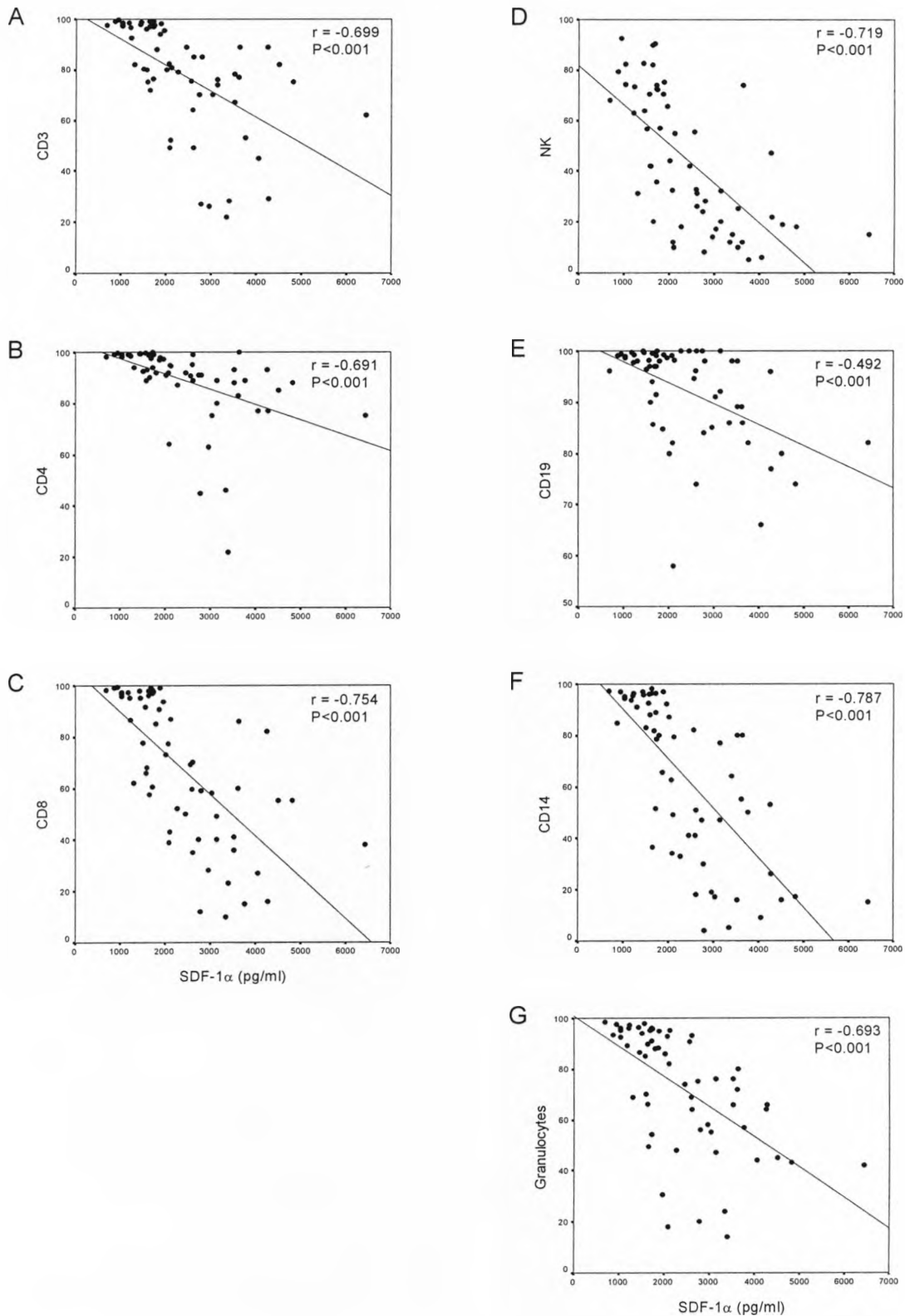


Figure 7.4 Relationship between levels of plasma SDF-1 α and CXCR4 expression on whole blood leukocytes from subjects in the ND, TB, HIV and HIV/TB study groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), CD14 (F) cells and granulocytes (G). Data are shown as the percentage of cells expressing CXCR4.

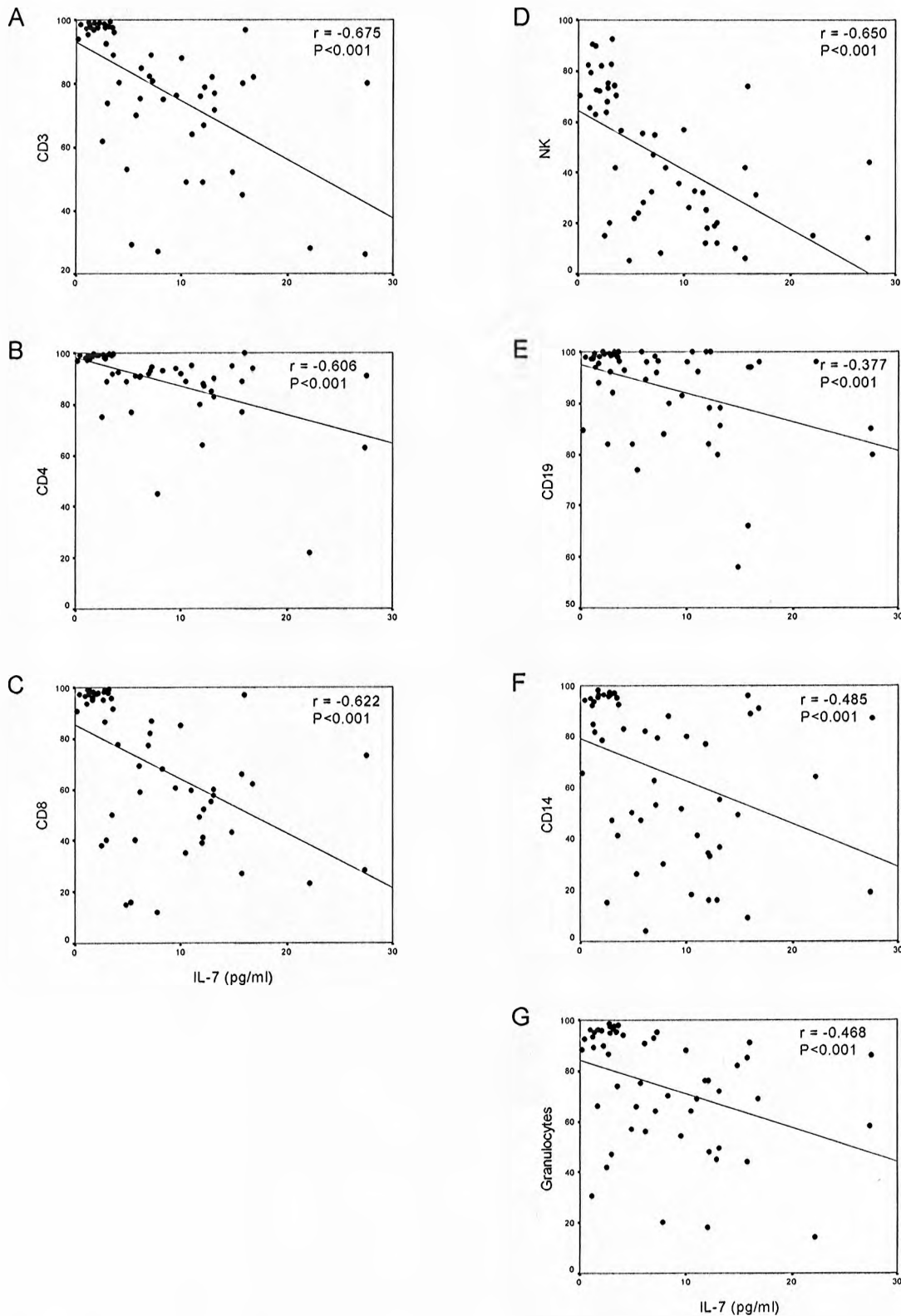


Figure 7.5 Relationship between levels of plasma IL-7 and CXCR4 expression on whole blood leukocytes from subjects in the ND, TB, HIV and HIV/TB study groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), CD14 (F) cells and granulocytes (G). Data are shown as the percentage of cells expressing CXCR4.

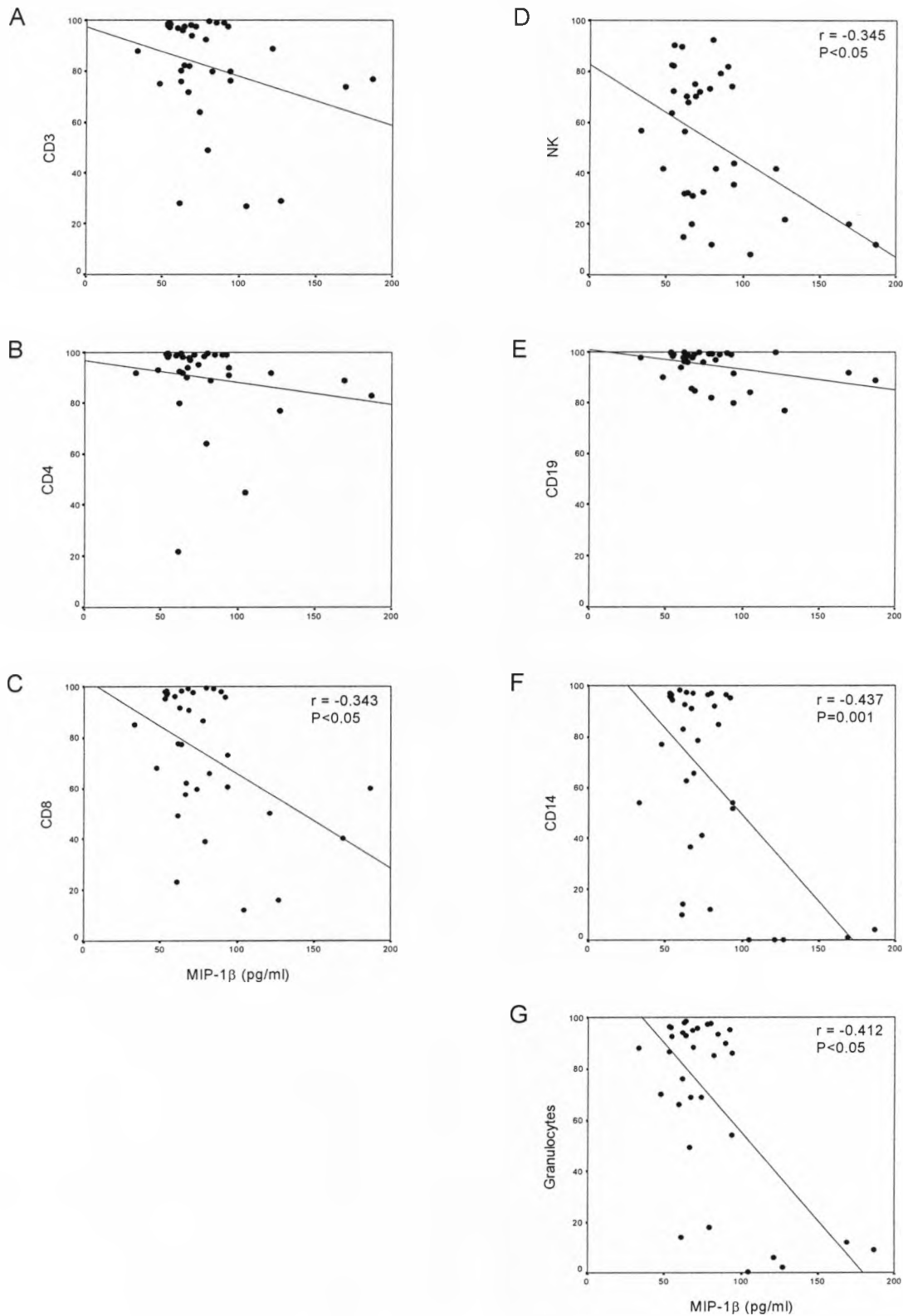


Figure 7.6 Relationship between levels of plasma MIP-1β and CXCR4 expression on whole blood leukocytes from subjects in the ND, TB and HIV study groups: CD3 (A), CD4 (B), CD8 (C), NK (D), CD19 (E), CD14 (F) cells and granulocytes (G). Data are shown as the percentage of cells expressing CXCR4.

the >14% group (Figure 7.7E). No correlation was found between MIP-1 β plasma levels and percentage of CD4⁺ T cells (Figure 7.7F). Again, small sample numbers here may mask the true relationships. Additionally, a positive correlation was found between both SDF-1 α and IL-7 levels and log₁₀ HIV-1 RNA copies/ml ($r = 0.408$, $P < 0.05$ and $r = 0.435$, $p < 0.05$, respectively).

7.3.5 Effect of HAART on plasma cytokine levels

The effect of antiretroviral therapy on the plasma levels of these cytokines in HIV-1 infected adults was determined by measuring plasma cytokine levels before starting HAART, and again eight weeks later. See Table 5.1 for characteristics of the study participants. SDF-1 α and IL-7 plasma levels were analysed for eight HIV-1 seropositive patients and MIP-1 β levels were only determined for six patients due to insufficient sample. SDF-1 α levels were decreased in four patients, unchanged in two patients and increased in two patients compared with pre-treatment levels. Overall, the median SDF-1 α level decreased from 1965.8 pg/ml pre-therapy to 1770.04 pg/ml eight weeks later (Fig 7.8A). IL-7 levels were reduced in six patients, and increased in two patients after eight weeks of HAART. Thus, the median IL-7 level decreased from 7.09 pg/ml pre-therapy to 5.2 pg/ml eight weeks later (Fig 7.8B). In contrast, MIP-1 β levels increased in four patients, remained unchanged in one, and increased in one patient compared with pre-treatment levels. Therefore, the median levels increased from 67.5 pg/ml to 80.6 pg/ml (Fig 7.8C).

7.4 Discussion

In this Chapter a cross-sectional analysis of SDF-1 α , IL-7 and MIP-1 β levels was performed on number of different cohorts in order to determine whether differences exist in levels of these cytokines due to age, the presence of the CCR5 Δ 32 polymorphism, infection with HIV-1 and/or *M.tuberculosis*, and treatment with HAART. In addition, the relationship between CXCR4 and CCR5 expression on various leukocyte subsets and levels of these cytokines was analysed.

Findings here have demonstrated lower SDF-1 α and IL-7 levels, and higher MIP-1 β levels in cord blood plasma in comparison with adult plasma. While previous studies have demonstrated that cord blood mononuclear cells have a reduced ability to produce cytokines (Lee *et al.*, 1996; Qian *et al.*, 1997; Wilson *et al.*, 1986), our results have shown that plasma levels of cytokines are

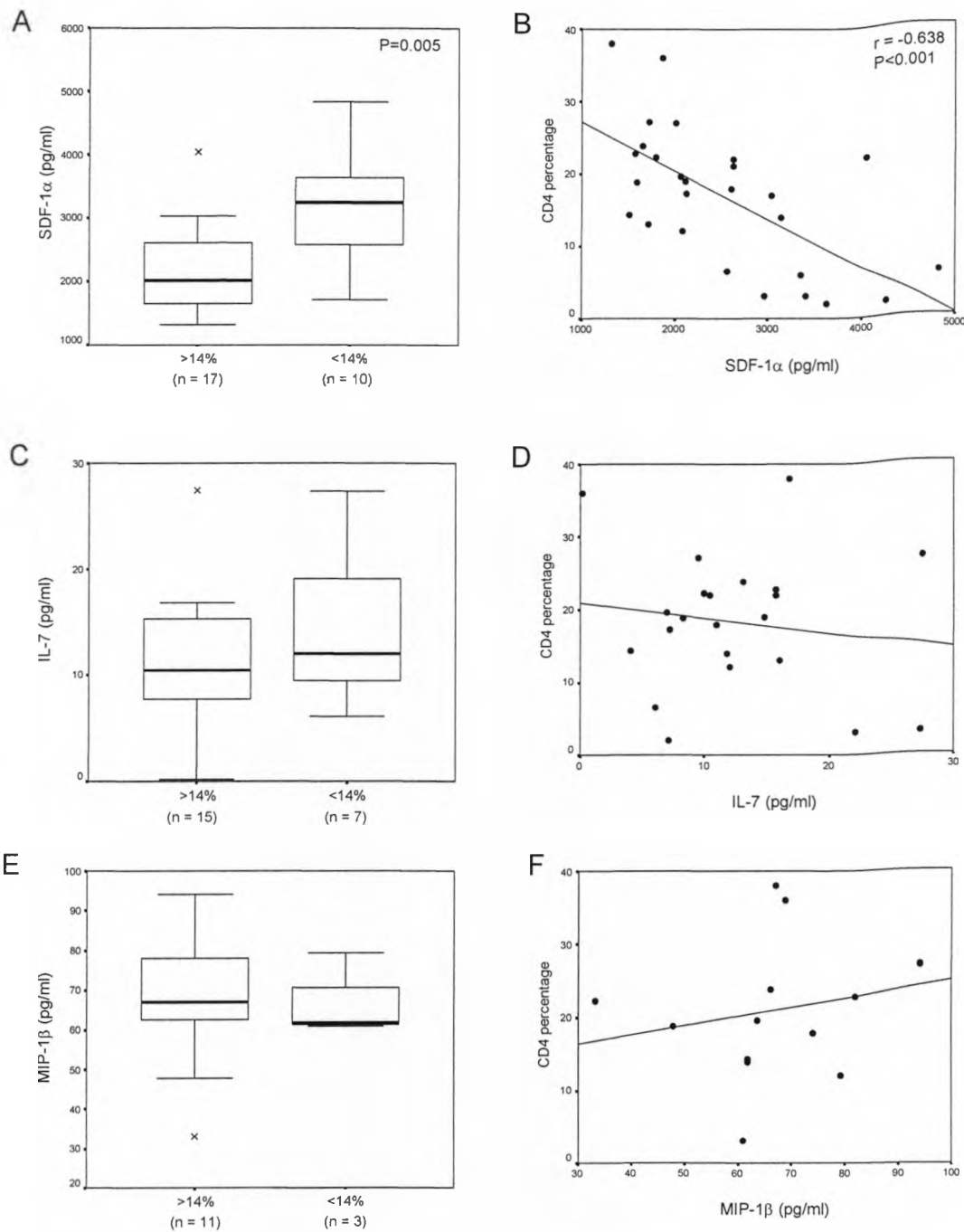


Figure 7.7 Relationship between the stage of HIV-1 disease and SDF-1 α (A), IL-7 (C) and MIP-1 β (E) plasma levels. This was determined by combining and then stratifying the HIV and HIV/TB groups (HIV only for MIP-1 β) according to a CD4⁺ T cell percentage of >14% and <14% (horizontal axis). Correlations between percentage of CD4⁺ T cells and circulating levels of SDF-1 α (B), IL-7 levels (D) and MIP-1 β (F) are shown.

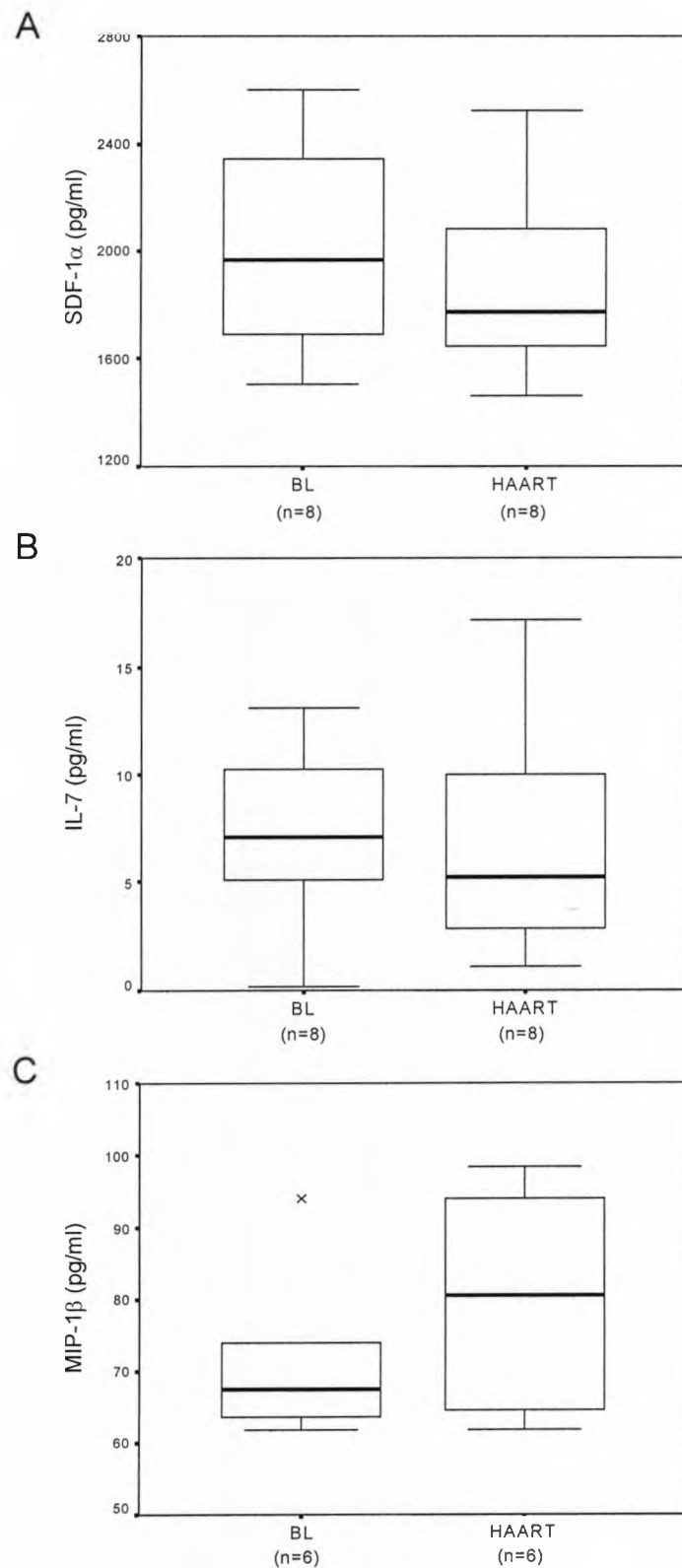


Figure 7.8 Comparison of plasma levels of SDF-1 α (A) and IL-7 (B) and MIP-1 β (C) from HIV-1 seropositive patients at baseline (BL) and after eight weeks of HAART. Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Sample numbers are indicated.

altered, but not necessarily reduced. Thus, these results provide further evidence for the immaturity of the infant's immune system as manifested by differences in cytokine levels between cord and adult blood.

Long-term non-progression, or delayed progression to AIDS is associated with a number of factors, including chemokine receptor polymorphisms, and chemokine production. Individuals who are homozygous for the $\Delta 32$ deletion are highly resistant to infection with HIV-1 (Dean *et al.*, 1996; Huang *et al.*, 1996; Samson *et al.*, 1996a), and those individuals who are heterozygous for the mutation have delayed progression to AIDS (Dean *et al.*, 1996; Huang *et al.*, 1996; Zimmerman *et al.*, 1997). Moreover, increased β -chemokine production is associated with long-term survival (Clerici *et al.*, 1996; Ullum *et al.*, 1998). Previous work has shown that individuals heterozygous for the 32 bp deletion have decreased percentages of CCR5-expressing CD4⁺ T cells as compared with individuals without the mutation (de Roda Husman *et al.*, 1999; Wu *et al.*, 1997b). Thus, this study was undertaken to determine the relationship between lower CCR5 surface expression in CCR5 $\Delta 32$ /wt individuals and the corresponding circulating levels of its ligand (MIP-1 β). Moreover, in Chapter Six it was demonstrated that these CCR5 $\Delta 32$ /wt individuals have higher CXCR4 ABC than CCR5wt/wt individuals. The results from this study are the first to demonstrate significantly lower circulating SDF-1 α levels in CCR5 $\Delta 32$ heterozygotes than CCR5wt/wt individuals. A trend towards an increase in MIP-1 β levels in the CCR5 $\Delta 32$ /wt individuals in comparison with the CCR5wt/wt individuals was additionally observed. As previous studies have shown that CCR5 $\Delta 32$ /wt individuals produce significantly higher levels of CC chemokine than CCR5 $\Delta 32$ heterozygotes (Ferbass *et al.*, 2000; Paxton *et al.*, 2001), the lack of significance is likely due to sample size. The mechanism for the reduction in SDF-1 α levels and increase in MIP-1 β levels in CCR5 $\Delta 32$ heterozygotes is currently unknown. One hypothesis may be that increased CXCR4 receptor density would result in increased receptor-mediated uptake of SDF-1 α leading to lower circulating SDF-1 α levels. On the other hand, lower SDF-1 α levels may result in less SDF-1 α available to bind to and subsequently downregulate CXCR4. Similarly, lower CCR5 receptor density in these individuals may result in increased extracellular MIP-1 β levels through decreased receptor-mediated uptake, or alternatively, increased MIP-1 β levels could result in increased binding to and internalisation of CCR5. IL-7 levels were not substantially altered in CCR5 $\Delta 32$ /wt individuals. Taken together, these results

suggest that receptor density as well as chemokine production/metabolism may play a role in long-term non-progression in CCR5 Δ 32/wt individuals.

Persons infected with HIV-1, pulmonary tuberculosis and those co-infected with HIV-1 and *M. tuberculosis*, have significantly reduced CXCR4 expression and increased CCR5 expression when compared with healthy persons (Chapter Four). Further, HAART plays a role in downregulation of CCR5 expression in HIV-1 seropositive individuals, while the effects of HAART on CXCR4 are more controversial (Giovannetti *et al.*, 1999; Giovannetti *et al.*, 2001; Nicholson *et al.*, 2001; Pierdominici *et al.*, 2002; Smith *et al.*, 2002). Results here have shown that SDF-1 α levels are elevated in all disease groups in comparison with healthy individuals. Furthermore, SDF-1 α plasma levels in both TB groups were significantly higher than levels in the HIV group, thus changes are the opposite of our previous results where there was a greater reduction in CXCR4 expression in the two TB groups when compared with the HIV group was observed. Additionally, a significant negative correlation between the expression of CXCR4 on CD3⁺, CD4⁺ and CD8⁺ memory cells, CD14⁺ and NK cells and the duration of treatment in the HIV/TB group was evident (Section 4.3.6). Moreover, CXCR4 expression did not increase with duration of anti-TB treatment despite resolution of infection. Thus, as all the patients in this study were receiving anti-TB treatment, the elevated SDF-1 α levels in the two TB groups, above that found for the HIV group, may be due to infection with *M. tuberculosis* or to anti-TB treatment.

A trend towards an increase in IL-7 levels was observed in the HIV group when compared with the TB group, and the HIV/TB group when compared with the HIV group, however, only IL-7 plasma levels in the HIV/TB group were significantly higher than the TB group (P=0.01). Interestingly, these are an inverse image of the absolute CD4 T cell count and percentage (Table 7.1), which are significantly higher in the TB group compared with both the HIV (P<0.001) and HIV/TB (P<0.001) groups. The CD4 T cell percentage is also significantly higher in the HIV group compared with the HIV/TB (P<0.05) group, with a trend to this effect for the absolute T cell count. IL-7 is important in modulating peripheral T cell expansion in states of T cell depletion through its potent effects on mature T cells. Recent reports have shown that T cell depletion is accompanied by increased production of IL-7 (Bolotin *et al.*, 1999; Mastroianni *et al.*, 2001; Napolitano *et al.*, 2001), suggesting a role for this cytokine in T cell regeneration.

Therefore, the low CD4 T cell counts in the HIV groups is most likely the reason for the increase in IL-7 plasma levels. It is noteworthy that while correlations were strong between SDF-1 α and all cell subsets (except CD19⁺ B cells), correlations between IL-7 and the T cell subsets (CD3⁺, CD4⁺ and CD8⁺ cells) and NK cells were stronger than correlations between IL-7 and CD19⁺, CD14⁺ cells and granulocytes, probably reflecting the function of this cytokine in T cell expansion.

MIP-1 β levels were significantly higher in the TB group when compared with both the ND and HIV group. These results are in accordance with a previous study which detected higher MIP-1 α levels in both the TB and HIV/TB groups than the HIV and control group (Mayanja-Kizza *et al.*, 2001). This finding is not surprising as chemokines play a critical role in the immune response to *M. tuberculosis*. They are potent leukocyte activators, chemoattractants and aid in granuloma formation. In addition, MIP-1 β and RANTES have been shown to suppress the intracellular growth of *M. tuberculosis* (Saukkonen *et al.*, 2002). There are a number potential mechanisms for this inhibition. The activation of macrophages by RANTES, MIP-1 α , and MIP-1 β induces strong tyrosine phosphorylation of several proteins. Further, MIP-1 β can modulate MIP-1 α -induced TNF- α production (Fahey *et al.*, 1992). Moreover, RANTES, MIP-1 α , and MIP-1 β were recently reported to enhance phagocytosis and killing of *Trypanosoma cruzi*, another intracellular organism (Lima *et al.*, 1997) through the induction of nitric oxide in macrophages (Villalta *et al.*, 1998). Thus, *M. tuberculosis*-induced CC chemokine could act in an autocrine manner to activate and regulate macrophage responses (Saukkonen *et al.*, 2002). CC chemokine, therefore constitute an important part of the innate immune response to *M. tuberculosis* infection.

In this study no significant difference in MIP-1 β levels between the ND and the HIV group was identified. However, when the HIV group was stratified into two groups according to the CD4⁺ T cell percentage, a trend towards a reduction in MIP-1 β levels in the <14% group when compared with the >14% group was found. These results presented here are preliminary, as the sample numbers are small, with 11 individuals in the >14% group and only three in the <14% group. A positive correlation between SDF-1 α and IL-7 plasma levels was observed, as well as strong negative correlations between both SDF-1 α and IL-7 plasma levels and CXCR4 expression on all cell subsets evaluated when the ND, TB, HIV and HIV/TB groups were combined.

Additionally, studies have suggested an association between SDF-1 (Ikegawa *et al.*, 2001) and IL-7 (Llano *et al.*, 2001b) and HIV-1 disease progression. Thus, these results provide further support for the role of both SDF-1 α and IL-7 in the pathogenesis of HIV-1 disease.

In contrast to the downregulating activity of SDF-1 α on CXCR4 expression shown *in vitro* (Amara *et al.*, 1997) which is consistent with what was found here *in vivo*, IL-7 has been shown to upregulate CXCR4 *in vitro* (Jourdan *et al.*, 2000; Llano *et al.*, 2001b; Pedroza-Martins *et al.*, 2002) and higher levels are associated with SI variants (Llano *et al.*, 2001b). That higher IL-7 levels are associated with lower CD4 T cell counts and higher viral loads is consistent with the likelihood of SI variants at later stages of HIV-1 disease. However, our *in vivo* findings are different to those obtained in *in vitro* studies, as increasing IL-7 levels were associated with reduced CXCR4. This could be explained in that *in vitro* models of cytokine/receptor interactions would not take into account the multiple factors that would play a role *in vivo*. To our knowledge this current study represents the first report that has directly assessed the relationship between IL-7 levels and CXCR4 expression *in vivo*.

A trend towards a reduction in SDF-1 α and IL-7 levels and an increase in MIP-1 β levels was observed after eight weeks of HAART. Other studies have reported no significant change in SDF-1 levels (Derdeyn *et al.*, 1999), reduction in IL-7 levels (Mastroianni *et al.*, 2001), and an increase in RANTES levels following HAART (Aukrust *et al.*, 1998). Although, results from our study were not significant, and sample numbers were small, they give an indication of the effect of HAART on circulating SDF-1 α , IL-7 and MIP-1 β levels, and need to be extended.

Work in Chapters Two and Four has shown a reciprocal relationship between CXCR4 and CCR5 receptor expression, and in this Chapter an inverse relationship between the ligands for these receptors, SDF-1 α and MIP-1 β has been demonstrated. The similarities in association between SDF-1 α and IL-7 plasma levels and levels of CXCR4 expression may be the result of functions that these cytokines have in common. In addition, a positive relationship between SDF-1 α and IL-7 levels was observed. SDF-1 α and IL-7 levels were significantly lower in cord than adult blood, while the opposite was true for MIP-1 β levels. SDF-1 α plasma levels in CCR5 Δ 32

heterozygotes were significantly lower than for CCR5wt/wt individuals, with a trend towards higher MIP-1 β levels in the CCR5 Δ 32 heterozygotes in comparison with the CCR5wt/wt individuals. In HIV-1 infected individuals (with and without TB) stratified according to CD4⁺ T cell percentage, those individuals with a CD4⁺ T cell percentage of <14% had significantly higher SDF-1 α levels than individuals with a CD4⁺ T cell percentage of >14% with a trend to that effect for IL-7 levels. When determining MIP-1 β levels in these study groups sufficient plasma was not available to include samples from individuals in the HIV/TB group, there was an indication that MIP-1 β levels might be reduced in the HIV-1 infected individuals with a CD4⁺ T cell percentage of <14%. On evaluation of chemokine levels in response to HAART in HIV-1 infected individuals, a trend towards a reduction in SDF-1 α and IL-7 levels and an increase in MIP-1 β levels was found after eight weeks of therapy. Exceptions to the relationships described above occurred with respect to infection with *M. tuberculosis*, where SDF-1 α , IL-7 and MIP-1 β plasma levels were significantly elevated in the TB group when compared to ND and HIV groups.

In summary, work in this Chapter has identified a positive relationship between SDF-1 α and IL-7 plasma levels, and an inverse relationship between SDF-1 α /IL-7 and MIP-1 β plasma levels (with the exception of infection with *M. tuberculosis*), and provides preliminary evidence for differences in the levels of these cytokines with respect to age, the CCR5 Δ 32 polymorphism, infection with HIV-1 and/or *M.tuberculosis*, HIV-1 disease progression and HAART. Whereas in some instances it was apparent that strong relationships existed between CXCR4 and CCR5 and plasma levels of their respective ligands, there were a few exceptions. Larger sample sizes for some comparisons will help to clarify these relationships. These findings have implications for the pathogenesis and treatment of HIV-1 infection and deserve to be further explored.

CHAPTER EIGHT

CONCLUDING REMARKS

CXCR4 and CCR5, are the major HIV-1 coreceptors, which together with CD4, are utilised for viral entry. These receptors play an important role in the immune response by mediating chemotaxis in response to their specific ligands, SDF-1 α (CXCR4) and MIP-1 α , MIP-1 β and RANTES (CCR5). Thus, changes in the cell surface expression of these coreceptors has implications for chemotaxis, as well as for viral entry. The overall aim of this study was to assess the cellular distribution of CXCR4 and CCR5, in peripheral blood of healthy individuals and HIV-1 seropositive individuals. This study reports on age-related differences in chemokine receptor expression, effects of *Mycobacterium tuberculosis* (TB group) and HIV-1 (HIV group), alone or in combination (HIV/TB group), as well as the effects of administration of HAART in HIV-1 infected adults. In addition, plasma levels of the ligands for CXCR4 and CCR5, SDF-1 α and MIP-1 β respectively, were measured.

What became apparent shortly after the initiation of the study, and continued as a theme throughout the work, with a number of exceptions, was the reciprocal modulation of the two major HIV coreceptors, and that between the coreceptors and their respective ligands, and between the ligands themselves. The first time this reciprocal relationship between the coreceptors was evident was in the initial experiments to determine the cutoff time for staining of samples for the expression of CXCR4 and CCR5. Here, results showed that the expression of CXCR4 and CCR5 were reciprocally modulated with standing time: the expression of CXCR4 was significantly increased, while the expression of CCR5 was significantly reduced. These changes were observed as early as three hours after venesection, with major changes occurring after 24h.

The next part of the work involved the study of coreceptor expression in infants. Children

infected perinatally undergo a faster progression to disease than do HIV-1 infected adults (Rogers *et al.*, 1987; Scott *et al.*, 1989). Therefore, it was important to investigate whether age-related differences in the major HIV-1 coreceptors exist, as this may have implications for understanding differences in disease pathogenesis between infants and adults. Here, the reciprocal relationship in coreceptor expression was evident in the cord blood samples where the percentage of CXCR4-expressing cells was very high, and the proportion of CCR5-expressing leukocytes very low. An exception to this reciprocal relationship was that the CXCR4 expression decreased at 4.5 months and then increased to adult levels. In contrast, the proportion of CCR5-expressing leukocytes gradually increased with age. One limitation of the study was that the uninfected infants studied were born to HIV-1 seropositive mothers, and so are defined as exposed-uninfected infants. These infants were studied as it is extremely difficult to obtain specimens from infants born to HIV-1 uninfected mothers. T lymphocyte maturation abnormalities have been detected in uninfected HIV-exposed infants (Clerici *et al.*, 2000b), and so results here must not be seen as necessarily representative of uninfected infants that are not exposed to HIV-1. Additionally, a longitudinal, rather than a cross-sectional study, as conducted here, may more accurately reflect age-related changes in coreceptor expression. However, these results contribute to the expanding body of research aimed at understanding the differences in disease pathogenesis between infants and adults.

At the time that this study was undertaken, only three-colour flow cytometry was available in our laboratory, and therefore coreceptor-expressing naive cells were characterised using only CD45RA. This marker does not accurately define naive cells, as mentioned previously. However, CXCR4 expression on lymphocytes of healthy control persons was associated predominantly with CD45RA-expressing (naive) cells, while CCR5 expression was associated predominantly with CD45RO-expressing (memory) cells. On further analysis, CD4⁺CXCR4⁺ cells expressed equal proportions of CD45RA and CD45RO, as did CD8⁺CCR5⁺ cells. By contrast, CD4⁺CCR5⁺ cells expressed much higher proportions of CD45RO than CD45RA, with the opposite true for CD8⁺CXCR4⁺ cells. It is now possible for four-colour flow cytometry to be carried out in our laboratory, and thus coreceptor-expressing naive and memory subsets can be more accurately characterised.

As both HIV-1 disease and tuberculosis, alone or in combination, are occurring increasingly, particularly in Africa, an understanding of the immune processes are necessary for constructive intervention. Thus, this study was undertaken to address the effect of infection of persons with *Mycobacterium tuberculosis* (TB group) and HIV-1 (HIV group), alone or in combination (HIV/TB group), on the expression of CXCR4 and CCR5 on different subsets of peripheral leukocytes (Chapter Four). The reciprocal relationship between CXCR4 and CCR5 expression was again evident in persons infected with HIV-1 and/or *M.tuberculosis* as, in general, the proportion of leukocytes expressing CXCR4 was significantly reduced in patients infected with HIV-1, *M. tuberculosis* and those with dual infection, whereas the proportions of CCR5-bearing leukocytes were significantly increased from that found for healthy individuals. In addition, CXCR4 expression was modulated to a greater extent by the presence of pulmonary TB or its treatment, whereas CCR5 expression was modulated to a greater extent by the presence of HIV-1 infection. Thus, the expression of CXCR4 and CCR5 on different leukocyte subsets are reciprocally altered in the presence of HIV-1 and TB disease.

Following from the study described above, it was important to analyse whether HAART would play a role in normalizing the expression of these receptors, and additionally to question whether HAART would modulate these receptors similarly, or in a reciprocal manner (Chapter Five). In general, after eight weeks of HAART, CXCR4 expression did not change significantly from pre-therapy levels, while CCR5 expression was significantly reduced in comparison with pre-therapy levels. As this is a more highly activated population, as measured by CD8⁺CD38⁺ cells, these receptors may take longer to normalize in this population than in North American or European populations. One limitation of this study was the small sample size. Thus, additional patients would have to be studied to confirm the effect of HAART on the expression of CXCR4 and CCR5. However, it was apparent that CCR5 may be an additional phenotypic marker of immune reconstitution.

As the work in previous Chapters involved the evaluation of the percentage of coreceptor-expressing cells, it was important to additionally evaluate the number of coreceptor ABC (Chapter Six). Quantitation was performed on cord blood and adult whole blood samples. Results from this work showed that as found for the percentage of cells expressing the coreceptors, the

number of CXCR4 ABC was higher in all cord blood leukocytes subsets (except NK cells) than adult leukocytes, while the opposite was true for CCR5 ABC i.e. the number of CCR5 ABC was lower on all cord blood leukocyte subsets with the exception of CD19⁺ B cells and CD14⁺ monocytes. Thus, here it was found that the number of CXCR4 and CCR5 ABC, in addition to the percentage of cells, were reciprocally expressed on cord blood and adult leukocytes.

Perhaps the most convincing evidence of reciprocal modulation of CXCR4 and CCR5 expression has come from a comparison of CCR5 Δ 32/wt adults with CCR5wt/wt adults. The CCR5 Δ 32/wt individuals had lower percentages of CCR5-expressing cells, as well as lower numbers of ABC than the CCR5wt/wt individuals. In addition, there was an increase in the number of CXCR4 ABC on CD3⁺ cells in these individuals, but not in the percentage CXCR4-expressing cells. The mechanism of this reciprocal increase in CXCR4 expression that occurs in persons with a lower CCR5 expression is presently unknown. SDF-1, as well as MIP-1 α , MIP1- β and RANTES induce chemotaxis of T cells. Therefore, individuals heterozygous for the CCR5 Δ 32, may have increased CXCR4 expression on T cells to compensate for the reduction in CCR5 expression on these cells. Although, delayed disease progression is associated with the CCR5 Δ 32 gene, this protective effect is lost when individuals harbour the SI CXCR4-utilizing HIV-1 strains (Michael *et al.*, 1997a), thus the greater CXCR4 receptor density may induce a more rapid disease progression in these individuals when they are infected with SI viruses. This work provides preliminary evidence for a compensatory increase in CXCR4 expression in individuals heterozygous for the CCR5 Δ 32 mutation. Further studies are required to identify the mechanism of this increase in CXCR4 ABC in CCR5 Δ 32 heterozygotes.

It is noteworthy that the quantitation technique was more sensitive than the standard analysis of percentages of cells expressing the coreceptor, especially with reference to CXCR4, where extremely high percentages of cells express the coreceptor, thus making it difficult to assess differences between cohorts. More in depth studies on coreceptor expression would value from the utilisation of both approaches.

The question of whether specific ligands for the receptors might regulate their expression *in vivo*

was next examined (Chapter Seven). This was analysed with respect to factors such as to age, presence of a CCR5 Δ 32 allele, infection with HIV-1 and *M. tuberculosis* and treatment with HAART. IL-7, which has functions in common with SDF-1 α , was included as a cytokine that does not bind either coreceptor. Initial studies to determine whether plasma cytokine levels in cord and adult blood were different, revealed significantly lower SDF-1 α and IL-7 levels, and higher MIP-1 β levels in cord blood plasma in comparison with adult plasma. Thus, in this study, the levels of ligands for CXCR4 and CCR5, i.e. SDF-1 α and MIP-1 β are reciprocally expressed relative to each other and to their respective chemokine receptors.

As individuals heterozygous for CCR5 Δ 32 allele have higher numbers of CXCR4 ABC and lower CCR5 expression (both percentage and number of ABC) in comparison with CCR5wt/wt individuals (Chapter Eight), the effect of the presence of this mutation on circulating levels of MIP-1 β and SDF-1 α were analysed. Significantly lower circulating SDF-1 α levels, and a trend towards an increase in MIP-1 β levels were found in CCR5 Δ 32 heterozygotes in comparison with CCR5wt/wt individuals. Thus, SDF-1 α and MIP-1 β levels are reciprocally expressed in the CCR5 Δ 32/wt individuals in comparison with the CCR5wt/wt individuals, and additionally, SDF-1 α and MIP-1 β levels mirrored the number of CXCR4 and CCR5 ABC in these individuals. The lack of significance in the increase in MIP-1 β levels is likely due to sample size. This is supported by previous studies where CCR5 Δ 32/wt individuals were shown to produce significantly higher levels of CC chemokine than CCR5 Δ 32 heterozygotes (Ferbass *et al.*, 2000; Paxton *et al.*, 2001). The mechanism for this reduction in SDF-1 α levels and increase in MIP-1 β levels in CCR5 Δ 32 heterozygotes is currently unknown, and thus future work to identify this mechanism is important.

The effect of infection with HIV-1 and/or *M. tuberculosis* on plasma cytokine levels was determined. SDF-1 α levels were significantly increased in all disease groups in comparison with healthy individuals, while MIP-1 β levels were significantly higher in the TB group when compared with both the ND and HIV group, and were not determined in the HIV/TB group due to insufficient sample. Thus, here a reciprocal relationship was observed between CXCR4 expression, which is reduced in infection with HIV-1 and/or *M. tuberculosis*, and levels of SDF-1 α , with strong negative correlations between SDF-1 α levels and CXCR4 on all cell subsets. Negative correlations were also evident for IL-7 plasma levels and CXCR4 on all cell subsets.

The same reciprocal relationship however, was not evident in this cohort for MIP-1 β levels and expression of CCR5. CCR5 expression is increased with infection with *M.tuberculosis* and MIP-1 β levels are also increased in comparison with healthy individuals. This is not surprising as CC chemokine play an important role in the innate immune response to infection with *M.tuberculosis*.

Further, the relationship between plasma levels of SDF-1 α , IL-7 and MIP-1 β and stage of HIV-1 disease was determined. Plasma levels of SDF-1 α were significantly higher in the study participants with a CD4⁺ T cell percentage of <14% when compared with those with a CD4 T cell percentage of >14%. In addition, a significant correlation was found between lower levels of SDF-1 α and a higher percentage of CD4⁺ T cells. In contrast, there was a trend towards a slight reduction in MIP-1 β plasma levels in the study participants with a CD4⁺ T cell percentage of <14% when compared with those with a CD4⁺ T cell percentage of >14%. Thus, in terms of stage of HIV-1 disease there was a trend towards a reciprocal relationship between SDF-1 α and MIP-1 β levels. One reason the reduction in MIP-1 β levels may not have been significant was that fewer specimens were analysed due to insufficient plasma, thus additional specimens are necessary to confirm whether SDF-1 α and MIP-1 β levels are reciprocally modulated over the course of HIV-1 disease.

Next, the effect of antiretroviral therapy on the plasma levels of these cytokines in HIV-1 infected adults was determined. Overall, the median SDF-1 α levels decreased and the median MIP-1 β levels increased during the study period, and although not found to be significant suggests some normalization of chemokine levels.

Taken together, work from this study has contributed to the study of CXCR4 and CCR5 expression, particularly with respect to age-related changes, presence of the CCR5 Δ 32 polymorphism, modulation of expression by HIV-1 and TB disease, stage of HIV-1 disease and treatment with HAART. Here a reciprocal relationship between CXCR4 and CCR5 receptor expression was identified, as well as between SDF-1 α and MIP-1 β levels which was evident in a number of different studies. Future work to analyse the mechanism for the reciprocal

modulation of CXCR4 and CCR5, and their ligands, has important implications for understanding the pathogenesis of HIV-1 disease.

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APPENDIX A: Abbreviations

μl	microlitre
°C	degrees Celsius
6Ckine	chemokine with 6 cysteines
ABC	antibodies bound per cell
ACD	acid citrate dextrose
AIDS	acquired immunodeficiency syndrome
AMAC	alternative activated macrophage associated CC-chemokine
AOP-RANTES	aminooxypentane RANTES
ART	antiretroviral therapy
ATAC	activation-induced T cell-derived and chemokine related
BCA-1	B cell-attracting Chemokine 1
BL	baseline
BLC	B lymphocyte chemoattractant
BRAK	breast and kidney derived chemokine
BSA	bovine serum albumin
CB	cord blood
ChemR	chemokine receptor
Ckβ	chemokine β
CTL	cytotoxic T lymphocyte
Cys	cysteine
DAG	diacylglycerol
DARC	duffy antigen receptor for chemokines
DC-CK1	dendritic cell-derived chemokine 1

ECL	extracellular loop
EDTA	ethylenediaminetetra-acetic acid
ELC	Epstein-Barr virus-induced receptor ligand chemokine
ELISA	human enzyme-linked immunosorbent assay
ELR	glutamic acid-leucine-arginine
ENA	epithelial cell-derived neutrophil-activating
ER	endoplasmic reticulum
ESkine	embryonic stem cell chemokine
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FL	fluorescence
FSC	forward scatter
GCP	granulocyte chemotactic protein
GDP	guanine diphosphate
gp	glycoprotein
GPR	G-protein-coupled receptor
GRO	growth related oncogene
GTP	guanine triphosphate
h	hour
HAART	highly-active antiretroviral therapy
HCC	haemofiltrate CC chemokine
HIV	human immunodeficiency virus
I-309	a human CC chemokine
I-TAC	interferon-inducible T cell alpha chemoattractant
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin

IP-10	interferon- γ -inducible protein 10
IP ₃	inositol triphosphate
kDa	kiloDalton
LAM	lipoarabinomannan
LARC	liver and activation regulated chemokine
LCC	liver CC chemokine
LEC	liver-expressed chemokine
LESTR	leukocyte-derived seven-transmembrane domain receptor
Lkn	leukotactin
LMC	lymphocyte and monocyte chemoattractant
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LTNP	long-term non-progressor
M-tropic	macrophage-tropic
mAb	monoclonal antibody
MCAF	monocyte chemotactic and activating factor
MCIF	macrophage cytotoxicity inducing factor
MCP	monocyte chemoattractant protein
MDC	macrophage-derived chemokine
MGSA	melanoma growth stimulating activity
Mig	monokine induced by γ -interferon
min	minutes
MIP	macrophage inflammatory protein
ml	millilitre
mM	millimolar
MPIF	myeloid progenitor inhibitory factor
mw	molecular weight

NAP	neutrophil activating protein
NCC	new CC chemokine
nd	not determined
ND	normal donor
NK	natural killer
NNRTI	non-nucleoside reverse transcriptase inhibitor
NNY-RANTES	N-nonanoyl-RANTES
NRTI	nucleoside reverse transcriptase inhibitor
NSI	non syncytium-inducing
ON	overnight
PARC	pulmonary and activation-regulated chemokine
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSF	pre-B cell growth stimulating factor
PE	phycoerythrin
PEP	post-exposure prophylaxis
PerCP	peridinin chlorophyll
PF	platelet factor
PHA	phytohaemagglutinin (lectin from <i>Phaseolus vulgaris</i>)
PLC	phospholipase C
RANTES	regulated on activation, normal T cell expressed and secreted
RBC	red blood cells
RFI	relative fluorescence intensity
RNA	ribonucleic acid
ROC	regulator of cullins
SCID	severe combined immunodeficiency
SCM	single C motif

SDF	stromal cell-derived factor
SI	syncytium-inducing
SIV	simian immunodeficiency virus
SLC	secondary lymphoid tissue chemokine
SSC	side scatter
T-tropic	T cell-tropic
t0	time 0
TAR	trans-activation response region
TARC	thymus- and activation-regulated chemokine
TB	tuberculosis
TCA-4	thymus-derived chemotactic agent-4
TECK	thymus-expressed chemokine
Th	T-helper
TNF	tumour necrosis factor
TYMSTR	T-lymphocyte-expressed seven-transmembrane domain receptor
UN	uninfected
VL	viral load
WBC	white blood cells
wt	wild type

APPENDIX B: Monoclonal antibody panels**Panel 1**

Tube number	FL1 (FITC)	FL2 (PE)	FL3 (PerCP)
1	CD45	CD14	
2	IgG ₁	IgG _{2a}	
3	CD19	CXCR4	
4	CD19	CCR5	
5	IgG _{2a}	IgG _{2a}	
6	CD14	CXCR4	
7	CD14	CCR5	
8	IgG ₁	IgG _{2a}	IgG ₁
9	CD16+CD56	CXCR4	CD3
10	CD16+CD56	CCR5	CD3
11	CD3	CXCR4	CD4
12	CD3	CXCR4	CD8
13	CD3	CCR5	CD4
14	CD3	CCR5	CD8

Panel 2

Tube number	FL1 (FITC)	FL2 (PE)	FL3 (PerCP)
1	CD45	CD14	
2	IgG ₁	IgG _{2a}	
3	CD19	CXCR4	
4	CD19	CCR5	
5	IgG _{2a}	IgG _{2a}	
6	CD14	CXCR4	
7	CD14	CCR5	
8	IgG ₁	IgG _{2a}	IgG ₁
9	CD16 + CD56	CXCR4	CD3
10	CD16 + CD56	CCR5	CD3
11	CD45RA	CXCR4	CD4
12	CD45RA	CXCR4	CD8
13	CD45RA	CCR5	CD4
14	CD45RA	CCR5	CD8
15	CD45RO	CXCR4	CD4
16	CD45RO	CXCR4	CD8
17	CD45RO	CCR5	CD4
18	CD45RO	CCR5	CD8
19	CD38	CXCR4	CD8
20	CD38	CCR5	CD8

APPENDIX C: Statistical evaluation of results

SPSS^R software version 11.0 for Windows (SPSS Inc., Chicago, Illinois) was used for all the statistical analyses. Values of $P < 0.05$ were considered to be statistically significant.

C1 Comparison of two samples

C1.1 Mann-Whitney U-test

The Mann-Whitney U-test is a nonparametric test that is used when data from two independent samples, possibly of different size, are being compared. This technique uses the ranks of data rather than their actual values. Comparison of CXCR4 and CCR5 expression on different leukocyte subsets between the different study groups was done using the Mann-Whitney U test.

C1.2 Wilcoxon signed ranks test

The Wilcoxon signed ranks test is a nonparametric technique that is used when data from two related samples are compared. It makes use of ranked data, and makes no assumptions about the shapes of the distributions of the two variables. This technique was used to analyze data from HIV-1 infected individuals before and 8 weeks after starting HAART.

C2 Spearman's rank correlation

Spearman's rank correlation is a commonly used nonparametric technique to test the direction and strength of association between two variables. This method uses ranked (relative) data, rather than the actual data values. The values of each of the variables are ranked from smallest to largest. It uses the statistic r which falls between -1 and +1. If $r = -1$, there is a perfect negative correlation, and similarly if $r = +1$ there is a perfect positive correlation between two sets of data. The Spearman correlation coefficient was calculated to determine correlations between various immune and viral parameters and expression of either chemokine receptor.

APPENDIX D: Ethical clearance

This study was approved by the University of the Witwatersrand Ethical Committee, protocol number M000113. Patients were recruited after informed consent was obtained and confidentiality of all records ensured.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)

Ref: R14/49 Shalekoff

CLEARANCE CERTIFICATE

PROTOCOL NUMBER M13

PROJECT

HIV Coreceptors: Distribution, Modulation & Function

INVESTIGATORS

Dr S Shalekoff

DEPARTMENT

Dept of Virology, National Institute for Virology

DATE CONSIDERED

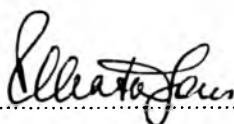
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DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 00/03/29

CHAIRMAN.....



.....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr C Tiemessen

Dept of Dept of Virology, National Institute for Virology

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DECLARATION OF INVESTIGATOR(S)

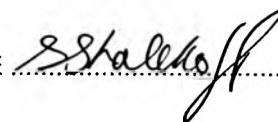
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

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

DATE

5/4/99

SIGNATURE



PROTOCOL NO.: M 13

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES