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The Regulation of CXCR4 and CCR5 Expression on Human Monocytes byTh1 and Th2 Cytokines: Functional Implications on Cell Migration and HIV Infection

by

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Master of Science

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Abstract

The chemokine receptors CXCR4 and CCR5 have recently been identified as the primary coreceptors that work in conjunction with CD4 to mediate infection by HIV. CCR5 is the coreceptor used by M-tropic HIV, which is important in transmission and dominate the early stages of infection. Later in infection there is a switch to a predominance of T-tropic virus, which use CXCR4 as a coreceptors, and this has been associated with progression to AIDS. Monocytes, which express both CXCR4 and CCR5, are susceptible to infection by HIV and act as a viral reservoir. Alterations in the expression of CXCR4 and CCR5 have been associated with corresponding changes in the susceptibility of these cells to HIV infection. The role of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines in the regulation of CXCR4 and CCR5 expression by monocytes, and the functional implications of Th1 and Th2 cytokine induced alterations in chemokine receptor expression on chemotaxis and HIV infection, are poorly understood and constituted the major aim of this investigation.

PBMC isolated from HIV negative and HIV positive adult peripheral blood, and HIV negative cord blood, were cultured in the presence and absence of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines. The expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was measured by flow cytometry. IL-2 decreased the expression of CXCR4, but did not alter the expression of CCR5 on monocytes. IFN-γ, in contrast, decreased the expression of both CXCR4 and CCR5 on the surface of monocytes. Despite the similarities in their effects on T cells, IL-4 and IL-13 exhibited differential effects on chemokine receptor expression by monocytes. IL-4 decreased CXCR4 expression on monocytes, but was unable to influence the expression of CCR5. In contrast to this, IL-13 decreased the expression of both CXCR4 and CCR5 on the surface of monocytes.

To understand the molecular mechanisms of chemokine receptor down regulation induced by Th1 and Th2 cytokines, the expression of mRNA encoding CXCR4 and CCR5 was examined. Alterations in chemokine receptor expression on the surface of monocytes by Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines were not associated with changes im mRNA expression, as determined by RT-PCR analysis. In order to investigate the possibility that Th1 and Th2 cytokines are inducing chemokine secretion that is responsible for the observed down regulation, the secretion of chemokines from HIV negative adult PBMC was examined using ELISA. The secretion of the CXCR4 ligand SDF-1 was increased by IL-2, IFN-γ, IL-4 and IL-13. In contrast, secretion of the CCR5 ligand RANTES was induced by only IFN-γ and IL-13. Stimulation with Th1 and Th2 cytokines may have caused chemokine receptor internalization following interaction with their endogenously produced ligands, which may be responsible for the observed down regulation. While the association of chemokine secretion with decreased chemokine receptor expression may explain the observed effect, further investigations are necessary to confirm this proposition.

The functional consequences of decreases in CXCR4 and CCR5 expression by Th1 and Th2 cytokines were investigated by chemotactic assays. Monocytes from HIV negative adults, HIV positive adults, and HIV negative cord blood were cultured in the presence and absence of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines before

their ability to migrate in response to the CXCR4 ligand SDF-1, and the CCR5 ligand MIP-1α, were measured. The decreases in chemokine receptor expression induced by Th1 and Th2 cytokines were associated with a corresponding inhibition of monocyte chemotaxis induced by these chemokines.

The consequences of decreases in CXCR4 and CCR5 expression by Th1 and Th2 cytokines were also investigated with respect to HIV entry and replication. Monocytes from HIV negative adults were incubated in the presence and absence of Th1 and Th2 cytokines before exposure to dual tropic HIV. Viral entry was detected by PCR specific for HIV *gag* DNA. Stimulation of monocytes with Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines was unable to influence entry of dual tropic HIV. To investigate the effects of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines on HIV replication, monocytes from HIV negative adults were cultured in the presence and absence of Th1 and Th2 cytokines before exposure to M-tropic HIV. HIV replication was monitored using ELISA to detect the production of p24 in the culture supernatants. Viral replication was inhibited by IFN-γ, IL-4 and IL-13, but was unaffected by IL-2. These results suggest that IFN-γ, IL-4 and IL-13 inhibit replication of HIV at points subsequent to entry.

In summary, these results suggest that decreases in CXCR4 and CCR5 expression on monocytes, induced by Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines, were associated with an inhibition of monocyte chemotaxis induced by the ligands of these chemokine receptors. However, the decreases in chemokine receptor expression by Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines were unable to inhibit HIV entry in monocytes. HIV replication in monocytes was inhibited by IL-4, IL-13 and IFN-γ, but this inhibition affects unknown post entry events in the viral life cycle.

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List of Abbreviations

A	Adenosine
AIDS	Acquired Immune Deficiency Syndrome
AOP-RANTES	Aminooxypentaine RANTES
APC	Antigen Presenting Cell
bp	Base pair
C-terminal	Carboxy terminal
CCR2b	CC chemokine receptor-2b
CCR3	CC chemokine receptor-3
CCR5	CC chemokine receptor-5
CD	Cluster of Differentiation
cDNA	Complementary DNA
CMV	Cytomegalovirus
CXCR4	CXC chemokine receptor-4
DARC	Duffy blood group antigen
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
ELISA	Enzyme Linked Immunosorbent Assay
ELR Motif	Glutamic acid - Leucine - Arginine Motif
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
G protein	Guanine nucleotide binding protein
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
gp	Glycoprotein
GRO	Growth Related Oncogene
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
hrs	Hours
IFN	Interferon
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
kDa	Kilo-Dalton
LTR	Long Terminal Repeat
M-tropic	Macrophage tropic
MCF	Mean Channel Fluorescence
MDM	Monocyte-Derived Macrophages
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
M-MLV	Murine-Maloney Leukemia Virus
MOI	Multiplicity of Infection

mRNA	Messenger RNA
N-terminal	Amino terminal
NF	Nuclear Factor
NK	Natural Killer
OPD	O-phenylenediamine
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PHA	Phytohemagglutinin
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RNA	Ribonucleic acid
rpm	Revolutions per Minute
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SDF	Stromal Derived Factor
STAT	Signal Transducer and Activator of Transcription
T-tropic	T cell tropic
TGF	Transforming Growth Factor
Th	T helper
TNF	Tumour Necrosis Factor
V3 Loop	Third Variable Loop

Introduction

HIV and AIDS

In 1981 acquired immune deficiency syndrome (AIDS) was identified as a susceptibility to opportunistic infections and rare cancers which resulted from a loss of $CD4^+$ T cells. This condition was found to be associated with the transmission of body fluids, and this led to the identification of human immunodeficiency virus (HIV) as the cause of this condition in 1983. In infected individuals, HIV can be found in $CD4^+$ T cells and macrophages, as well as in body fluids such as blood, semen, vaginal fluid, and breast milk. The consequences of this are that HIV transmission can occur through the use of contaminated needles or infected blood products, sexual intercourse, and from mother to child during delivery or by breast feeding. HIV exists in two major forms which differ in their virulence and geographical localization. HIV-1 is the most virulent form and the cause of most of the AIDS cases in the world, while the less virulent HIV-2 is confined mostly to Africa and India.

Typically, viral infections follow one of two general courses. Some viruses cause an acute infection which is quickly eliminated by the immune system, and immunological memory results. Other viruses can establish a chronic or latent infection, in which the immune system is unable to eliminate the virus, but the infection is under control. HIV infection does not follow either of these typical courses, as it establishes a persistent infection which the immune system is unable to control. Initially, HIV infection seems to be subdued by the immune system, but the virus continues to replicate and destroys the cells that control the immune response.

The first stages of infection with HIV are usually asymptomatic, but in some cases infected individuals experience symptoms that resemble influenza (1). At this early

stage of infection there is a great deal of virus in the blood (viremia), and a significant decrease in the number of $CD4^+$ T cells (1). This is the acute stage of infection, and is the stimulus for the production of antibodies (seroconversion) and $CD8^+$ T cell activation (2;3). These immune responses, particularly the activation of $CD8^+$ T cells, are responsible for bringing the infection under apparent control and reducing the amount of virus in the blood, which allows $CD4^+$ T cells to recover to almost normal levels.

It is at this point that the infection enters the stage known as clinical latency or the asymptomatic phase. This stage of infection is characterized by continued virus replication, and the vast majority of the virus found in the blood at this point is produced by newly infected cells (1). The level of viral replication and the subsequent plasma viral load is increased by immune activation caused by HIV itself, or infection by other pathogens (4). The persistent viral replication and T cell death that occurs in this period ultimately results in a severe immunodeficiency, which allows the appearance of opportunistic infections and rare cancers that would normally be eliminated (1). It is the appearance of these illnesses, which are rarely seen in immunocompetent individuals, and the depletion of $CD4^+$ T cells to less than 200 per mm^3, that mark the transition to AIDS (1).

While the mortality rate of HIV infection continues to be high, there have been groups of infected individuals who provide hope that this virus can be overcome. One such group, termed long term non-progressors, is composed of individuals who have undergone seroconversion but fail to show signs of a compromised immune system. They tend to maintain very low levels of virus in their blood and are presumed to have

mounted an immune response that effectively controls the infection, thereby preventing progression to AIDS (2;3).

Another group of individuals have had confirmed HIV exposures, but have failed to seroconvert or show signs of infection. These individuals do not develop signs of immunodeficiency or clinical AIDS, and there is some evidence that they have mounted a cell-mediated immune response against the virus (3;5). The fact that they do not become immunocompromised, or show any other signs of HIV infection, suggests that they have eliminated the virus with this response.

Finally, a small number of individuals seem to have some protection from HIV infection provided by a mutated form of the chemokine receptor CCR5 (5-7). The anomaly is a 32 base pair (bp) deletion that results in a non-functional, truncated receptor that is not expressed on the surface of cells (7). Individuals that are homozygous for this mutant have been shown to be resistant to infection by macrophage (M)-tropic strains of HIV (6;7).

Pediatric AIDS

The length of the latent period of HIV infection can vary widely, averaging 9 years in adults (8). In contrast to this, the average length of the latent period is significantly shorter in newborns infected by maternal-infant transmission, as most progress to AIDS in a year (8;9). The reasons for this difference in latent phase length have not been determined, and investigation is complicated by the fact that immune system development is not completely understood.

Cord blood, drawn from the umbilical cord at birth, has been the subject of intense investigation due to its possible uses as a source of stem and progenitor cells. This work has subsequently provided considerable insight into the immune systems of infants, and the cells of which it is comprised. The most obvious discrepancy between cord blood and adult peripheral blood is the presence of stem and progenitor cells, which are considerably more abundant in cord blood (9;10). There are also a great deal of $CD45RO^+$ memory T cells in adult blood, which are essentially absent in cord blood (10). These memory T cells have already been exposed to antigen, and have been maintained by the immune system to ensure a quick response should that particular antigen be encountered again. The T cells in cord blood are mostly naïve $CD45RA^+$ T cells, which have not yet been exposed to an antigen (10). This is likely to be an important factor in the immaturity of immune responses observed in infants. Cord blood T cells have been shown to have a decreased ability to produce cytokines, such as interleukin (IL)-2, IL-4, and interferon (IFN)-γ, and decreased ability to proliferate in response to cytokines or other forms of stimulation (9). Immune cells derived from cord blood have also been shown to have a decreased ability to respond to cytokines (11), and to differ from adult cells in their ability to migrate in response to certain chemokines (12). This lack of response to various stimuli is likely to be a consequence of the immaturity of these cells. In comparison to adult blood, cord blood also has an increased CD4/CD8 ratio, less $CD3^+$ T cells, low expression of human leukocyte antigen (HLA)-DR, and small populations of $CD3^-CD8^+$ and $CD3^-CD7^+$ cells which are absent in adults (10). All of these deficiencies in the expression of important immune molecules, and the presence of immature cells that are not found in adults, contribute to the immunological immaturity of infants.

	Cord Blood	Adult Blood
CD4:CD8 Ratio	3.2	1.7
CD3+ Cells	15.7%	62%
CD45RA:CD45RO Ratio	8	<1
CD5+ CD19+ Cells	2.14%	1%
CD3- CD8+ Cells	1.5%	Not Detectable
CD3- CD7+ Cells	5%	Not Detectable
HLA-DR Expression	3.4%	14%

Table 1: Comparison of Lymphocyte Populations and Expression of Surface Molecules in Umbilical Cord Blood and Adult Peripheral Blood (10).

The Human Immunodeficiency Virus

HIV is an enveloped retrovirus, which belongs specifically to a group referred to as lentiviruses. Like all retroviruses, HIV has an RNA genome. Each virus contains two copies of its RNA genome which, upon entry into target cells, is reverse transcribed into complementary DNA (cDNA) that is subsequently integrated into the genome of the host cell. This is accomplished by two enzymes that are present in the viral particle, the viral reverse transcriptase and integrase, respectively. This integrated viral cDNA, called the provirus, first provides a template for the production of the messenger RNA (mRNA) used to produce viral proteins, and then for the production of new RNA genomes. Once assembled, these newly formed viruses leave the host cell by budding, and take a portion of the plasma membrane which forms the viral envelope.

The genome of HIV is composed of nine genes, three of which are common to all retroviruses. These are known as *gag* (which is expressed as the structural proteins of the viral core), *pol* (which when expressed forms the enzymes required for integration and replication) and *env* (which contains the information required to make the glycoproteins that reside in the viral envelope). The other genes encode proteins involved in viral replication and infectivity, and include *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef*. These nine genes are found between two structures called long terminal repeats (LTR), which are necessary for integration of the provirus, and later for the expression of the viral genes since the LTR contains the binding sites for transcription factors (1). The most important transcription factor for the expression of HIV genes is nuclear factor (NF)-κB, which binds to these promoters in the LTR (1;4). This transcription factor is abundant in activated $CD4^+$ T cells, and its purpose is the induction of RNA polymerase which is used by the cell to transcribe genes, but in this case also transcribes those belonging to the virus (4). After transcription, viral mRNA is subjected to numerous splicing pathways which generate the mRNA required for the production of viral proteins.

The ability of HIV to infect cells is due to the expression of a non-covalently linked complex of glycoprotein (gp)120 and gp41 on the surface of the viral envelope. These two viral glycoproteins are encoded by the *env* gene, and expressed as a single protein called gp160 that is subsequently cleaved to form gp120 and gp41 by a protease from the host cell (13). The high affinity of gp120 for CD4, expressed on the surface of human T cells, monocytes, and macrophages, causes the viral particles to target these cells specifically. The first step in the infection of a cell by HIV is the interaction of gp120 with CD4. Due to their affinity for one another, these molecules form a stable non-

covalent bond, which causes a conformational change in gp120 that exposes a region of this molecule called the third variable (V3) loop (13-16). It is this V3 loop that is responsible for chemokine receptor binding, and its exposure allows interaction with a chemokine receptor on the surface of the host cell (13;14). Chemokine receptor binding by the V3 loop brings viral gp41 close to the host cell membrane, and this allows gp41 to mediate fusion of the viral particle with the target cell, permitting the viral genome and proteins to enter the cytoplasm (13). It is the specificity of sequences in the V3 loop of gp120 for various chemokine receptors that determines the cellular tropism of the virus (14;16).

Although $CD4^+$ T cells are the primary target of HIV, during the early stages of infection and the asymptomatic phase, viral isolates from patients are able to infect and replicate in macrophages, but not established T cell lines (17-21). These HIV strains are referred to as M-tropic, and are the viral phenotype primarily responsible for transmission (17-21). Viral isolates from the early stages of infection also fail to induce the formation of syncytia, and are therefore referred to as non-syncytium inducing (17-19). Monocytes and macrophages do not suffer the same cytopathic effects of HIV that T cells endure, and therefore provide a constant viral reservoir (17;18;22). These infected monocytes and macrophages can be found in the blood, as well as in numerous tissues such as the brain, and because of this these cells are thought to be important in the spread of the virus throughout the body (17).

With disease progression there is a switch in viral phenotype, and the virus acquires the ability to infect and replicate within established T cell lines (17-21). Viral isolates of this type are called T cell (T)-tropic, and these strains have usually lost the

ability to productively infect macrophages while acquiring a syncytium inducing phenotype (17-21). The reasons for the switch in viral phenotype are largely unknown, but it is presumed to be due to the appearance of a selective advantage for T-tropic strains. However, there have been many instances in which clinical isolates from patients in the advanced stages of disease have been dual tropic, and shown the ability to productively infect both macrophages and T cell lines to different degrees (17;18;21).

Th1 and Th2 Immune Responses

Naïve $CD4^+$ T cells are activated by antigen presenting cells (APC) to develop into T helper (Th) cells belonging to either the Th1 or Th2 subset. This classification is based on the cytokines that the activated Th cells produce. Th1 cells secrete IL-2 and IFN-γ, and are responsible for promoting cell-mediated immunity (23-25). Th2 cells secrete predominantly IL-4, IL-5, IL-10 and IL-13, and are vital to the humoral immune response (23-25). The Th1 cytokine IFN-γ has also been shown to inhibit the production of Th2 cytokines, and therefore the production of Th2 cells (23;26). Similarly, the Th2 cytokines IL-4 and IL-10 inhibit the production of Th1 cytokines, and Th1 cell development (23;26). The direction that the immune response will take is determined by numerous factors whose complex interactions are not completely understood. It is known that the type of costimulation used by the APC, the characteristics of the antigen, and the type of major histocompatibility complex (MHC) molecule used to present the antigen all have an impact on this polarization (27;28). In addition to these influences, the cytokines that are produced early in the response, by cells that first encounter the antigen, seem to be of particular importance in determining the type of immune response that will result

(29;30). The polarization of the immune response in this manner can be vital to its effectiveness.

Chemokines and Chemokine Receptors

Chemoattractant cytokines, or chemokines, are small (8-12 kDa) polypeptides that are secreted during infection, inflammation, or tissue damage (13). They are secreted by many cell types, including phagocytes, endothelial cells, and fibroblasts. Chemokines work in conjunction with chemokine receptors to activate and recruit selected immune cells to specific sites in the body (13). Chemokines share anywhere from 20 to 90 % of their amino acid sequence, and the entire family has been divided into classes according to the arrangement of four conserved cysteine residues. In CXC, or α chemokines, there is an amino acid separating the first two of four conserved cysteines, while CC, or β chemokines lack the separation at this position (13;31;32). The two most recently identified groups include the C, or γ chemokines in which the first and third cysteines are absent, and the CX3C chemokines in which there are three amino acids separating the first two cysteines (13).

The CXC chemokines can be further subdivided into two groups. One of which contains a three amino acid sequence of glutamic acid-leucine-arginine (ELR) immediately before the first of the four conserved cysteines (33). These chemokines, which include IL-8, growth related oncogene (GRO) α, GROβ and GROγ, predominantly cause the activation and chemotaxis of neutrophils (33). The CXC chemokines that do not contain the ELR motif, such as stromal derived factor (SDF)-1α and SDF-1β, the CC chemokines, including macrophage inflammatory protein (MIP)-1α,

MIP-1β, and RANTES (regulated upon activation normal T cell expressed and secreted), and the C chemokine lymphotactin cause the activation and chemotaxis of monocytes, T cells, B cells, dendritic cells, natural killer (NK) cells, eosinophils and basophils (33). The study of chemokines *in vitro* has revealed redundancy and overlap in their functions, but it remains to be seen if this is the situation *in vivo*. Some chemokines have additional functions which include effects on hematopoietic cells, smooth muscle cells, fibroblasts, and keratinocytes, in addition to roles in angiogenesis, wound healing and viral infection (33).

Chemokine receptors are named according to the type of chemokine that they bind (13). For example, CC chemokines are bound by CC chemokine receptors, and CXC chemokines bind to CXC chemokine receptors. There is considerable ligand overlap within chemokine receptor groups, and expression of specific chemokine receptors on particular leukocyte subsets is responsible for determining which chemokines recruit or effect these cells (13;25). Typically, leukocytes express more than one type of chemokine receptor, which allows them to be responsive to numerous chemokines (13). In addition to leukocytes, erythrocytes have been found to express a chemokine receptor called the Duffy blood group antigen (DARC) which can bind many CC and CXC chemokines (13). Cytomegalovirus (CMV) and herpes saimiri virus both have chemokine receptors encoded in their genomes which bind chemokines (13). There are presently nine CC chemokine receptors (CCR1-CCR9) and four CXC chemokine receptors (CXCR1-CXCR4), and this list is continually growing as more receptors and chemokines are discovered (13).

All chemokine receptors share a highly conserved seven transmembrane domain structure, and are coupled to signaling pathways that are mediated by guanine nucleotide binding (G) proteins (13;32). Structurally, chemokine receptors have an extracellular amino (N)-terminal section composed of loops containing conserved cysteine residues (13). These cysteine residues form the disulphide bonds that are responsible for the structure of this portion of the receptor (13). These extracellular loops also contain the regions of highest variability between chemokine receptors, which are believed to play a vital role in determining the specificity of chemokine binding (13). There are also numerous residues in the N-terminal section of chemokine receptors which are glycosylated (13). The seven hydrophobic transmembrane domains are highly conserved among both chemokine receptors and other G protein coupled receptors (13). Chemokine receptors are different from all other seven transmembrane receptors in that they have smaller third intracellular domains (13). This domain is also believed to play a role in G protein binding, but the consequences of this change in its size are not defined (13). The intracellular carboxy (C)-terminal domain of chemokine receptors is only 40-50 amino acids in length, which is smaller than that of other G protein coupled receptors (13). The cytoplasmic domain also contains serine and threonine residues that are available for phosphorylation, in addition to sequence elements that are involved in interactions with G proteins and other cell signaling molecules (13).

Chemokine and chemokine receptor expression are modulated by factors such as the state of activation, the stage of differentiation, the functional subset to which the cell belongs, and the presence of cytokines (13). The mechanisms responsible for the immediate regulation of chemokine receptor expression include receptor recycling

between the plasma membrane and intracellular vesicles, and the synthesis and destruction of receptors (13). This recycling mechanism seems to be of particular importance to the chemotaxis of immune cells, as there may be a link between chemokine induced endocytosis of chemokine receptors and the ability of these cells to migrate (13). Prolonged regulation of chemokine receptor levels can be accomplished by alterations in transcription or translation (13).

Chemokine Receptors and HIV Infection

The chemokine receptors CXCR4 and CCR5 have been identified as the primary coreceptors that, in conjunction with CD4, allow the fusion of HIV with human monocytes and T cells (4;13). A small number of HIV isolates have proven to use other chemokine receptors as coreceptors, such as CCR2B and CCR3, but the majority of HIV isolates use CXCR4 or CCR5 at some point in the course of an infection (4;17;31). CXCR4 and CCR5 are the coreceptors responsible for allowing infection by T-tropic and M-tropic HIV-1 strains, respectively (4). CXCR4 is also known as LESTR and fusin, and was identified because of its sequence and structural homology to other chemokine receptors before its natural ligand was discovered. Later research has demonstrated that its natural ligand is SDF-1α (4;13). CXCR4 is primarily expressed on haematopoietic progentitor cells, thymocytes, and resting naïve T cells that have the phenotype $CD26^{low}CD45RA^{+}CD45RO^{-}$, but is also found on B cells, monocytes and macrophages (4;13). In its role as a chemokine receptor, CCR5 binds MIP-1α, MIP-1β, and RANTES (4;13). It has been shown to be present on activated memory T cells that are characteristically $CD26^{high}CD45RA^{low}CD45RO^{+}$, dendritic cells, monocytes, and

macrophages (4;13). The natural ligands of each chemokine receptor are responsible for the induction of chemotaxis in cells expressing their respective receptor (13).

The importance of these chemokines and chemokine receptors in HIV infection was realized when unknown factors secreted by $CD8^+$ T cells, which inhibited M-tropic HIV infection, were found to be MIP- 1α, MIP-1β, and RANTES (4). Later work identified CCR5 as the receptor for these chemokines (4). Interestingly, these factors failed to inhibit infection by T-tropic strains. This encouraged work leading to the discovery that CXCR4 was the coreceptor for these virus strains, and that the ligand of this receptor was able to block infection by T-tropic viruses (4). Experiments from numerous groups followed these initial discoveries, and demonstrated conclusively that CXCR4 and CCR5 were the coreceptors for T-tropic and M-tropic HIV, respectively, and that infections with each virus could be inhibited by the ligands of their respective coreceptor (4). This resistance to infection seems to be due to either the transient down regulation of the chemokine receptor from the cell surface as a result of ligand binding, or the ability of the bound chemokine to prevent interaction of the virus with the chemokine receptor (13). The ability of chemokines to inhibit HIV infection has been impressive enough to cause the development and testing of modified chemokines, such as aminooxypentane (AOP)-RANTES, for the prevention and treatment of HIV (13). These studies are in the early stages, but have shown some promise. Despite the excitement generated by this research, there is still considerable caution in this area. Since these experimental treatments are specific for a particular viral strain, they may accelerate the switch in viral phenotype that has been correlated with disease progression (4).

An important, and yet poorly understood aspect of HIV infection is the switch in viral phenotype that appears to correlate with the onset of progressive disease. As previously discussed, initial infection with HIV is usually established by an M-tropic virus. Later, there is a switch in viral phenotype, and a T-tropic strain emerges as the dominant form of the virus. This switch in viral strain is linked with an increase in T cell loss, increased viral replication, and progression to AIDS (1;4;17;34;35). This switch in viral tropism may occur due to mutation in the V3 loop of gp120, which is the region responsible for binding chemokine receptors (4). Due to the high rate of viral replication during infection, and the low fidelity of the viral reverse transcriptase, mutations such as these may be common (4). Early in infection, viruses that mutate into a T-tropic strain do not experience any selective advantage and are likely to be out-competed by M-tropic strains, but later in infection conditions may change and provide an environment in which these T-tropic strains can prosper (4). Host factors such as chemokine and cytokine secretion, and situations like the introduction of other pathogens, may all play a role in establishing an environment that is conducive to the replication of T-tropic HIV (4;19;21). The reasons for this switch in viral tropism are not fully understood, but a number of factors that can provide selective pressure are being investigated.

Cytokines and Viral infections

Cytokines are an important part of the immune response to pathogens such as viruses, and there has been considerable work done on the use of cytokines for treatment of viral infections, and as a component of vaccines directed against them. Cytokines have roles in the induction of the immune response, its polarization towards cell mediated or

humoral immunity, the coordination of an ongoing response, and the formation of immunological memory (36). Some of the mechanisms utilized to accomplish this control are the regulation of the activation state of specific immune cells and their attraction to sites of infection, as well as the direction of the expression of adhesion molecules, costimulatory molecules, and MHC molecules (36). In vaccinia virus infection of mice, for example, IL-2 was shown to be effective in controlling the infection due to its ability to attract NK cells to the site of infection, while IL-12 was effective in eliminating the virus by inducing a Th1 immune response (36). Cytokines such as tumour necrosis factor (TNF)-α and IFN-γ have also proven to have anti-viral activity by directly inhibiting viral replication in infected cells, and mice that were unable to produce these cytokines showed particular susceptibility to viral infections (36). Th2 cytokines, such as IL-4, which push the immune system towards a humoral response, were ineffective in controlling this vaccinia infection in the murine model (36).

Cytokines have been demonstrated to be an important factor in HIV infection, as the virus itself induces the expression of proinflammatory cytokines which provide it with a stimulus for replication (4). Infection with HIV also results in a chronic state of immune activation which causes disorder in the cytokine control system (4). The net result of this effect on the control of the cytokine network is the suppression of an effective anti-viral response to a rapidly reproducing virus, along with damage caused by the potent effects of long term exposure to high levels of these immune mediators. Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are present at high levels in most cases of chronic immune stimulation, such as that induced by HIV infection (4). These cytokines are highest in the late stages of infection, and have been found in the

tissues, serum, and cerebrospinal fluid, in addition to the lymphoid tissues where they are found with elevated levels of IL-10 and IFN-γ (4). The primary sources of these proinflammatory cytokines are believed to be chronically activated macrophages and $CD8^+$ T cells (4). In addition to the chronic immune activation, there is some suggestion that the virus itself, or HIV proteins such as gp120 and tat, can cause increased production of these cytokines from immune cells (4).

There is also evidence which indicates HIV infection causes suppression of the synthesis and secretion of cytokines important for immune regulation, specifically IL-2 and IL-12 (4). These cytokines are essential in the development of cell mediated immune responses that are responsible for the elimination of viral infections. The importance of this Th1 mediated immune response in the effective control of HIV infection is highlighted by the controversial link between disease progression and a Th2 response. This correlation was established when it was discovered that the secretion of Th2 cytokines from the peripheral blood mononuclear cells (PBMC) of HIV positive individuals was increased with disease progression (4). In contrast to this, others have demonstrated a reversion to a Th0 state in which there is no polarization, and both Th1 and Th2 cytokines are secreted (4). This issue has not been resolved, but since HIV has proven to inhibit Th1 cytokine production and replicate more effectively in Th0/Th2 type conditions, this has become an important area of research.

Due to these discoveries, there has been considerable interest in the effects of cytokines on HIV replication in activated T cells and macrophages. This work has shown that HIV replication *in vitro* can be increased by IL-1β, IL-2, IL-3, IL-6, IL-7, IL-12, IL-15, TNF-α, TNF-β, macrophage colony-stimulating factor (M-CSF) and granulocyte-

macrophage colony-stimulating factor (GM-CSF), and decreased by IFN-α, IFN-β, and IL-16 (4). Cytokines such as IL-4, IL-10, IL-13, IFN-γ, and transforming growth factor (TGF)-β have variable effects that depend on the cell type being investigated and the experimental conditions (4). It should also be noted that the effects of some of these cytokines occur in both T cells and macrophages, while others are more specific to a particular cell type. As can be expected from the complex system of cytokine regulation, some cytokines have been shown to work together to amplify their effects, while others do the opposite (4). The result of this is that the net effect of a cytokine is a balance between its ability to promote and inhibit HIV replication in a particular system.

The exact mechanisms of the modulation of HIV replication by these cytokines are only known in a few examples. Increased HIV replication due to the proinflammatory cytokines TNF-α and IL-1β is due to their activation of the transcription factor NF-κB, which then causes the transcription of the provirus (4). IL-6 seems to increase HIV replication by a post-translational mechanism that is poorly understood (4). The inhibition of replication caused by IL-16 appears to be caused by a decrease in transcription of the provirus, which may be related to the ability of this cytokine to prevent T cell activation (4).

Cytokines are also able to influence HIV infection and replication by inducing the production of chemokines which modulate the expression of chemokine receptors (13;34;37-39). This significantly complicates the ability of cytokines to influence HIV infection and replication, as now the direct effects of cytokines on viral replication must be considered in addition to their impact on chemokine secretion and the expression of chemokine receptors. The consequences of alteration in chemokine and chemokine

receptor expression are specific to the particular viral strain which uses the effected receptor, and this further increases the complexity of these effects. Experiments dealing with the effects of cytokines on the production of chemokines have demonstrated that the proinflammatory cytokines TNF-α and IL-1β, in addition to cytokines such as IL-2, IL-15, and IFN-γ, promote the secretion of chemokines (4;31;34). These chemokines can then induce changes in cell activation states and chemokine receptor expression, both of which can impact HIV infection and replication.

Previous work on chemokine receptor expression by T cells from HIV negative individuals has found that CXCR4 is expressed at high levels on these cells, while CCR5 expression was very low, only being present on 0.1-2% of thymocytes (20). T cells from adult and cord blood have similar levels of CXCR4 expression which is seen on both memory and naïve cells (40). CCR5 expression is limited to memory T cells, and due to the lack of these cells in cord blood, there are few CCR5 expressing T cells present (40).

IL-4 has consistently been shown to cause an increase in the expression of CXCR4 on $CD4^+$ T cells (41-43). This effect has been associated with an increase in chemotaxis of these cells induced by the CXCR4 ligand SDF-1 (41;42), and an increased susceptibility of these cells to infection by T-tropic HIV (42). Cytokines such as IL-2 (20;42;43), IL-7 (20;42), IL-10 (43), IL-12 (43), and IL-15 (42) have also been shown to increase the expression of CXCR4 on the surface of T cells, and consequently increase the chemotaxis of these cells (42;43), and their susceptibility to T-tropic HIV infection (20;42). CXCR4 expression on T cells has been shown to be down regulated by IL-10 (41) and IL-2 (40), with a corresponding decrease in SDF-1 induced chemotaxis (41). CCR5 levels on the surface of T cells have been increased by culture with IL-2 (40;44),

and decreased by IL-12 (38). Decreases in CCR5 expression have been associated with secretion of MIP-1α and MIP-1β (38), and inhibition of M-tropic HIV infection (38). The expression of CCR5 on T cells was unaffected by culture with IL-4, IL-10, or IL-13 (38). Overall, the study of chemokine receptor expression on T cells has linked increased CXCR4 expression with Th2 cells and cytokines, while CCR5 expression seems to be favoured by Th1 conditions (24).

There is comparatively less information on the regulation of chemokine receptor expression on the surface of monocytes by cytokines. Freshly isolated monocytes from HIV negative individuals do not seem to express high levels of CCR5, but the levels of this chemokine receptor increase for the first few days of culture as these cells differentiate into macrophages (14;45). Macrophages themselves express CCR5 at high levels (14;45). The expression of CXCR4 is low on the surface of both monocytes and macrophages, but levels are sufficient to allow infection of these cells with a T-tropic HIV (39). There appears to be no significant difference between the levels of these chemokines receptors on monocytes from adult and cord blood (40).

The effects of the proinflammatory cytokine TNF-α have been thoroughly examined, and results indicate that this cytokine increases the replication of HIV in monocytes by stimulating viral transcription (46;47). There have also been studies which have shown that culture with TNF-α decreases the ability of HIV to infect these cells (22;46). Work which demonstrates the ability of TNF-α to decrease the susceptibility of macrophages to HIV infection has linked this inhibition to a decrease in CCR5 expression mediated by the secretion of chemokines such as RANTES (46). Investigation of IL-16 and its effects on MDM have demonstrated that this cytokine decreases the

transcription of both CXCR4 and CCR5 mRNA in these cells (48). Work done on the influence of IL-10 on CCR5 expression by monocytes has shown that this cytokine is capable of increasing the transcription and surface expression of CCR5 (49). This increase in CCR5 expression by IL-10 was associated with increases in functional measures such as calcium mobilization and chemotaxis induced by MIP-1β (49). These results were duplicated by a similar study which found that IL-10 increased CCR5 expression by monocytes, and was linked to increases in both chemotaxis induced by CCR5 ligands and infection by M-tropic HIV (50).

The Regulation of CXCR4 and CCR5 Expression by IL-2 and IFN-γ, and their Effects on Chemotaxis and HIV Infection

Although the ability of HIV to infect macrophages is well known, the factors which regulate the susceptibility of monocytes and macrophages to HIV are not well defined. In addition to this, the importance of these cells in the progression from HIV infection to clinical AIDS is only beginning to be investigated. The impact of Th1 and Th2 cytokines on the expression of CXCR4 and CCR5 by monocytes and macrophages, and the consequences of this with respect to chemotaxis and HIV infection, are poorly understood.

The primary function of IL-2 is to promote the proliferation of T cells, but this cytokine also plays a minor role in the growth of B cells and NK cells (23). IL-2 is also involved in the activation of monocytes, and stimulates the effector and secretory activities of these cells (51;52). IL-2 is secreted by naïve T cells and Th1 cells in response to stimulation by an APC (23), and has proven to stimulate the replication of

HIV (4). There has been little investigation of the effects of IL-2 on CXCR4 and CCR5 expression by monocytes and macrophages, or the effects of this cytokine on HIV replication in these cells. IL-2 has been demonstrated to increase HIV replication in a monocytic cell line (53) and in PBMC from HIV negative donors (54).

The principal physiological effect of IFN-γ is macrophage activation, but this cytokine also induces naïve $CD4^+$ T cells to become Th1 cells, activates NK cells, and increases the expression of MHC molecules by somatic cells (23). This cytokine is produced by T cells in response to infection by microbes, and is a vital part of host defense against these pathogens (55). IFN-α, IFN-β, and IFN-γ have all been demonstrated to have anti-viral, and specifically anti-HIV properties, in many cell types and numerous investigations (56-61).

IFN-γ has been shown to have controversial effects on HIV infection and replication. IFN-γ has been demonstrated to inhibit HIV replication in monocytes (31;62), macrophages (56), monocyte-derived macrophages (MDM) (63), and monocytic cell lines (64). The proposed reasons for this inhibition of replication include inhibition of late stages in the life cycle of HIV (63), an inhibition of viral gene expression (62), and decreases in CD4 expression (31;65). This cytokine has also demonstrated the ability to inhibit HIV entry in monocytes (62;65;66), macrophages (56), and monocytic cell lines (64), when these cells were cultured with IFN-γ prior to exposure to the virus. Contrary to this evidence, IFN-γ stimulation has also been shown to promote HIV replication when added after HIV infection (31), or to a latently infected monocytic cell line (67). After the identification of chemokine receptors as coreceptors for HIV, investigation of the effects of IFN-γ on these molecules revealed that this cytokine increases CCR5

expression in a monocytic cell line (68) and primary monocytes (31), but had no effect on CXCR4 expression (68). These changes in CCR5 expression were linked to corresponding increases in chemotaxis induced by CCR5 ligands (31;68). Recent investigation of the effects of IFN-γ have shown that this cytokine increases the replication of T-tropic HIV in MDM, while inhibiting the replication of an M-tropic virus (34). These results were associated with decreases in CD4 and CCR5 expression caused by IFN-γ, and no change in CXCR4 expression (34).

The Regulation of CXCR4 and CCR5 Expression by IL-4 and IL-13, and their Effects on Chemotaxis and HIV Infection

IL-4 and IL-13 are both secreted by Th2 cells, and have been shown to have many similar effects including the inhibition of the inflammatory functions of monocytes (69). The similarity in the actions of these cytokines may be due to the fact that they share receptor components and signaling pathways (70). Under normal physiological circumstances, IL-4 is responsible for B cell growth and activation, the induction of immunoglobulin class switching, the development of naïve T cells into members of the Th2 subset, and the inhibition of macrophages (71). IL-13 has effects similar to IL-4 on B cells and macrophages, but does not directly affect T cells (72).

Early work on the effects of IL-4 found that this cytokine generally promoted HIV infection and replication in monocytes (69;73), MDM (47;74;75), and monocytic cell lines (69;74), by increasing the expression of HIV mRNA (74;76). Contrary to this, other studies indicated that IL-4 inhibited early steps in viral replication in monocytes (73), and inhibited HIV replication if monocytes were exposed to this cytokine before

infection (76). IL-13 was also able to inhibit HIV infection in monocytes (69), and monocytic cell lines (69). Recent study of the effects of IL-4 and IL-13 have continued to give contradictory results. In some cases these cytokines have been shown to decrease HIV replication in monocytes (77), and MDM (19;22;72;78). These decreases in HIV replication were due to decreases in HIV mRNA expression (19;77), or a reduction of CD4 (22), CXCR4 (22), or CCR5 (22;78) expression. IL-13 was also shown to inhibit HIV replication by decreasing reverse transcription of the virus (72). However, both IL-4 and IL-13 have recently been demonstrated to increase HIV replication in MDM (77).

A clear understanding of the effects of Th1 and Th2 cytokines on chemokine receptor expression, and its subsequent impact on chemotaxis and HIV infection, has not yet emerged. This information may influence our understanding of the interaction between HIV and the immune system, and the switch in viral phenotype that has been linked to the progression from HIV to AIDS. Elucidating the role of monocytes in HIV infection and disease progression may also be of value as the importance of these cells in this context is only beginning to be understood. If the consequences of CXCR4 and CCR5 regulation by cytokines on HIV infection are understood, it could lead to improved treatments involving cytokines and chemokines.

Hypothesis

It was hypothesized that the culture of monocytes with Th1 and Th2 cytokines will alter the expression of CXCR4 and CCR5 on the surface of these cells. This modulation of chemokine receptor expression will cause corresponding changes in both the chemotaxis of these cells towards the respective ligands of these chemokine receptors, and the susceptibility of these cells to HIV entry and replication.

Objectives and Approach

The goal of this work is to understand the regulation of CXCR4 and CCR5 expression on resting human monocytes by cytokines, and examine the functional effects of this regulation. Specifically, the objectives of this investigation are:

1) to examine the impact of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines on the expression of CXCR4 and CCR5 on monocytes from the blood of HIV negative cord blood, HIV negative adults, and HIV positive adults;
2) to investigate possible methods for this regulation, such as changes in mRNA expression and chemokine secretion;
3) to determine the functional impact of this regulation on the chemotaxis of these cells in response to the ligands of CXCR4 and CCR5;
4) to investigate the effects of this CXCR4 and CCR5 regulation on the ability of HIV to enter monocytes, and replicate in them.

These objectives were accomplished by examining the effect of Th1 and Th2 cytokines on the expression of CXCR4 and CCR5 using flow cytometry. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the expression of mRNA

encoding these chemokine receptors was also performed. The effects of cytokines on the secretion of chemokines was examined by enzyme linked immunosorbent assay (ELISA). The physiological impact of CXCR4 and CCR5 regulation by Th1 and Th2 cytokines was then examined with chemotactic assays that measured the ability of these cells to migrate in response to the respective ligands of these chemokine receptors. The functional effects of alterations in CXCR4 and CCR5 expression on monocytes, induced by Th1 and Th2 cytokines, were further investigated using polymerase chain reaction (PCR) to detect HIV entry, and p24 ELISA to monitor the HIV replication.

Methods and Materials

Isolation and Culture of PBMC from Cord Blood and Adult Peripheral Blood

HIV negative adult peripheral blood was obtained from healthy adult volunteers. Cord blood was drawn from the umbilical cords of full term healthy newborns shortly after birth (this was approved by the Ethics board of the Ottawa Hospital, General Campus). HIV positive adult peripheral blood was obtained from consenting volunteers with $CD4^+$ T cell counts ranging from 100-400 cells/μl, who were undergoing anti-retroviral therapy. All blood samples were collected in sodium heparin containing blood collection tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). PBMCs were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Baie D'Urf 130, Quebec). Blood was layered on top of Ficoll-Hypaque (at a ratio of 1/3 Ficoll-Hypaque to 2/3 blood), and centrifuged for 30 minutes at 20°C at 1600 rpm. The mononuclear cell layer was collected and washed three times with phosphate buffered saline (PBS), before being resuspended in Iscove's Modified Dulbecco's Medium (IMDM; Sigma Chemical Company, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml gentamicin (Sigma, Oakville, ONT). PBMC ($1x10^6$ cells/ml) were cultured at 37°C with 5% CO_2 for 24 and 48 hrs in 24 well plates (Falcon, Becton-Dickinson, Lincoln Park, NJ) in the presence or absence of recombinant human IL-2, IL-4, IL-7, IL-10, IL-12, IL-13, TNF-α, and IFN-γ (Research Diagnostics, Flanders, NJ), as time course experiments showed that the optimal effects of these cytokines occurred at these time points. All cytokines were used at a concentration of 1 ng/ml, as this was the concentration that induced maximal effects in chemokine receptor expression.

Purification of Monocytes from PBMC by Negative Selection

Purified populations of monocytes were produced by negative selection using anti-CD2 (pan T) and anti-CD19 (pan B) antibody coated magnetic dynabeads (Dynal, Lake Success, NY) according to manufacturer's instructions. Briefly, after isolation of PBMC as previously described, cells were suspended in a solution of 2% FBS in PBS and enumerated. The number of anti-CD2 and anti-CD19 magnetic beads required was calculated to be a 10 times excess of the number of T cells (40%) and B cells (20%) estimated to be present in the PBMC, respectively. The necessary amount of beads were washed three times with 2% FBS in PBS, and incubated with PBMC for 30 minutes on ice. After incubation the beads were allowed to equilibrate in a magnetic rack for 5 minutes, after which the purified monocytes were removed from the beads and cultured as previously described.

Analysis of CXCR4 and CCR5 Expression by Flow Cytometry

PBMC were harvested from culture plates after 24 or 48 hrs of culture. They were washed once with a solution of 0.1% sodium azide in PBS, and then distributed into flow cytometry tubes (Falcon, Lincoln Park, NJ). The cells were then incubated for 20 minutes with a gamma immunoglobulin solution to block non-specific binding, and stained for three colour flow cytometric analysis with fluorochrome labeled monoclonal antibodies. Monocytes were labeled with FITC conjugated anti-CD14 antibodies (Leu-M3; Becton Dickinson, Franklin Lakes, NJ). Monocytes were also labeled with PE conjugated anti-CXCR4 antibodies (12G5; Pharmingen, San Diego, CA) and Cy-Chrome conjugated anti-CCR5 antibodies (2D7; Pharmingen, San Diego, CA). After the addition of the

fluorochrome labeled antibodies, the cells were incubated for 30 minutes at 4°C in the dark. PBMC were then washed once with PBS/0.1% sodium azide, and resuspended in PBS/0.1% azide for flow cytometric analysis. Autofluoresence and isotype controls were included.

Monocytes were gated based on forward scatter/side scatter characteristics, and CD14 labeling. A total of 20 000 events were recorded for each sample. CXCR4 and CCR5 expression was then measured in these gated populations. Data was acquired on a Becton-Dickinson FACScan Flow Cytometer. The validity of comparisons in the expression of CXCR4 and CCR5 between different patients and populations was ensured by the use of Calbrite™ Beads (Becton Dickinson, Franklin Lakes, NJ). This data was analyzed using the WinMDI software package (J. Trotter, Scripps Institute, San Diego, CA).

RNA Isolation and RT-PCR Analysis

Monocytes were cultured with IL-2, IL-4, IL-13 or IFN-γ (Research Diagnostics, Flanders, NJ) for 12 hrs, and isolated immediately prior to RNA extraction by positive selection with anti-CD14 antibody coated magnetic dynabeads (Dynal, Lake Success, NY), according to manufacturer's instructions. Total cellular RNA was then extracted using Tri Reagent (Molecular Research Center, Inc, Cincinnati, OH), a monophase solution containing guanine isothiocyanate and phenol, as described by the manufacturer. Total cellular RNA (1 μg) was reverse-transcribed in a 20 μl reaction mixture containing 2.5 μM of random hexamers, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM each of dGTP, dATP, dCTP, and dTTP, 20 units of RNAse inhibitor, and 500 units

of M-MLV reverse transcriptase (all RT-PCR reagents obtained from Roche Diagnostic Systems, Laval, Quebec) at 42°C for 60 minutes. The resulting cDNA was separated into 5 µl aliquots equivalent to 100 ng of RNA. These aliquots of cDNA were subjected to PCR analysis using 1.25 units of Amplitaq DNA polymerase, 1µM of each of the appropriate sense and anti-sense primers, 2 mM $MgCl_2$, and 0.5 mM each of dGTP, dATP, dCTP, and dTTP, (all PCR reagents obtained from Roche Diagnostic Systems, Laval, Quebec) in a total volume of 50 µl. The primer sequences used were: β-actin sense 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'; β-actin anti-sense 5'-CTA GAA GCA TTG CGG TGG CAG ATG GAG GG-3'; CXCR4 sense 5'-AGC TGT TGG CTG AAA AGC TGG TCT ATG-3'; CXCR4 anti-sense 5'-GCG CTT CTG GTG GCC CTT GGA GTG TG-3' (79); CCR5 sense 5'-GCT CTC TCC CAG GAA TCA TCT TTA C-3'; CCR5 anti-sense 5'-TTG GTC CAA CCT GTT AGA GCT ACT G-3' (48). The amplification conditions for CXCR4 were 35 cycles of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. For CCR5 the conditions were 35 cycles of denaturing at 94°C for 2 minutes, annealing at 56°C for 2 minutes and extension at 72°C for 2 minutes. The PCR products for β-actin (610 bp), CXCR4 (250 bp) and CCR5 (320 bp) were resolved by electrophoresis on 1.2% agarose gels and visualized with ethidium bromide staining. Densitometry was performed to normalize the expression of CXCR4 and CCR5 to β-actin using the GS-670 densitometer and the Molecular Analyst image analysis software (Bio-Rad, Hercules, CA).

Analysis of Chemokine Secretion by ELISA

Blood was obtained from 5 healthy adult volunteers, and PBMC were isolated as previously described. PBMC were cultured in the presence and absence of IL-2, IL-4, IL-13 or IFN-γ (Research Diagnostics, Flanders, NJ) for 24 and 48 hrs, as described previously. At these time points, supernatants were collected and frozen at -80°C. ELISA was then performed to determine the concentrations of SDF-1, MIP-1α, and RANTES in the supernatants. ELISA plates (Nunc Immunomodules, Roskilde, Denmark) were coated overnight with primary monoclonal antibodies against each chemokine (SDF-1 MAB-310, 25 μg/ml; MIP-1α MAB-670, 10 μg/ml; RANTES MAB-678, 10 μg/ml; R&D Systems, Minneapolis, MN), diluted in PBS. Plates were washed with PBS-Tween 20 and blocked with a solution of 10% FBS in PBS. The detection step was performed using biotinylated monoclonal antibodies against each chemokine diluted in a solution of 10% FBS in PBS (SDF-1 BAF-310, 25 ng/ml; MIP-1α BF-270, 25 ng/ml; RANTES BAF-278, 5 ng/ml; R&D Systems, Minneapolis, MN). After washing, streptavidin-peroxidase (Jackson Immuno Research, West Grove, PA) was added to achieve a final dilution of 1:1000. Plates were washed again before the addition of the o-phenyenediamine substrate (OPD; Sigma, Oakville, ONT), and the colour formed by the reaction was read at 450 nm. Serially diluted recombinant chemokines were used as standards (SDF-1, 350-NS-010, 2000 pg/ml; MIP-1α, 270-LD-010, 2000 pg/ml; RANTES, 278-RN-010, 5000 pg/ml; R&D Systems, Minneapolis, MN). Plate washes and reading were automated (Bio-Rad Laboratories, Hercules, CA), and standard curves and concentrations were calculated using Microplate Manager 4.0 software (Bio-Rad Laboratories, Hercules, CA).

Analysis of Monocyte Chemotaxis

Chemotaxis was performed in disposable 96 well ChemoTx plates (NeuroProbe Inc, Gaithersburg, MD). The lower chambers of the plate were filled with 30 μl of PBS, or solutions of PBS with the CXCR4 ligand SDF-1 (1000 ng/ml; BD PharMingen, Mississauga, ON) or the CCR5 ligand MIP-1α (100ng/ml; BD PharMingen, Mississauga, ON). A polycarbonate filter with 5 μm pores was placed on top of the lower wells, separating them from the upper wells. PBMC were cultured in the presence or absence of IL-2, IL-4, IL-13 or IFN-γ (Research Diagnostics, Flanders, NJ) for 24 or 48 hrs, as previously described, washed and resuspended in media. Cells were enumerated and $1x10^5$ PBMC were added to the upper chamber of each well. Plates were incubated for 1.5 hrs at 37°C and 5% CO_2. After incubation, fluid and cells from the lower wells were removed and added to equal amounts of PBS/0.1% azide. Cells were stained with fluorochrome labeled anti-CD14 antibodies (Leu-M3; Becton Dickinson, Franklin Lakes, NJ) to identify monocytes, and counted by flow cytometry. The number of migrated cells was calculated as the difference between the number of cells that moved in response to a chemokine solution and those that moved towards media alone. Migration of cytokine stimulated monocytes was expressed as a percentage of the number of migrated monocytes in the media control.

Propagation and Titration of HIV

M-tropic HIV-1 Bal and dual tropic HIV-1 clinical isolate #204 stocks were propagated in PHA (Sigma, Oakville, ONT) and IL-2 stimulated PBMC, and titred by the Spearman-Karber method, using a commercial p24 ELISA kit.

Analysis of p24 Concentration by ELISA

Analysis of HIV infected culture supernatants was carried out using a p24 ELISA kit (Mandel, Guelph, ONT) according to manufacturer's instructions. Briefly, p24 standards and sample supernatants were added to Triton X-100 containing anti-p24 antibody coated plates and incubated for 2 hours at 37°C. Plates were washed 6 times with p24 wash buffer, and biotin labeled detector antibody was added to the plates before they were incubated for 1 hour at 37°C. Plates were washed 6 times with p24 wash buffer, and incubated for 30 minutes at room temperature after the addition of streptavidin conjugated horse-radish peroxidase. Plates were washed 6 times with p24 wash buffer, and incubated for 30 minutes at room temperature in the dark with the OPD substrate. Colour formed by the reaction was read at 450 nm. Plate washes and reading were automated (Bio-Rad Laboratories, Hercules, CA), and standard curves and concentrations were calculated using Microplate Manager 4.0 software (Bio-Rad Laboratories, Hercules, CA).

Analysis of HIV Entry in Monocytes by PCR

PBMCs were isolated from the blood of HIV negative adult donors, and monocytes were negatively selected with magnetic dynabeads (Dynal, Lake Success,

NY) as previously described. Monocytes were cultured in the presence and absence of IL-2, IL-4, IL-13 and IFN-γ (Research Diagnostics, Flanders, NJ) for 48 hrs, and were then exposed to HIV Clinical Isolate #204 at a multiplicity of infection (MOI) of 0.1 for 8 hrs, as time course experiments indicated this was optimal for *gag* detection. After infection, monocytes were subjected to DNA isolation using Tri Reagent (Molecular Research Center, Inc, Cincinnati, OH) as directed by the manufacturer. An aliquot of 1 μg of this DNA was used as the template for a PCR reaction which amplified the HIV proviral *gag* DNA. PCR reaction mixtures contained 1.25 units of Amplitaq DNA polymerase, 2 mM $MgCl_2$, 0.5 mM each of dGTP, gATP, dCTP, and dTTP (all PCR reagents obtained from Roche Diagnostic Systems, Laval, Quebec), and 1μM of each of the appropriate sense and anti-sense primers for β-actin or 250 μM of the sense and antisense primers for HIV *gag*, in a total volume of 50 μl. The primer sequences used were: β-actin sense 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'; β-actin anti-sense 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'; gag sense 5'-ATA ATC CAC CTA TCC CAG TAG GAG AAA T-3'; gag anti-sense 5'-TTT GGT CCT TGT CTT ATG TCC AGA ATG C-3' (80). The amplification conditions for *gag* were 35 cycles of denaturing at 94°C for 1 minute, annealing at 56°C for 2 minutes and extension at 72°C for 2 minutes. The amplification conditions for β-actin were 30 cycles of denaturing at 94°C for 1 minute, annealing at 56°C for 2 minutes and extension at 72°C for 2 minutes. The PCR products for HIV *gag* (114 bp), and β-actin (610 bp) were resolved by electrophoresis on 2% agarose gels and visualized with ethidium bromide staining. Densitometry was performed to normalize the expression of CXCR4

and CCR5 to β-actin using the GS-670 densitometer and the Molecular Analyst image analysis software (Bio-Rad, Hercules, CA).

Analysis of HIV Replication in Monocytes

Monocytes from HIV negative adults were negatively selected from PBMC and cultured in media with and without IL-2, IL-4, IL-13 and IFN-γ (Research Diagnostics, Flanders, NJ) in 24 well tissue culture plates (Falcon, Becton-Dickinson, Lincoln Park, NJ), as previously described. After 48 hrs of culture, monocytes were washed with PBS, and resuspended in media and the appropriate cytokine. Cells were then infected at an MOI of 0.1 with HIV-1 Bal for 12 hrs, washed three times with PBS, and resuspended in media again with the appropriate cytokine. Monocytes were then cultured in 24 well tissue culture plates (Falcon, Becton-Dickinson, Lincoln Park, NJ), as previously described, and supernatants were collected and frozen at -80°C on days 0, 5, 10, and 15, and analyzed by p24 ELISA.

Statistical Analysis

Statistical analysis was done using the student's *t*-test, taking probability values less than 0.05 as significant.

Results

Analysis of the Effects of Th1 and Th2 Cytokines on the Expression of CXCR4 and CCR5 on Human Monocytes:

Initially, the effects of the Th1 and Th2 cytokines IL-2, IL-4, IL-7, IL-10, IL-12, IL-13, TNF-α, and IFN-γ on the expression of CXCR4 and CCR5 by monocytes were examined. Many of these cytokines, such as IL-7, IL-10, IL-12, and TNF-α, had no effect on these chemokine receptors, or had effects that were highly variable, and therefore were not further investigated. Subsequent work focused on the Th1 cytokines IL-2 and IFN-γ, and the Th2 cytokines IL-4 and IL-13. These cytokines had consistent effects and are thought to play a vital role in HIV immunopathogenesis.

The Effect of Th1 Cytokines on CXCR4 and CCR5 Expression:

Analysis of CXCR4 and CCR5 Expression on Monocytes by Flow Cytometry

The expression of CXCR4 and CCR5 on monocytes from HIV negative adults, HIV positive adults, and HIV negative cord blood was investigated by flow cytometry. PBMC were isolated from 10 HIV negative cord blood samples, 10 HIV negative adult blood samples, as well as blood of 6 HIV positive adult donors. These cells were cultured in the presence and absence of IL-2 and IFN-γ for 24 and 48 hrs, and subjected to analysis by flow cytometry.

In order to determine both the optimal doses of IL-2 and IFN-γ, and the time points where these cytokines exhibited optimal biological effects, dose response and time course experiments were performed. Studies on the effects of various cytokine doses on chemokine receptor expression indicated that 1 ng/ml was sufficient to elicit the observed

effects of IL-2 and IFN-γ, and was used for all subsequent experiments (Figure 1A). Investigation of the kinetics of these effects revealed that they were maximal after 24 and 48 hrs following stimulation with IFN-γ, so these time points were the focus of further experiments (Figure 1B).

Investigation of the effects of IL-2 and IFN-γ on HIV negative adult monocytes revealed that both of these cytokines reduced the expression of CXCR4 on the surface of these cells. The down regulation of CXCR4 caused by IL-2 and IFN-γ was detectable after 24 hrs of culture, but was not significant. The effects of these cytokines were most dramatic and significant after 48 hrs, with IL-2 decreasing the MCF from the unstimulated value of 21.78 ± 4.9 to 10.7 ± 1.9 ($p = 0.031$) and IFN-γ decreasing it from 21.78 ± 4.9 to 7.4 ± 1.3 ($p = 0.029$). Culture of monocytes from HIV negative adults with IL-2 for 24 and 48 hrs did not result in a significant change in the expression of CCR5. In contrast, IFN-γ caused significant decreases in CCR5 expression at both time points [47.8 ± 11.0 versus 29.5 ± 2.0 ($p = 0.049$) after 24 hrs; 55.9 ±8.9 versus 20.9 ± 4.3 ($p = 0.0003$) after 48 hrs] (Figure 2A).

To eliminate the possibility that the effects of IL-2 and IFN-γ were mediated by contact with, or secretions by, other cell types in PBMC cultures, these experiments were performed on purified monocytes isolated by negative selection from the PBMC of 5 HIV negative adults, and then incubated in the presence or absence of IL-2 or IFN-γ for 48 hrs. IL-2 and IFN-γ both significantly decreased CXCR4 expression on the surface of these purified monocytes after 48 hrs [8.2 ± 1.2 in untreated versus 5.3 ± 0.6 ($p = 0.015$) for IL-2 treated and 3.9 ± 1.0 ($p = 0.007$) for IFN-γ treated monocytes].

Figure 1:
A) The Effects of IL-2 and IFN-γ Concentration on the Expression of CXCR4 and CCR5 on Monocytes. Adult PBMC were incubated with increasing doses of IL-2 or IFN-γ for 48 hrs, and the effects of these cytokines on CXCR4 and CCR5 expression on the surface of $CD14^+$ monocytes were monitored by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor.
B) Timing of the Effects of IFN-γ on the Expression of CXCR4 and CCR5 on Monocytes. Adult PBMC were incubated with IFN-γ (1 ng/ml) for the indicated lengths of time, and the effects of this cytokine on the expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was monitored by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor.

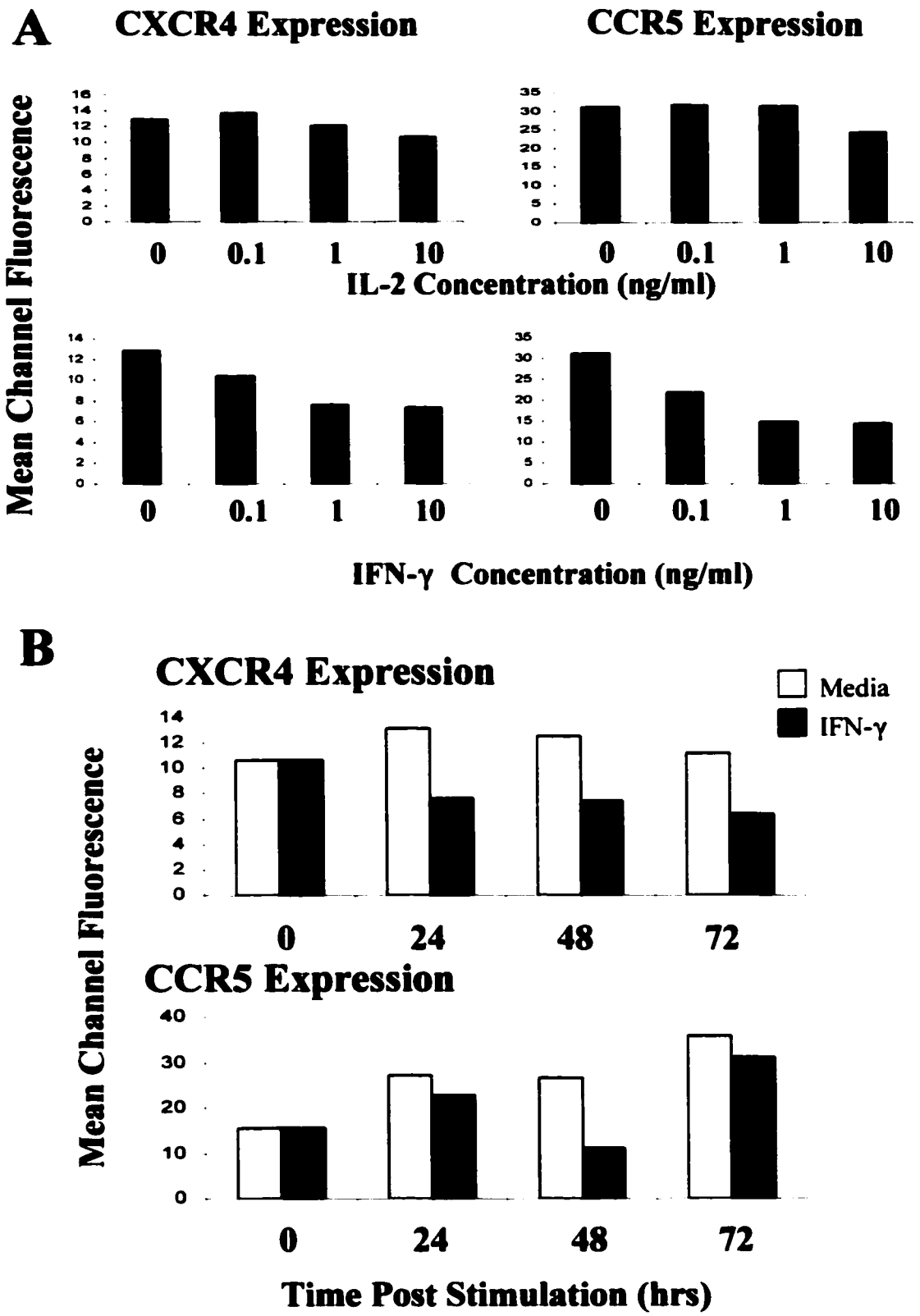
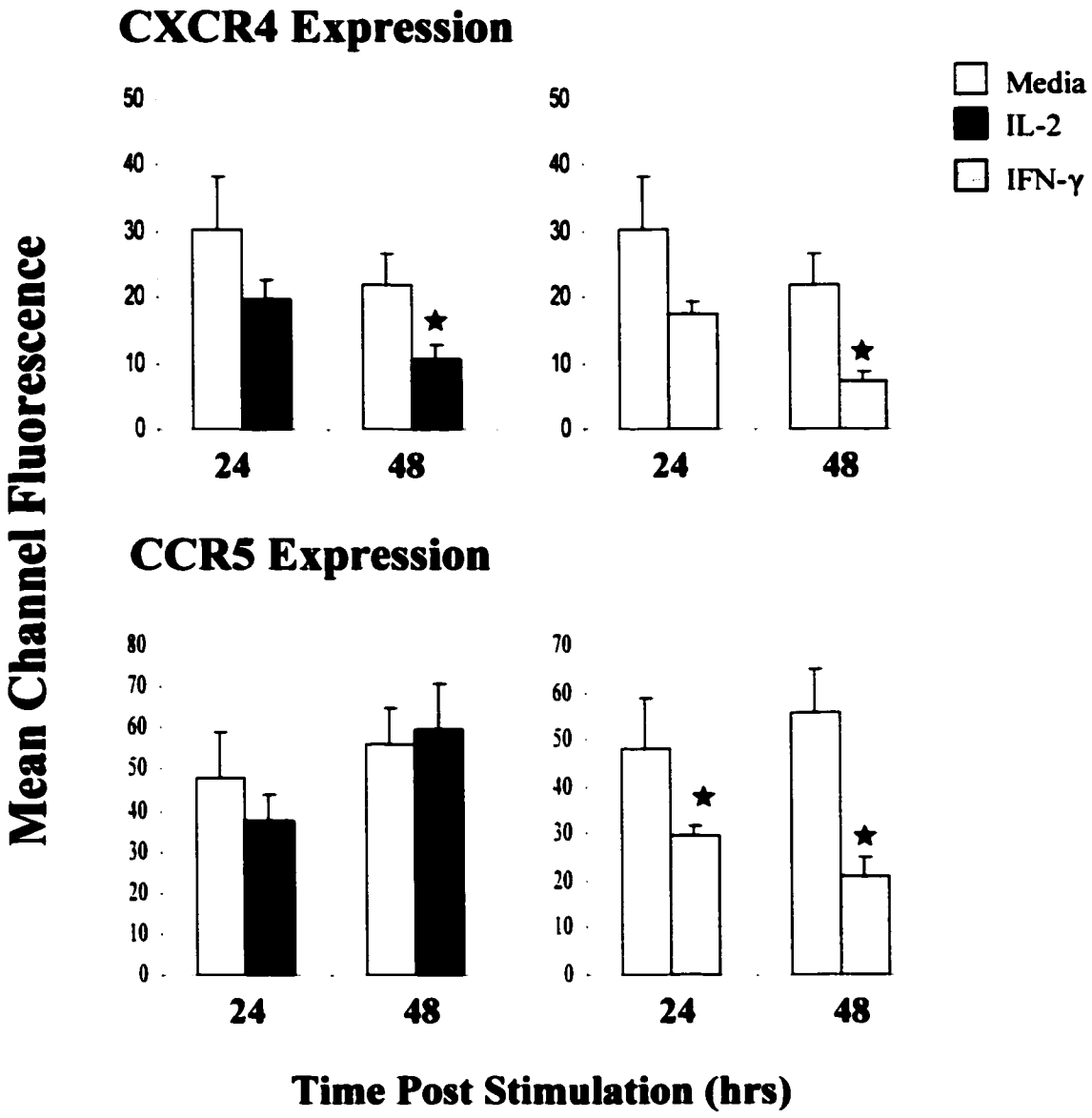

A
CXCR4 Expression
CCR5 Expression
Mean Channel Fluorescence
0
0.1
1
10
IL-2 Concentration (ng/ml)
IFN-γ Concentration (ng/ml)
B
CXCR4 Expression
Media
IFN-γ
CCR5 Expression
Mean Channel Fluorescence
0
24
48
72
Time Post Stimulation (hrs)

Figure 2A: The Effects of IL-2 and IFN-γ on the Expression of CXCR4 and CCR5 on Monocytes from HIV Negative Adult Blood. PBMC from 10 HIV negative adults were cultured for 24 and 48 hrs in the presence and absence of IL-2 or IFN-γ. The expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was examined by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor. The average mean channel fluorescence (MCF), standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).

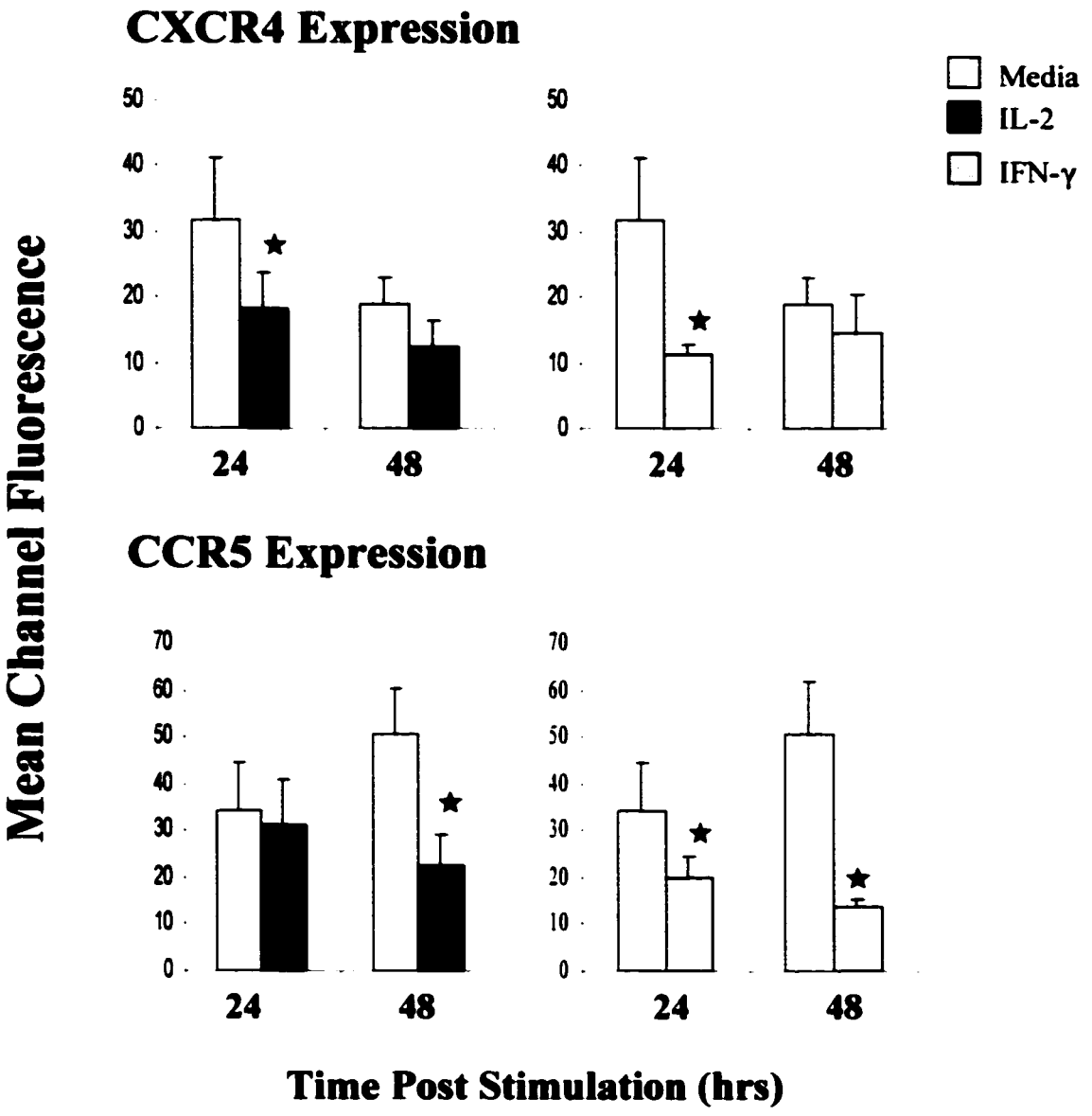

CXCR4 Expression
Media
IL-2
IFN-γ
Mean Channel Fluorescence
50
40
30
20
10
0
24
48
CCR5 Expression
80
70
60
50
40
30
20
10
0
Time Post Stimulation (hrs)

CCR5 expression on purified monocytes treated with IL-2 for 48 hrs was unaffected [20.3 ± 2.4 in untreated monocytes versus 17.3 ± 2.3 ($p = 0.07$) in IL-2 treated monocytes]. In contrast, IFN-γ caused a significant decrease in the expression of CCR5 from a MCF of 20.3 ± 2.4 to 5.4 ± 0.4 ($p = 0.001$). The effects of IL-2 and IFN-γ on chemokine receptor expression in cultures of purified monocytes were identical to those observed in monocytes cultured as PBMC (data not shown). Therefore, in subsequent experiments PBMC cultures were used to determine the effects of cytokines on CXCR4 and CCR5 expression, and their biological functions.

Monocytes isolated from HIV negative cord blood showed similar results to those obtained in HIV negative adult monocytes. Significant decreases in CXCR4 expression were observed after culture with IL-2 and IFN-γ, but only after 24 hrs. At this time point, MCF values decreased from 31.7 ± 9.4 in unstimulated monocytes to 18.1 ± 5.5 ($p = 0.033$) in IL-2 treated monocytes and to 11.3 ± 1.3 ($p = 0.047$) in those cultured with IFN-γ. The effects of IL-2 and IFN-γ on CXCR4 expression were lost after 48 hrs of culture. IL-2 did not affect CCR5 expression at 24 hrs, but caused a significant decrease in its expression after 48 hrs of culture [50.6 ± 11.3 in untreated versus 22.3 ± 6.9 ($p = 0.01$) in IL-2 treated monocytes]. IFN-γ caused a significant decrease in the expression of CCR5 after both 24 and 48 hrs of culture, decreasing the MCF from 34.0 ± 10.3 to 19.8 ± 4.6 ($p = 0.034$) and 50.6 ± 11.3 to 13.6 ± 1.9 ($p = 0.004$), respectively (Figure 2B).

Monocytes from HIV positive individuals showed some differences from that of HIV negative individuals in that the decrease of CXCR4 by IL-2 seen in uninfected

Figure 2B: The Effects of IL-2 and IFN-γ on the Expression of CXCR4 and CCR5 on Monocytes from HIV Negative Cord Blood. PBMC from 10 HIV negative cord blood samples were cultured for 24 and 48 hrs in the presence and absence of IL-2 or IFN-γ. The expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was examined by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor. The average mean channel fluorescence (MCF), standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).


CXCR4 Expression
Media
IL-2
IFN-γ
Mean Channel Fluorescence
50
40
30
20
10
0
24
48
CCR5 Expression
70
60
50
40
30
20
10
0
Time Post Stimulation (hrs)

patients was not observed. In fact, culture with IL-2 for 24 hrs resulted in a significant increase in CXCR4 expression from 41.8 ± 6.6 to 49.0 ± 8.0 ($p = 0.041$). Culture with IL-2 for 48 hrs had no effect on CXCR4 expression. IFN-γ also had little effect on CXCR4 expression by monocytes from HIV positive blood following 24 hrs of treatment. However, IFN-γ stimulation decreased CXCR4 expression after 48 hrs [46.2 ± 12.0 in unstimulated monocytes versus 21.45 ± 6.6 ($p = 0.078$) in those treated with IFN-γ], but this decrease was not statistically significant. The influence of IL-2 and IFN-γ on the expression of CCR5 on monocytes isolated from the blood of HIV positive patients was similar to that observed in monocytes from HIV negative adults. In comparison to media controls, CCR5 expression on monocytes cultured with IL-2 was not significantly different after 24 or 48 hrs. IFN-γ did not effect CCR5 expression after 24 hrs, but caused a significant decrease in CCR5 expression after 48 hrs of treatment, from 94.7 ± 21.0 to 52.7 ± 10.3 ($p = 0.003$) (Figure 2C).

A representative histogram showing the general effects of IL-2 and IFN-γ on CXCR4 and CCR5 expression is shown in Figure 3.

Analysis of CXCR4 and CCR5 mRNA Expression in Monocytes by RT-PCR

To determine whether IL-2 of IFN-γ decrease CXCR4 and CCR5 expression at the transcriptional level, the expression of mRNA encoding these chemokine receptors was examined by semi-quantitative RT-PCR analysis. Monocytes were isolated from HIV negative adult blood by positive selection and a time course analysis of chemokine receptor mRNA expression was performed. CXCR4 and CCR5 mRNA levels were unaffected by IFN-γ over a range of time points up to 24 hrs post stimulation (Figure 4).

Figure 2C: The Effects of IL-2 and IFN-γ on the Expression of CXCR4 and CCR5 on Monocytes from HIV Positive Adult Blood. PBMC from 6 HIV positive adults were cultured for 24 and 48 hrs in the presence and absence of IL-2 or IFN-γ. The expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was examined by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor. The average mean channel fluorescence (MCF), standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).

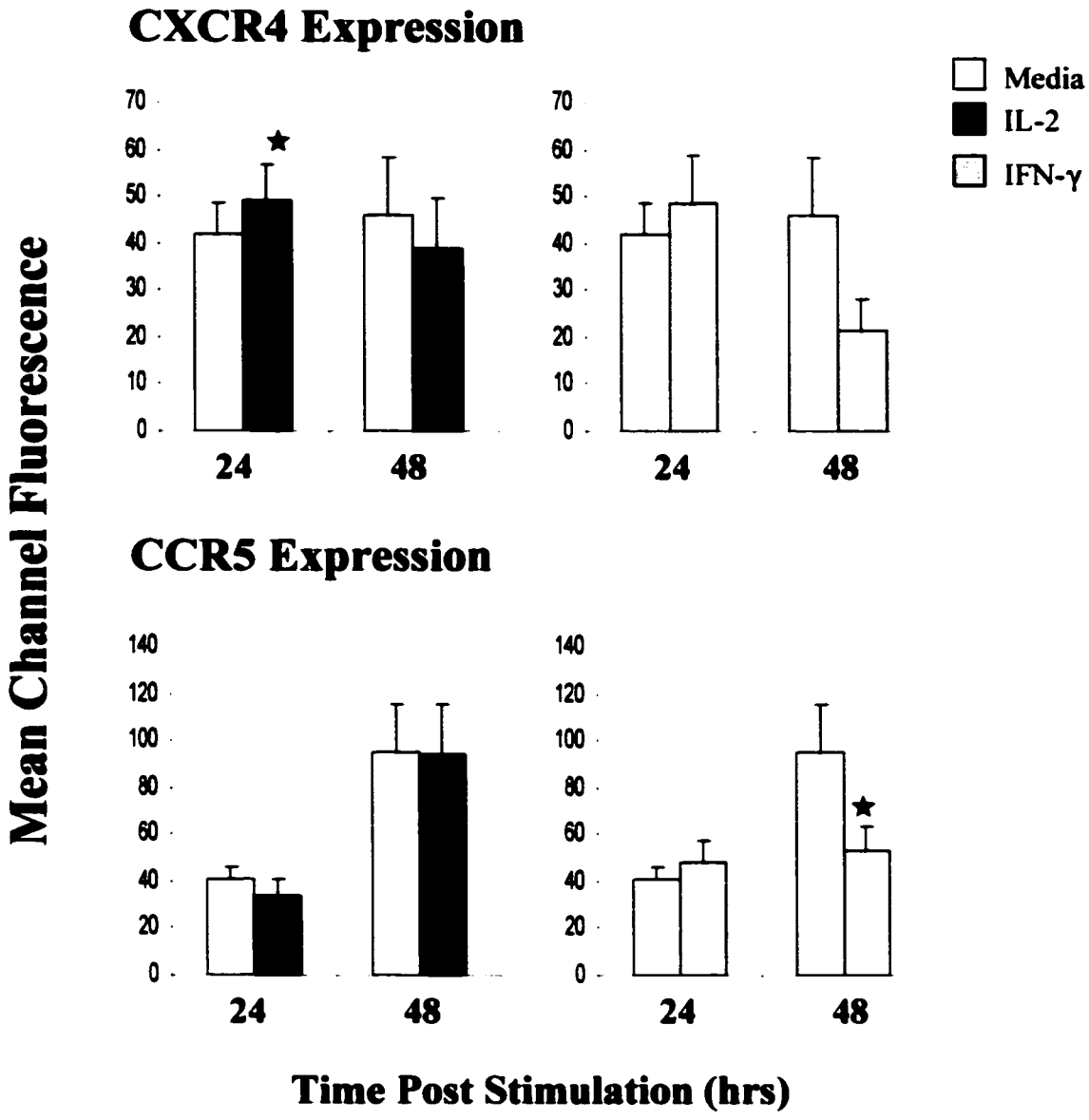

CXCR4 Expression
Media
IL-2
IFN-γ
Mean Channel Fluorescence
70
60
50
40
30
20
10
0
24
48
CCR5 Expression
140
120
100
80
60
40
20
0
Time Post Stimulation (hrs)

Figure 3: The Effects of IL-2 and IFN-γ on the Expression of CXCR4 and CCR5 on Monocytes. Representative flow cytometry histograms of the general effects of IL-2 and IFN-γ on the surface expression of CXCR4 and CCR5 on $CD14^+$ monocytes from HIV negative adults, HIV positive adults, and HIV negative cord blood. Shaded curves represent control monocytes cultured with media alone, while open curves represent monocytes treated with the indicated cytokines.

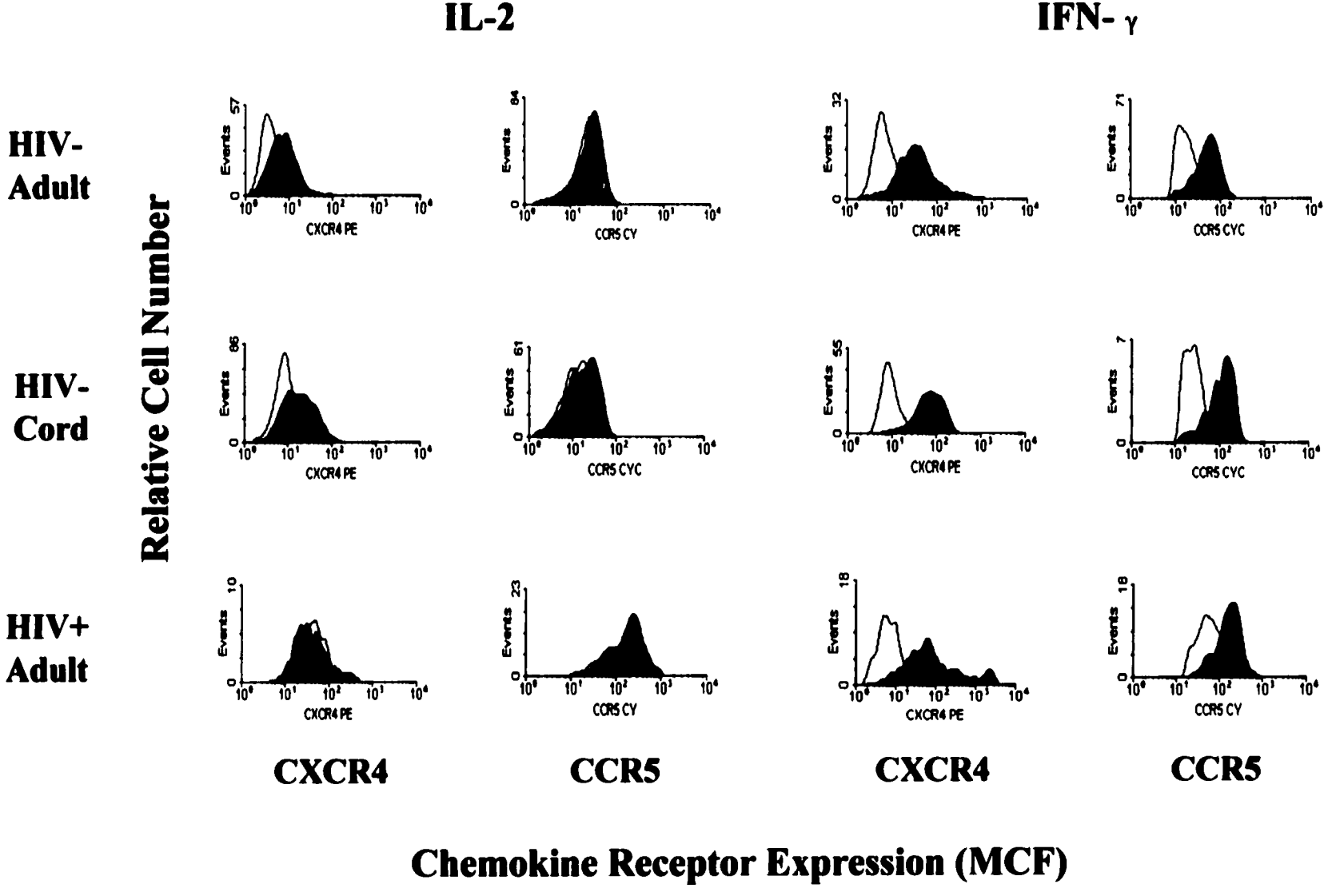
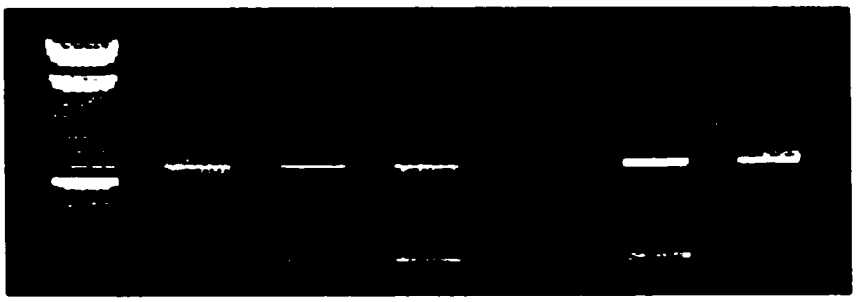

IL-2
IFN- γ
HIV- Adult
HIV- Cord
HIV+ Adult
Relative Cell Number
Events
CXCR4 PE
CCR5 CY
CCR5 CYC
CXCR4
CCR5
CXCR4
CCR5
Chemokine Receptor Expression (MCF)

Figure 4: Timing of the Effect of IFN-γ on the Transcription of mRNA encoding CXCR4 and CCR5 in Monocytes. PBMC were isolated from HIV negative adult blood, and incubated with IFN-γ for the indicated lengths of time. At these points, $CD14^+$ monocytes were positively selected with anti-CD14 antibody coated magnetic beads, RNA was removed from these cells and subjected to semi-quantitative RT-PCR analysis using specific primers for mRNA encoding CXCR4 or CCR5, and β-actin. Amplified products were run on an agarose gel, and analyzed by densitometry using β-actin as an internal control.

CXCR4



β-Actin (610 bp)

CXCR4 (250 bp)

0.53	0.69	0.72	0.68	0.68	0.65

Ratio of CXCR4/β-Actin

CCR5

β-Actin (610 bp)

CCR5 (320 bp)

0.64	0.65	0.63	0.79	0.57	0.70

Ratio of CCR5/β-Actin

0	2	4	8	12	24

Time Post Stimulation (hrs)

Due to the lack of differences observed in the kinetics of chemokine receptor mRNA expression, the time point of 12 hrs was chosen for subsequent investigation.

Monocytes were isolated from three HIV negative adults and three HIV negative cord blood samples, and subjected to RT-PCR analysis after culture in the presence and absence of IL-2 or IFN-γ. The ratio of the expression of CXCR4 mRNA to β-actin mRNA in adult and cord blood monocytes was unaffected by culture with IL-2 and IFN-γ. Similarly, the levels of CCR5 mRNA, in monocytes from adult and cord blood, determined in this manner also proved to be unaffected by culture with IL-2 and IFN-γ. One representative sample of each is shown (Figure 5). These results suggest that stimulation of monocytes with IL-2 or IFN-γ down regulates CXCR4 and CCR5 expression, but does not affect the expression of mRNA encoding either CXCR4 or CCR5.

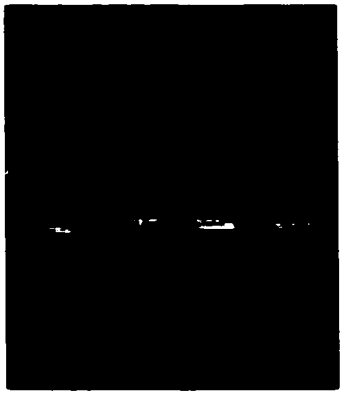
Analysis of Chemokine Secretion

The absence of transcriptional regulation prompted investigation of the possibility that IL-2 and IFN-γ induce the secretion of chemokines which caused internalization of CXCR4 and CCR5, resulting in the down regulation of these receptors observed by flow cytometry. To investigate this possibility, PBMC from 5 HIV negative adults were cultured with IL-2 or IFN-γ, and supernatants were analyzed for the secretion of the CXCR4 ligand SDF-1, and the CCR5 ligands MIP-1α, and RANTES by ELISA.

After 24 hrs, culture of PBMC with IL-2 increased production of SDF-1 from an average of 90 ± 30 pg/ml to 207 ± 124 pg/ml ($p = 0.231$), although this increase was not statistically significant. Similarly, culture with IFN-γ enhanced SDF-1 production from

Figure 5: The Effect of IL-2 and IFN-γ on the Transcription of mRNA encoding CXCR4 and CCR5 in Monocytes. PBMC from 3 HIV negative adults and 3 HIV negative cord blood samples were incubated in the presence and absence of IL-2 or IFN-γ for 12 hrs, and then $CD14^+$ monocytes were positively selected using anti-CD14 antibody coated magnetic beads. RNA was isolated from these monocytes, and subjected to semi-quantitative RT-PCR analysis using specific primers for mRNA encoding CXCR4 or CCR5, and β-actin. Amplified products were run on an agarose gel, and analyzed by densitometry using β-actin as an internal control. One representative sample from adult and cord blood is shown.

CXCR4



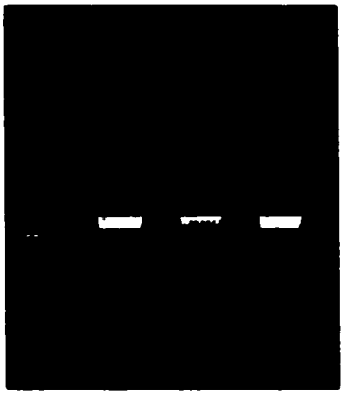
β-Actin (610 bp)

CXCR4 (250 bp)

0.73 0.81 0.79

Media IL-2 IFN-γ

Ratio of CXCR4/β-Actin



β-Actin (610 bp)

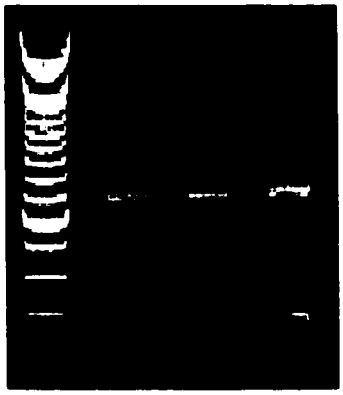
CXCR4 (250 bp)

0.61 0.70 0.69

Media IL-2 IFN-γ

Ratio of CXCR4/β-Actin

CCR5



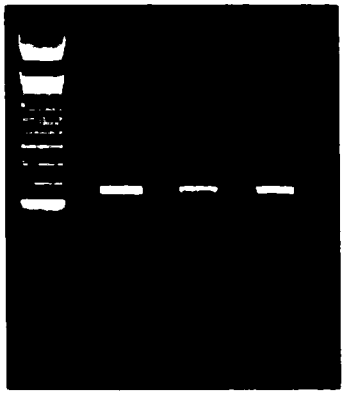
β-Actin (610 bp)

CCR5 (320 bp)

0.89 0.88 0.91

Media IL-2 IFN-γ

Ratio of CCR5/β-Actin



β-Actin (610 bp)

CCR5 (320 bp)

0.47 0.54 0.55

Media IL-2 IFN-γ

Ratio of CCR5/β-Actin

Adult Blood

Cord Blood

an average of 90 ± 30 pg/ml to 392 ± 112 pg/ml ($p = 0.023$) (Figure 6). The concentration of the CCR5 ligand RANTES in the cell supernatants was not significantly increased by culture with IL-2. In contrast, IFN-γ enhanced RANTES production from an average of 3277 pg/ml to 4990 ± 1085 pg/ml ($p = 0.0002$) after 24 hrs of culture, and from 483 ± 348 pg/ml to 1925 ± 680 pg/ml ($p = 0.017$) after 48 hrs of culture (Figure 6). The concentration of the CCR5 ligand MIP-1α was also examined, but was not detectable in the supernatants of cells cultured in the presence or absence of IL-2 or IFN-γ (data not shown).

These results suggest that the increases in chemokine secretion caused by IL-2 and IFN-γ may cause the internalization of CXCR4 and CCR5, which in turn may result in the down regulation of chemokine receptor expression.

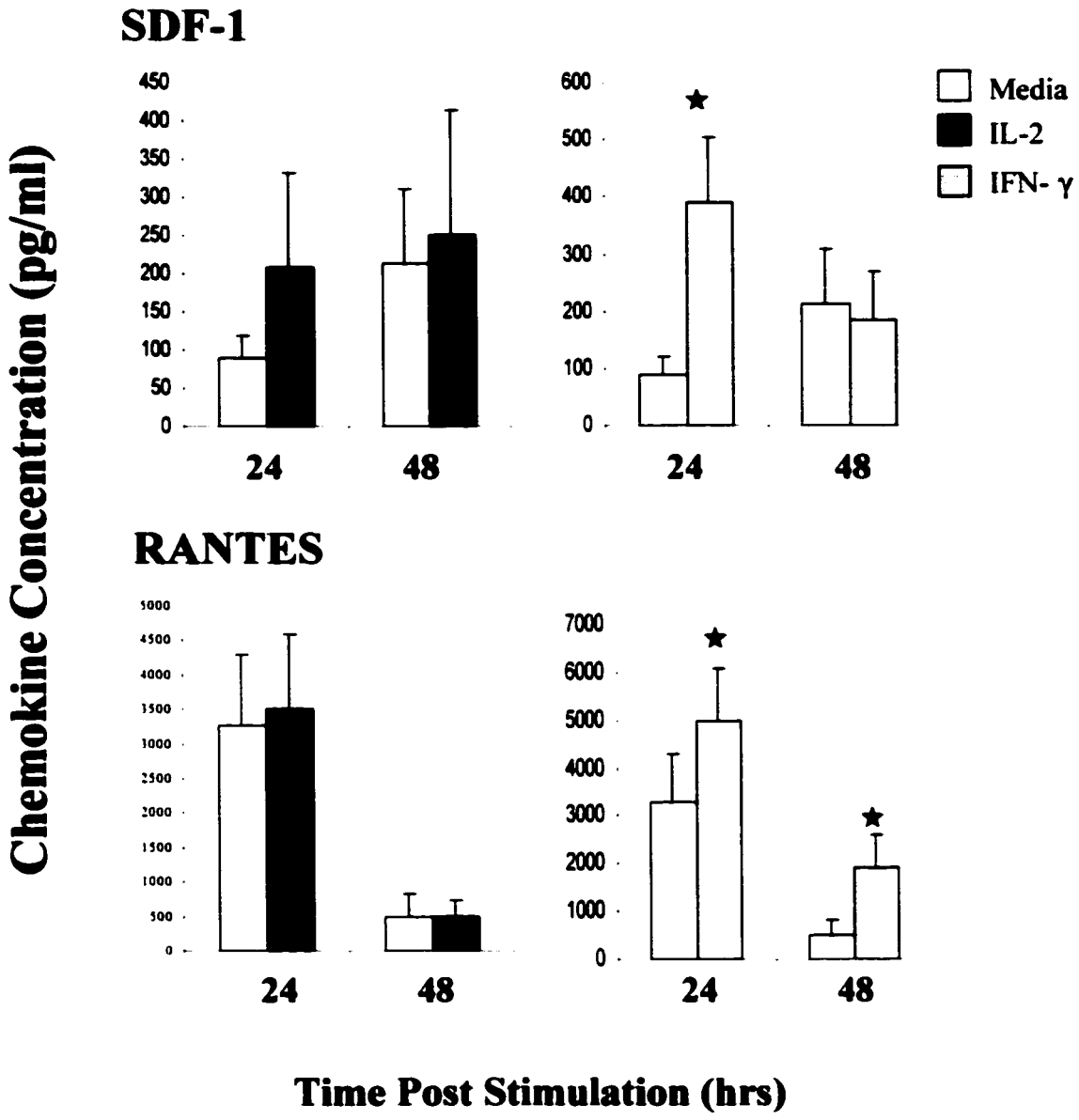
Implications of the Down Regulation of CXCR4 and CCR5 Expression on Monocytes by IL-2 and IFN-γ

Chemokine receptors play a vital role in chemokine induced cell migration, and act as coreceptors for HIV infection of its target cells. Down regulation of CXCR4 and CCR5 on monocytes, by IL-2 and IFN-γ, may influence chemotaxis attributed to these chemokine receptors. In addition, down regulation of chemokine receptors may result in decreased HIV entry.

Analysis of Monocyte Chemotaxis

To investigate the effects of CXCR4 and CCR5 down regulation on monocytes by IL-2 and IFN-γ, PBMC were isolated from the blood of 6 HIV negative adults, 6 HIV negative cord blood samples, and 6 HIV positive adults.

Figure 6: The Effect of IL-2 and IFN-γ on the Secretion of Chemokines. PBMC were isolated from the blood of 5 HIV negative adult donors, and incubated for 24 and 48 hrs in the presence and absence of IL-2 or IFN-γ. Supernatants were collected at these time points and subjected to ELISA for the detection of the CXCR4 ligand SDF-1, and the CCR5 ligands RANTES, and MIP-1α (not shown). The average chemokine concentration, standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).


SDF-1
RANTES
Media
IL-2
IFN- γ
Chemokine Concentration (pg/ml)
Time Post Stimulation (hrs)
24
48
24
48
24
48
24
48

These cells were cultured in the presence and absence of IL-2 or IFN-γ, and subjected to chemotactic analysis. These chemotaxis experiments were performed using SDF-1, a natural ligand for CXCR4, and MIP-1α , one of the ligands of CCR5. Concentrations for each of these ligands that would cause chemotaxis of monocytes were optimized by performing dose response experiments. These showed that 100 ng/ml of MIP-1α and 1000 ng/ml of SDF-1 induced maximal chemotaxis, and therefore these concentrations were used for subsequent experiments (Figure 7A). Cytokine dose response experiments again confirmed that the maximal effects of IL-2 and IFN-γ on chemotaxis induced by both SDF-1 and MIP-1α occurred at a cytokine concentration of 1 ng/ml (Figure 7B).

IL-2 significantly decreased the migration of adult monocytes to 57.2 ± 20.8 % ($p = 0.05$) and 47.7 ± 14.7 % ($p = 0.012$) after 24 and 48 hrs, respectively. Similarly, SDF-1 induced chemotaxis of these monocytes was also significantly and dramatically reduced following stimulation with IFN-γ [14.0 ± 4.9 % ($p = 0.001$) after 24 hrs; 14.0 ± 5.8 % ($p = 0.001$) after 48 hrs]. IL-2 induced a significant decrease in chemotaxis mediated by the CCR5 ligand MIP-1α after 24 hrs of culture, decreasing migration to 40.0 ±15.3 % ($p = 0.008$) of control monocytes. However, there was no significant effect of IL-2 on this chemotaxis after 48 hrs of culture. Similarly, stimulation of HIV negative adult monocytes with IFN-γ caused a significant, and almost complete, inhibition of chemotaxis induced by MIP-1α [10.2 ± 6.7 % ($p = 0.001$) after 24 hrs; 4.3 ± 2.2 % ($p = 0.0001$) after 48hrs] (Figure 8A).

Figure 7:
A) The Effects of Chemokine Concentration on the Chemotaxis of Monocytes. PBMC from HIV negative adult blood were subjected to chemotaxis using increasing doses of SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5) in order to determine the optimal dose of these chemokines for use in subsequent experiments. Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and are expressed as a percentage of the control with no added chemokines.
B) The Effects of IL-2 and IFN-γ Concentration on the Chemotaxis of Monocytes. PBMC from HIV negative adult blood were subjected to chemotaxis induced by SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5) after culture with increasing concentrations of IL-2 or IFN-γ for 48 hrs. Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and are expressed as a percentage of a control cultured with media alone.

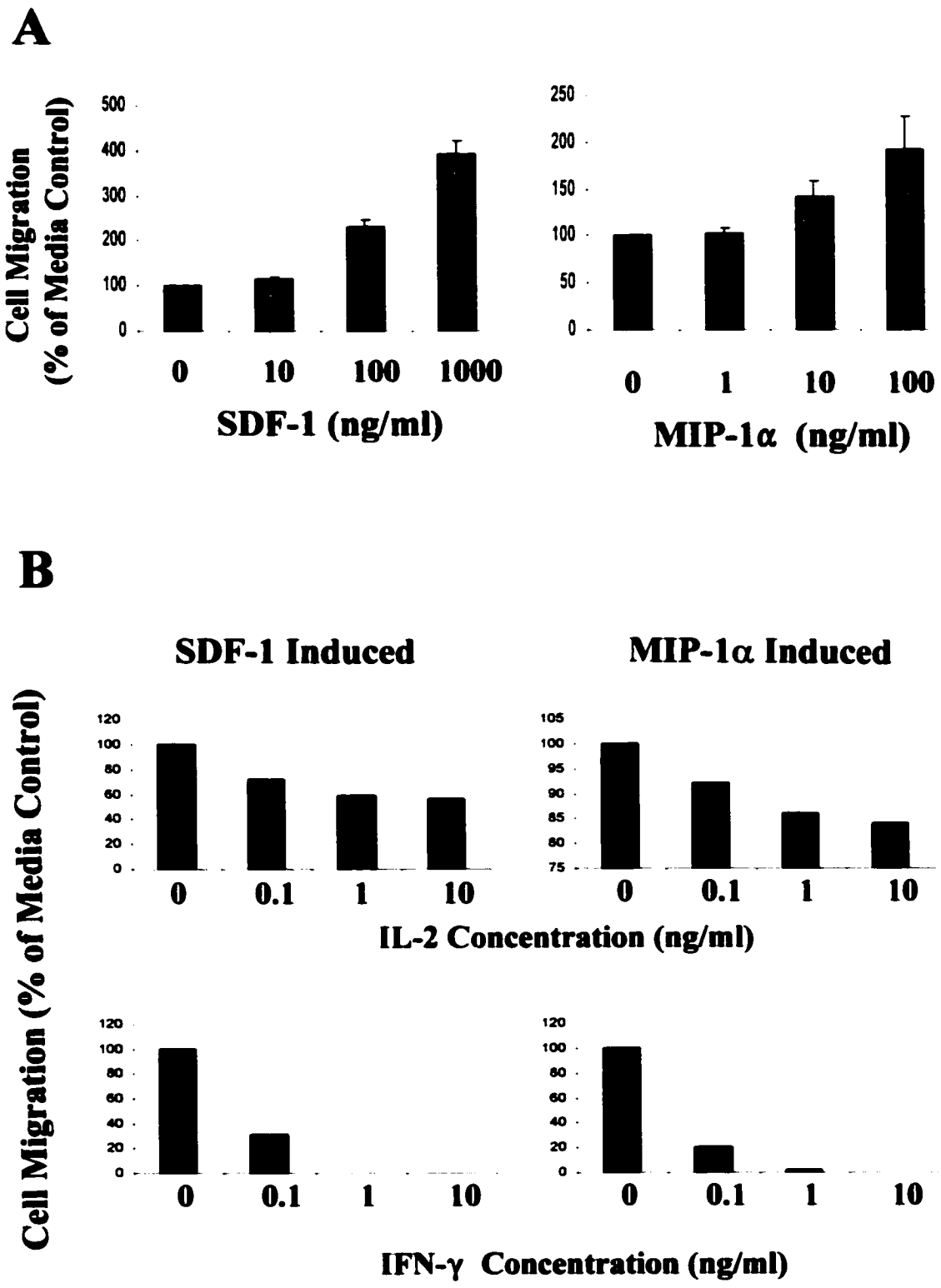
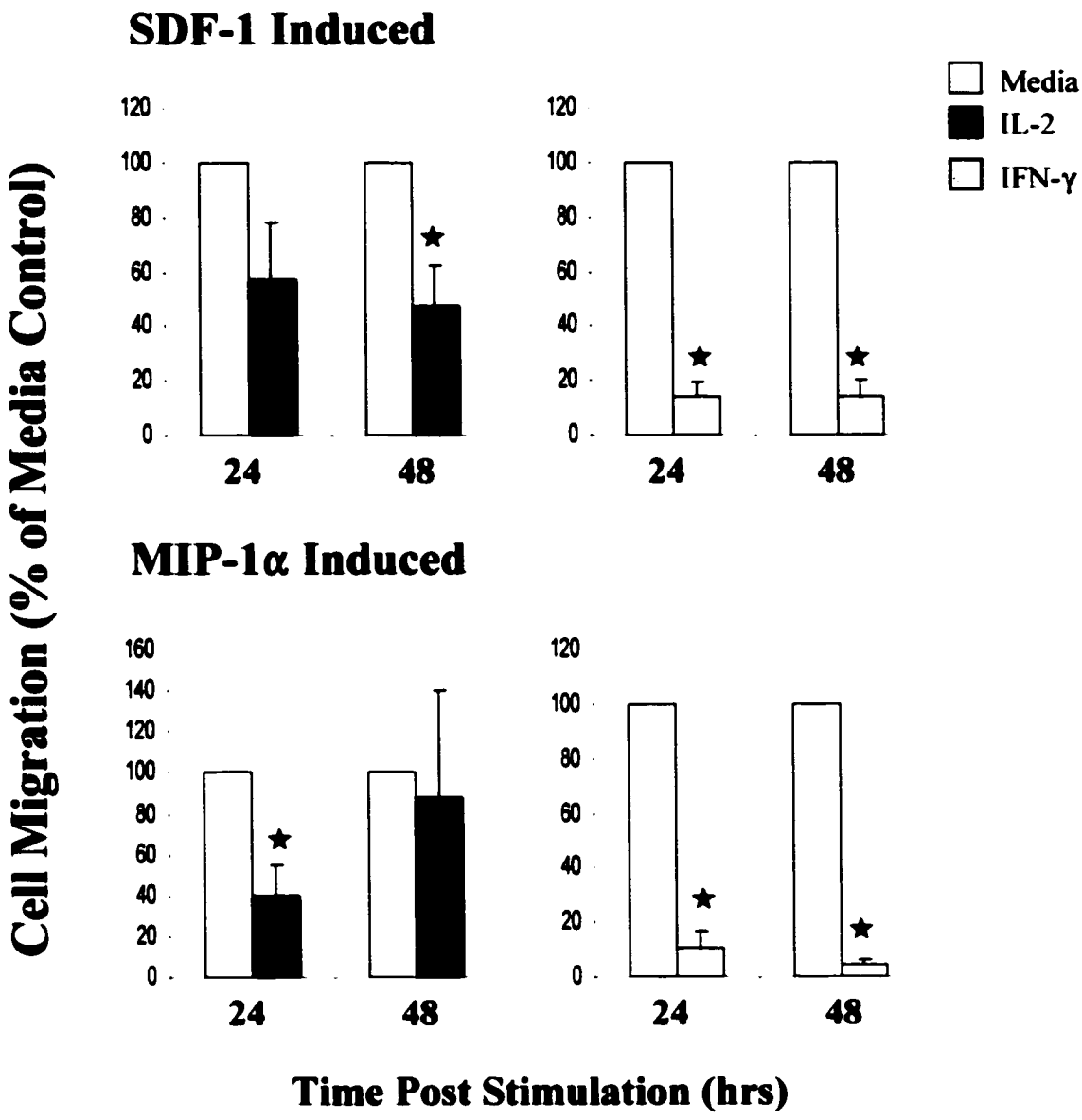

A
Cell Migration (% of Media Control)
500
400
300
200
100
0
0
10
100
1000
SDF-1 (ng/ml)
250
200
150
100
50
0
0
1
10
100
MIP-1α (ng/ml)
B
SDF-1 Induced
MIP-1α Induced
Cell Migration (% of Media Control)
120
100
80
60
40
20
0
0
0.1
1
10
105
100
95
90
85
80
75
IL-2 Concentration (ng/ml)
IFN-γ Concentration (ng/ml)

Figure 8A: The Effects of IL-2 and IFN-γ on the Chemotaxis of Monocytes from HIV Negative Adult Blood Induced by the Ligands of CXCR4 and CCR5. PBMC from 6 HIV negative adults were cultured for 24 and 48 hrs in the presence and absence of IL-2 or IFN-γ, and then subjected to chemotaxis induced by SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5). Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and expressed as a percentage of a control cultured with media alone. The average migration percentage of monocytes, standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).


SDF-1 Induced
MIP-1α Induced
Media
IL-2
IFN-γ
Cell Migration (% of Media Control)
Time Post Stimulation (hrs)
24
48
0
20
40
60
80
100
120
140
160

Monocytes derived from HIV negative cord blood also showed a significant decrease in SDF-1 induced chemotaxis to 48.5 ± 18.2 % ($p = 0.03$) of the media control after 24 hrs of culture with IL-2. This effect was lost after 48 hrs of culture. Similarly, chemotaxis of IFN-γ treated cord blood monocytes was decreased to 3.2 ± 1.6 % ($p = 0.001$) of the media control after 24 hrs, but this effect was subsequently lost. Monocytes from cord blood cultured with IL-2 demonstrated a significant decrease in MIP-1α induced chemotaxis to 65.0 ±12.4 % ($p = 0.024$) after 24 hrs, but this inhibition was not maintained at 48 hrs. Culture of cord blood monocytes for 24 hrs with IFN-γ caused a significant and severe decrease in chemotaxis to 3.4 ± 2.4 % ($p = 0.0001$) of the media control, which was again lost after 48 hrs (Figure 8B).

Monocytes from HIV positive adults also showed a decrease in SDF-1 induced chemotaxis after 24 hrs of culture with IL-2 to 33.0 ± 19.9 % ($p = 0.014$) of the media cultured control. This effect of IL-2 was no longer significant after 48 hrs of culture with this cytokine. Like monocytes from uninfected adults, those from infected individuals also showed significant decreases in chemotaxis to SDF-1 after both 24 and 48 hrs of culture with IFN-γ, decreasing to 20.8 ± 12.4 % ($p = 0.002$) and 27.3 ± 17.8 % ($p = 0.01$) of control monocytes, respectively. Culture with IL-2 did not cause a significant decrease in MIP-1α induced chemotaxis after 24 or 48 hrs. However, IFN-γ caused a significant and almost complete inhibition of chemotaxis in response to MIP-1α at both time points, inhibiting chemotaxis of these cells to 23.8 ± 12.9 % ($p = 0.005$) after 24 hrs, and 9.0 ± 4.6 % ($p = 0.0001$) after 48 hrs (Figure 8C).

Figure 8B: The Effects of IL-2 and IFN-γ on the Chemotaxis of Monocytes from HIV Negative Cord Blood Induced by the Ligands of CXCR4 and CCR5. PBMC from 6 HIV negative cord blood samples were cultured for 24 and 48 hrs in the presence and absence of IL-2 or IFN-γ, and then subjected to chemotaxis induced by SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5). Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and expressed as a percentage of a control cultured with media alone. The average migration percentage of monocytes, standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).

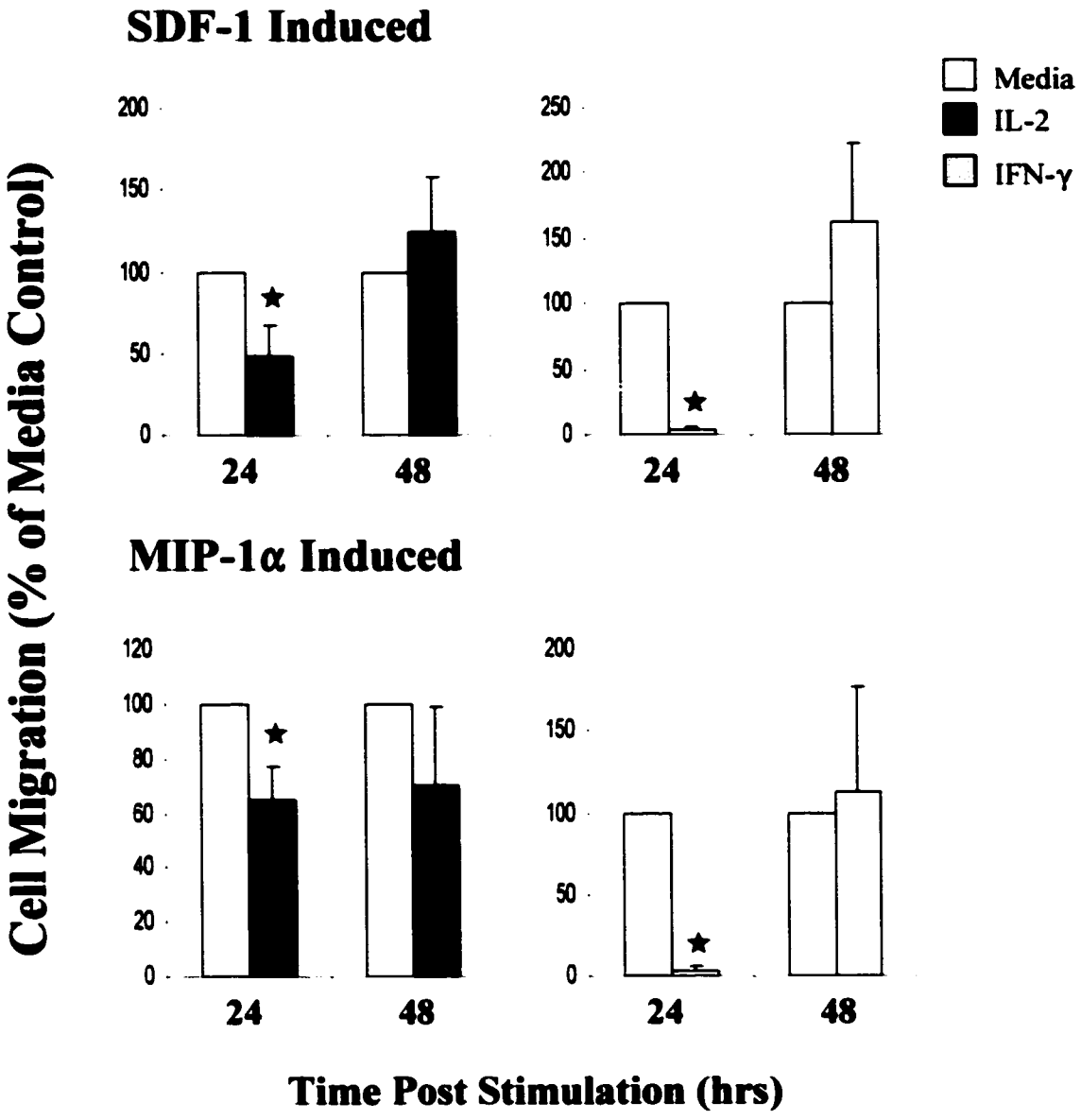
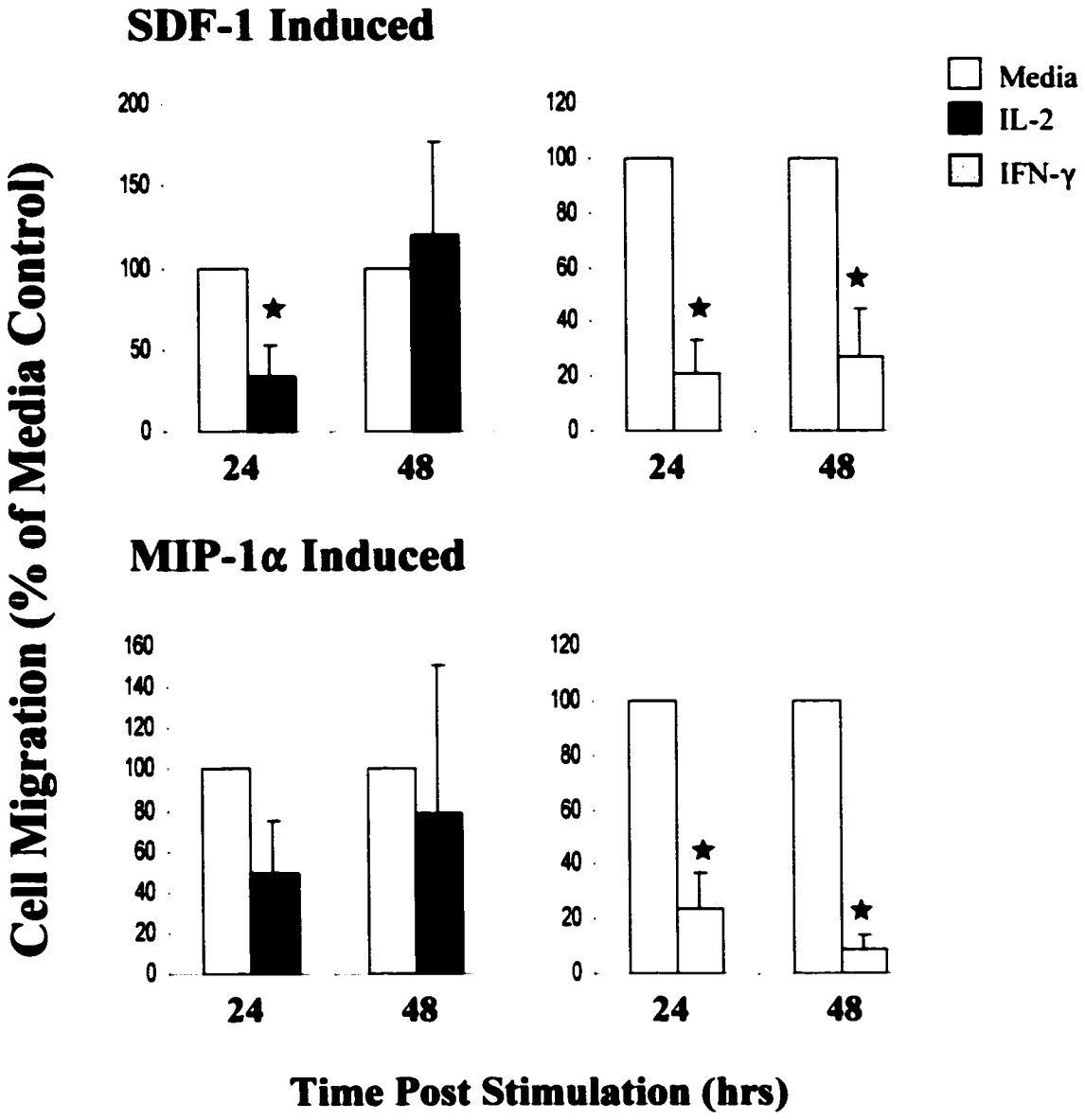

SDF-1 Induced
Media
IL-2
IFN-γ
Cell Migration (% of Media Control)
200
150
100
50
0
24
48
250
200
150
100
50
0
24
48
MIP-1α Induced
120
100
80
60
40
20
0
24
48
200
150
100
50
0
24
48
Time Post Stimulation (hrs)

Figure 8C: The Effects of IL-2 and IFN-γ on the Chemotaxis of Monocytes from HIV Positive Adult Blood Induced by the Ligands of CXCR4 and CCR5. PBMC from 6 HIV positive adults were cultured for 24 and 48 hrs in the presence and absence of IL-2 or IFN-γ, and then subjected to chemotaxis induced by SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5). Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and expressed as a percentage of a control cultured with media alone. The average migration percentage of monocytes, standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).


SDF-1 Induced
MIP-1α Induced
Media
IL-2
IFN-γ
Cell Migration (% of Media Control)
Time Post Stimulation (hrs)
24
48
200
150
100
50
0
120
80
60
40
20
160
140

These results suggest that decreases in chemokine receptor expression by IL-2 and IFN-γ are associated with decreases in monocyte migration induced by their respective chemokine ligands.

Analysis of HIV Entry in Monocytes

To determine the ability of HIV to enter monocytes exhibiting decreases in CXCR4 and CCR5 expression caused by IL-2 and IFN-γ, PCR was used to specifically detect HIV *gag* DNA that is present in the unintegrated forms of viral DNA and the integrated proviral DNA (80;81). The presence of HIV *gag* DNA is a suitable measure for viral entry in the present investigation because the DNA forms detected by this method are present early in the life cycle of the virus.

To determine the optimal time of HIV *gag* expression in monocytes negatively selected from the PBMC of an HIV negative individual, a time course of HIV infection with the dual tropic clinical isolate #204 was performed from 0 to 12 hrs after infection. Results indicated that maximal HIV *gag* DNA was detectable after 8 hrs of infection, so this time point was used in subsequent experiments (Figure 9A).

Once the timing of the appearance of this marker was defined, monocytes were isolated by negative selection from the PBMC of 5 HIV negative adults. These monocytes were incubated in the presence and absence of IL-2 or IFN-γ for 48 hrs before being infected with the dual tropic HIV clinical isolate #204. Results showed that culture of monocytes with IL-2 or IFN-γ had no effect on the amount of HIV *gag* DNA that was detected in these cells, and therefore did not affect the ability of this HIV isolate to enter monocytes. The results of one representative sample is shown (Figure 9B).

Figure 9:
A) Timing of HIV Entry in Monocytes. Monocytes were isolated from the PBMC of an HIV negative adult by negative selection with antibody coated magnetic beads. These monocytes were incubated with dual tropic HIV Clinical Isolate #204 for the indicated amounts of time. The DNA was extracted from these monocytes, and the HIV *gag* sequence was amplified with specific primers. An equal amount of DNA was also amplified with primers specific for β-actin. Amplified products were run on an agarose gel, and analyzed by densitometry using β-actin as an internal control.
B) The Effects of IL-2 and IFN-γ on HIV Entry in Monocytes. Monocytes were isolated from the PBMC of 5 HIV negative adults by negative selection with antibody coated magnetic beads. These monocytes were incubated with IL-2 or IFN-γ for 48 hrs before being incubated with dual tropic HIV Clinical Isolate #204 for 8 hrs. The DNA was extracted from these monocytes, and the HIV *gag* sequence was amplified with specific primers. An equal amount of DNA was also amplified with primers specific for β-actin. Amplified products were run on an agarose gel, and analyzed by densitometry using β-actin as an internal control. One representative sample is shown with the average ratios of HIV *gag* DNA to β-actin. The average ratios of HIV *gag* DNA to β-actin, standard error, and probability values were calculated by standard methods.

A

β-Actin (610 bp)

HIV *gag* (114 bp)

0.0	0.36	0.45	0.40	Ratio of HIV *gag*/β-Actin

0 4 8 12

Time Post Infection (hrs)

B

β-Actin (610 bp)

HIV *gag* (114 bp)

0.01 ± 0.006	1.49 ± 0.3	1.52 ± 0.4	1.49 ± 0.4	Ratio of HIV *gag*/β-Actin

Uninfected Control	Media	IL-2	IFN-γ

Analysis of HIV Replication in Monocytes

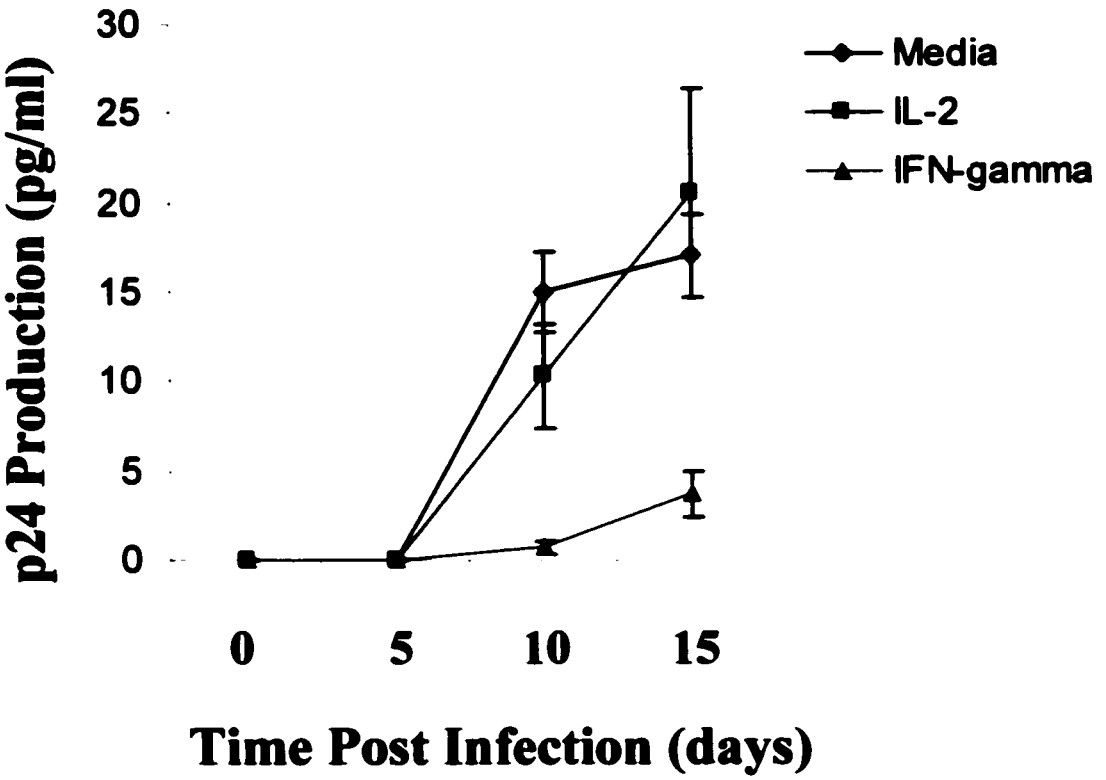
The investigation of viral entry revealed the inability of IL-2 and IFN-γ to inhibit HIV entry in monocytes, but IFN-γ has previously been shown to inhibit HIV replication. Since IL-2 and IFN-γ inhibit chemokine receptor expression and chemokine induced chemotaxis, but do not affect the entry of HIV into monocytes under these conditions, these cytokines may be influencing viral replication at steps subsequent to entry. Monocytes isolated by negative selection from 5 HIV negative adult blood samples were incubated with IL-2 or IFN-γ for 48 hrs prior to infection with M-tropic HIV Bal. In unstimulated monocytes, the p24 production was undetectable 5 days after infection, but it increased to an average of 15.0 ± 2.2 pg/ml by day 10, and reached a maximal level of 17.0 ± 2.3 pg/ml by day 15. Stimulation of monocytes with IL-2 did not effect p24 production, and gave results that were similar to those observed for unstimulated monocytes. In contrast, p24 production by HIV replicating in monocytes cultured with IFN-γ was significantly reduced when compared to that observed in unstimulated monocytes (Figure 10).

The Effects of Th2 Cytokines on CXCR4 and CCR5 Expression:

Analysis of CXCR4 and CCR5 Expression on Monocytes by Flow Cytometry

In the next series of experiments, the effects of Th2 cytokines (IL-4 and IL-13) on CXCR4 and CCR5 expression, and the implications of this in chemotaxis, HIV entry, and viral replication, were investigated. The expression of CXCR4 and CCR5 were examined on the surface of monocytes from 10 HIV negative cord blood samples, 10 HIV negative

Figure 10: The Effects of IL-2 and IFN-γ on HIV Replication in Monocytes. Monocytes were isolated from the PBMC of 5 HIV negative adults by negative selection with antibody coated magnetic beads. These monocytes were then incubated in the presence and absence of IL-2 or IFN-γ for 48 hrs before being infected with M-tropic HIV Bal for 12 hrs. After infection, monocytes were washed extensively and cultured for the indicated amounts of time. Supernatants were collected 0, 5, 10, and 15 days after infection and subjected to analysis of p24 production by ELISA. Average p24 production, standard error, and probability values were calculated by standard methods.

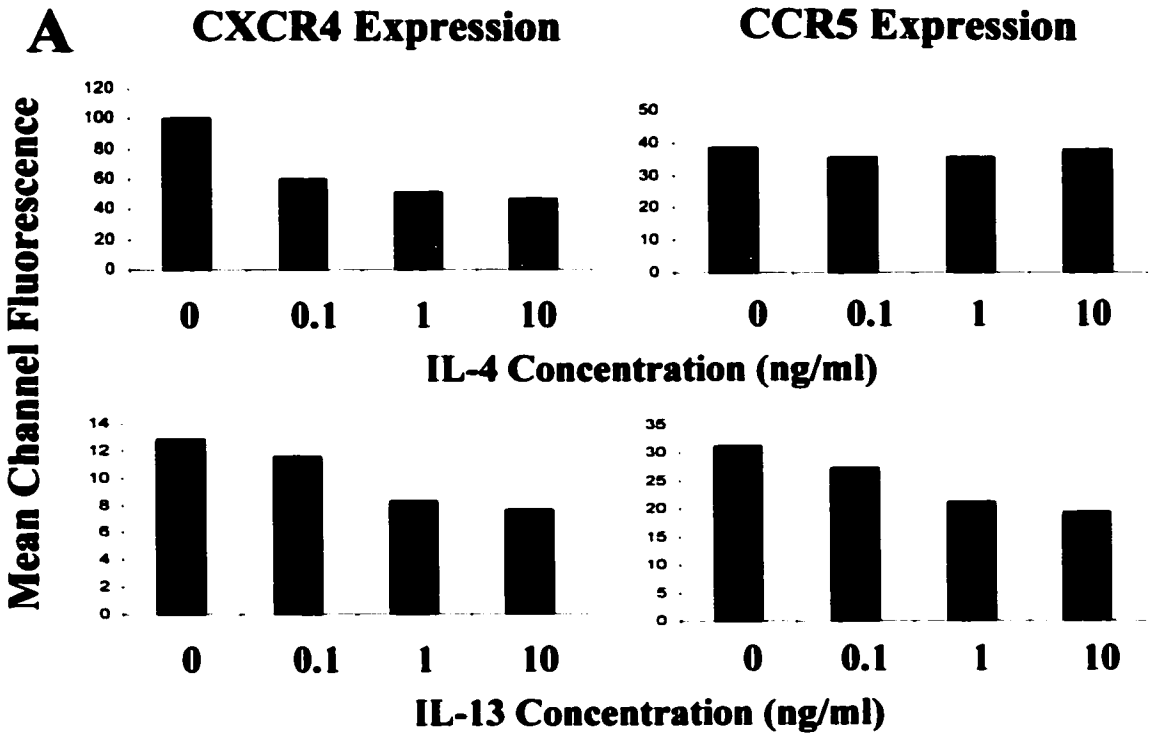

30
25
20
15
10
5
0
p24 Production (pg/ml)
0
5
10
15
Time Post Infection (days)
Media
IL-2
IFN-gamma

adults, and 6 HIV positive adults. These cells were cultured in the presence and absence of IL-4or IL-13 for 24 and 48 hrs, and examined by flow cytometry. Optimization experiments were performed to determine the doses of IL-4 and IL-13 required to elicit an effect, and the time point at which this effect occurs. Results indicated that 1 ng/ml of IL-4 and IL-13 was sufficient to cause the observed effects on chemokine receptor expression (Figure 11A). Investigation of the kinetics of these effects revealed that they occurred after 24 and 48 hrs of culture (Figure 11B).

The study of monocytes from the blood of HIV negative adults demonstrated that IL-4 caused significant decreases in CXCR4 expression from a MCF of 30.2 ± 8.3 to 15.0 ±1.6 ($p = 0.049$), and from 21.8 ± 4.9 to 9.5 ± 2.1 ($p = 0.016$) after 24 and 48 hrs, respectively. Similarly, IL-13 decreased CXCR4 expression by monocytes from 30.2 ± 8.3 to 18.0 ± 0.2 ($p = 0.068$), and from 21.8 ± 4.9 to 14.8 ±1.4 ($p = 0.09$) after 24 and 48 hrs, respectively, but these decreases were not statistically significant. Culture of monocytes from HIV negative adults with IL-4 for 24 hrs caused a significant increase in CCR5 expression [47.8 ± 11.0 in unstimulated monocytes versus 80.6 ± 13.8 ($p = 0.038$) after IL-4 treatment], but this effect was lost after 48 hrs. IL-13 had no effect on CCR5 expression after 24 hrs of culture, however this cytokine caused a significant decrease in CCR5 expression from a MCF of 55.9 ± 8.9 to 28.0 ± 2.6 ($p = 0.049$) after 48 hrs (Figure 12A).

To ensure that the observed effects of IL-4 and IL-13 were not mediated by other cell types, these experiments were performed on purified monocytes isolated from the PBMC of HIV negative adults and incubated with IL-4 or IL-13 for 48 hrs. Culture of purified monocytes with IL-4 and IL-13 resulted in significant decreases in CXCR4

Figure 11:
A) The Effects of IL-4 and IL-13 Concentration on the Expression of CXCR4 and CCR5 on Monocytes. Adult PBMC were incubated with increasing doses of IL-4 or IL-13 for 48 hrs, and the effects of these cytokines on CXCR4 and CCR5 expression on the surface of $CD14^+$ monocytes were monitored by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor.
B) Timing of the Effects of IL-4 on the Expression of CXCR4 and CCR5 on Monocytes. Adult PBMC were incubated with IL-4 (1 ng/ml) for the indicated lengths of time, and the effects of this cytokine on the expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was monitored by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor.


A
CXCR4 Expression
CCR5 Expression
Mean Channel Fluorescence
120
100
80
60
40
20
0
50
40
30
20
10
0
0
0.1
1
10
IL-4 Concentration (ng/ml)
14
12
10
8
6
4
2
0
35
30
25
20
15
10
5
0
IL-13 Concentration (ng/ml)

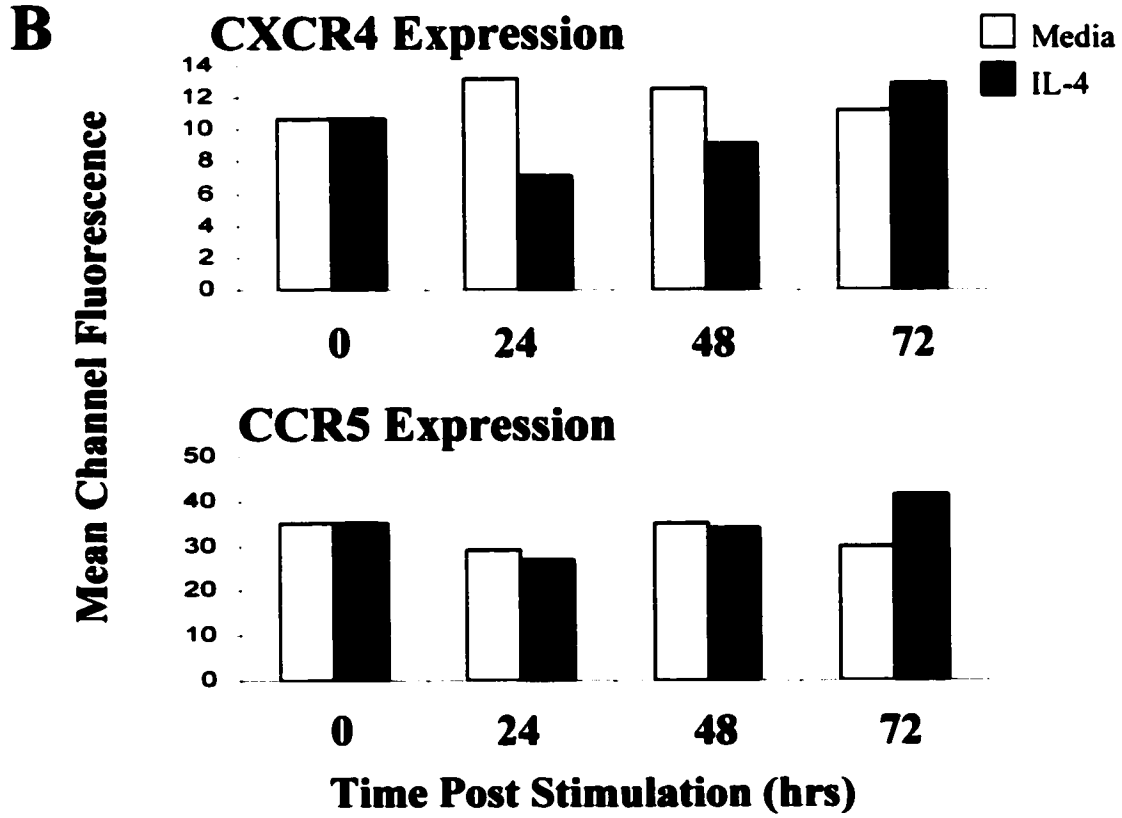
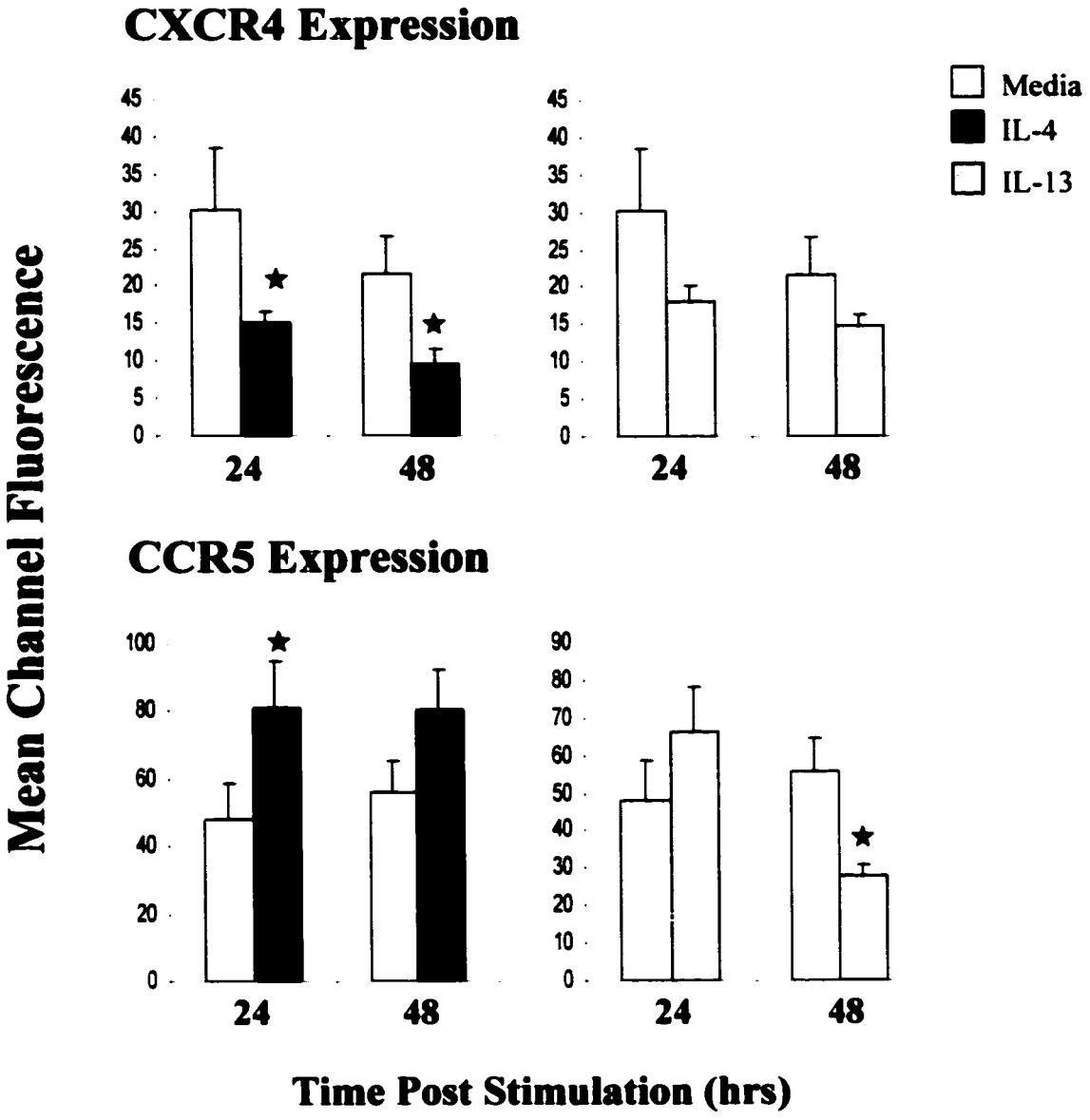

B
CXCR4 Expression
Media
IL-4
Mean Channel Fluorescence
14
12
10
8
6
4
2
0
0
24
48
72
CCR5 Expression
50
40
30
20
10
0
Time Post Stimulation (hrs)

Figure 12A: The Effects of IL-4 or IL-13 on the Expression of CXCR4 and CCR5 on Monocytes from HIV Negative Adult Blood. PBMC from 10 HIV negative adults were cultured for 24 and 48 hrs in the presence and absence of IL-4 or IL-13. The expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was examined by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor. The average mean channel fluorescence (MCF), standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).

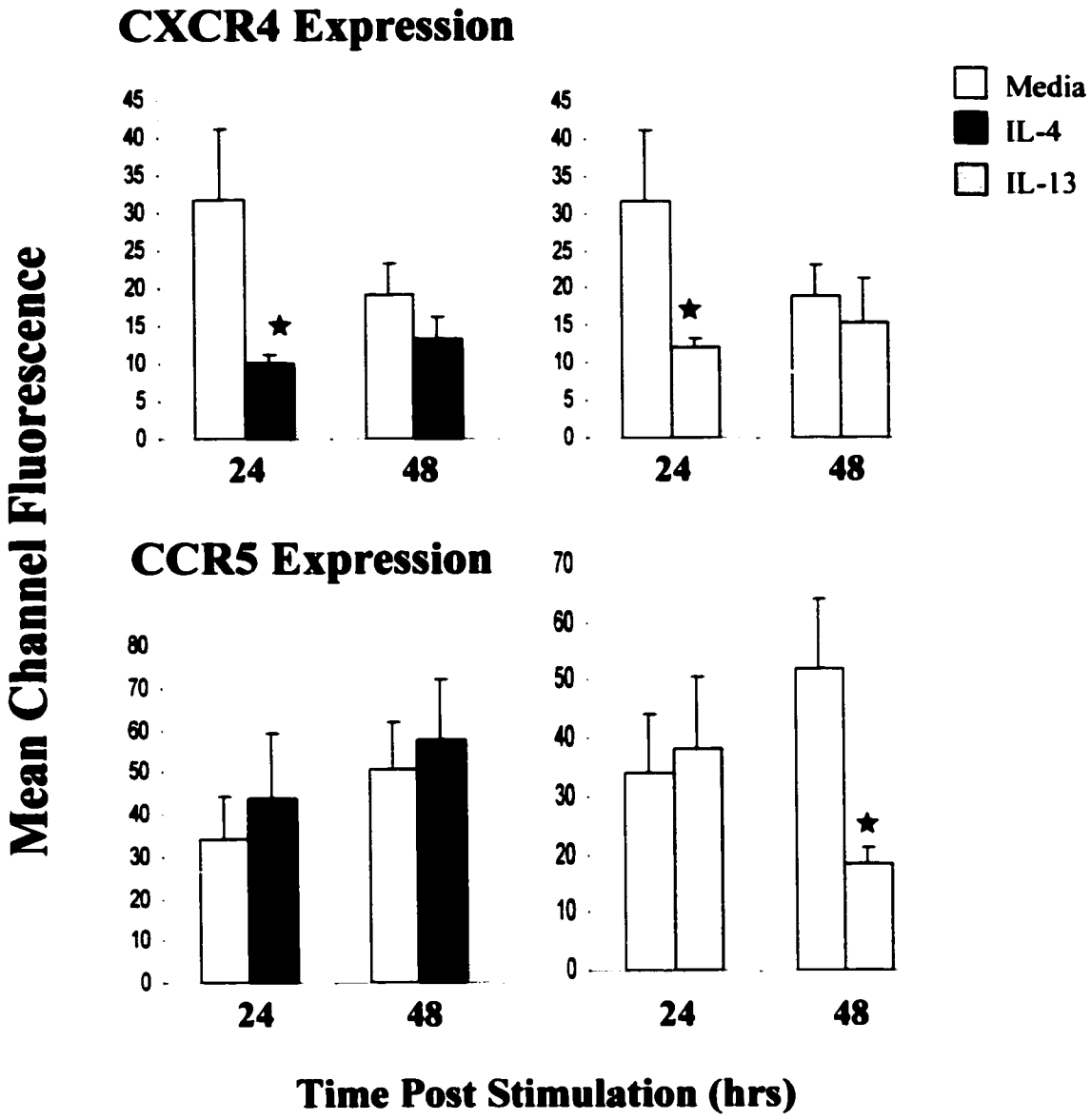

CXCR4 Expression
Media
IL-4
IL-13
45
40
35
30
25
20
15
10
5
0
24
48
CCR5 Expression
100
80
60
40
20
0
90
70
50
30
10
Mean Channel Fluorescence
Time Post Stimulation (hrs)

expression after 48 hrs, when compared to unstimulated monocytes [8.2 ± 1.2 in unstimulated monocytes versus 3.28 ± 0.4 ($p = 0.002$) after IL-4 treatment and 4.9 ± 1.5 ($p = 0.007$) after IL-13 treatment]. CCR5 expression on purified monocytes was not affected by stimulation with IL-4 [20.3 ± 2.4 in unstimulated monocytes versus 16.8 ± 2.0 ($p = 0.063$) in IL-4 treated]. In contrast to this, purified monocytes cultured with IL-13 demonstrated a decrease in CCR5 expression, from a MCF of 20.3 ± 2.4 seen in the media control to 12.8 ± 1.3 ($p = 0.014$) in those treated with IL-13. The effects observed in this experiment were identical to those previously described for monocytes from HIV negative adult blood cultured as PBMC (data not shown). Therefore, in subsequent experiments PBMC were used to determine the effects of IL-4 and IL-13 on chemokine receptor expression and their biological implications.

Culture of cord blood monocytes with IL-4 caused a significant decrease of CXCR4 expression after 24 hrs [31.7 ± 9.4 in unstimulated monocytes versus 10.1 ± 1.1 ($p = 0.047$) after IL-4 treatment]. The effect of IL-4 on CXCR4 expression was lost after 48 hrs. IL-13 also caused a significant decrease in CXCR4 expression after 24 hrs of stimulation [31.68 ± 9.4 in media control monocytes versus 12.2 ± 1.0 ($p = 0.046$) in those cultured with IL-13], but this effect was lost after 48 hrs. IL-4 had no significant effect on CCR5 expression after 24 or 48 hrs of culture. IL-13 also did not affect CCR5 expression after 24 hrs, but did cause a significant decrease in CCR5 expression from 52.21 ± 11.9 to 18.3 ± 2.9 ($p = 0.031$) after 48 hrs of culture (Figure 12B).

The expression of CXCR4 on monocytes from the blood of HIV positive individuals was unaffected by 24 hrs of culture with IL-4, but was significantly decreased

Figure 12B: The Effects of IL-4 and IL-13 on the Expression of CXCR4 and CCR5 on Monocytes from HIV Negative Cord Blood. PBMC from 10 HIV negative cord blood samples were cultured for 24 and 48 hrs in the presence and absence of IL-4 or IL-13. The expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was examined by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor. The average mean channel fluorescence (MCF), standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).


CXCR4 Expression
Media
IL-4
IL-13
Mean Channel Fluorescence
45
40
35
30
25
20
15
10
5
0
24
48
CCR5 Expression
80
70
60
50
40
30
20
10
0
70
60
50
40
30
20
10
0
Time Post Stimulation (hrs)

from 46.2 ± 11.9 to 14.4 ± 1.9 ($p = 0.039$) after 48 hrs of culture. Stimulation with IL-13 did not significantly alter CXCR4 expression by monocytes from HIV positive blood after 24 hrs. However, after 48 hrs of culture with IL-13, CXCR4 expression by monocytes was significantly decreased [46.2 ± 11.9 in untreated monocytes versus 24.4 ± 1.9 ($p = 0.009$) in those cultured with IL-13]. Similar to the results observed in HIV negative adults, IL-4 had no significant impact on CCR5 expression by monocytes from HIV infected individuals. Culture of monocytes from HIV positive blood with IL-13 did not significantly change the expression of CCR5 after 24 hrs, but did significantly decrease the expression of this chemokine receptor from 94.7 ± 21.0 to 63.5 ± 9.9 ($p = 0.0001$), after 48 hrs (Figure 12C).

Representative histograms depicting the general effect of both IL-4 and IL-13 on CXCR4 and CCR5 expression on monocytes from all sources are shown (Figure 13).

Analysis of CXCR4 and CCR5 mRNA Expression in Monocytes by RT-PCR

To determine if IL-4 and IL-13 decreased CXCR4 and CCR5 expression at the transcriptional level, the expression of mRNA encoding these chemokine receptors was investigated by semi-quantitative RT-PCR analysis. Monocytes from three adults and three cord blood samples were subjected to this analysis. Monocytes isolated from HIV negative cord blood and adult peripheral blood both showed no change in the level of mRNA encoding CXCR4 after culture with IL-4 or IL-13, as determined by densitometry. Similarly, the levels of CCR5 encoding mRNA in monocytes derived from cord blood and adult blood also proved to be unaffected by culture with IL-4 or IL-13. One representative of each is shown (Figure 14).

Figure 12C: The Effects of IL-4 and IL-13 on the Expression of CXCR4 and CCR5 on Monocytes from HIV Positive Adult Blood. PBMC from 6 HIV positive adults were cultured for 24 and 48 hrs in the presence and absence of IL-4 or IL-13. The expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes was examined by flow cytometry with fluorochrome labeled monoclonal antibodies specific for each chemokine receptor. The average mean channel fluorescence (MCF), standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).

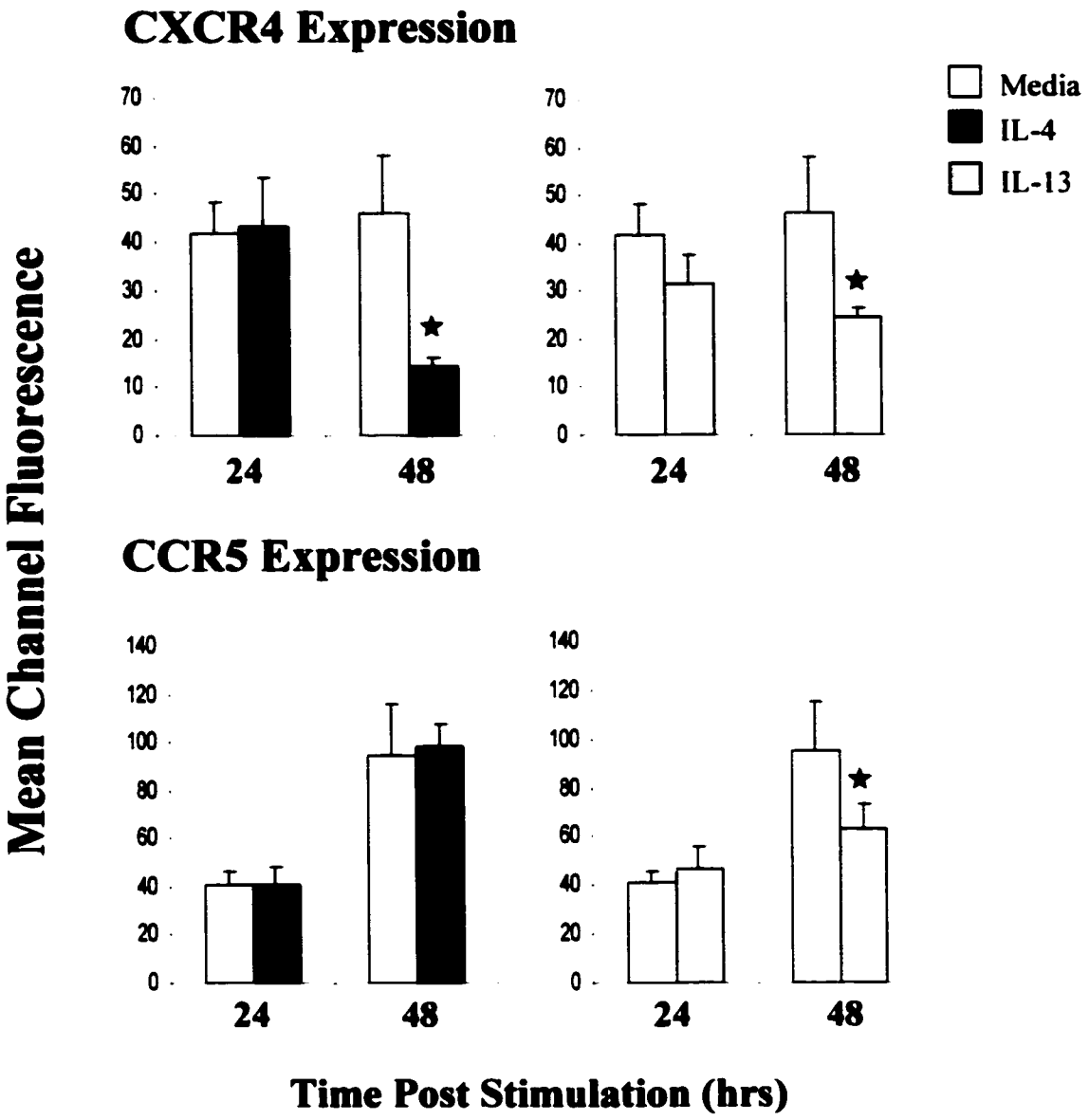

CXCR4 Expression
Media
IL-4
IL-13
70
60
50
40
30
20
10
0
24
48
CCR5 Expression
140
120
100
80
60
40
20
0
Mean Channel Fluorescence
Time Post Stimulation (hrs)

Figure 13: The Effects of IL-4 and IL-13 on the Expression of CXCR4 and CCR5 on Monocytes. Representative flow cytometry histograms of the general effects of IL-4 or IL-13 on the expression of CXCR4 and CCR5 on the surface of $CD14^+$ monocytes from HIV negative adults, HIV positive adults, and HIV negative cord blood samples. Shaded curves represent control monocytes cultured with media alone, while open curves represent monocytes treated with the indicated cytokines.

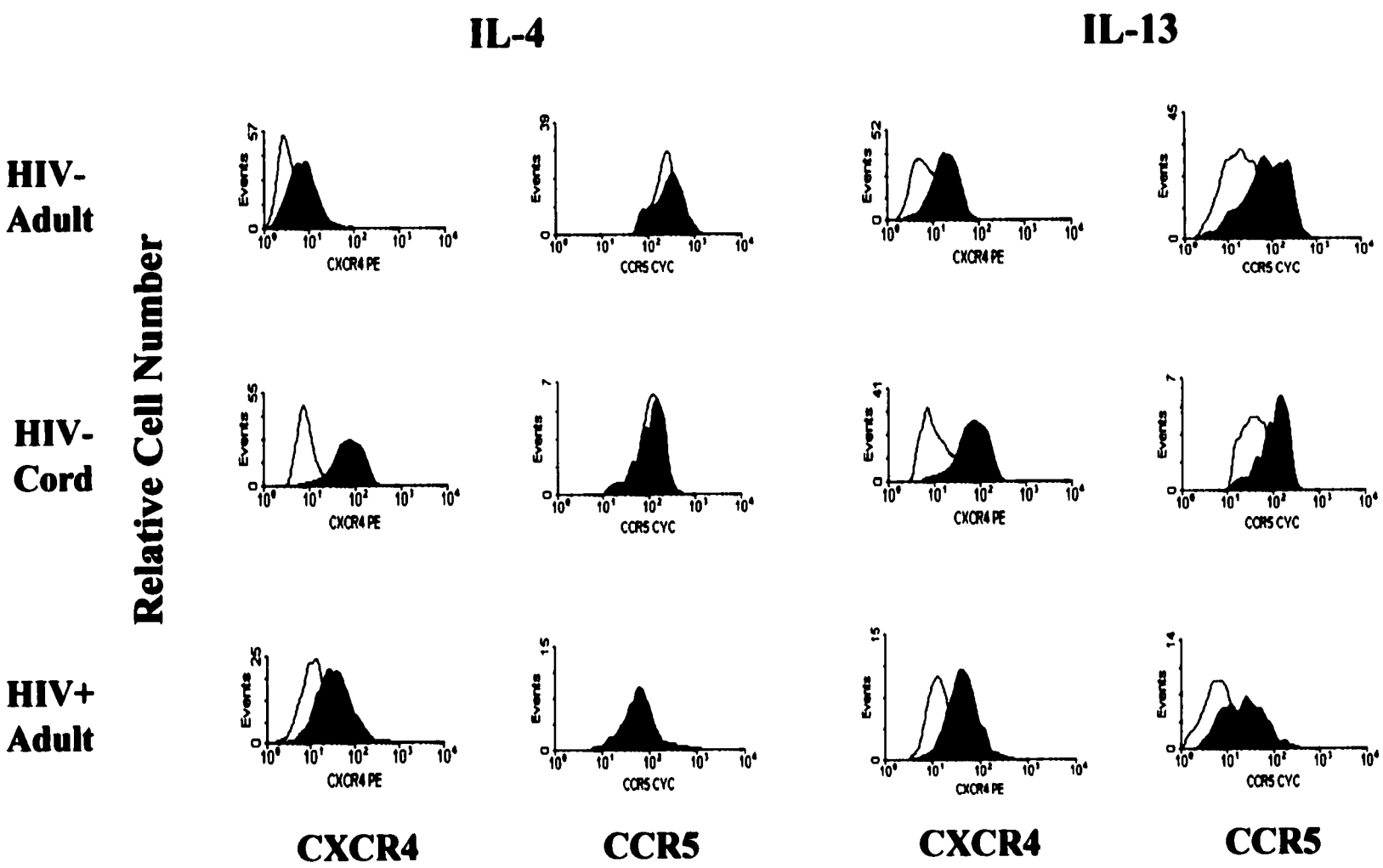
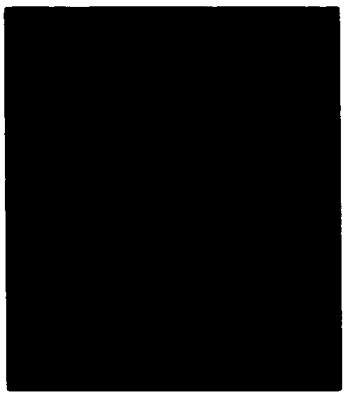

IL-4
IL-13
HIV-
Adult
HIV-
Cord
HIV+
Adult
Relative Cell Number
Events
CXCR4 PE
CCR5 CYC
CXCR4
CCR5
CXCR4
CCR5
Chemokine Receptor Expression (MCF)

Figure 14: The Effect of IL-4 and IL-13 on the Transcription of mRNA encoding CXCR4 and CCR5 in Monocytes. PBMC from 3 HIV negative adults and 3 HIV negative cord blood samples were incubated in the presence and absence of IL-4 or IL-13 for 12 hrs, and then $CD14^+$ monocytes were positively selected using anti-CD14 antibody coated magnetic beads. RNA was isolated from these monocytes, and subjected to semi-quantitative RT-PCR analysis using specific primers for mRNA encoding CXCR4 or CCR5, and β-actin. These amplified products were run on an agarose gel, and subjected to densitometric analysis using β-actin as an internal control. One representative sample from adult and cord blood is shown.

CXCR4



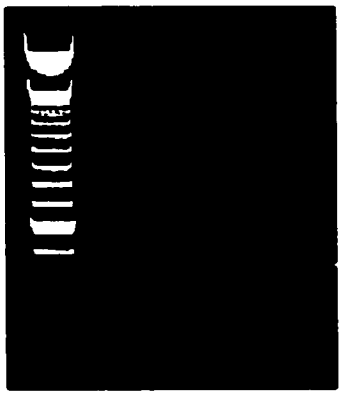
β-Actin (610 bp)

CXCR4 (250 bp)

0.98 0.97 0.94

Media IL-4 IL-13

Ratio of CXCR4/β-Actin



β-Actin (610 bp)

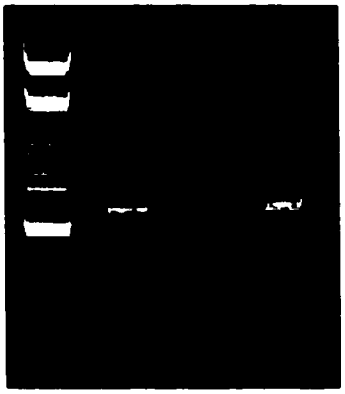
CXCR4 (250 bp)

1.02 0.97 1.03

Media IL-4 IL-13

Ratio of CXCR4/ β-Actin

CCR5



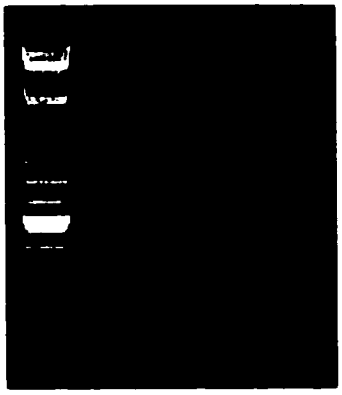
β-Actin (610 bp)

CCR5 (320 bp)

0.65 0.73 0.68

Media IL-4 IL-13

Ratio of CCR5/β-Actin



β-Actin (610 bp)

CCR5 (320 bp)

1.06 1.04 1.09

Media IL-4 IL-13

Ratio of CCR5/β-Actin

Adult Blood

Cord Blood

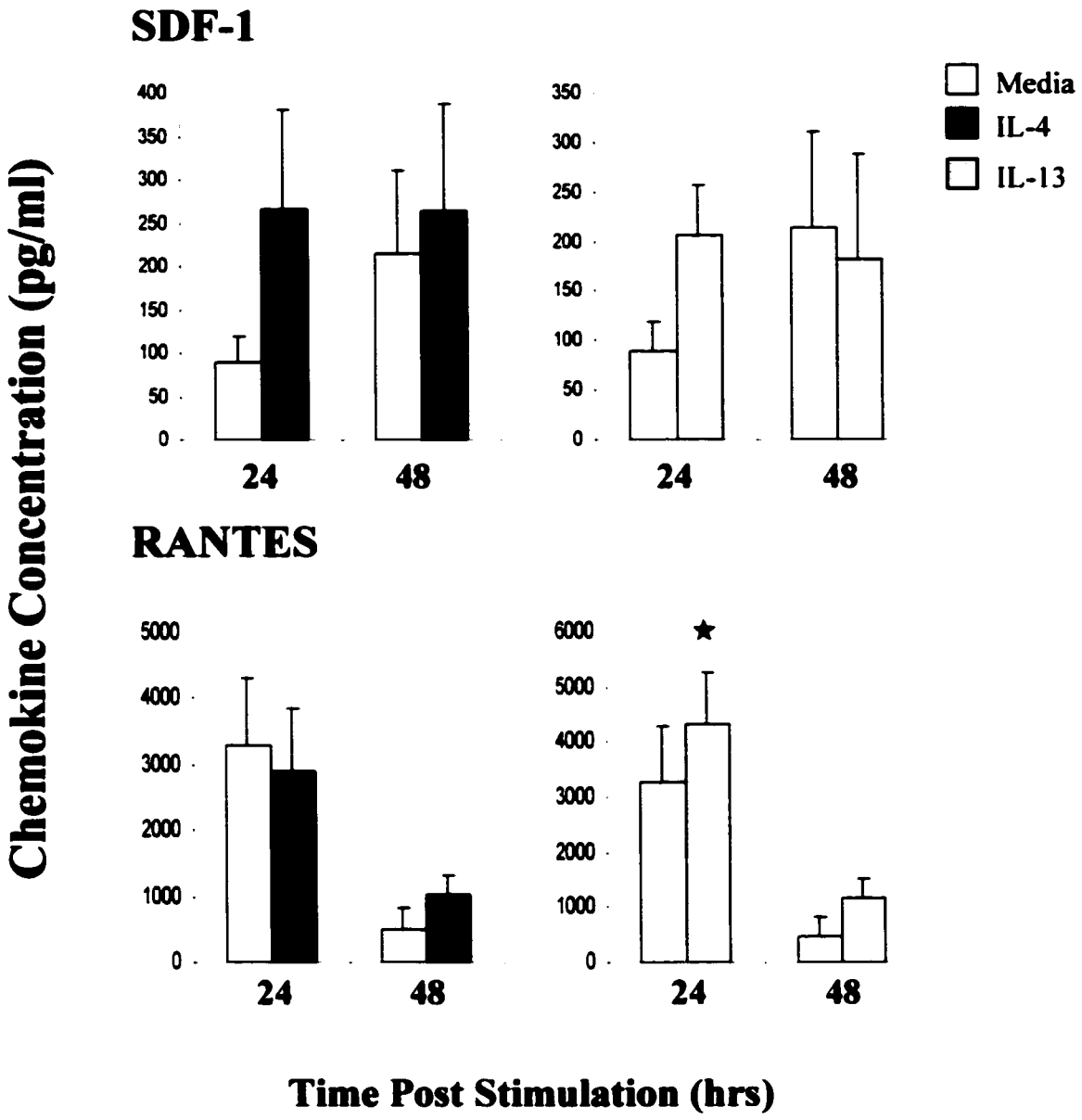
These results suggest that IL-4 and IL-13 down regulate CXCR4 and CCR5 expression on monocytes, but do not affect the expression of mRNA encoding these chemokine receptors.

Analysis of Chemokine Secretion

The absence of an effect at the transcriptional level lead to the examination of the possibility that IL-4 and IL-13 are inducing chemokine secretion which is responsible for the down regulation of CXCR4 and CCR5. PBMC from 5 HIV negative individuals were cultured with IL-4 or IL-13, and their supernatants were examined for chemokine secretion by ELISA. Culture of PBMC with IL-4 caused an increase in the production of SDF-1 from an average of 90 ± 30 pg/ml to 267 ± 51 pg/ml ($p = 0.134$) after 24 hrs. This increase in SDF-1 concentration by IL-4 was not present after 48 hrs of culture. Similarly, culture of PBMC with IL-13 also caused an increase in the secretion of SDF-1 after 24 hrs, from 90 ± 30 pg/ml to 207 ± 51 pg/ml ($p = 0.175$), but this effect was lost at 48 hrs (Figure 15).

The concentration of the CCR5 ligand RANTES in culture supernatants was not significantly effected by IL-4 after 24 or 48 hrs of culture [3277 ± 1016 pg/ml in unstimulated PBMC versus 2893 ± 946 pg/ml ($p = 0.059$) after 24 hrs of IL-4 treatment; 483 ± 349 pg/ml in unstimulated PBMC versus 1019 ±285 pg/ml ($p = 0.160$) after 48 hrs of IL-4 treatment]. In contrast to this, IL-13 caused an increase in RANTES production from 3277 ± 1016 pg/ml to 4342 ± 939 pg/ml ($p = 0.023$) after 24 hrs, but this effect was lost after 48 hrs (Figure 15). The concentration MIP-1α was also measured in

Figure 15: The Effects of IL-4 and IL-13 on the Secretion of Chemokines. PBMC were isolated from the blood of 5 HIV negative adult donors, and incubated for 24 and 48 hrs in the presence and absence of IL-4 or IL-13. Supernatants were collected at these time points and subjected to ELISA for the detection of the CXCR4 ligand SDF-1, and the CCR5 ligands RANTES, and MIP-1α (not shown). The average chemokine concentration, standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).


SDF-1
Media
IL-4
IL-13
400
350
300
250
200
150
100
50
0
24
48
350
300
250
200
150
100
50
0
24
48
RANTES
5000
4000
3000
2000
1000
0
24
48
6000
5000
4000
3000
2000
1000
0
24
48
Chemokine Concentration (pg/ml)
Time Post Stimulation (hrs)

supernatants of cells cultured in the presence and absence of IL-4 and IL-13, but this chemokine was not present in detectable amounts (data not shown). These results suggest that the observed decreases in CXCR4 and CCR5 expression by IL-4 and IL-13 may be the result of the internalization of these receptors caused by the presence of chemokines.

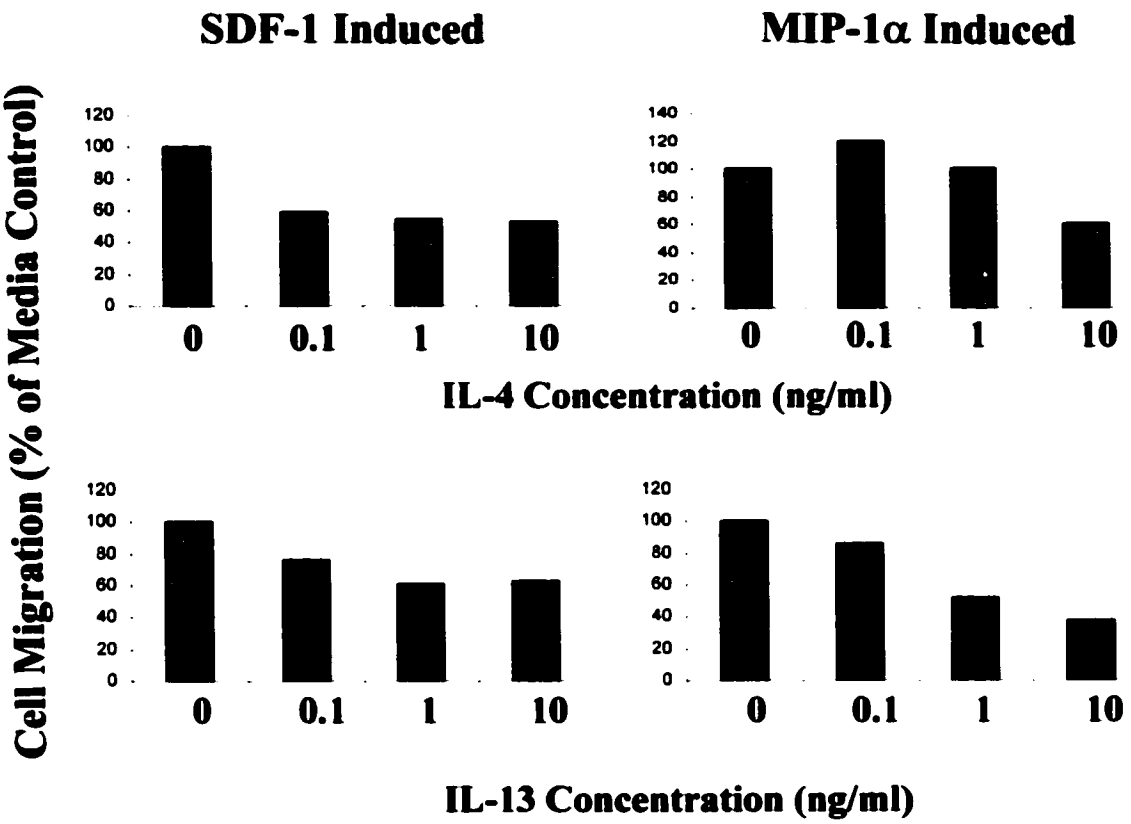
Implications of the Down Regulation of CXCR4 and CCR5 Expression on Monocytes by IL-4 and IL-13:

Chemokine receptors are responsible for the ability of cells to undergo migration induced by chemokines, and have been identified as the coreceptors for HIV. The decrease in CXCR4 and CCR5 expression by monocytes, caused by treatment with IL-4 and IL-13, may have implications in chemotaxis and the ability of HIV to infect these cells.

Analysis of Monocyte Chemotaxis

Given the observed decreases in CXCR4 and CCR5 expression by IL-4 and IL-13, the impact of these changes on the functional ability of monocytes to undergo chemotaxis was examined. PBMC were isolated from the blood of 6 HIV negative cord blood samples, 6 HIV negative adults, and 6 HIV positive adults, and cultured in the presence and absence of IL-4 or IL-13 before being subjected to chemotactic analysis. Cytokine dose response experiments confirmed that a cytokine concentration of 1 ng/ml was sufficient to observe the effects of IL-4 and IL-13 on chemotaxis induced by both SDF-1 and MIP-1α (Figure 16).

Figure 16: The Effects of IL-4 and IL-13 Concentration on the Chemotaxis of Monocytes. PBMC from HIV negative adult blood were subjected to chemotaxis induced by SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5) after culture with increasing concentrations of IL-4 or IL-13 for 48 hours. Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and are expressed as a percentage of a control cultured in media alone.


SDF-1 Induced
MIP-1α Induced
Cell Migration (% of Media Control)
120
100
80
60
40
20
0
140
120
100
80
60
40
20
0
0
0.1
1
10
IL-4 Concentration (ng/ml)
IL-13 Concentration (ng/ml)

IL-4 had no significant effect on the chemotaxis of monocytes isolated from the blood of HIV negative adults after 24 hrs of culture. However, after 48 hrs of culture with IL-4 the chemotaxis of adult monocytes was decreased to 30.9 ± 16.6 % ($p = 0.006$) of the media control. Treatment of HIV negative adult monocytes with IL-13 did not significantly affect the chemotaxis of these cells after 24 hrs, but chemotaxis induced by SDF-1 was significantly decreased to 36.0 ± 24.4 % ($p = 0.047$) of the media control after 48 hrs. Culture of HIV negative adult monocytes with IL-4 resulted in a significant increase in MIP-1α induced chemotaxis to 421.0 ± 129.6 % ($p = 0.034$) after 24 hrs compared to the media control, but this effect was lost after 48 hrs. IL-13 did not have a significant impact on MIP-1α induced chemotaxis of HIV negative adult monocytes after 24 hrs of culture, but this cytokine caused a significant inhibition of migration to 37.6 ± 16.7 % ($p = 0.009$) of the media control after 48 hrs (Figure 17A).

Monocytes derived from HIV negative cord blood showed a significant decrease in SDF-1 induced chemotaxis to 37.8 ± 4.9 % ($p = 0.0001$) of the media control after 24 hrs of culture with IL-4, but this effect was subsequently lost. IL-13 also caused a decrease in SDF-1 induced chemotaxis of cord blood monocytes, when compared to the media control, that was significant after 24 and 48 hrs [37.6 ± 8.8 % ($p = 0.001$) and 61.3 ± 11.3 % ($p = 0.014$) of the media control, respectively]. Monocytes from cord blood treated with IL-4 demonstrated no significant change in MIP-1α induced chemotaxis when compared to monocytes cultured with media alone. In contrast to this, culture with IL-13 caused a significant inhibition of MIP-1α induced chemotaxis after both 24 and 48 hrs of culture, decreasing the migration of these monocytes to 43.0 ± 7.7 % ($p = 0.0001$) and 70.1 ± 17.2 % ($p = 0.047$) of the media controls, respectively (Figure 17B).

Figure 17A: The Effects of IL-4 and IL-13 on the Chemotaxis of Monocytes from HIV Negative Adult Blood Induced by the Ligands of CXCR4 and CCR5. PBMC from 6 HIV negative adults were cultured for 24 and 48 hrs in the presence and absence of IL-4 or IL-13, and then subjected to chemotaxis induced by SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5). Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and expressed as a percentage of a control cultured in media alone. The average migration percentage of monocytes, standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).

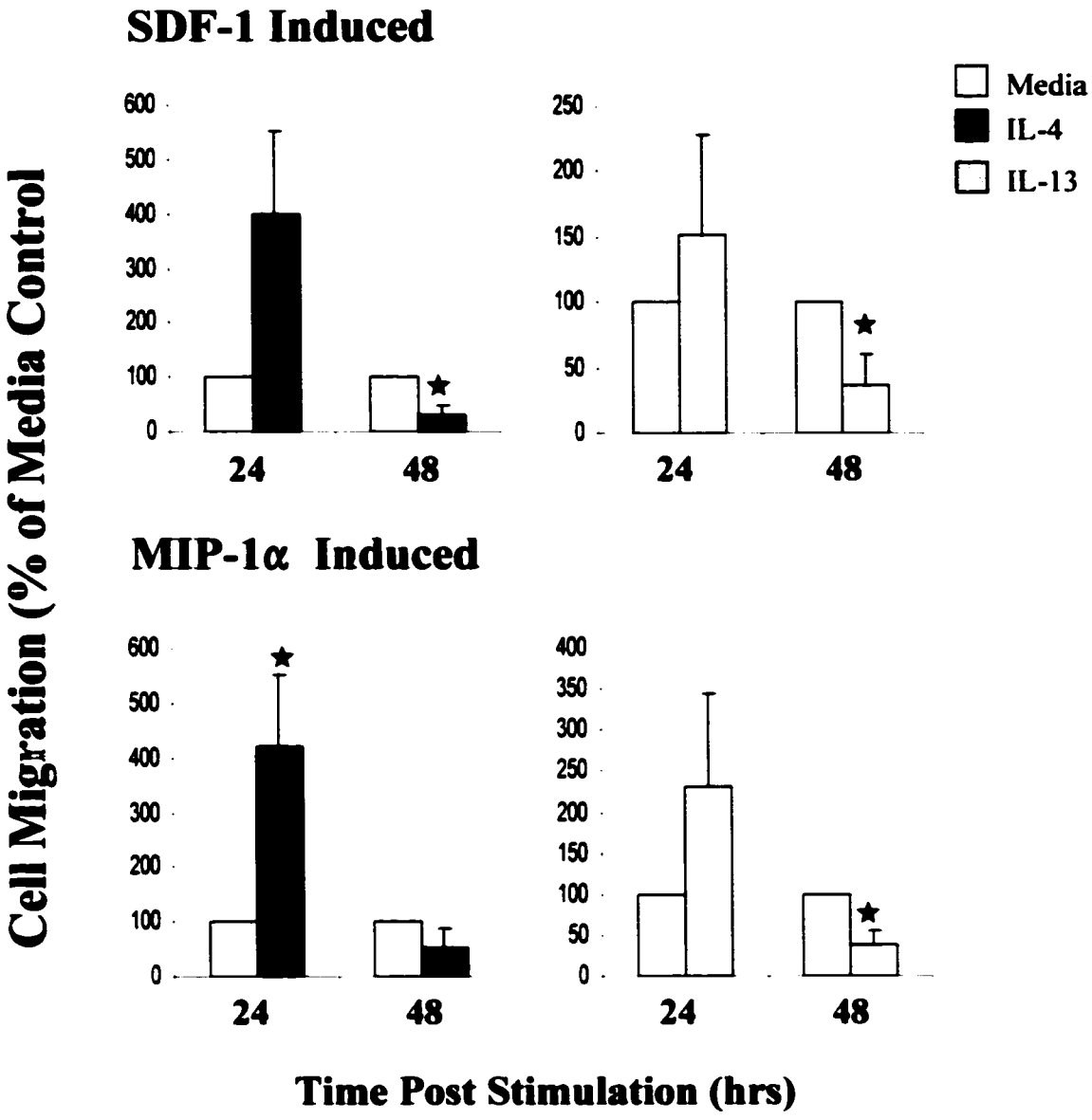
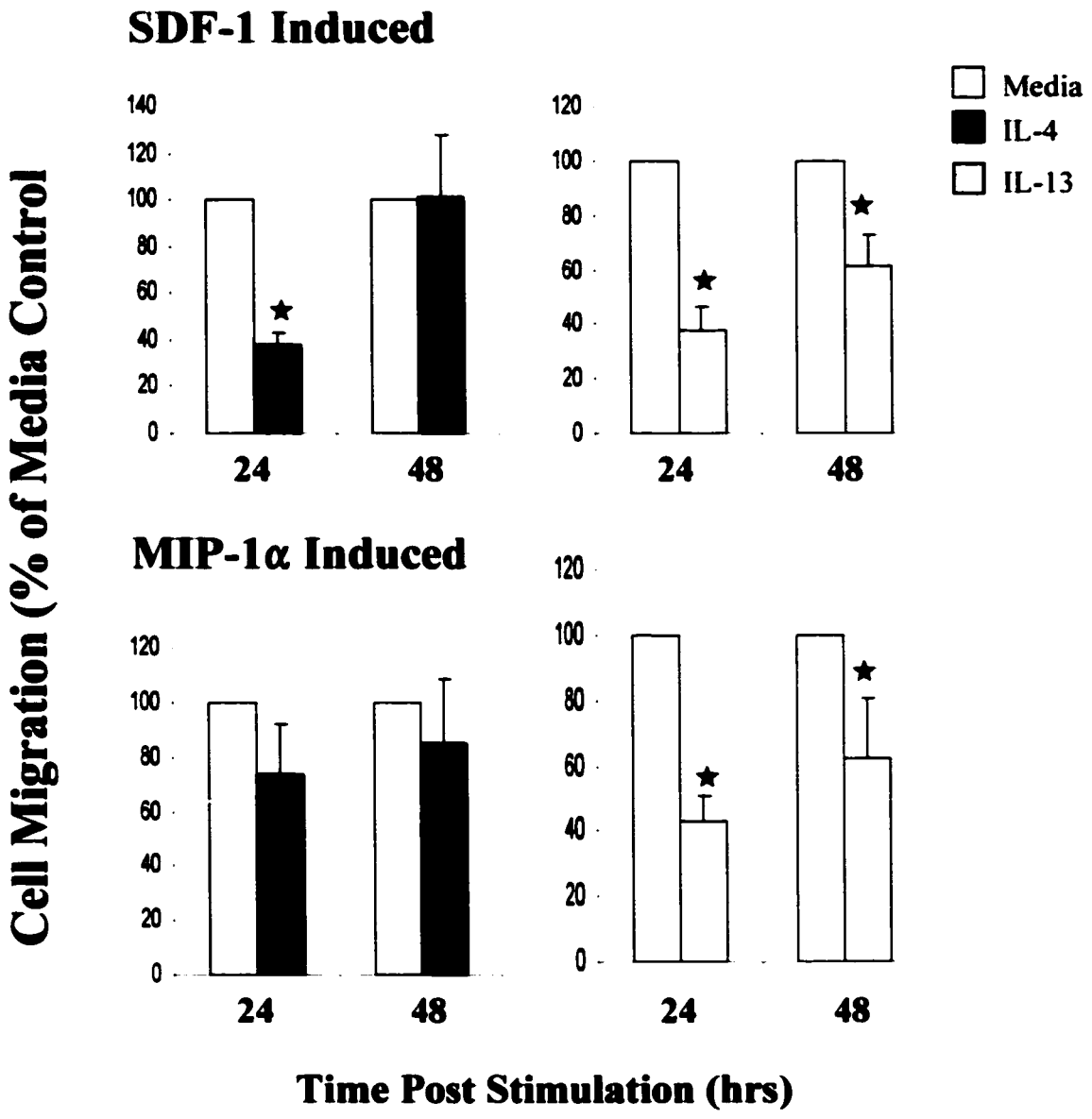

SDF-1 Induced
Media
IL-4
IL-13
600
500
400
300
200
100
0
24
48
250
200
150
100
50
0
24
48
MIP-1α Induced
600
500
400
300
200
100
0
24
48
400
350
300
250
200
150
100
50
0
24
48
Cell Migration (% of Media Control
Time Post Stimulation (hrs)

Figure 17B: The Effects of IL-4 and IL-13 on the Chemotaxis of Monocytes from HIV Negative Cord Blood Induced by the Ligands of CXCR4 and CCR5. PBMC from 6 HIV negative cord blood samples were cultured for 24 and 48 hrs in the presence and absence of IL-4 or IL-13, and then subjected to chemotaxis induced by SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5). Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and expressed as a percentage of a control cultured in media alone. The average migration percentage of monocytes, standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).


SDF-1 Induced
MIP-1α Induced
Media
IL-4
IL-13
Cell Migration (% of Media Control)
Time Post Stimulation (hrs)
24
48
0
20
40
60
80
100
120
140

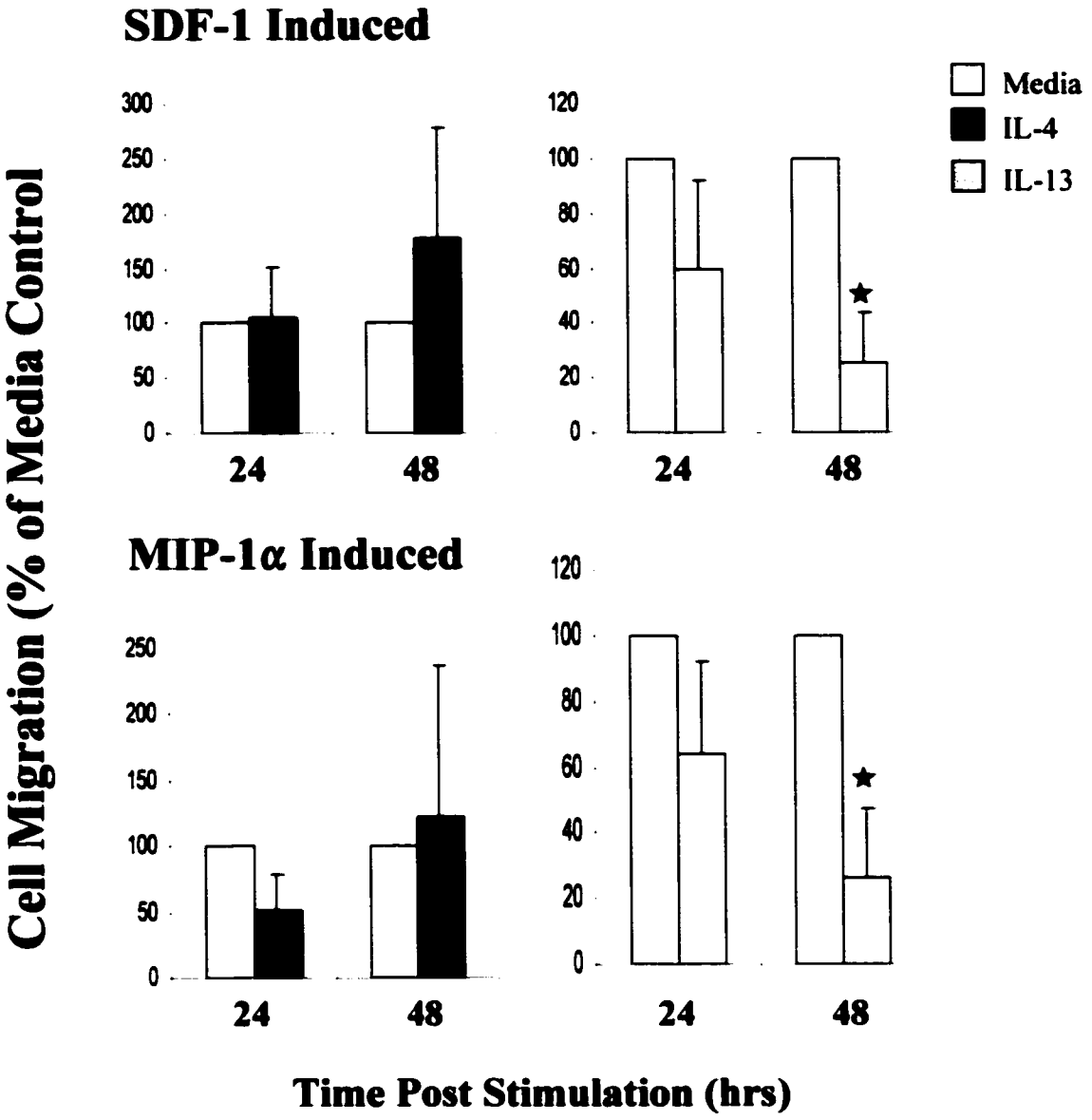
The migration of monocytes from the blood of HIV positive adults towards SDF-1 was not significantly altered by treatment with IL-4 after 24 or 48 hrs. Stimulation of monocytes from HIV positive adults with IL-13 for 24 hrs had no significant impact on SDF-1 induced chemotaxis. However, similar to monocytes from uninfected adults, those from infected individuals also showed a significant decrease in SDF-1 induced chemotaxis after 48 hrs of culture with IL-13, to 52.8 ± 31.3 % ($p = 0.013$) of the media control. Culture of monocytes from HIV positive individuals with IL-4 for 24 and 48 hrs did not change MIP-1α induced chemotaxis. IL-13 caused a significant inhibition of chemotaxis in response to MIP-1α after 48 hrs of culture [41.8 ± 36.6 % ($p = 0.02$) of media control], but this decrease was not present after 24 hrs (Figure 17C).

These results suggest that the decreases in chemokine receptor expression caused by IL-4 and IL-13 stimulation are associated with decreases in chemotaxis induced by their respective chemokine ligands.

Analysis of HIV Entry in Monocytes

To examine the ability of HIV to enter monocytes exhibiting decreases in CXCR4 and CCR5 expression caused by IL-4 and IL-13, PCR was used to detect HIV *gag* DNA. Monocytes were isolated by negative selection from the PBMC of 5 HIV negative individuals, and cultured in the presence and absence of IL-4 or IL-13 for 48 hrs before exposure to dual tropic HIV clinical isolate #204. Results demonstrated that culture of monocytes with IL-4 or IL-13 was unable to decrease the appearance of HIV *gag* DNA in these cells. These results suggest that, despite the ability of IL-4 and IL-13

Figure 17C: The Effects of IL-4 and IL-13 on the Chemotaxis of Monocytes from HIV Positive Adult Blood Induced by the Ligands of CXCR4 and CCR5. PBMC from 6 HIV positive adults were cultured for 24 and 48 hrs in the presence and absence of IL-4 or IL-13, and then subjected to chemotaxis induced by SDF-1 (a ligand for CXCR4), or MIP-1α (a ligand for CCR5). Migrated $CD14^+$ monocytes were enumerated by flow cytometry, and expressed as a percentage of a control cultured in media alone. The average migration percentage of monocytes, standard error, and probability values were calculated by standard methods. Significant values are indicated by a star (★).


SDF-1 Induced
MIP-1α Induced
Media
IL-4
IL-13
Cell Migration (% of Media Control
Time Post Stimulation (hrs)
300
250
200
150
100
50
0
120
100
80
60
40
20
0
24
48

to decrease the expression of CXCR4 and CCR5 on the surface of monocytes, these cytokines were unable to decrease viral entry by a dual tropic HIV isolate. The results of one representative sample is shown (Figure 18).

Analysis of HIV Replication in Monocytes

Since IL-4 and IL-13 decrease chemokine receptor expression and inhibit chemotaxis induced by chemokine ligands, but were unable to inhibit the entry of HIV in monocytes, these cytokines may be inhibiting HIV replication at points after viral entry. Monocytes were isolated from the PBMC of 5 HIV negative adults by negative selection, and incubated in the presence or absence of IL-4 or IL-13 for 48 hrs prior to infection with M-tropic HIV Bal. HIV infected monocytes cultured in only media did not produce detectable amounts of p24 after 5 days of culture, but it increased to an average of 15.0 ± 2.2 pg/ml and 17.0 ± 2.3 pg/ml after 10 and 15 days, respectively. The production of p24 by monocytes cultured with IL-4 and IL-13 was also not detectable 5 days post infection, and was significantly inhibited on subsequent days in comparison unstimulated monocytes (Figure 19).

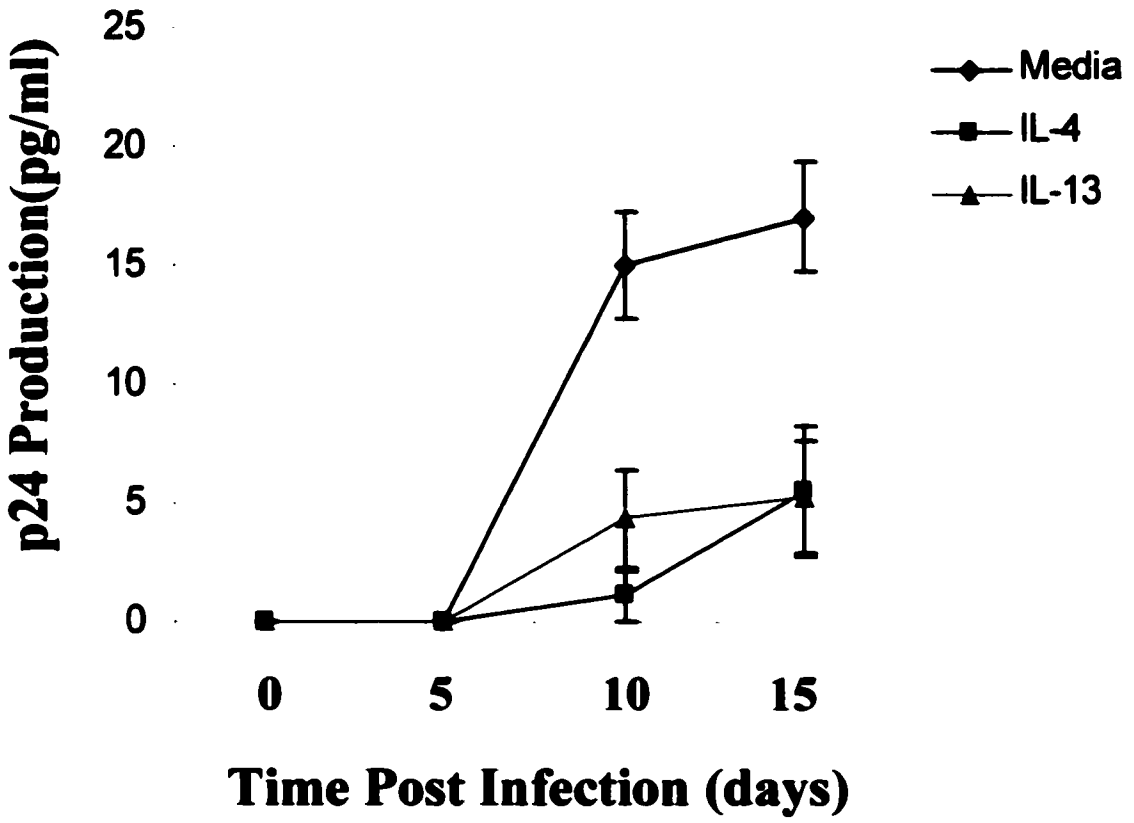
Figure 18: The Effects of IL-4 and IL-13 on HIV Entry in Monocytes. Monocytes were isolated from the PBMC of 5 HIV negative adults by negative selection with antibody coated magnetic beads. These monocytes were incubated with IL-4 or IL-13 for 48 hrs before being incubated with dual tropic HIV Clinical Isolate #204 for 8 hrs. The DNA was extracted from these monocytes, and the HIV *gag* sequence was amplified with specific primers. An equal amount of DNA was also amplified with primers specific for β-actin. Amplified products were run on an agarose gel, and analyzed by densitometry using β-actin as an internal control. One representative sample is shown with the average ratios of HIV *gag* DNA to β-actin. The average ratios of HIV *gag* DNA to β-actin, standard error, and probability values were calculated by standard methods.

β-Actin (610 bp)

HIV *gag* (114 bp)

0.01	1.36	1.49	1.31	Ratio of
± 0.004	± 0.4	± 0.3	± 0.3	HIV *gag*/β-Actin
Uninfected Control	Media	IL-4	IL-13	

Figure 19: The Effect of IL-4 and IL-13 on HIV Replication in Monocytes. Monocytes were isolated from the PBMC of 5 HIV negative adults by negative selection with antibody coated magnetic beads. These monocytes were then incubated in the presence and absence of IL-4 or IL-13 for 48 hrs before being infected with M-tropic HIV Bal for 12 hrs. After infection, monocytes were washed extensively before being cultured for the indicated amounts of time. Supernatants were collected 0, 5, 10, and 15 days after infection and subjected to analysis of p24 production by ELISA. Average p24 production, standard error, and probability values were calculated by standard methods.


25
20
15
10
5
0
p24 Production(pg/ml)
0
5
10
15
Time Post Infection (days)
Media
IL-4
IL-13

Discussion

Monocytes are mononuclear phagocytes that circulate in the blood until they are induced to move into tissues by factors, such as chemokines, that are released at the site of infection or injury. Monocytes migrate to the site of infection or injury, directed by a chemokine gradient within the tissue, where they are induced to differentiate into macrophages. Macrophages are activated by interactions with Th1 cells, and IFN-γ that has been secreted by activated Th1 cells or NK cells. Activated macrophages have increased abilities to produce nitric oxide and oxygen radicals, fuse their lysosomes with phagosomes, and eliminate pathogens (82). Monocytes and macrophages are the primary targets of M-tropic HIV strains which dominate the early stages of infection and are responsible for transmission (17-21). HIV infected monocytes and macrophages do not suffer the same cytopathic effects as T cells, and because of this provide a constant source of virus (17;18;22). The infection of monocytes and macrophages by HIV is mediated by interaction of viral gp120 with CD4 and a chemokine receptor such as CXCR4 or CCR5. Chemokine receptors are subject to regulation by cytokines present in the microenvironment. The alteration of the expression of CXCR4 and CCR5 on monocytes may thus influence the ability of HIV to enter these cells. The impact of alterations in CXCR4 and CCR5 expression on the migration of monocytes, and the role of these chemokine receptors in the infection of monocytes and macrophages with HIV are not well understood.

The objectives of this study were: (1) to investigate the effects of Th1 and Th2 cytokines on the expression of the chemokine receptors CXCR4 and CCR5 on monocytes, and understand the molecular mechanisms of this regulation; and (2) to study the implications of CXCR4 and CCR5 regulation on monocytes by Th1 and Th2

cytokines, with respect to the migration of monocytes in response to chemokines, and their susceptibility to HIV entry and replication. The results of this study demonstrate that Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-13) cytokines, in general, decreased the expression of chemokine receptors on monocytes obtained from HIV negative adults, HIV positive adults, and HIV negative cord blood. Decreases in CXCR4 and CCR5 expression on monocytes, induced by Th1 and Th2 cytokines, were not accompanied by corresponding changes in mRNA expression. Th1 and Th2 cytokines caused the secretion of chemokines, and the observed down regulation of CXCR4 and CCR5 may be due to ligand induced internalization of these chemokine receptors following interaction with endogenously produced chemokines like SDF-1 and RANTES. In addition, decreases in CXCR4 and CCR5 expression were generally associated with an inhibition of chemotaxis induced by their respective chemokine ligands. Th1 and Th2 cytokines, particularly IFN-γ, significantly inhibited the expression of CXCR4 and CCR5, and chemotaxis induced by their respective ligands, but were unable to inhibit entry of HIV in monocytes. However, IL-4, IL-13 and IFN-γ, in contrast to IL-2, were able to inhibit HIV replication in these cells. Taken together, these results suggest that both Th1 and Th2 cytokines inhibited chemokine receptor expression, and these decreases are associated with an inhibition of monocyte chemotaxis induced by their respective chemokine ligands. However, decreases in chemokine receptor expression by Th1 and Th2 cytokines were unable to inhibit HIV entry, indicating that the reduced CXCR4 and CCR5 expression was not sufficient to decrease entry of the virus.

The Role of IL-2 in Chemokine Receptor Expression, Cell Migration, and HIV Infection:

IL-2 is a cytokine that is predominantly secreted by Th0 and Th1 cells, and plays a vital role in T cell responses by promoting the growth and proliferation of these cells (23). This cytokine is also involved in the growth of B cells, and activation of cytotoxic activities of $CD8^+$ T cells and NK cells (83). In addition to its role in T cells, IL-2 has been shown to cause the activation of monocytes (51;52), and the secretion of the chemokine IL-8 from these cells (84). HIV infected individuals have been shown to have defects in their ability to produce Th1 cytokines, like IL-2 (38). This has lead to the use of Th1 cytokines, such as IL-2, as an immunotherapeutic agent, which has been shown to increase the numbers of $CD4^+$ T cells and restore immune function in HIV infected individuals (85). *In vitro* experiments, and studies in HIV infected individuals undergoing IL-2 therapy in the absence of antiviral drugs, have shown that IL-2 increases viral replication (85;86). This increase in HIV replication may be due to the ability of IL-2 to activate T cells, and increase the expression of CCR5 on these cells (38). Despite the use of IL-2 in HIV therapy, the effects of this cytokine on CXCR4 and CCR5 expression by monocytes and the implications of chemokine receptor regulation on monocyte migration and HIV infection are not well understood.

The present investigation demonstrates that IL-2 decreases the expression of CXCR4 on the surface of monocytes from HIV negative adult and cord blood. Although IL-2 has been shown to increase CCR5 expression on lymphocytes, results of this experiment show that IL-2 did not influence CCR5 expression on monocytes. The down regulation of CXCR4 is not due to changes in the expression of mRNA encoding this

chemokine receptor. Stimulation of monocytes with IL-2 may have caused CXCR4 internalization following interaction of CXCR4 with its endogenously produced ligand, SDF-1, which results in this down regulation. However, IL-2 did not influence CCR5 expression, which may be due to the inability of IL-2 to induce RANTES secretion. While the association of chemokine secretion with decreased chemokine receptor expression may explain the observed effect, further investigations are necessary to confirm this proposition.

The decrease in CXCR4 expression by IL-2 was associated with an inhibition of chemotaxis induced by SDF-1. There was some minor variation observed in the effects of IL-2 on chemokine receptor expression and chemotaxis with respect to the kinetics. The effects of IL-2 were generally similar in monocytes from HIV negative adults and HIV negative cord blood, except for the timing of these effects. The decreased chemotaxis observed in cord blood monocytes, relative to that observed in adult blood, may be related to the fact that cord blood cells have a decreased ability to secrete and respond to cytokines (11;87).

CXCR4 expression on monocytes from HIV positive individuals was not significantly decreased by IL-2. The reasons for this loss of response to IL-2 in monocytes from HIV positive blood is not clear. It is likely that HIV infection may result in alterations in the surface expression of the IL-2 receptor, or changes in IL-2 induced signaling. This is supported by recent observations in which IL-2 receptor expression has been shown to be altered on the surface of monocytes, T cells, and B cells obtained from HIV positive blood (83). In addition, $CD8^+$ T cells from HIV infected blood exhibited increased IL-2 receptor expression, but are not responsive to IL-2, suggesting a defect in

IL-2 induced signaling (83). This finding is in agreement with an earlier investigation that found decreased STAT 5 expression in PBMC from infected blood, indicating a defect in IL-2 induced signaling (88). The molecular mechanism for the loss of IL-2 responsiveness in HIV infected individuals is not clear at present. HIV gp120 has also been implicated in alterations of IL-2 receptor expression and its associated signaling (89). It is also possible that antiretroviral therapy may alter IL-2 receptor expression and signaling.

It has been established that PBMC, monocytes, and T cells from HIV positive individuals have a decreased ability to secrete Th1 cytokines such as IFN-γ, IL-2 and IL-12 (4;36;58;61;90;91) and these effects are more pronounced with disease progression (4;36;92;93). In contrast, the secretion of inflammatory cytokines like IL-1α, IL-1β, TNF-α (4;94), and Th2 cytokines like IL-4, IL-10, and IL-13 (4;36;92;93) are increased. These alterations in the cytokine system of HIV positive patients may also contribute to the inability of monocytes from these individuals to respond to IL-2.

IL-2 did not significantly alter the expression of CCR5 on monocytes from HIV negative and positive adults. However, variable results were observed in cord blood monocytes as IL-2 caused a significant decrease in CCR5 expression after 48 hrs. Similarly, this variability was observed in chemotaxis, as IL-2 decreased chemotaxis towards MIP-1α after 24 hrs in HIV negative adult and cord blood monocytes. The reasons for these variable results are not clear, but they may be due to variability in the effects of IL-2 on monocytes from different individuals, or the immaturity of cord blood monocytes. It is also possible that MIP-1α serves as the ligand for an unidentified

chemokine receptor that may be modulated by IL-2, and decreases in the expression of this receptor may be responsible for the decreased chemotaxis.

The IL-2 induced decrease in CXCR4 expression was unable to inhibit the entry of dual tropic HIV into monocytes stimulated with this cytokine. This may be due to the inability of IL-2 to decrease the expression of CCR5 on monocytes. As expected, HIV replication in monocytes was also not affected by IL-2.

The Role of IFN-γ in Chemokine Receptor Expression, Cell Migration, and HIV Infection:

IFN-γ is a pleiotropic cytokine produced by Th0 cells, Th1 cells, $CD8^+$ T cells, and NK cells, and plays a vital role in host defense against pathogens such as bacteria and viruses. The primary biological effects of IFN-γ include the activation of NK cells, the development of Th1 cells from naïve $CD4^+$ T cells (26), and increasing the MHC expression and anti-viral activities of somatic cells (36). IFN-γ has also been shown to activate monocytes and macrophages (55), induce the differentiation of monocytes into macrophages (67), and increase the expression of MHC class I and II molecules on the surface of these cells (67).

The role of IFN-γ in the modulation of HIV infection is well established. IFN-γ has been shown to both augment and inhibit HIV replication by mechanisms that are poorly understood (31). Culture of primary mononuclear phagocytes with IFN-γ prior to infection with HIV was shown to inhibit viral replication, while IFN-γ treatment after HIV infection increased replication of the virus (31;95). Investigation of the effects of IFN-γ on HIV infection have demonstrated the ability of this cytokine to inhibit both

HIV entry (31;34;56;62;64-66), and virus replication in monocytes and macrophages (31;34;53;56;62-66).

IFN-γ may exert anti-HIV effects by inhibiting chemokine receptor expression, thereby inhibiting virus entry in host cells. Recently, the effects of IFN-γ on chemokine receptor expression have been investigated, but results have been controversial. In one study, IFN-γ did not significantly alter the expression of CXCR4 on MDM (34). However, IFN-γ has been shown to decrease CXCR4 expression on monocytes in another report (96). Similarly, controversial results of the effects of IFN-γ on CCR5 expression on monocytes have been reported. IFN-γ has been shown to increase (31;68) and decrease (34) the expression of CCR5 on the surface of monocytes and MDM. Similarly, the effects of IFN-γ on chemotaxis have shown that IFN-γ increases MIP-1α induced chemotaxis of monocytes (31), while another study has shown that this cytokine has effects which result in a general inhibition of monocyte migration (97).

This study investigated the effects of IFN-γ on CXCR4 and CCR5 expression on monocytes, and the implications of this in chemotaxis and HIV infection. Contrary to previously published observations, this study showed that IFN-γ decreased the expression of both CXCR4 and CCR5 on monocytes. These decreases in chemokine receptor expression were not accompanied by corresponding changes in the expression of CXCR4 and CCR5 encoding mRNA, but IFN-γ did cause increased RANTES and SDF-1 secretion that may be responsible for down regulating CXCR4 and CCR5. The down regulation of chemokine receptor expression induced by IFN-γ was ssociated with decreases in the chemotaxis of monocytes induced by their respective ligands.

Differences in the effects of IFN-γ on chemokine receptor expression and chemotaxis observed in this study and those reported by other laboratories are likely to be due to differences in experimental conditions. The cell type under investigation, and the duration of culture, may influence IFN-γ mediated responses. The differentiation of monocytes into MDM is accompanied by alterations in the expression of several surface receptors, including chemokine (14;45) and IFN-γ receptors (98). Macrophages exhibit higher levels of IFN-γ receptor expression compared to monocytes, and this may explain the increased effects of IFN-γ on chemokine receptor expression in these cells (31). The presence of additional cytokines to induce differentiation, or the use of different monocyte isolation methods which result in activation, may also affect the response of these cells to cytokines. For example, the use of gelatin coated flasks for monocyte isolation in one investigation may have resulted in the activation of these cells, and this may be responsible for the elevated levels of CCR5 that were observed in this study (31). High concentrations of IFN-γ used in different studies may also contribute to the inconsistency observed in the effects of this cytokine. For example 50 - 100 ng/ml of IFN-γ, which may exceed physiological concentrations, have been used in some studies (31). Investigations of the effects of cytokines on HIV infection are also influenced by the tropism of the virus in question, as CXCR4 and CCR5 using viruses may be affected differently by cytokines like IFN-γ. A study of the effects of IFN-γ on the replication of HIV in MDM demonstrated that this cytokine increased the replication of T-tropic HIV, while inhibiting the replication of M-tropic HIV in these cells (34). The opposing effects of IFN-γ on these viral strains was attributed to a lack of change in CXCR4 expression, and a decrease in CCR5 expression induced by this cytokine, respectively (34). It has also

been suggested that differences in isolation and culture methods may result in various levels of maturation in MDM, and this may influence the susceptibility of these cells to infection by HIV strains (34).

Similar to results obtained with IL-2, a loss of IFN-γ mediated decreases in chemokine receptor expression, and chemotaxis induced by their respective ligands, was observed in monocytes from HIV positive individuals. Monocytes and macrophages derived from the blood of HIV positive individuals have been shown to have a decreased ability to respond to IFN-γ (91), in addition to decreased functional abilities including super-oxide anion formation (99), phagocytosis of particular antigens (100;101), anti-tumour activity (91), and chemotaxis (102). The reasons for this IFN-γ mediated functional impairment in monocytes from HIV infected individuals are not understood, but may be a consequence of antiretroviral therapy or HIV infection. Alterations in IL-2 receptor expression and IL-2 mediated signaling have been observed in cells obtained from HIV infected individuals (83;89). The possibility of alterations in IFN-γ mediated signaling, and the effects of this on chemokine receptor expression and monocytes chemotaxis, remain to be investigated.

The *in vivo* significance of the observed decreases in chemokine receptor expression induced by IL-2 and IFN-γ, and the associated inhibition of chemotaxis induced by their respective ligands, are not clear. These changes may be a consequence of the activation and differentiation of monocytes, induced by cytokines. The expression of CXCR4 has been shown to be decreased on monocytes as they differentiate (14;45), and both IL-2 and IFN-γ induce the activation and differentiation of these cells. The expression of chemokine receptors, such as CXCR4 and CCR5, may be required

primarily for the migration of monocytes to sites of infection or injury. Once there, monocytes are activated by cytokines like IL-2 and IFN-γ to become effector cells in which chemokine induced migration is less important. This would make these activated monocytes less responsive to chemokines and prevent migration away from the site of infection or injury (103). This may limit damage to surrounding healthy tissue, while allowing monocytes and macrophages to perform their effector functions.

Despite the decreases in the expression of CXCR4 and CCR5 caused by IFN-γ, this cytokine was unable to inhibit entry of dual tropic HIV. This suggests that viral entry requires a threshold amount of chemokine receptor expression, below which HIV entry cannot occur. Despite the significant reductions in CXCR4 and CCR5 expression caused by IFN-γ, this cytokine may have been unable to reduce chemokine receptor expression below this threshold. Previous work on HIV entry has shown that, while completely blocking CD4 expression on MDM with monoclonal antibodies completely reduced M-tropic HIV entry, completely blocking CCR5 by similar methods was able to reduce entry of M-tropic HIV by only 60% (104). This observation has been supported by a recent study which suggests that when CD4 expression is high, maximal levels of viral entry can occur even in the presence of minimal levels of CCR5 expression (105). Decreases in CCR5 expression are only able to significantly affect HIV entry when CD4 expression is very low (105). These observations suggests that other factors, like CD4 expression, may be more important than chemokine receptor expression in determining the susceptibility of monocytes to HIV entry. The inability of IFN-γ to alter the expression of CD4 by monocytes in the present study (data not shown) may be responsible for their susceptibility to HIV entry, although the expression of CXCR4 and CCR5 were

significantly reduced by this cytokine. In this investigation, the lack of a decrease in *gag* DNA likely indicates that viral entry, and subsequent events leading up to the appearance of HIV *gag* DNA, were not inhibited by decreases in chemokine receptor expression.

Given the inability of IFN-γ to inhibit HIV entry, the effects of this cytokine on HIV replication were examined. The results of this study are in agreement with previously published reports which suggested that cytokines may cause a post-entry block which inhibits HIV replication, while still permitting viral entry (106;107). The present investigation revealed that IFN-γ significantly inhibited viral replication, but these results provide no information about the particular points in the viral life cycle, subsequent to entry, that are inhibited by this cytokine.

The Role of IL-4 and IL-13 in Chemokine Receptor Expression, Cell Migration, and HIV Infection:

IL-4 and IL-13 are produced by Th2 cells, and both direct the immune system towards a humoral immune response. IL-4 plays a vital role in Th2 cell proliferation, and directs antibody isotype switching, activation, and proliferation in B cells (23). IL-13 has been shown to have similar effects to those caused by IL-4, as these cytokines have proven to share a component of their respective receptors and some signaling pathways (70). The effects of IL-4 and IL-13 on monocytes and macrophages are poorly understood, but these cytokines have been shown to inhibit their activation (69;71;108), and differentiation from monocytes into macrophages (73;77).

The ability of IL-4 to increase CXCR4 expression by T cells is well documented (19;20;41;42), but the effects of this cytokine on chemokine receptor expression by

monocytes is not well understood. IL-4 has been shown to down regulate CCR5 expression on the surface of MDM (78). However, another investigation shows this cytokine has no effect on CCR5 expression by these cells (19). The effects of IL-13 on CXCR4 and CCR5 expression by monocytes and macrophages are also poorly understood. There is evidence that IL-13 decreases CXCR4 (22) and CCR5 (22;78) expression by MDM.

Little is known about the effects of IL-4 on HIV entry in monocytes, but IL-13 has been shown to decrease the entry of this virus in MDM (22;69;72). IL-4 and IL-13 have both been shown to inhibit HIV replication in monocytes and macrophages (22;69;72;77;78;109), by mechanisms that include decreases in chemokine receptor levels (78), and inhibition of reverse transcription and viral gene expression (72;77). Contrary to this, there is also some indication that these cytokines, particularly IL-4, increase the replication of HIV in these cells (19;47;69;74-77;109), by increasing viral transcription (19;74;76;77;109). There are additional studies which suggests these cytokines increase HIV replication in monocytes, but decrease it in MDM (77), and that high concentrations of IL-4 increase viral replication while lower concentrations inhibit it (109).

This investigation showed that IL-4 had differential effects on CXCR4 and CCR5 expression on monocytes. IL-4 significantly inhibited the expression of CXCR4 on monocytes, whereas CCR5 expression was not affected by this cytokine. In addition, IL-4 and IL-13 exhibited distinct effects on the expression of CCR5 on monocytes. In contrast to IL-4, IL-13 caused a significant decrease in CCR5 expression on monocytes. The down regulation of chemokine receptors by IL-4 and IL-13 did not involve changes in

mRNA expression, but may be due to internalization caused by the induction of RANTES and SDF-1 secretion by these cytokines. As discussed previously, the contributions of other mechanisms to this down regulation cannot be ruled out, and the association of IL-4 and IL-13 induced chemokine secretion with chemokine receptor internalization requires further study. We also show for the first time that IL-4 and IL-13 induced decreases in CXCR4 and CCR5 expression were associated with an inhibition of monocyte migration in response to the respective chemokine ligands of these receptors.

IL-4 and IL-13 decreased the expression of CXCR4 on the surface of monocytes from all sources. The down regulation of CXCR4 expression by IL-4 and IL-13 was generally associated with an inhibition of monocyte migration induced by SDF-1. However, this was not observed in monocytes from HIV positive adults, as the migration of these cells was unaffected by IL-4 stimulation. As previously discussed, this may be due to the high variability seen in the migration of monocytes from HIV positive blood, the disorder of the cytokine system in infected individuals (4;36;92-94), or the functional impairment of these cells observed in previous investigations (91;99-102).

Investigation of the effect of IL-4 pretreatment on HIV entry in monocytes revealed that, although IL-4 inhibited CXCR4 expression and SDF-1 induced chemotaxis, this cytokine was unable to inhibit entry of dual tropic HIV. Since IL-4 decreased CXCR4 expression, but was unable to alter that of CCR5, the use of a T-tropic virus for the investigation of viral entry would have been ideal. However, under the conditions used in this study, unstimulated monocytes were unable to be infected by T-tropic HIV (data not shown). A dual tropic HIV strain was used for the study of viral entry, and IL-4 was unable to inhibit the infection of monocytes by this virus. Despite the

decreases in CXCR4 and CCR5 expression induced by IL-13, this cytokine was also unable to inhibit the entry of dual tropic HIV in monocytes. However, as observed in other investigations, both IL-4 and IL-13 caused a significant inhibition of HIV replication in monocytes, but the steps in the viral life cycle that are inhibited by these cytokines are not clear. As suggested by previous work, these cytokines may be inhibiting steps in the viral life cycle subsequent to entry (106;107)

The physiological role of the decrease in CXCR4 expression induced by IL-4 and IL-13 is not known, but may be related to their role in preventing the activation and differentiation of monocytes (69;71;73;77;108). Under the influence of Th2 cytokines like IL-4 and IL-13, the immune system polarizes itself towards a humoral response in which monocytes and macrophages play a limited role. As a consequence of the development of this humoral response, in which SDF-1 may be used as a signal to attract T and B cells (both of which express high levels of CXCR4) to the site of infection of injury, the chemotaxis of monocytes towards this ligand will be reduced.

Culture of monocytes with IL-4 generally did not cause a significant change in the expression of CCR5, or the migration of monocytes induced by its chemokine ligand MIP-1α. However, IL-4 did cause a significant increase in CCR5 expression by monocytes from HIV negative adult blood after 24 hrs of culture. This increase in CCR5 expression was also associated with an increase in the migration of monocytes induced by MIP-1α at this time point. The reasons for this anomaly are not clear. In contrast to IL-4, IL-13 caused a significant down regulation of CCR5 expression by monocytes from all sources. This decrease in CCR5 expression was associated with an inhibition of monocyte chemotaxis induced by MIP-1α after IL-13 stimulation.

Cytokine induced decreases in chemokine receptor expression have only been observed and investigated *in vitro*, and the occurrence of these changes *in vivo* has yet to be proven. With this in mind, these results suggest that Th1 and Th2 cytokines have similar effects on chemokine receptor expression and chemotaxis, but these may occur for different physiological reasons. Once monocytes have migrated to a site of infection or injury, and are activated by Th1 cytokines like IL-2 and IFN-γ, the decrease in chemokine receptor expression and migration may prevent these cells from damaging healthy tissue. However, these same effects are induced by Th2 cytokines like IL-4 and IL-13, and in this case may be responsible for limiting the migration of monocytes in response to a signal intended for lymphocytes. In the context of HIV infection, the decreases in chemokine receptor expression are unable to inhibit the infection of these cells. However, some Th1 and Th2 cytokines are able to inhibit replication of the virus. Although cytokines are unable to inhibit HIV infection of monocytes, the effects of chemokine receptor regulation in these cells on the emergence of T-tropic viral strains remains to be determined. The loss of the ability of monocytes to migrate in response to chemokines may also suppress the ability of these cells to combat opportunistic infections, in addition to HIV itself. The dysregulation of the cytokine system observed in HIV infection may contribute to the pathogenesis of AIDS by inhibiting the activity and migration of monocytes, while allowing them to be infected and serve as viral reservoirs.

Summary

This study further defines the impact of Th1 and Th2 cytokines on CXCR4 and CCR5 expression by monocytes, and the implications of this regulation on the chemotaxis and HIV infection of these cells. It is clear from the limited amount of conflicting studies that this area requires further investigation before the role of cytokines in these processes is understood. This study demonstrates that IL-2 decreases the expression of CXCR4 on monocytes, but is unable to alter the expression of CCR5. However, IFN-γ was able to significantly decrease the expression of both CXCR4 and CCR5 on the surface of monocytes. Despite their similar effects on T cells, IL-4 and IL-13 had distinct effects on the expression of chemokine receptors on monocytes. IL-4 was unable to alter the expression of CCR5 on the surface of monocytes, but the expression of this chemokine receptor was decreased by IL-13. Both IL-4 and IL-13 decreased the expression of CXCR4 on monocytes. These alterations in chemokine receptor expression were not associated with changes in mRNA expression, but may be due to internalization caused by the secretion of chemokines induced by Th1 and Th2 cytokines. The down regulation of CXCR4 and CCR5 expression is associated with an inhibition of monocyte chemotaxis induced by the respective ligands of these chemokine receptors. Decreases in the expression of CXCR4 and CCR5 induced by Th1 and Th2 cytokines were not sufficient to inhibit the ability of HIV to enter monocytes, although subsequent unknown steps in HIV replication are inhibited by IL-4, IL-13 and IFN-γ.

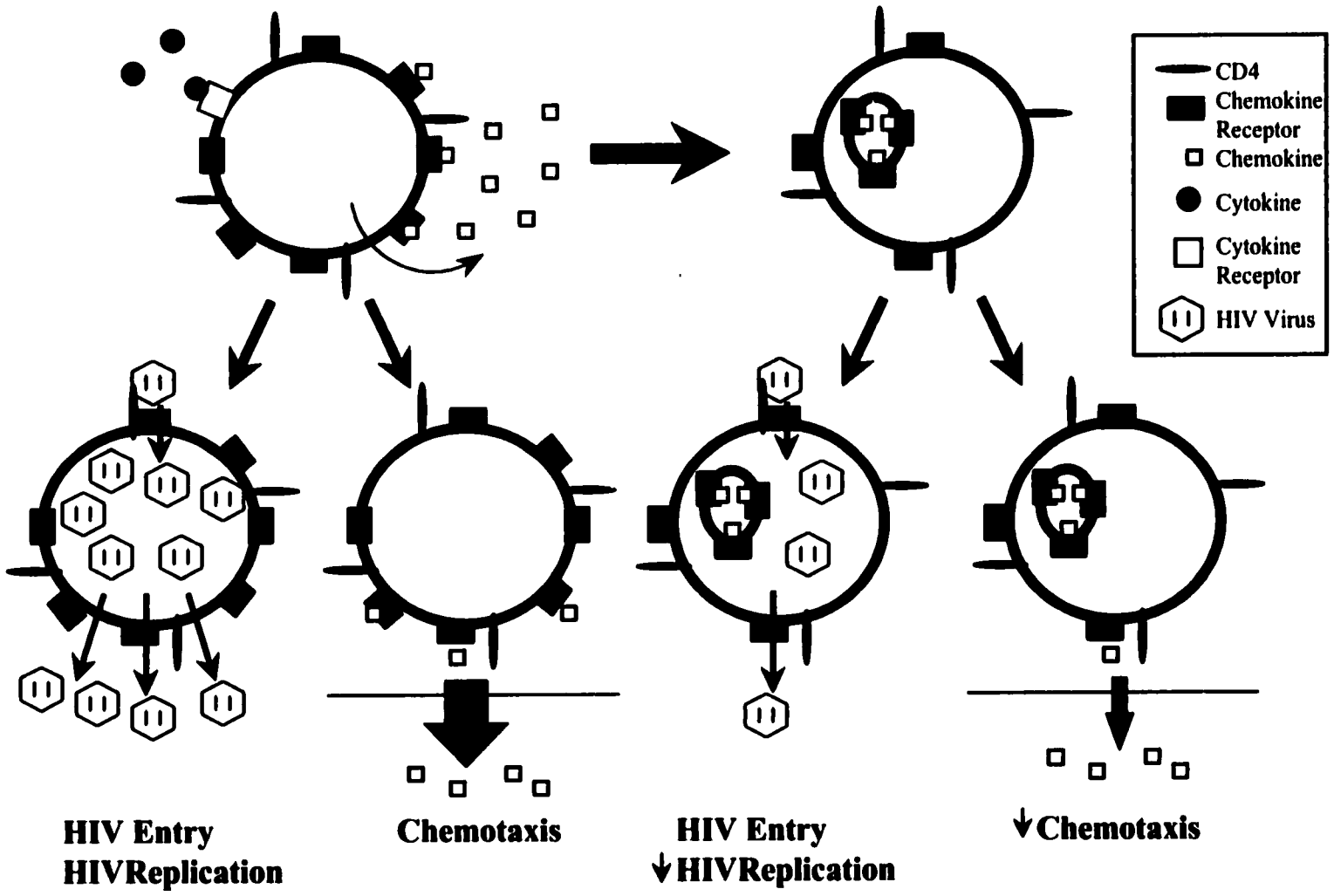
Table 2: Summary of the Effects of the Th1 Cytokines IL-2 and IFN-γ on the Expression of CXCR4 and CCR5 by Monocytes, and the Impact of this on Chemotaxis and HIV Infection.

	IL-2	IFN-γ
CXCR4 Expression	Decreased	Decreased
CXCR4 mRNA Expression	No Effect	No Effect
CXCR4 Ligand Secretion	Increased SDF-1	Increased SDF-1
CXCR4 Ligand Induced Chemotaxis	Decreased	Decreased
CCR5 Expression	No Effect	Decreased
CCR5 mRNA Expression	No Effect	No Effect
CCR5 Ligand Secretion	No Effect	Increased RANTES
CCR5 Ligand Induced Chemotaxis	No Effect	Decreased
Dual Tropic HIV Entry	No Effect	No Effect
M-Tropic HIV Replication	No Effect	Decreased

Table 3: Summary of the Effects of the Th2 Cytokines IL-4 and IL-13 on the Expression of CXCR4 and CCR5 by Monocytes, and the Impact of this on Chemotaxis and HIV Infection.

	IL-4	IL-13
CXCR4 Expression	Decreased	Decreased
CXCR4 mRNA Expression	No Effect	No Effect
CXCR4 Ligand Secretion	Increased SDF-1	Increased SDF-1
CXCR4 Ligand Induced Chemotaxis	Decreased	Decreased
CCR5 Expression	No Effect	Decreased
CCR5 mRNA Expression	No Effect	No Effect
CCR5 Ligand Secretion	No Effect	Increased RANTES
CCR5 Ligand Induced Chemotaxis	No Effect	Decreased
Dual Tropic HIV Entry	No Effect	No Effect
M-Tropic HIV Replication	Decreased	Decreased

Figure 20: Model Depicting the Effects of Cytokines on Chemokine Receptor Expression on Monocytes, and the Implications of these Alterations in Chemotaxis and HIV Infection. Th1 and Th2 cytokines may induce the secretion of chemokines, which bind to their respective chemokine receptors and cause their internalization. The down regulation of chemokine receptor expression results in an inhibition of chemotaxis induced by the chemokine ligands of these receptors. Decreases in chemokine receptor expression were unable to inhibit HIV entry in monocytes, but HIV replication was inhibited by some cytokines at points subsequent to this event.


CD4
Chemokine Receptor
Chemokine
Cytokine
Cytokine Receptor
HIV Virus
HIV Entry
HIVReplication
Chemotaxis
HIV Entry
↓HIVReplication
↓Chemotaxis

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