CHEMOTACTIC PEPTIDES ENCODED BY THE HIV-1 *NEF* GENE

by

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(Under the Direction of Ethan Will Taylor)

ABSTRACT

Preliminary experimental evidence showed the potential for formation of a new isoform of the nef gene in HIV-1 by –1 ribosomal frameshifting. We have named this hypothetical *nef* transframe protein as *nef-fs*. Previous sequence analysis and molecular modeling data strongly support the possibility that this *nef-fs* may encode a chemokine-homologue. To prove this modeling result, we synthesized a series of peptides by solid phase synthesis. These peptides are located about one third of the way into the *nef* coding region, downstream of an internal methonine that is the beginning of a potential signal peptide of *nef-fs*. A calcium mobilizaion assay and a chemotaxis assay were performed in order to test these peptides' chemotactic activity. The calcium mobilizaion assay showed that two of the peptides could induce calcium mobilization in a *Jurkat* cell at certain concentrations which indicated chemotacticity. The chemotaxis assay proved the two peptides can induce chemotactic movement of a *Jurkat* cell with a characteristic bell shape curve. The remaining peptides have little or none of this chemotactic ability. Both of these experiments proved that these peptides encoded by *nef-fs* are chemotactic peptides and support our modeling result that this peptide could be a chemokine

homologue. Our results showed only two out of the five peptides having significant chemotactic ability and the remaining of peptides lack this property although much of the sequences is identical. This suggested that certain conformations maybe required for this *nef-fs* to be chemotactic *in vivo*.

INDEX WORDS: Frameshift; HIV-1; *nef*; *nef-fs*; chemokine; solid phase synthesis; chemotaxis; calcium mobilizaion; synthesize; peptide

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DEDICATION

TO

MY DEAREAST DAD, MOM

AND

WIFE, SON, BROTHER

WITH LOVE

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Chemokines

Chemokines are small polypeptides whose principal function is to recruit leukocytes from the circulation to sites of infection. They are powerful activators; nanomolar concentrations are often sufficient to elicit biological activities. The chemokines fall into four classes, CC, CXC, C, and CX3C as shown in Figure 1.1. The C used in the classification scheme refers to cysteine, which forms structurally important disulfide bonds, two in all classes except C, which contains only one. In the CC family of chemokines an adjacent pair of cysteines is involved in disulfide bridging; one additional amino acid separates the pair in the CXC family, as do three in the CX3C family. The human chemokine genes are located on chromosome 17 and 4 [1, 2]. Although the primary sequences of the chemokines are known, only a handful of three-dimensional structures have been elucidated to date. The CXC chemokines platelet factor 4 (PF4) and interleukin 8 (IL-8), are both monomers comprised of an amino-terminal loop, three antiparallel β strands connected by loops, and a COOH-terminal α-helix [3-5]. Growth related CXC proteins α (GROα) and neutrophil activating peptide-2 (NAP-2) have a similar structure[6, 7]. The structures of two CC chemokines, macrophage inflammatory protein 1β (MIP-1β) and regulated-upon-activation, normal T cell expressed and secreted (RANTES) have also been determined [8]. In this case, the three-dimensional structures of eotaxin-3, Stromal cell-derived

factor 1 (SDF-1) and human lymphotactin have also been recently determined using nuclear magnetic resonance [9-11].

The chemokine receptors are structurally related proteins within the superfamily of receptors that signal through heterotrimeric, GTP-binding, seven-transmembrane-domain (7TM) proteins [12]. Similar to the chemokine classification scheme, the receptors have four subfamilies, CCR and CXCR, which constitute the majority of known receptors, and XCR and CX3CR as shown in figure 1.1. The CCR's all contain a pair of cysteines likely to be involved in structurally important disulfide bridging between the amino terminal domain and the third extracellular loop. They all have two conserved cysteines, one in the third extracellular loop; these two cysteines form a disulfide bond critical for the conformation of the ligand binding site. CC chemokines frequently recognize two or more receptors whereas CXC chemokines usually have high affinity for single receptors (with at least one exception: IL-8 binds both CXCR1 and CXCR2).

Individual chemokines act as ligands for different receptors. For example, human monocyte chemotactic protein-3 (MCP-3) acts as a ligand for CCR1, CCR2, CCR3 and CCR5 [13-16].

The CC chemokine receptors include CCR1 and CCR2, originally designated MIP-1α/RANTES and MCP-1 receptor, respectively, based on their ligands [17]. Now, eleven chemokines are reported to bind to the CCR1 receptor, including MIP-1α (macrophage inflammatory protein 1α), MIP-1β, MIP-1δ, RANTES (regulated on activation normal T cell expressed and secreted), MCP-1 (monocyte chemotactic peptide 1), MCP-2 [18], MCP-3 [19], MCP-4 [20], Lkn-1 (leukotactin-1)[21], MPIF-1 (myeloid progenitor inhibitory factor 1) [20] and HCC-1 (hemofiltrate CC chemokine 1) [20], with varying affinities and acting with different

degrees of agonism. CCR2 is divided into CCR2a and CCR2b, which are RNA-splicing variants. CCR2 also recognize MCP-2 and MCP-3 [12]. CCR3, which is the eotaxin receptor, is prominently expressed in eosinophils and functions in the recruitment of these cells in allergic reactions [22]. CCR4 binds mainly to MIP-1α and RANTES while CCR5 recognizes MIP-1α, RANTES and MIP-1β [23]. CCR6 and CCR7 recognize CCL20 and CCL19/CCL21 [24, 25]. CCR8 is expressed on monocytes and T lymphocytes and is the sole receptor for the human CC chemokine 1 (CCL1, I-309) and for the viral chemokine, vCCL1 (viral macrophage inflammatory protein 1 [vMIP-1]) [26]. CCR9 can recognize CCL25 [27]. CCR10 binds to CCL27 [28].

Among the CXC chemokine receptors for neutrophils, the IL-8 receptor (IL-8R) [29] is expressed as two subtypes: CXCR1 and CXCR2 [12]. CXCR1 recognizes only IL-8, while CXCR2 recognizes all other CXC neutrophil chemokines as well. Immunoflouresence studies have shown that CXCR1 and CXCR2 are expressed on neutrophils and monocytes, and in a small proportion of lymphocytes [12]. While the amino terminal domain determines ligand selectivity, several other extracellular sites function as IL-8 binding and signaling domains [30] as shown in Figure 1.3. CXCR3, which shares 36% homology with the IL-8 receptors, is a new chemokine receptor that recognizes interferon inducible protein 10 (IP10) and monokine induced by interferon γ (Mig) [31]. It is highly expressed in IL-2 activated T lymphocytes (including $CD4^+$ and $CD8^+$ cells), but not in resting T lymphocytes, B lymphocytes monocytes, or granulocytes [32]. CXCR4, formerly known as LESTR and Fusin [33], is now known as the ligand for SDF-1 [34]. It has a wide distribution in tissues including brain, heart, liver and colon. CXCR5 and its ligand CXCL13 regulate compartmentalization of B- and T-cells in secondary lymphoid organs [35]. CXCL16 is a recently discovered chemokine that is expressed in soluble

and transmembrane forms, ligates CXCR6 chemokine receptor, and guides migration of activated Th1 and Tc1 (Cell Mediated Lympholytic-1 cells) cells [36].

Many 7-TM receptor molecules with high similarity to chemokine receptors have been identified, but specific chemokine receptor functions have yet to be defined. These include BLR1 (Burkitt's lymphoma-derived receptor 1), MDR15 (monocyte derived receptor 15), EBI1 (Epstein Barr Virus infected lymphoma) [37], and CMBRL1 (chemokine beta receptor like 1) [38]. Several receptor molecules encoded by viruses that bind to chemokines have also been identified, including US28 of cytomegalovirus [39] and ECRF3 of herpesvirus saimiri [40].

The chemokine/chemokine receptors network forms a complex and sophisticated biochemical signaling system involved in the regulation of leukocyte and lymphocyte trafficking that is necessary for functions of the host inflammatory defense responses, including homing, diapedesis, and disposal of inflammatory cells [12]. Although it was originally thought that the components of this system are distributed on hematopoietic cells, a large amount of evidence that shows that tissue chemokines and tissue chemokine receptors also exist.

Several signaling pathways are involved in the chemokine/chemokine receptor system as shown in Figure 1.4. Activation of Gi/o-family (G protein-coupled) heterotrimeric proteins by CCRs may inhibit or activate ATP cyclase (AC) depending on the relative strength of the Gα and Gβγ-mediated signals, as well as the tissue-specific pattern of expression of receptors, G proteins and AC isozymes [41, 42]. Chemokine/chemokine receptor also can regulate phosphoinositide-specific phospholipase C (PLC) which catalyses the hydrolysis of phosphatidylinositol to inositol-trisphosphates (IP3) and diacylglycerol (DAG) [43]. These second messengers trigger numerous downstream events, including the mobilization of Ca^{2+} from intracellular stores and the activation of protein kinase C (PKC) [44]. Two groups of Ca^{2+} channels, voltage-dependent

Ca^{2+} channels (VDCCs) and second messenger-operated Ca^{2+} channels (SMOCs), are regulated by chemokine/chemokine receptor pathways [45, 46]. Chemokines are also found to regulate phospholiase A2 activity and mitogen-activated-protein-kinase (MAPK) pathways [47].

The activity of chemokine receptors is usually assayed by measuring changes in intracellular Ca^{2+} levels following challenge of agonists [48]. Mobilization of Ca^{2+} from intracellular stores indicates that PLC-β has been activated; although it does not define which G protein family has transduced the chemokine message from receptor to effector. However, increases in intracellular Ca^{2+} levels do not necessarily prove that the PLC/IP3 pathway has triggered Ca^{2+} release from intracellular stores. An alternative explanation would be the chemokine-mediated opening of channels in the outer cell membrane allowing Ca^{2+} flux into the cell. In addition, CC chemokines are able to promote Ca^{2+} fluxes *via* the cyclic adenosine diphosphate-ribose (cADPR)/ryanodine receptor pathway and the sphingosine kinase/sphingosine 1-phosphate cascade [49].

MCP-1 was the first CC chemokine to be discovered. It has been shown to attract monocytes [12]. MCP-2, MCP-3 and MCP-4 were discovered subsequently [50, 51]. MCP-1 today is known to attract monocytes, T lymphocytes and basophilic leukocytes. MCP-2, 3 and 4 also attract eosinophils. All four MCPs are potent attractants for activated T lymphocytes [52-54]. MCPs also enhance target cell lysis by NK cells [55]. Eotaxin, another CC chemokine, has a powerful effect on eosinophils and it is there fore considered to be a most important chemokine in the pathophysiology of allergic conditions including asthma [56]. IP-10, a chemokine induced by Interferon Gamma (IFN-γ) was originally identified expressed in delayed type hypersensitivity reactions of the skin [57, 58]. Another IFN-γ induced chemokine, Mig, was later described [59, 60]. These two chemokines are involved in the regulation of lymphocytic

infiltration observed in autoimmune lesions, delayed-type hypersensitivity responses, some viral infections, and certain tumors. Stromal cell derived factor-1 (SDF-1) was described as the ligand molecule for CXCR4 [34, 61]. It stimulates monocytes, neutrophils and peripheral blood lymphocytes. Mice that have been engineered not to express the SDF-1 gene are not able to survive, indicating that this chemokine may have additional important functions [62].

1.2 Chemokine/chemokine receptors in HIV infection

Human immunodeficiency virus (HIV) has been at the forefront of research focused on immune evasion by RNA viruses. A lot of chemokines are found to be involved in the HIV infections and several chemokine receptors are proved to be critical to HIV. In addition, several HIV encoded proteins are found to be associated with chemokine/chemokine receptor pathways, which benefits viruses' survival and replication.

1.2.1 Chemokine anti-HIV defense mechanisms

In both the innate and adaptive immune responses, the chemokine system mediates amplification of these responses by enhancing immune cells to be cytolytic or producing antiviral factors.

Chemokines can recruit and activate natural killer cells [63]. The natural killer (NK) cells can kill virus-infected cells without the requirement for antigen specificity [64, 65], which is very important for the anti-HIV defense in the early stages of infection. NK cells of HIV-infected individuals have been demonstrated to be an important source of CC-chemokines, such as RANTES and macrophage inflammatory proteins 1α and 1β, that potently suppress replication of R5 strains of HIV [66, 67]. Furthermore, there is an inverse correlation between

the level of plasma viremia and the ability of NK cells and NK cell-derived supernatants to suppress endogenous HIV replication in $CD4^+$ T cells *ex vivo* [68].

Chemokines also can recruit and activate eosinophils [69]. Recent work has provided evidence on the beneficial role of eosinophils in viral clearance. This antiviral activity is mediated at least in part by the eosinophil secretory Rnases [70-72].

Chemokines play important roles in adaptive responses against HIV. They were found to recruit and activate $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells inhibit HIV-1 replication through cytolytic and non-cytolytic pathways [73, 74]. One of the major defense mechanisms of $CD8^+$ T cells is virus-specific cytolysis that eliminates infected cells and thus controls HIV infection [75, 76]. The cytolytic pathways involve calcium-dependent exocytosis involving perforin, granzyme and Fas-mediated programmed cell death [77]. The non-cytolytic pathways involve the production of chemokines that inhibit viral entry [78]. $CD8^+$ T cells are also an important source of chemokines that were shown to inhibit HIV entry into CD4-bearing cells and limit infection both *in vivo* and *in vitro* [78]. Furthermore, chemokines are potent attractants of T cells and NK cells and could, therefore, enhance antiviral effector functions by stimulating cytotoxicity. For example, it has previously been shown that CCL5 enhanced the HIV-specific cytotoxicity of $CD8^+$ T cells. Hadida *et al.* demonstrated that the HIV-specific cytotoxic T lymphocytes (CTLs) derived from peripheral blood mononuclear cells (PBMCs) of HIV-infected patients could kill target cells expressing major HIV proteins, such as Gag, Pol, or Env epitopes, by a mechanism that depends on the Fas-Fas ligand (FasL) pathway, and that CCL5 regulates the expression of FasL in these cells [79, 80]. CCL5 is essential in this process and acts *via* interaction with CCR3 because the expression of FasL and the Fas-FasL killing observed in cytotoxicity assays were markedly decreased by neutralizing Abs against either CCL5 or CCR3. Cell surface expression

of FasL protein in HIV-specific CTLs is also upregulated by CCL11, a ligand specific for CCR3. Together, these findings demonstrate that the action of CCL5 via CCR3 is necessary to regulate FasL expression on HIV-specific $CD8^+$ T cells that kill through the Fas-FasL pathway. Furthermore, Wagner and colleagues showed that granzyme A and chemokines CCL3 and CCL5 are both localized in the cytolytic granules of HIV-1 specific $CD8^+$ cytotoxic cells [81]. It is interesting that the production of chemokines has been associated with cytolytic function. For example, CXCL12, was shown to induce a progressive increase of apoptosis when added to the *Jurkat* $CD4^+$/$CXCR4^+$ T cell line [82]. CXCL12 significantly increased the expression of surface Fas and intracellular FasL. The ability of CXCL12 to induce apoptosis was inhibited by an anti-Fas neutralizing Ab. These findings suggest a role for CXCL12 in the homeostatic control of $CD4^+$ T cell survival or apoptosis mediated by the Fas-FasL pathway.

Many chemokines were found to have anti-HIV activity. Interleukin-8 (IL-8) reduced virus replication in a dose-dependent fashion [83]. Snyderman *et. al* demonstrated that IL-8 led to the cross-phosphorylation and cross-desensitization of both CCR5 and CXCR4. However, IL-8 down-regulated and inhibited HIV-1 infection to CCR5 but not CXCR4 [84]. Since CCR5 is a target for the entry of primary viruses in monocytes these results suggest a selective role for IL-8 in limiting HIV-1 infection through this receptor [85]. Markovitz *et. al.* demonstrates that IL-8 also stimulates HIV-1 replication in macrophages and T lymphocytes. They also found that compounds which inhibit the actions of IL-8 and GRO-α *via* their receptors, CXCR1 and CXCR2, also inhibit HIV-1 replication in both T lymphocytes and macrophages [84].

β Chemokines including RANTES, macrophage inflammatory protein-1α (MIP-1α), and MIP-1β, were shown to induce a dose-dependent inhibition of different strains of HIV-1 and HIV-2 in lymphocytes [78]. They inhibit HIV-1 infection of $CD4^+$ T cells by inhibiting

interactions between the virus and CCR5 receptors. However, while virus entry is inhibited at the moment of infection, in macrophages, the amount of virus produced by RANTES-treated macrophages is similar to the untreated cultures, suggesting an enhanced viral replication. In lymphocytes, exposure to RANTES significantly increases the pool of inhibitory β-chemokines through intracellular signals that result in increased production of MIP-1α and MIP-1β, thereby amplifying the antiviral effects of RANTES. In macrophages this amplification step does not occur. In fact, RANTES added to the macrophages is efficiently cleared from the culture, without inducing synthesis of β-chemokines. Since macrophages serve as a reservoir of HIV-1, this may contribute to the failure of endogenous chemokines to successfully eradicate the virus [86]

Stro cell-derived factor-1 (SDF-1) inhibits HIV-1 infection of $CD4^+$ and some CD4 cells by inhibiting interactions between the virus and CXCR4 receptors [34, 61].

The role of macrophage-derived chemokine (MDC) in the control of HIV infections has been controversial. MDC was originally identified as a CD8 T cell protein product capable of blocking HIV infection by using non-R5 and R5 virus isolates [87]. An immortalized T-cell clone was used, and the molecule with anti-HIV activity lacked the first two amino acids [87]. This original finding has been difficult to reproduce. Also, CD26-processed MDC had a somewhat enhanced anti-HIV activity but it is still relatively modest [88]. The anti-HIV potential of MDC was recently revisited [89]. It was found that MDC inhibits the replication of R5 HIV Bal in monocyte-derived macrophages, but not in T cells, although there was considerable donor-to-donor variability [89]. Interestingly, MDC did not affect the virus entry or reverse transcription but acted at a later post-entry step. In the same study, emphasis was put on the use of carefully controlled MDC preparations to obtain reproducible antiviral activity [89]

because some of the commercially available preparations gave inconsistent results due to the poor quality of the protein.

Monocyte chemotactic protein-2 (MCP-2) could exert its viral inhibitory activity on virus by competitive binding to CCR5[90].

Lymphotactin inhibits HIV by involving mechanisms other than competition for the receptors [90]. However, XCL1 (lymphotactin/single C motif-1alpha/activation-induced, T cell-derived and chemokine-related cytokine) was identified as upregulated by *tat* expression [91]. XCL1 is a C chemokine and plays a specific and important role in tissue-specific recruitment of T lymphocytes that benefits the virus [91].

1.2.2 The role of chemokine receptors in HIV infection

After a long search for a molecule that confers species restriction to HIV infection, several members of the chemokine receptor family have been identified to serve as co-factors or co-receptors for HIV entry into $CD4^+$ and some CD4 cells. CCR5, CCR3 and CCR2b, members of the β-chemokine receptor family, function as co-receptors for M-tropic strains or R5 viruses [92-96]. CXCR4, an CXC-chemokine receptor, acts as a co-receptor for T-tropic strains, or X4 viruses [97]. CCR8, CCR9 or CX3CR1, as well as several orphan receptors (such as GPR1, GPR15/BOB and APJ) have been shown to function as co-receptors for HIV-1 infection [98-104].

CCR5 is a 7TM protein containing four extracellular domains: an amino-terminal domain and three extracellular loops. Although many studies have focused on the interaction of CCR5 with HIV-1 120 kD glycoprotein (gp120), the corresponding interaction sites in CCR5 and gp120 have not been characterized. Identified receptor chimeras and point mutants have been

studied to identify regions of CCR5 used by different viral. GP120-CCR5 interactions are conformationally complex involving residues in all four extracellular domains of CCR5 [105]. The N-terminal domain of CCR5 is particularly important due to its ability to confer co-receptor function to a variety of other chemokine receptors [106]. It has been shown that a 32 base pair deletion in the human CCR5 gene (CCR5 Δ32) causes a frame shift at amino acid 185 and produces a mutant protein which is severely truncated and is not expressed on the cell surface [107-109]. Selection against this allele must be almost absent since people who have inherited two copies of the mutant CCR5 gene do not appear to have any observable deleterious phenotype yet they are remarkably resistant to infection by HIV-1 [107-109]. The high prevalence of the mutant CCR5 Δ32 allele, approximately 20% in some populations, has fueled speculation that it might have become fixed as the result of selective pressure brought about by exposure to an unknown pathogen that utilized CCR5 at some time in the past [108, 109]. CCR5 Δ32 homozygotes have no known health problem and have been shown in several studies to have delayed progression of acquired immunodeficiency syndrome (AIDS), lower viral load, delayed HIV-1 replication in vitro, and a selective pressure for the syncytium syncytium-syncytium inducing HIV 1 phenotype [110].

Late in 1995 Cocchi *et al*. showed that the chemokines MIP-1α, MIP-1β and RANTES could inhibit HIV in vitro [78]. However, these chemokines only blocked viral infectivity mediated by macrophage-tropic (M-tropic) viruses. They did not block viral infectivity mediated by T-cell tropic (T-tropic) strains of virus. M-tropic HIV-1 strains are responsible for virus transmission and are the prevalent virus type presented in early infections [111-113]. Typically, after years of infection T-tropic virus strains emerge that are better at infecting certain CD4+ cells. The emergence of T-tropic virus strains in infected individuals correlates with accelerated

disease progression [114, 115]. Shortly after the discovery that certain chemokines exhibited anti-viral properties, Feng et al. demonstrated that an orphan receptor, then known as LESTR and now known as CXCR4, was able to act as the co-receptor required for cellular entry by T-tropic but not M-tropic strains of HIV-1 [116]. CXCR4, originally named as LESTR, is an HIV-1 co-receptor required for cellular entry by T-tropic but not M-tropic strains of HIV-1. It is ubiquitously expressed on a wide variety of cell types including most hematopoietic cell types [117]. It is also expressed at high levels on vascular endothelial cells [118], neurons [119], and microglia and astrocytes [120]. Richard Horuk showed direct CD4-independent association of the gp120 with the CXCR4. In contrast to CCR5, the N-terminal domain of CXCR4 is considerably less important for co-receptor function. Rather, the first and second extracellular loops of CXCR4, especially the second extracellular loop, are crucial determinants for all viral strains. In addition, studies with a human neuronal cell line hNT show that gp120 can cause apoptosis of neurons mediated via the chemokine co-receptor CXCR4 [121].

CCR3 was shown by several groups to be a HIV-1 co-receptor [93, 95] and it is expressed on microglial cells of the brain [120] where it could potentially promote infection of the CNS by HIV-1, contributing to diseases like AIDS dementia. In addition, CCR3 has also been shown to be expressed on dendritic cells [122] and could play a role there in HIV-1 infection. Kaposi's sarcoma-associated herpesvirus encodes two chemokine-like proteins (vMIP-I and vMIP-II). vMIP-II was shown to block infection of HIV-1 on a CD4-positive cell line expressing CCR3 and to a lesser extent on one expressing CCR5, whereas both vMIP-I and vMIP-II partially inhibited HIV infection of peripheral blood mononuclear cells [123]. Like eotaxin, vMIP-II activated and chemoattracted human eosinophils via CCR3.

It has been reported that growth of both M and T-tropic strains of HIV-1 in activated T cells could be enhanced by the CCR7 chemokine secondary lymphoid-tissue chemokine SLC [124]. This enhancing effect of SLC was inhibited by pretreatment of cells with pertussis toxin, suggesting that this inhibition involves signaling via a receptor coupled with a G-alpha class of G-protein. In addition, SLC was also found to enhance the promoter activity of HIV-1 LTR. These results indicate that signaling via CCR7 has a strong positive effect on HIV growth [124]. Thus, SLC may contribute to persistent infection of HIV in the secondary lymphoid tissues by promoting viral replication in activated T cells.

CX3CR1, previously named V28 which was shown previously to be expressed in neutrophils, monocytes, T lymphocytes, and several solid organs, including brain [125], has also been shown to function as an HIV-1 co-receptor.

1.3. HIV encoded chemokine/chemokine receptor mimics and chemokine-binding proteins

Gp120 is a glycoprotein located on the viral membrane surface. HIV-1 infection requires initial interactions between the viral envelope surface glycoprotein gp120, the cell-surface protein CD4, and a chemokine receptor CCR5 or CXCR4 as shown in Figure 1.5. HIV-1 gp120 has been shown to activate signaling pathways through the chemokine receptors in several cell types including lymphocytes, neurons, and astrocytes [126]. This may cause cellular injury in some cell types [127, 128]. These responses include K^+, Cl^-, and nonselective cation currents, intracellular Ca^{2+} increases, and activation of several kinases including the focal adhesion-related tyrosine kinase Pyk2, mitogen-activated protein kinases (MAPK), and phosphoinositol-3 kinase [126, 129-132]. Activation of the MAPK leads to gp120-induced expression of chemokines such as monocyte chemoattractant protein-1 and macrophage-inflammatory protein-1β and the

proinflammatory cytokine tumor necrosis factor α [133]. These responses establish a complex cytokine network, which may enhance or suppress HIV-1 replication. In addition, dysregulation of macrophage function by gp120/chemokine receptor signaling may contribute to local inflammation and injury and further recruit additional inflammatory and/or target cells. Although engagement of CD4 and a chemokine receptor by gp120 is the initial step required for entry and infection, this interaction can also occur apart from infection as a result of defective virions or soluble gp120 shed from virus particles or infected cells. Recent studies demonstrate that gp120 can activate intracellular signals in multiple cell types, which may provide an additional mechanism of pathogenesis along with direct infection. In some CD4-negative cell types, gp120 has been shown to activate signals by binding to the chemokine receptor directly [132, 133], although high-affinity, efficient gp120–chemokine interactions typically require that gp120 first binds to CD4 to undergo conformational changes that induce the chemokine receptor binding site [134]. GP120-triggered signaling has been extensively explored in $CD4^+$ T lymphocytes where it has been implicated in causing dysfunction and apoptosis [127, 128]. In $CD4^+$ T cells many gp120-elicited responses are activated through CD4, which is principally linked to the Src kinase $p56^{lck}$, so it is important to distinguish signals mediated by the chemokine receptors apart from those activated through CD4. Primary macrophages express CD4 and chemokine receptors but lack $p56^{lck}$ [128].

41 kD Glycoprotein (Gp41) is a 345-amino acid protein located on the viral membrane. There is no agreement on the exact mechanism by which Gp41 (or other viral proteins) accomplish membrane fusion. The present view is that they first contact the target cell membrane by their amino-terminal hydrophobic domains, termed fusion peptides, and then undergo conformation changes in order to bring the viral and cellular lipid bilayers in proximity,

allow their external leaflets to merge, thereby forming a semifusion intermediate as shown in Figure 1.5. Next, an aqueous connection, a fusion pore, must open across the internal leaflets of the merged membranes and expand in order to maintain an open passage to the nucleocapsid [135-137]. Gp41 consists of an amino-terminal extracellular domain (or ectodomain), a membrane-spanning domain, and a carboxy-terminal cytoplasmic (or intraviral) domain. Mutations in the cytoplasmic domain can modify the efficiency of membrane fusion in either a positive or a negative way, but the protein still can continue to function despite the cytoplasmic domain's complete deletion, at least in syncytium-formation assays [138, 139]. When the gp41 ectodomain is anchored by the membrane-spanning domain of an unrelated protein, such as CD4, fusion activity is also observed [140]. Therefore, only the ectodomain of GP41 seems to have a direct role in the membrane fusion process. Like other viral fusogenic proteins, such as the influenza virus HA2 [141], the Ebola virus GP2 [142], or the paramyxovirus F protein [143], retroviral ectodomains are characterized by the presence of two α-helix regions with propensity to form coiled coils that are called heptad repeats [136]. In the case of HIV-1 gp41, synthetic peptides corresponding to the amino-terminal (HR1) and carboxy-terminal (HR2) heptad repeats associate in solution to yield a tight protease-resistant 3:3 complex [144]. X-ray crystallography revealed a six-helix bundle structure with a central coiled coil formed by three parallel HR1s against which three HR2s are packed in antiparallel orientation [145-147]. A similar structure was observed by nuclear magnetic resonance or X-ray crystallography in the ectodomain of other retroviral transmembrane proteins, the simian immunodeficiency virus (SIV) gp41 [148, 149], the human T-lymphotropic virus type 1 gp21 [150], and the Moloney murine leukemia virus p15E [151]. In all retroviruses, the two helices are separated by a region of variable length with a highly characteristic Cys-$(X)_{5-7}$-Cys motif, in which the two Cys residues are engaged in a

disulfide link [152]. This loop region also allows reversal of the chain orientation necessary for the packing of the HR2 helices on the central coiled coil. The two helices and the intervening loop region can therefore be envisioned as forming a hairpin, with the amino-terminal fusion peptide pointing toward the viral membrane and the disulfide-bonded Cys motif oriented toward the SU-a finding which is in agreement with the role of several residues of the loop region in the stability of the SU/TM complex [153, 154]. The loop region of gp41 must be accessible at some stage, since it corresponds to an immunodominant B epitope in HIV-1 and other lentiviruses [155, 156]. These results confirm the role of the gp41 ectodomain in the late steps of the membrane fusion process. *Tat* protein, the transactivating factor of the human immunodeficiency virus type 1 (HIV-1), is a small cationic polypeptide that can be released from HIV-1 infected cells. Released from *tat* expressing cells into the extracellular milieu through a non-canonic, Golgi-independent secretion pathway, extracellular Tat binds to specific receptors on the cell membrane and triggers different signal transduction pathways. *Tat* interacts with β-chemokine receptors CCR2 and CCR3 and acts as a potent chemoattractant for various leukocytes [157-160]; binds chemokine receptor CXCR4 and competes with X4-HIV-1 virus infection on T-cells [161, 162]

HIV negative factor (*Nef*), a 206-amino acid, N-terminally myristoylated protein, but not *tat*, gp120, and gp160, provoked leukocyte recruitment into the CNS in a rat model [163]. These suggest a mechanism by which HIV-1 *nef* protein may be essential for AIDS neuropathogenesis, as a mediator of the recruitment of leukocytes that may serve as vehicles of the virus and perpetrators for disease through their production of neurotoxins. The strong reduction of bioactivity by heat treatment of *nef* and the blocking effect of the monoclone antibody (mAb) 2H12, which recognizes the carboxy-terminal amino acid (aa) residues 171–190 (but not of mAb

3E6, an anti-*nef* Ab of the same isotype, which maps the aa sequence 168–175, as well as a mixture of mAbs to CD4) provided evidence for the specificity of the observed *nef* effects [163]. Using a modified Boyden chamber technique, *nef* exhibited chemotactic activity on mononuclear cells in vitro. Coadministration of the anti-*nef* mAb 2H12 as well as heat treatment of *nef* inhibited *nef*-induced chemotaxis. Besides soluble *nef*, chemotaxis was also induced by a *nef*-expressing human astrocytoma cell line but not by control cells. These data suggest a direct chemotactic activity of soluble *nef* [164-167].

1.4. Anti-HIV therapy based on chemokine co-receptors

A total of 20 drugs have been approved by FDA for the treatment of HIV-1 infection. Nineteen of them fall into three major therapeutic classes: the nucleoside (and nucleotide) analog RT inhibitors (NRTIs), the nonnucleoside RT inhibitors (NNRTIs), and the protease inhibitors (PIs). The emergence of viral resistance to one drug frequently results in cross-resistance to other because of their similar chemical structures and mechanisms of action. The development of viral resistance requires new and effective drugs that will be convenient, well-tolerated, and capable of suppressing viruses that are resistant to existing drugs with new mechanisms other than the three major classes. On the basis of these facts, efforts have been focused on the development of antagonists of CCR5 and CXCR4, as well as GP120 and GP41 inhibitor as antiviral agents.

Several steps are involved in the process of Env mediated entry of HIV-1 proceeds as shown in Figure 1.6. Each step represents a potential target for inhibition by novel drugs. In a first step, HIV-1 binds to the cell through interactions with non-specific attachment factors such as dendritic cell-specific intercellular adhesion molecule (ICAM)-grabbing nonintegrin (DC-

SIGN) [168, 169]. These interactions may function to not only concentrate virus at the cell surface, but also increase the infectivity of the bound particles and constitute a potential target for topical microbicides. Next, high affinity interactions with CD4 result in massive conformational changes within gp120 that result in the formation and exposure of a co-receptor binding site and allows for subsequent interactions with a member of the chemokine receptor family, typically CCR5 or CXCR4. Both of these critical binding events have been targeted by new generations of inhibitors. Neutralizing antibodies and soluble forms of receptors have been used to target the CD4-Env interaction. Myriad small molecules that antagonize the utilization of chemokine receptors by HIV-1 have also been developed and are being evaluated clinically. The interaction of Env with a receptor also triggers conformational changes in the gp41 that results in the formation of a highly stable six-helix bundle coincident with membrane fusion [170, 171]. Targeting intermediates of this process using peptides derived from gp14, such as T20, has been demonstrated to be highly effective at blocking fusion both *in vitro* and in humans

The first reported non-peptidyl, small-molecule, antagonist of CCR5 is TAK-779 [172]. This compound binds to CCR5 with an IC_{50} of 1 nM, as measured by competition against RANTES, and to CCR2 with an IC_{50} of 27 nM. It has little affinity for other chemokine receptors, which means good selectivity. More importantly, it inhibits fusion and viral entry with IC_{50} of 2–100 nM depending on the assay used. Although poor oral bioavailability is still a problem to overcome in clinical use, TAK-779 has proved that small antagonists of CCR5 can have antiviral activity. A second CCR5 antagonist, Schering C (structure undisclosed) [173], has an IC_{50} for the CCR5 receptor of 1 nM, appears to be a more potent antiviral than TAK-779, is orally bioavailable, and should enter phase I clinical trials during the second half of 2000. Additional CCR5 antagonists described by Merck include the 4-(carbamoyl)-piperidines, the

most potent of which have affinities for the receptor of <1 nM and moderate antiviral activities with IC_{95}s of 100–200 nM as measured in peripheral blood mononuclear cell spread assays [174].

The bicyclams are a series of highly potent and selective inhibitors of X4-tropic HIV-1 replication and are now known to be CXCR4 antagonists [175]. The most efficacious of these is AMD 3100, an antagonist of replication with IC_{50} of 2–50 nM against a variety of X4 strains. This is currently in clinical trials [176], however, the clinical trials showed that the compound had no oral bioavailability, which will present a problem for clinical use. A variety of peptide antagonists of CXCR4 have been described, the most potent of which is the 18 amino acid cationic peptide T22 ([Tyr5,12,Lys7]-polyphemusin II), and its analogs T134 and T140 all of which have IC_{50} of less than 10 nM in antiviral assays [177]. Studies with blocking CXCR4 monoclonal antibodies suggest that these peptides bind to the amino terminus and two of the extracellular loops of CXCR4 [178]. A serious concern for the development of any CXCR4 antagonist, however, is the possibility of mechanism-based toxicity suggested by the results of *in vivo* studies. Mice were lethally irradiated and then reconstituted with hematopoietic cells from mice lacking CXCR4. These studies showed that continued presence of CXCR4 is required for normal hematopoiesis in adult mice [179-181], raising the possibility that long-term antagonism of CXCR4 could result in impairment of hematopoiesis.

An alternative strategy to inhibit HIV-1 is to reduce surface expression levels of HIV-1 co-receptors. In fact, the antiviral activity of the chemokines themselves is related in part to their ability rapidly to downregulate surface expression of their cognate receptors [182]. Two recent communications have taken this principle of cell-surface-receptor depletion a logical step forward and devised a way to trap the HIV-1 co-receptors CXCR4 and CCR5 in the endoplasmic

reticulum (ER), thus preventing their transport to the cell surface [175, 183]. The tetrapeptide sequence KDEL, which is an ER retrieval sequence[184], was engineered onto the C-terminal end of the CC chemokines MIP-1α and RANTES, and the CXC chemokine SDF-1. These intracellularly retained chemokines or "intrakines" were then able to prevent the surface expression of newly synthesized CCR5 or CXCR4, probably by forming intracellular complexes. The lymphocytes transduced to express the intracellular chemokine were found to be viable and resistant to HIV-1 infection.

PRO542, first described by Maddon and colleagues [185] and developed by Progenics, Inc., is tetravalent, i.e., it contains four gp120 binding sites. PRO542 blocks replication of numerous HIV-1 isolates in cell culture with typical IC_{90} values of 25 μg/ml or less, although some isolates are resistant [186, 187]. With two completed clinical trials, PRO542 has been proved to be able to achieve sustained levels of PRO542 in people and that such sustained levels can exert an antiviral effect. These findings open the door for further clinical use.

GP41 inhibitors are also a focus for AIDS drug discovery. At first, synthetic peptides overlapping the two heptad repeat regions HRI [e.g., DP-107] and HRII [e.g., DP-178] were found to block HIV-1 replication in cell culture [188, 189]. In particular, DP-178 had remarkable potency, with an IC_{90} of about 0.3 nM [189]. The eventual understanding that these two regions of gp41 play an important role in viral fusion and entry provided an attractive model to explain the antiviral activity of the synthetic peptides [190]. The discovery of an extremely potent inhibitor that blocks HIV-1 replication by a novel mechanism raised the possibility that DP-178 itself might be clinically useful. As a relatively large (36 amino acids) synthetic peptide, however, DP-178 has had problem particularly regarding manufacturing and formulation. Despite these challenges, Trimeris Inc and its partner, F. Hoffmann-La Roche Inc, have

developed this agent, now called T-20, into a FDA approved therapeutic agent for HIV-1 infection. The second-generation gp41 peptide inhibitor, T-1249, is a 39-mer representing a hybrid of sequences from HIV-1 and HIV type 2 that overlaps the T-20 region and contains a pharmacokinetic-enhancing domain present in T-20. The peptide is reportedly more potent than T-20 *in vitro* and is active against T-20-resistant HIV-1 strains. Results of a phase I/II trial of T-1249, conducted in HIV-1-infected subjects, showed that T-1249 can mediate a decrease of virus levels in patients following twice-daily subcutaneous administration[191]. Expanded clinical trials are ongoing.

The connection between HIV and the chemokine system also have critical implications both for the evaluation of vaccine efficacy and for the development of more effective vaccination protocols.

An increasing body of evidence supports the concept that chemokine levels measured *in vivo* or *ex vivo* provide accurate correlates of protection elicited by anti-SIV vaccines in non-human primate models. This idea has been pioneered by Lehner et al.[192, 193], who documented an increased production of RANTES, MIP-1α and -1β by lymph-node $CD8^+$ T cells in macaques immunized by the targeted-iliac-lymph-node route. Despite the lack of universal consensus due to the wide variety of experimental conditions used, other reports have subsequently confirmed the original observation[194]. In case of HIV, chemokines have been widely used to enhance the vaccine efficiency[195].

A second potential implication for vaccines regards a "transdisease" principle that may have a broad impact on the vaccinology field. It stems from the concept that chemokines, by virtue of their unique immunomodulatory properties, may represent a new class of "intelligent" adjuvants for vaccines, capable of finely tuning protective immune responses by recruiting and

activating specific cells at the site of immunization[196]. In particular, it is emerging that chemokines play a pivotal role in the Th1/Th2 polarization of $CD4^+$ and $CD8^+$ T cell cytokine-secretion patterns, which is increasingly recognized as an important determinant of vaccine efficacy. For example, protective responses against viruses are believed to require Th1 polarization which is linked to IFN-γ production and vigorous CTL induction. Preliminary results from indicate that the CC chemokine RANTES is able to induce IFN-γ secretion by human cord blood naive T cells while suppressing IL-4 production (Smith et al., unpublished data). Thus, a single chemokine has a skewing capability on the cytokine-secretion program. If validated *in vivo*, this concept may permit the development of novel vaccine adjuvants with diverse immunomodulatory specificity for HIV[197].

1.5. Conclusions

The close relationship between chemokine and HIV promote rapid advances in both during the past few years. Although different chemokine/chemokine receptors involved in HIV infection explains viral tropism and some of the HIV-1 pathogenesis, a lot of questions remain unanswered. For example, what is the co-receptor in $CD4^-$ cells for HIV entry? How does the availability of chemokine receptors in different cells and the pattern of use of the various co-receptors by HIV strains influence HIV infection? What are the implications of virus-induced receptor signaling?

Addressing these questions is likely to provide new insights and new therapies that will enable us to better understand and to combat HIV-1.

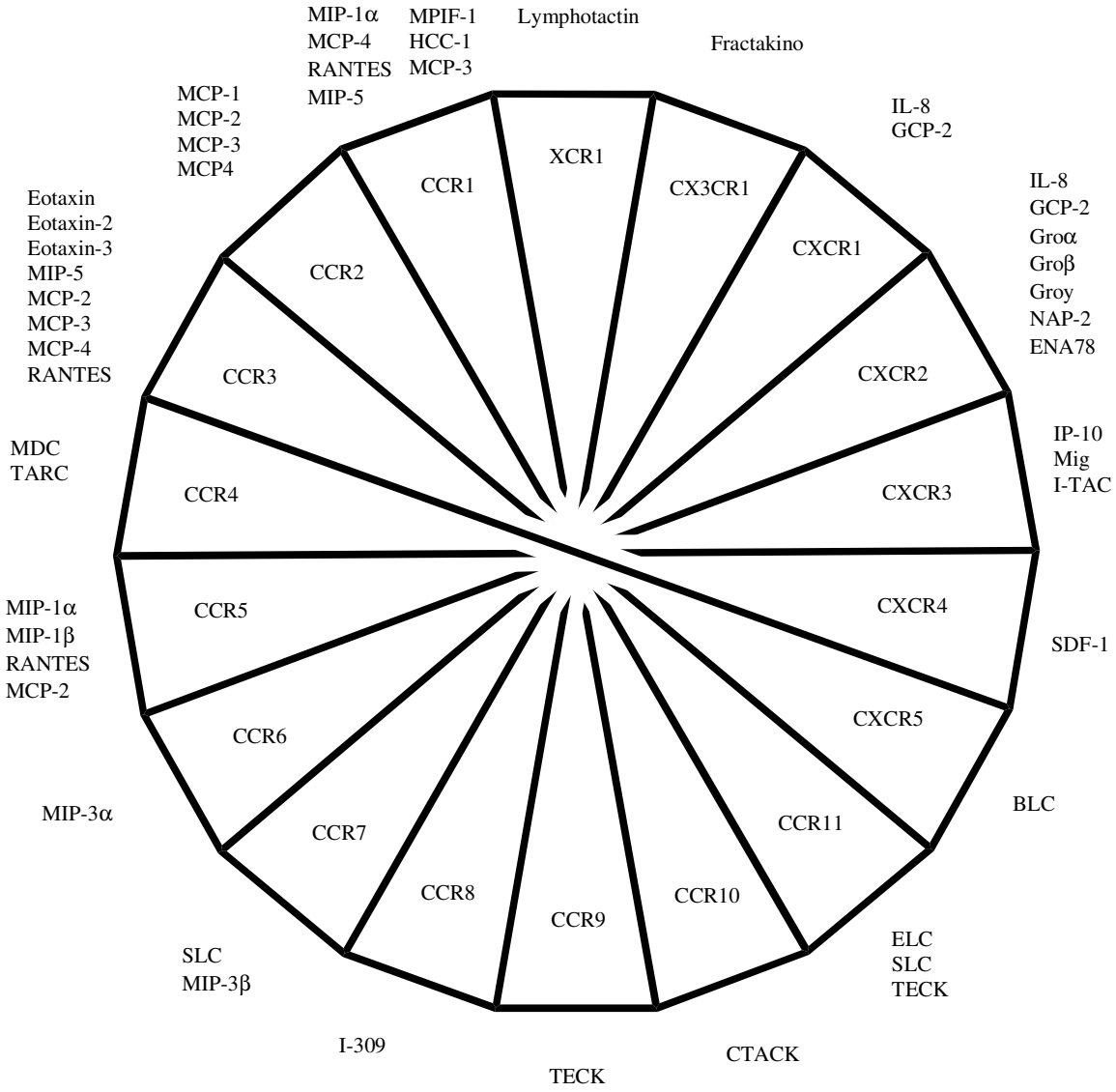


Figure 1.1 The chemokine/chemokine-receptor family.

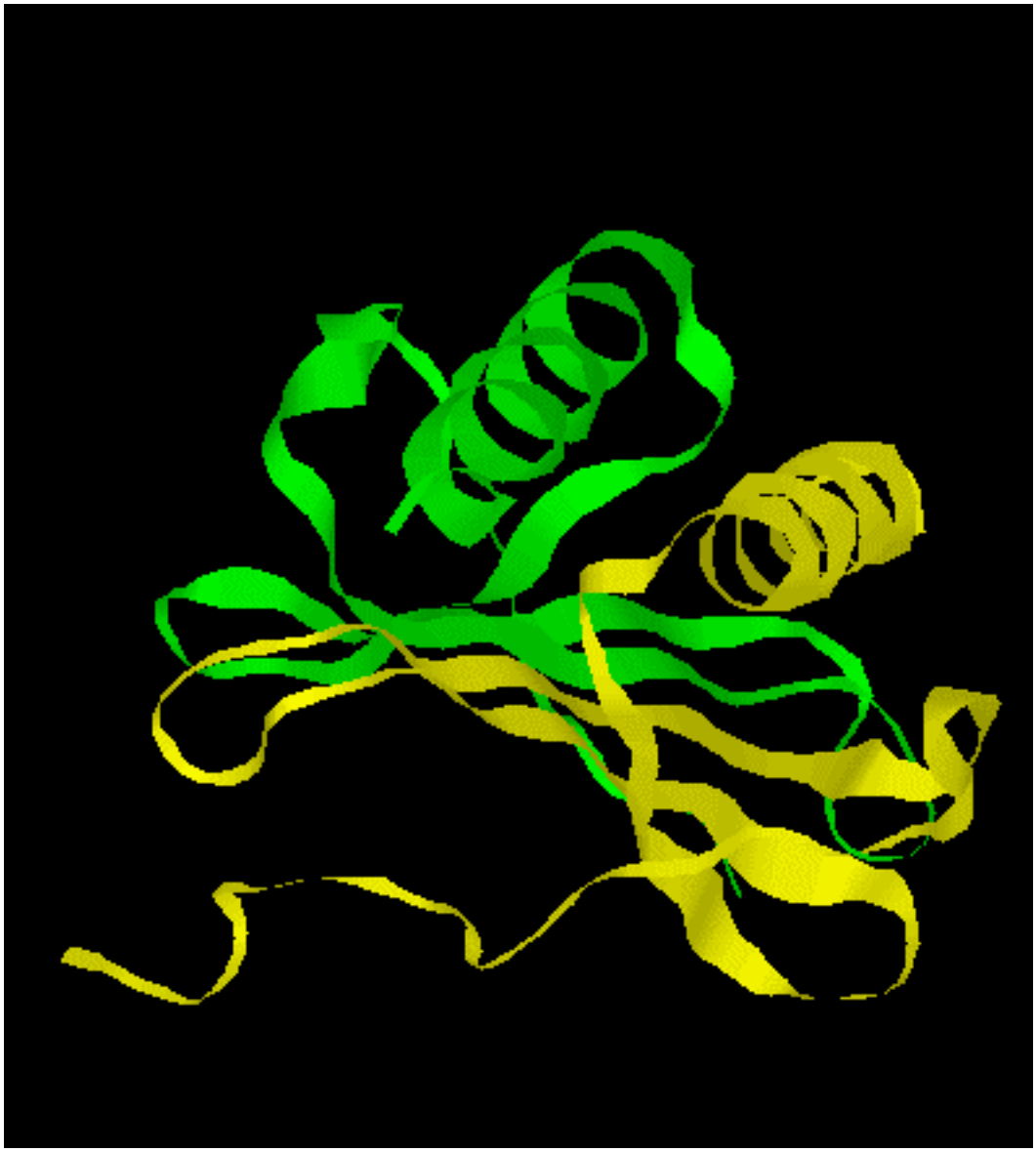


Figure 1.2 Three-dimensional structure of IL-8

Figure 1.3 CXCR1 binding IL-8.

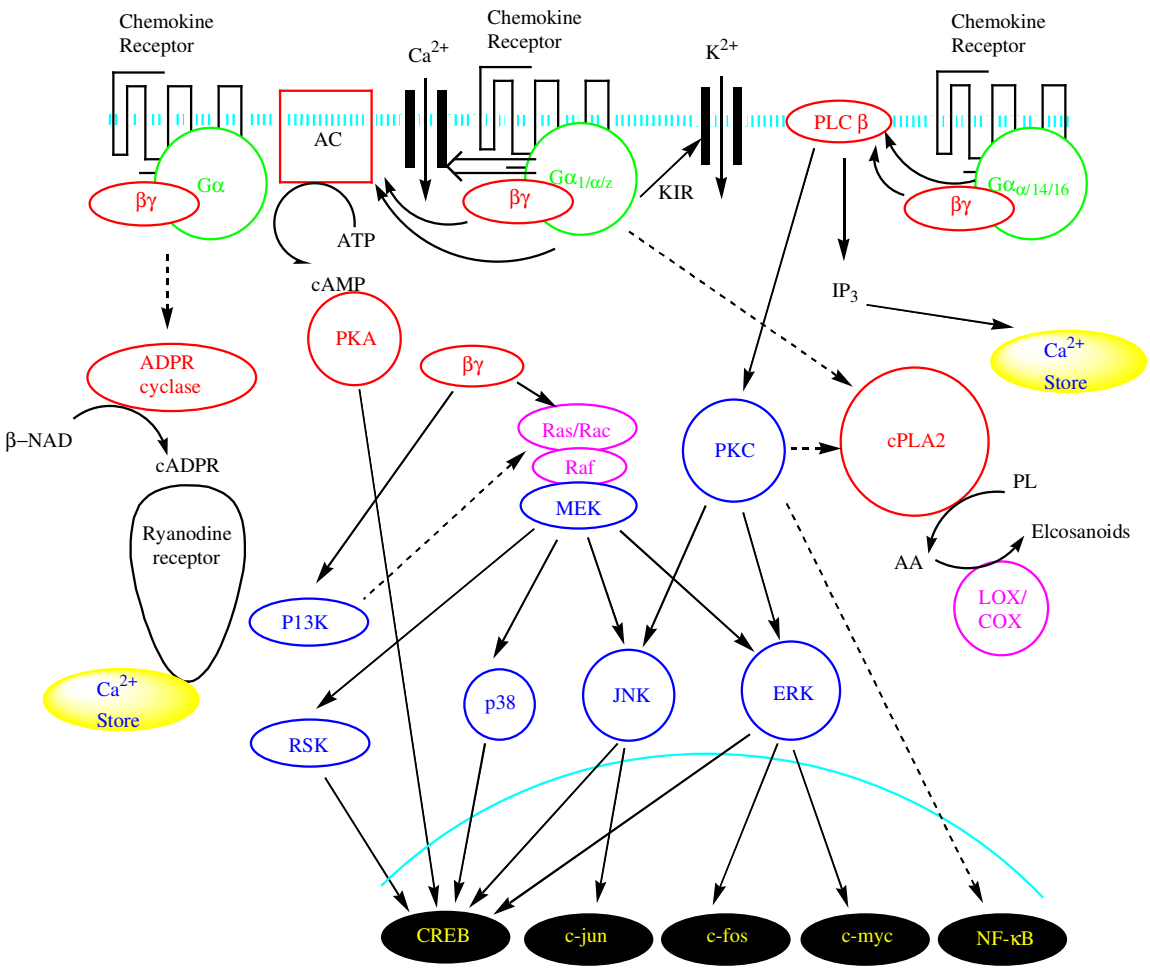


Figure 1.4 Intracellular signaling pathways activated by chemokine receptors.

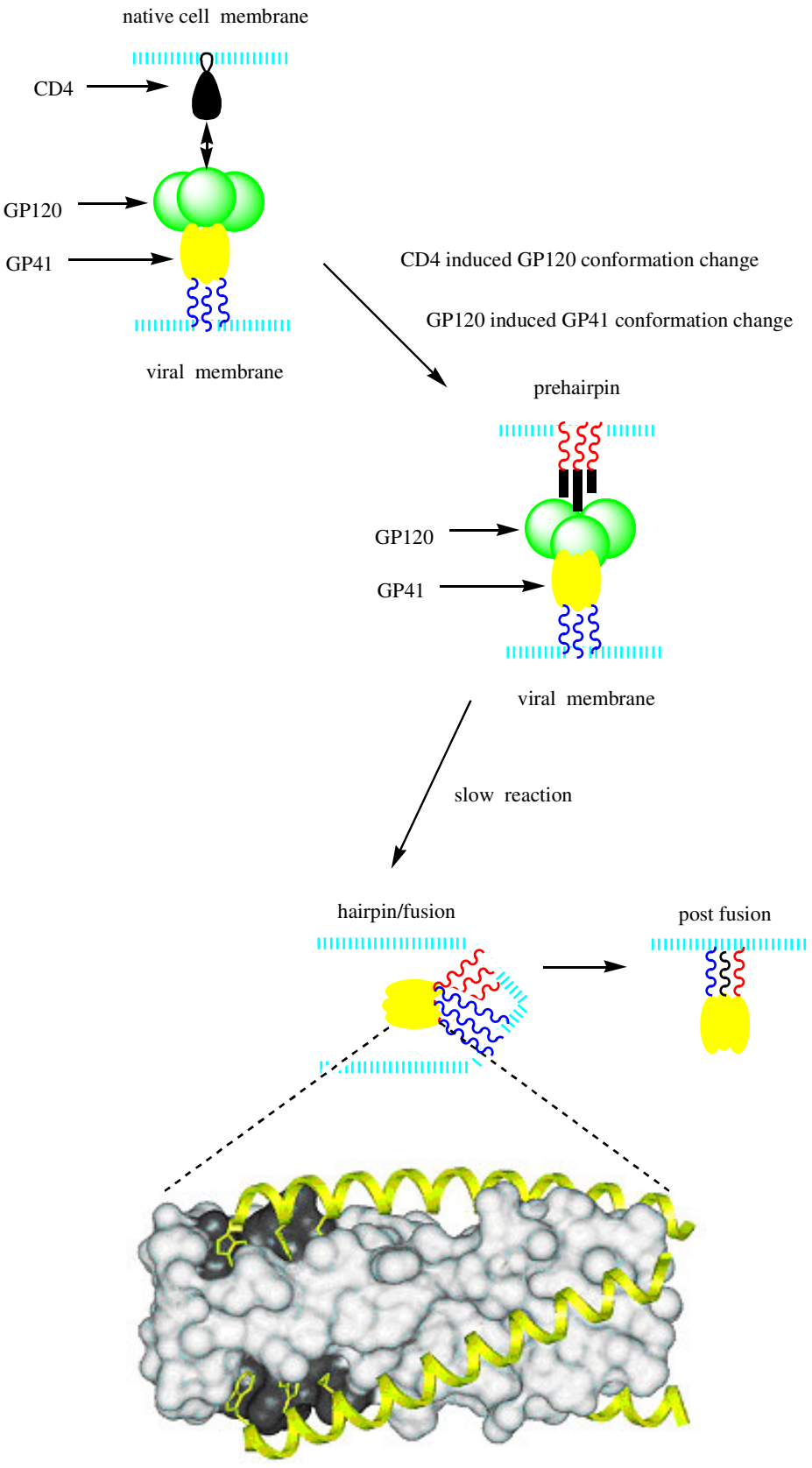

native cell membrane
CD4
GP120
GP41
viral membrane
CD4 induced GP120 conformation change
GP120 induced GP41 conformation change
prehairpin
GP120
GP41
viral membrane
slow reaction
hairpin/fusion
post fusion

Figure 1.5 Schematic representation of the current working model for viral membrane fusion.

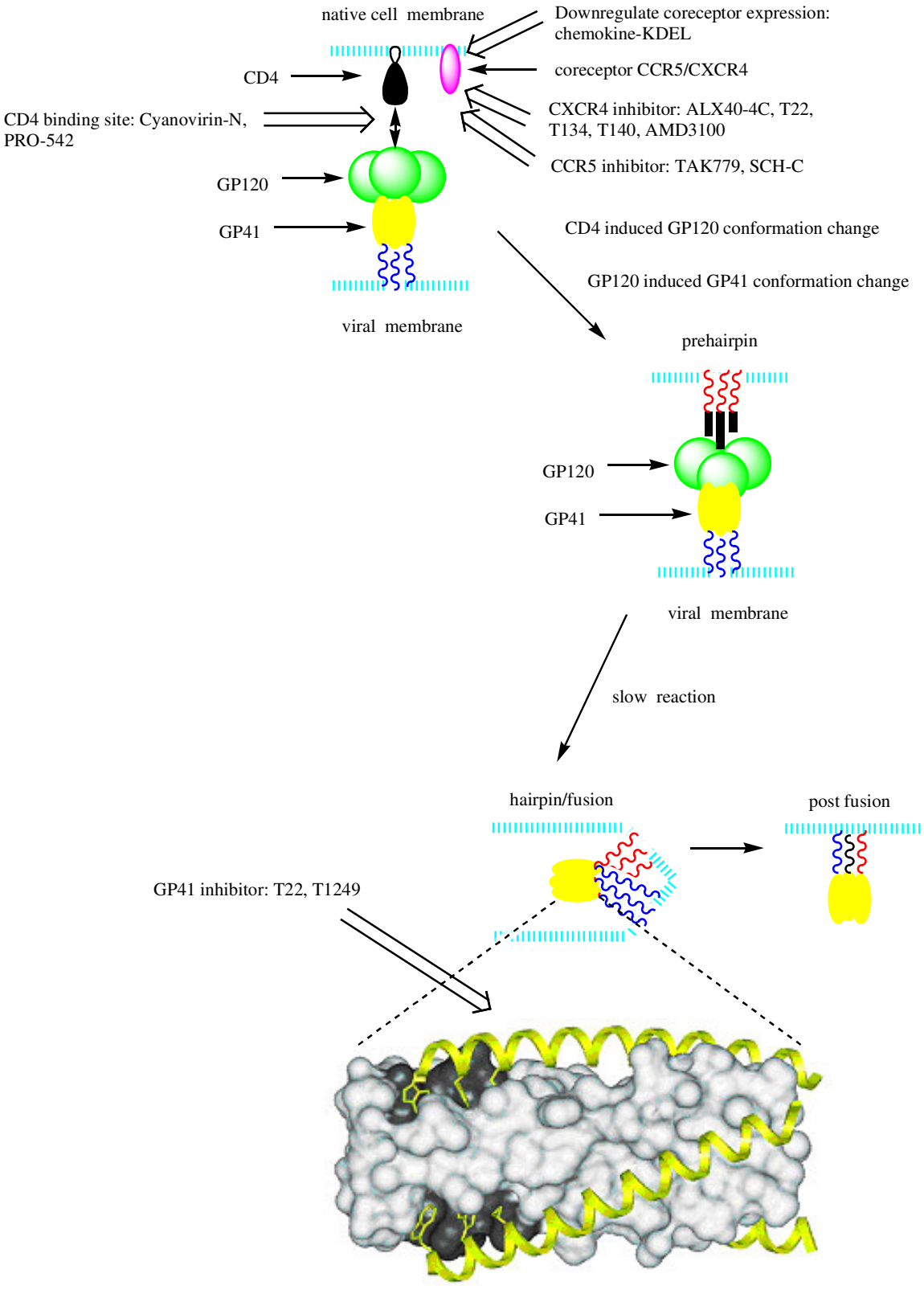


Figure 1.6 The dynamic process of Env mediated entry of HIV-1 proceeds.

CHAPTER 2

SOLID PHASE SYNTHESIS OF *NEF-FS* PEPTIDES

Introduction.

The *nef* gene from the HIV-1 encodes a myristylated 206-amino acid length protein, which is an auxiliary and non-structural protein, produced early during the viral life cycle in the host[198]. Because translation can occur at two start sites in the 5' region of the gene, it can encode two isoforms[199].

It is widely accepted that *nef* contributes substantially to disease pathogenesis by augmenting virus replication and significantly down-modulating T-cell function and CD4 expression[200]. *Nef* is also found to be chemoattractant.

In the vast majority of retroviral mRNAs, overlapping open reading frames (ORFs) encode the *gag* gene (for viral structural proteins), the *pro* gene (which encodes the viral protease), and the *pol* gene (*pol* encodes the replicative enzymes of the virus including integrase and reverse transcriptase) or a *pro/pol* gene depending on the virus. Since translational initiation signals which could be used to synthesize the Pol and/or Pro proteins are not present in the genomic mRNA, these retroviruses employ -1 ribosomal frameshifting to create Gag-Pol or Gag-Pro-Pol fusion proteins from a single *gag-pol* (or *gag-pro-pol*) translational unit[201]. At some intrinsic frequency (1 to 50 %), the translating ribosome shifts into the -1 reading frame at a heptanucleotide sequence conforming to the general sequence X XXY YYZ (termed the slippery

sequence) in the *gag-pol* (or *gag-pro*) overlapping region[202, 203]. The simultaneous-slippage model for -1 ribosomal frameshifting, is consistent with many experiments and explains the nucleotide sequence determinants of the slippery sequence at the frameshift site[204] as shown in figure 2.1. In HIV-1, a simple 3' hairpin appears to stimulate -1 frameshifting at the *gag-pol* junction[205-207].

Using computational genomic analysis our group previously identified a highly conserved –1 frameshift sequence in the HIV-1 *nef* coding region and showed that this frameshift site is functional in an *in vitro* assay. We have named this hypothetical *nef* transframe protein *nef-fs*. Sequence analysis of this peptide indicates that there is a conserved internal methionine at position 79, about 15 residues upstream of the –1 frameshift site, located about one third of the way into the *nef* coding region. The sequence immediately following this internal Met has an obvious similarity to a secretory signal peptide sequence and is especially similar to that of the CXC chemokine interleukin 8 (IL-8); the internal *nef* sequence begins MTYKAAV, as compared to MTSKLAV for IL-8. Subsequent alignment of *nef-fs* and various chemokines revealed significant sequence and topological similarities with two conserved UGA codons aligning with the two absolutely conserved cysteine residues of chemokines(Figure 2.2). As can be seen in the multiple alignment, although the alignment of chemokine sequences is exactly the same the alignment of the *nef-fs* sequence varies; in alignment A the residue encoded by UGA codon (either a selenocysteine or Cys) aligns with the second Cys in the CXC modif region which participates in a disulfide bridge with a Cys in the C-terminal region of the sequence. The significance score for alignment A was >4 SD higher than the average score of randomized sequences of identical size and composition. Using modified gap parameters our group obtained slightly different alignment with a higher significance score of >5.2 SD (see *nef-fs* alignment B

in Figure 2.2, in which the UGA codon aligns with the first Cys in the CXC motif). Alignment A of the *nef-fs* sequence appears to be structurally the more favorable of this twobecause in lymphotactin the single Cys in the CXC region matches with the second Cys as in the *nef-fs* sequence, which can form a disulfide bridge with the Cys in the C-terminal region. Using the IL-8 structure as the template, a 3D homology model was generated for the *nef-fs* sequence as shown in figure 2.3 and Figure 2.4. The resulting model appears to be as energetically favorable as the native structure and even the dimer interaction energy suggests that the mutant model dimer could be as stable as the native structure. In this model, several residues, including the two cysteines, are spatially in exactly the same location as in the template structure, thus supporting the model based upon the IL-8 structure. Threading of the *nef-fs* sequence through the protein fingerprints in the structural database (our group used the topology finger-print based inverse folding program Matchmaker, developed by Skolnick's group at the Scripps Research Institute) resulted in IL-8 as the top-most hit, at over 5 standard deviations above the mean as shown in table 2.1. It is significant to note that in this region the Matchmaker program has given an alignment (not shown) identical to the alignment A of the *nef-fs* sequence in Figure2.2. Parsimony analysis indicates that the *nef-fs* sequence is closer evolutionarily to a γ-chemokine. This is particularly significant due to the fact that the *nef-fs* sequence, like lymphotactin, also has only one cysteine in the CXC region.

Several later experiments confirmed that HIV-1 *nef* is a chemoattractant. Using a modified Boyden chamber assay, Pfister *et al.* showed that HIV-1-*nef* protein (compared with *Tat*, gp120, and gp160) can induce cellular trafficking into the CNS *in vivo* in a rat model, and *nef* is a chemoattractant for human blood-derived mononuclear and polymorphonuclear leukocytes (PMN)[163]. They failed to identify the regions of the protein involved although

they assayed all of the peptides from 140-206 and 17-21 and nothing showed significant chemotactic activity. David R.S *et al.* also found that *nef* had chemotactic activity[208], but their data showed that *nef* needs HIV *tat* to become a chemoattractant. Based on these findings and our modeling results, we believe that the sequence immediately following the 79 internal Met is the peptide that actually has the chemotactic activity. In this chapter, we will describe the synthesis of these peptides.

Generally, three methods are available for peptide synthesis, cell expression, solution phase synthesis and solid phase synthesis. We decided to use solid phase synthesis. There are several reasons for choosing a solid-phase approach to peptide synthesis, the ease of the procedure, the acceleration of the overall process, and the ability to achieve good yields of purified products. After unanticipated discovery of many new biologically active peptides and the expanded need for synthetic peptides to help solve problems in virtually all disciplines of biology, the solid-phase technique has been the method of choice. This approach, of course, does not replace the classic solution synthesis methods but rather supplements them. The choice of techniques depends on the objectives of the synthesis. The solid-phase method can yield many large active peptides compared with the solution phase method. It is particularly useful when large numbers of analogs, in relatively small quantities, are required as in structure-function studies on hormones, growth factors, antibiotics, and other biologically active peptides or for determining the antigenic epitopes of proteins. When carefully worked out, the solution methods can give high yields of highly purified products. Many superb syntheses of active peptides have been achieved in this way, but our assay only requires very small amount of peptides, therefore, we did not need to use solution phase peptide synthesis. We also need to consider follow up studies once we find these chemotactic peptides and the ability to expand it to

a peptides library. This is of great practical importance in rapid drug discovery. The solid phase method can satisfy all of these requirements and became our final choice.

Experiment Section

Mass spectra were recorded on a Micromass Autospec high-resolution mass spectrometer. HPLC analysis was performed with a WATERS Delta Prep 4000.

Amino acids used in synthesis

N_{α}-Fmoc-Nω-MTR-L-arginine, Nα-Fmoc-Nε-Boc-L-lysine, Fmoc-O-*tert*-butyl-L-threonine, Fmoc-O-*tert*-butyl-L-tyrosine, Fmoc-L-proline, Fmoc-L-leucine, Fmoc-O-*tert*-butyl-L-serine. All of these amino acids were purchased by SIGMA.

Chemicals

4-Benzyloxybenzyl alcohol, polymer-bound, Dichloromethane (DCM), *N*,*N*'-dimethylformamide (DMF), piperidine, trifluoracetic acid (TFA), *O*-benzotriazole-1-yl-*N*,*N*,*N*',*N*'-tetra methyl-uronium-tetrafluoroborate (TBTU), diisopropylethylamine (DIPEA), diisopropylcarbodiimide (DICI) and phenol were from Aldrich (Milwaukee, WI).

Kaiser test

A: Ninhydrin (5%, w/v) in ethanol (or benzyl alcohol)

B. Phenol (4:1, w/v) in ethanol

C. Potassium cyanide (2%, v/v, of a 1 mmol/liter aqueous solution) in pyridine.

The test is carried out by adding 4 drops of A, 2 drops of B, and 2 drops of C to the test sample (usually 4-5 mg of peptide-resin) contained in a small glass vial and heating at 100°C for 5 minutes. A blue coloration indicates a positive result. The coupling reaction is incomplete.

Isatin test (for peptide with Proline/Serine as terminal residue)

Isatin solution: Add isatin (2 g) to benzyl alcohol (60 ml) and stir the mixture for 2 hr at ambient temperature. Filter to remove insoluble isatin and add Boc-Phe-OH (2.5 g) to the filtrate.

The test is carried out by adding 2-3 drops of the above isatin solution to the sample (usually 4-5 mg of peptide-resin) contained in a small glass vial and heating at 100°C for 5 minutes. A blue coloration indicates a positive result. The coupling reaction is incomplete.

Loading of C-Terminal amino acids to hydroxy-functionalized resins

The resin (4-Benzyloxybenzyl alcohol, polymer-bound, 500 mg) was placed in a 100 ml reactor, followed by the addition of Fmoc-L-Proline (506.07 mg, 1.5mmol). Add DMF (7 ml) to make the resin just mobile to shake. DIPCDI (252 mg, 2 mmol) can then be added followed by the dropwise addition of a solution of DMAP (1 ml, 0.05 mmol/ml) dissolved in DMF. Allow the reaction to proceed for 60 min, draw off the reaction solution, and wash the resin using DMF (two times, 5ml). Recouple the amino acid using the above procedure.

Wash the resin with DMF (five times, 5ml) and acetylate any remaining hydroxy functions (before Fmoc removal). Add DMF (7 ml) to make the swollen resin just mobile to shake. Acetic anhydride (0.283 ml, 3mmol) then is added followed by a solution of DMAP (1 ml, 0.05 mmol/ml) in DMF. After 1 hour draw off the reaction solution, wash the resin with DMF (ten times, 5ml).

Removal of 9-Fluorenylmethoxycarbonyl (Fmoc) Amino-Terminal Protecting Group

Piperidine/DMF (5 ml, 20%, v/v) was added to the resin contained in the 100 ml reactor and shake for 3 min. Draw off the solvents and repeat this procedure for a further 7 min. Draw off the solvents and wash the resin with DMF (ten times, 5 ml). Submit a portion of the resin (5 mg) to the Isatin test and strong blue color appear (removal completed).

Peptide Bond Formation

Place the Fmoc-amino acid (2.5 equivalents) and TBTU (2.38 equivalents) in a beaker. Add DMF (minimum to obtain solution) and DIEA (5 equivalents), and allow the mixture to react for 2-3 min. Add this pre activated amino acid solution to the swollen resin and add further DMF if necessary until the resin is just mobile to shake. Use a spatula to gently stir the mixture if necessary and allow the reaction to proceed with monitoring by the Kaiser test or isatin test. Begin monitoring after approximately 15 min, then at regular intervals (15 min intervals) using the following procedure. Withdraw a small sample (5 mg) of the resin from the reactor and place it in a vial. Wash the sample (by filtration) using DMF (twice, 1 ml), CH_2Cl_2 (1 ml), and diethyl ether (twice, 1 ml). Submit this sample to the Kaiser test (or isatin test in the case of Pro-terminating peptides). A negative test should be obtained to show the coupling is completed. When the reaction is complete draw off the reaction solution and wash the resin with DMF (ten times, 5 ml).

Peptide Lys-Thr-Arg-Tyr-Pro (KTRYP)

Add piperidine/DMF (2 ml, 20%, v/v) to the resin (80 mg) contained in the 100 ml reactor and shook for 3 min. Draw off the solvents and repeat this procedure for a further 7 min. Draw off the solvents and wash the resin with DMF (ten times, 2 ml). Submit a portion of the resin (5 mg) to the Kaiser test and strong blue color appears (removal completed). Then wash it with CH_2Cl_2 (five times, 5ml) and dry. After removal of DMF, place the peptide-resin into a round-bottom flask. Prepare the cleavage mixture with TFA (95%), EDT (2.5%), phenol (2.5%) and purge with nitrogen for 5 min. Add the cleavage mixture (2 ml) to the resin and shake gently for 18 hours. Filter into a round-bottom flask and wash the resin with TFA (two times, 4 ml). Remove the TFA by rotary evaporation under reduced pressure to produce a thick, oily residue.

Add diethyl ether (5 ml) to precipitate the peptide and extract the scavengers. Allow the peptide to settle and then remove the diethyl ether by decantation. Wash the solid with diethyl ether (two times, 5 ml) and dry under vacuum (cleavage procedure). The peptide is further purified by HPLC (TFA/water 0.1%, TFA/CH_3CN 0.1%, TFA/methanol 0.1%) with C-18 columns.

Peptide Thr-Lys-Thr-Arg-Tyr-Pro (TKTRYP)

Add piperidine/DMF (2 ml, 20%, v/v) to the resin (80 mg) contained in the 100 ml reactor and shake for 3 min. Repeat the cleavage procedure described above. The peptide is further purified by HPLC (TFA/water 0.1%, TFA/CH_3CN 0.1%, TFA/methanol 0.1%) with C-18 columns.

Peptide Pro-Thr-Lys-Thr-Arg-Tyr-Pro (PTKTRYP)

Add piperidine/DMF (2 ml, 20%, v/v) to the resin (80 mg) contained in the 100 ml reactor and shake for 3 min. Repeat the cleavage procedure described above. The peptide is further purified by HPLC (TFA/water 0.1%, TFA/CH_3CN 0.1%, TFA/methanol 0.1%) with C-18 columns.

Peptide Leu-Pro-Thr-Lys-Thr-Arg-Tyr-Pro (LPTKTRYP)

Add piperidine/DMF (2 ml, 20%, v/v) to the resin (80 mg) contained in the 100 ml reactor and shake for 3 min. Repeat the cleavage procedure described above. The peptide is further purified by HPLC (TFA/water 0.1%, TFA/CH_3CN 0.1%, TFA/methanol 0.1%) with C-18 columns.

Peptide Ser-Leu-Pro-Thr-Lys-Thr-Arg-Tyr-Pro (SLPTKTRYP)

Add piperidine/DMF (2 ml, 20%, v/v) to the resin (80 mg) contained in the 100 ml reactor and shake for 3 min. Repeat the cleavage procedure described above. The peptide was

further purified by HPLC (TFA/water 0.1%, TFA/CH_3CN 0.1%, TFA/methanol 0.1%) with C-18 columns.

Result and discussion

Standard Fmoc (9-fluorenylmethoxycarbonyl) protocols were chosen for our solid phase peptide synthesis. In this strategy, the Fmoc group was used for α-amino protection in the solid phase [209, 210] which can be rapidly cleaved with a secondary base, particularly piperidine diluted with *N,N*-dimethylformamide (DMF), and has little or no side reactions with other potentially sensitive amino acid derivatives. Comparing with Boc (*tert*-butoxycarbonyl) procedure, this method is operationally simple and chemically less complex than the Boc procedure.

The solid support has a large influence on the outcome of solid-phase synthesis [211]. In general, the resins used in solid-phase peptide synthesis should be compatible with acidic and basic conditions used in the synthesis, do not interfere with the reactions and swell well in various solvents. There are huge amounts of resins that can be used in Fmoc strategy peptide synthesis. Many of them are suitable for our experiment since our peptides are relative small without side chains or any other special amino acids. We decided to use 4-Benzyloxybenzyl alcohol, polystyrene-bound (100-200 mesh, polystyrene cross-linked with 1% DVB, 1.0-1.5mmol OH/g resin) as the solid support, which is compatible with the reaction conditions and swells well in DMF.

Because of the base lability of the Fmoc group, acid-labile side-chain protecting groups are employed in our synthesis. The guanidine group of Arg is protected by the 4-methoxy-2,3,6-

trimethylbenzenesulphonyl (Mtr) group. The Boc group was used for lysine's side chain protection. Threonine, tyrosine and serine side chains were protected by *tert*-butyl group.

Linkage agents are now commonly used in Fmoc solid-phase synthesis. The common resins (with linker) used for preparing acid peptides are Wang and 2-chlorotrityl resins. The common resins for preparing peptide amides are Rink, Knorr, DCHD and PAL resins. We choose Wang resins with 4-Benzyloxybenzyl alcohol as a linker.

Because of absence of an automated machine, we used a manually operated reactor for the solid-phase peptide synthesis as shown in Figure 2.5. The resin and the reactant will be loaded in the upper column. Then, nitrogen will be charged and start the reaction. Vacuum the column once the reaction is finished

After fixing the strategy, protected amino acids and resin, we start the addition of C-terminal amino acid. However, the racemization of the C-terminal amino acid during the acylation reaction could damage the successful synthesis. By using diisopropylcarbodiimide (DIPCDI) for activation and a catalytic amount of 4-dimethylaminopyridine (DMAP) for ester bond formation, we minimized racemization to almost undetectable levels. Our C-terminal amino acid is Pro and this made the loading easier compared with His and Cys.

Fmoc group removal is very simple and many different methods have been proposed [212]. Our standard method utilizes piperidine in DMF (20%, v/v) that gave a clean removal of Fmoc group without interfering with other compounds.

Several coupling procedures have been developed. For normal coupling reactions traditional reagents such as DIPCDI and active esters such as pentafluorophenyl esters [213] have been effective in amide bond formation on the solid-phase. The phosphonium- and uronium-based coupling agents have gained in prominence. These reagents activate Fmoc-

amino acids in an efficient manner, lead to rapid amide bond formation, are easy to use, and are devoid of side reactions if the activation is carried out properly. We used *O*-benzotriazole-1-yl-*N*,*N*,*N*',*N*'-tetra methyl-uronium-tetrafluoroborate (TBTU) [214] and diisopropylethylamine (DIPEA) in our standard coupling procedure with good results.

The peptide bond formation has been monitored by a Kaiser test or Isatin test. For the primary amino groups, the ninhydrin-based Kaiser test [215]was used in real time to control the quality of the reaction. For secondary amino function groups, the isatin test was used to monitor the reaction.

The final step of the solid-phase synthesis is cleavage of the peptide from the solid-support. The procedure usually releases the peptide from the resin while simutaeously removing the side chain protecting groups. Addition of scavenger was required to prevent Met to add back interamolecularly to resin-bound carbocations or other problematic side reaction. The scavenger mixture is very much dependent on the constituent amino acids. The cocktail we used contains TFA (95%), EDT (2.5%) and phenol (2.5%). The general scheme of the synthesis was shown in Scheme 2.1.

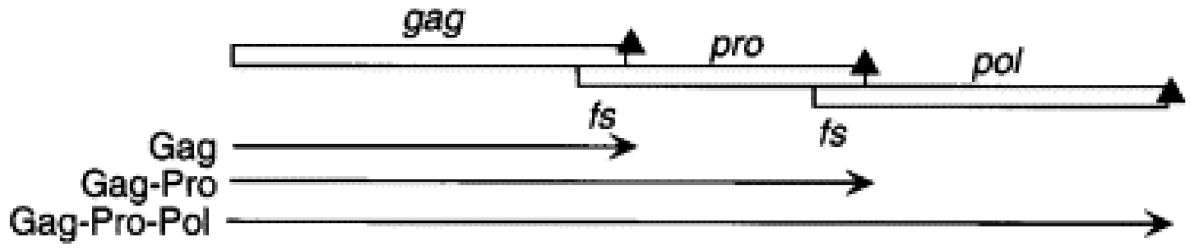
The crude product were purified by HPLC and characterized by Mass spectroscopy. In total, we synthesized 5 peptides by Fmoc solid-phase peptide synthesis with relative good yield as shown in Table 2.2.

Conclusion

We successfully synthesized a series of peptides, which are a part of the *nef-fs* with Fmoc solid-phase peptide synthesis. These peptides will be further explored for their biological property in later experiments, particularly their chemotactic property. If these

peptides do show strong biological activity they will be tested for anti-HIV activity and used as leads compounds in future drug discovery.

A


gag
pro
pol
fs
fs
Gag
Gag-Pro
Gag-Pro-Pol

B

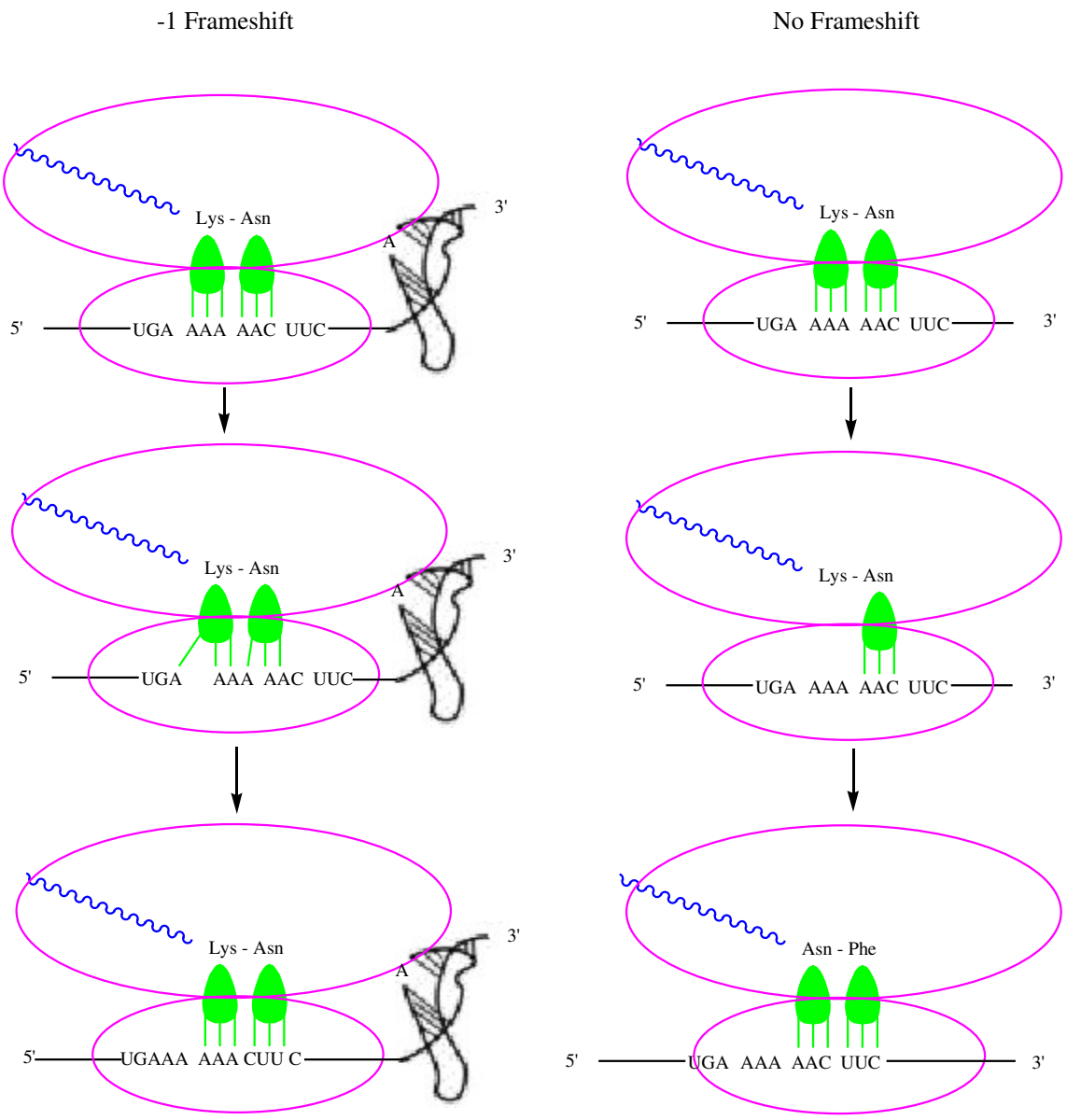

-1 Frameshift
No Frameshift
Lys - Asn
A
3'
5'
UGA AAA AAC UUC
Lys - Asn
3'
5'
UGA AAA AAC UUC
3'
Lys - Asn
A
3'
5'
UGA AAA AAC UUC
Lys - Asn
3'
5'
UGA AAA AAC UUC
3'
Lys - Asn
A
3'
5'
UGAAA AAA CUU C
Asn - Phe
5'
UGA AAA AAC UUC
3'

Figure 2.1 -1 ribosomal frameshift in retrovirus: (A) Production of Gag, Gag-Pro, and Gag-Pro-Pol fusion proteins from a single mRNA by way of two isolated -1 ribosomal frameshifting events. (B) Simultaneous slippage model for translational regulation of ribosomal frameshifting.

MULTIPLE SEQUENCE ALIGNMENT OF HIV-1 *NEF* TRANSFRAME PEPTIDE WITH CHEMOKINES

nef-fs A	MTYKAAVDLSH. FLKRKGGTGRANSLP. . . . . TKTRYPCSVDLPHTRLLP. CLAELHTRARGQ. . ISTDLWMVLQA. ST. SCAR. EVRRSQ
nef-fs B	MTYKAAVDLSH. FLKRKGGTGRANSLPT. . . KTRYPC. . SVDLPHTRL. LPCLAELHTRARGQ. . . ISTDLWMVLQ. ASTSCA. REVRRSQ
IFIP	MK. KSGVLFLLGII. LLVLIG. VQGTPVV. . . RKGRCSCISTNQG. TIHLQSLKDLKQFAPSPSCEKIEIIATLKN. GVQTCLNPDSADVK
IP-10	MNQTAIL. ICC. LI. FLTLSG. IQGVPLS. . . RTVRCTCISISNQ. PVNPRSLEKLEIIPASQFCPRVEIIATMKKKGEKRCLNPESKAIK
ENA-78	SSLCALLVLLL. LL. TQPG. PIASAGPAAAVLRELRCVCLQTTQG. . VHPKMISNLQVFAIGPQCSKVEVVASLKN. GKEICLDPEAPFLK
GRO-alpha	NPRLLRVALLL. LL. LVAA. GRRAAGASVA. . TELRCQCLQTLQG. . IHPKNIQSVNVKSPGPHCAQTEVIATLKN. GRK. CLNPASPIVK
Gro-Rat	MVSATRSLLCA. AL. PVLATSRQATGAPVA. . NELRCQCLQTVAG. . IHFKNIQSLKVMPPGPHCTQTEVIATLKN. GREACLDPEAPMVQ
Chm-Rat	TRRLLNAALLL. LL. LLMATSHQPSGTVVA. . RELRCQCLKTLPR. . VDFENIQSLTVTPPGPHCTQTEVIATLKD. GQEVCLNPQAPRLQ
PBP	SLLTALASSTKGQTKRNLAKGKEESLDSDL. YAELRCMCIKTTSG. . IHPKNIQSLEVIGKGTHCNQVEVIATLKD. GRKICLDPDAPRIK
PBSF	MDAKV. VAVLA. LV. LAALCI. SDGKPVS. . . LSYRCPCRFFESH. . IARANVKHLKI. LNTPNCA. LQIVARLKNNNRQVCIDPKLKWIQ
IL-8	MTSKLAVALLAAFLISAALCE. GAVLPRSA. . KELRCQCIKTYSK. PFHPKFIKELRVIESGPHCANTEIIVKLSD. GRELCLDPKENWVQ
Ltactin	MRLLL. . . LT. . FLGVCCLT. PWVVEGVGT. EVLEESSC. VNLQTQRLPVQKIKTYII. WEGA. . . MRAVIFVTKR. GLKICADPEAKWVK
Rantes-Hu	MKVSAAA. LAV. ILIATALCAPASASPYSS. DT. TPC. CFAYIAR. PLPRAHIKEYFY. TSGK. CSNPAVVFVTRK. NRQVCANPEKKWVR
Rantes-Mo	MKISAAA. LTI. ILTAAALCAPAPASPYGS. DT. TPC. CFAYLSL. ALPRAHVKEYFY. TSSK. CSNLAVVFVTRR. NRQVCANPEKKWVQ
MIP-1a-Hu	MQVSTAA. LAV. LLCTMALCNQ. FSASLAA. DTPTAC. CFSYTSR. QIPQNFIADYFE. TSSQ. CSKPGVIFLTKR. SRQVCADPSEEWVQ
MIP-1b-Hu	MKLCVTV. LSL. LMLVAAFCSPALSAPMGS. DPPTAC. CFSYTAR. KLPRNFVVDYYE. TSSL. CSQPAVVFQTKR. SKQVCADPSESWVQ
MCP-1	MKVSAAL. LCL. LLIAATFIPQGLAQPDAI. NAPVTC. CYNFTNR. KISVQRLASYRRITSSK. CPKEAVIFKTIV. AKEICADPKQKWVQ
MCP-2	MQVSAAL. LCL. LLTTAAFSTQVLAQPDSV. SIPITC. CFGLVNG. KIPFKKLESYTRITNSQ. CPQEAVIFKTKA. DKEVCADPQQKWVQ
MCP-3	MKASAAL. LCL. LLTAAAFSPQGLAQPVGI. NTSTTC. CYRFINK. KIPKQRLESYRRTTSSH. CPREAVIFKTKL. DKEICADPTQKWVQ
GFIC	MRISATL. LCL. LLIAAAFSIQVWAQPDGP. NAS. TC. CYVKKQK. . IPKRNLKSYRRITSSR. CPWEAVIFKTKK. GMEVCREAHQKWVE
Eotaxin	MKVSTAF. LCL. LLTVSAFSAQVLAHPG. . . IPS. AC. CFRVTNK. KISFQRLKSYKIITSSK. CPQTAIVFEIKP. DKMICADPKKKWVQ
I-309	MQIITTALVCL. LL. AGMWPEDVDSKSMQV. PFS. RC. CFSFAEQ. EIPLRAILCYRN. TSSI. CSNEGLIFKLKR. GKEACALDTVGWVQ
TCA-3	MKPTAMALMCL. LL. AAVWIQDVDSKSMLT. . VSNSC. CLNTLKK. ELPLKFIQCYRKMGSS. . CPDPPAVVFRLNKGRESCASTNKTWVQ

Fugure 2.2 Multiple alignment of HIV-1 *nef* fusion peptide resulting from –1 ribosomal frameshifting with CXC, CC and C type chemokines. At the top in the alignment, *nef-fs* A and *nef-fs* B represent the alternate alignments of *nef-fs* vs. chemokines. Alignment A is more consistent with the structure-based alignment of CXC vs. CC chemokines, based upon the X-ray crystal structures of IL-8 and MIP1.

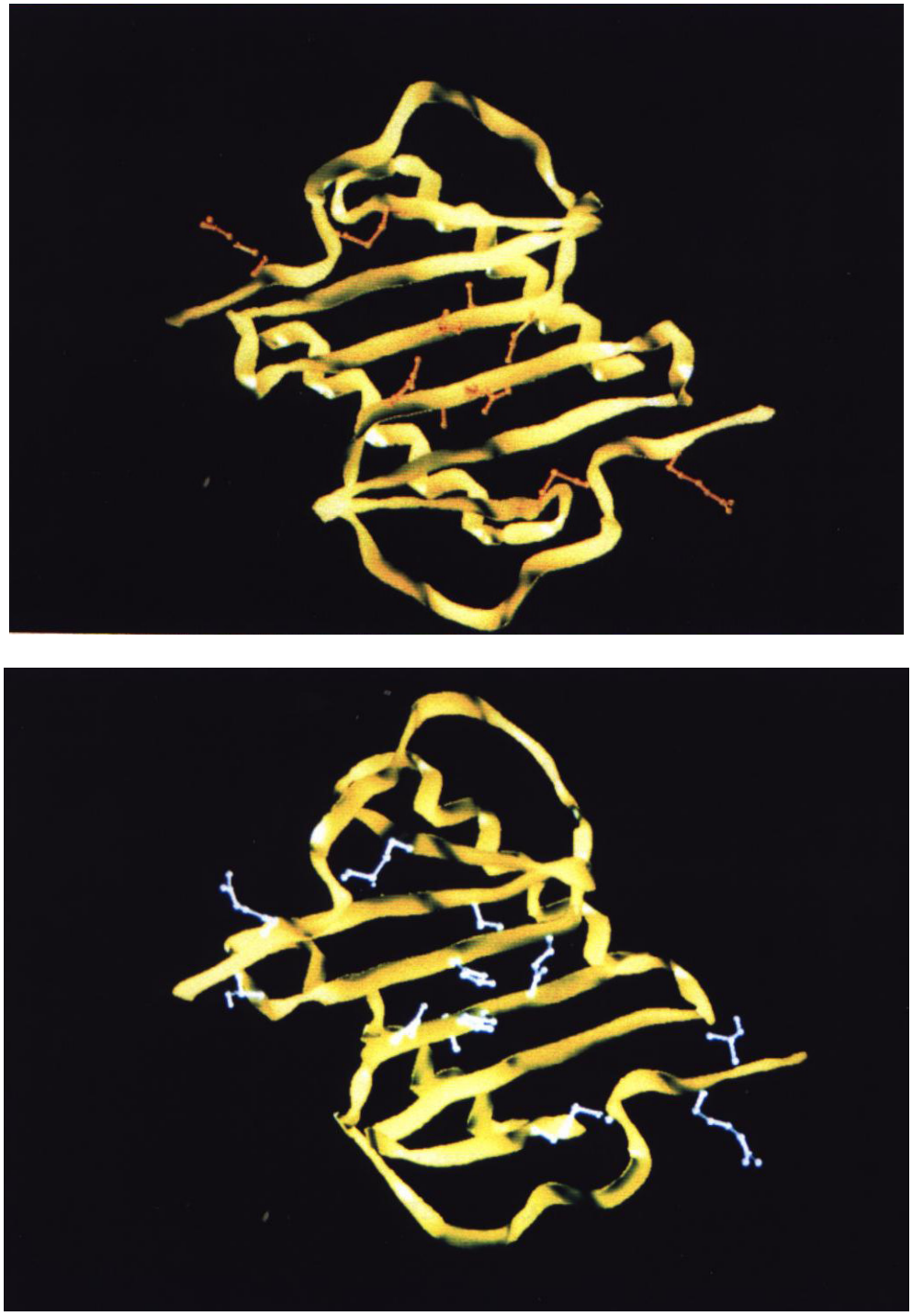
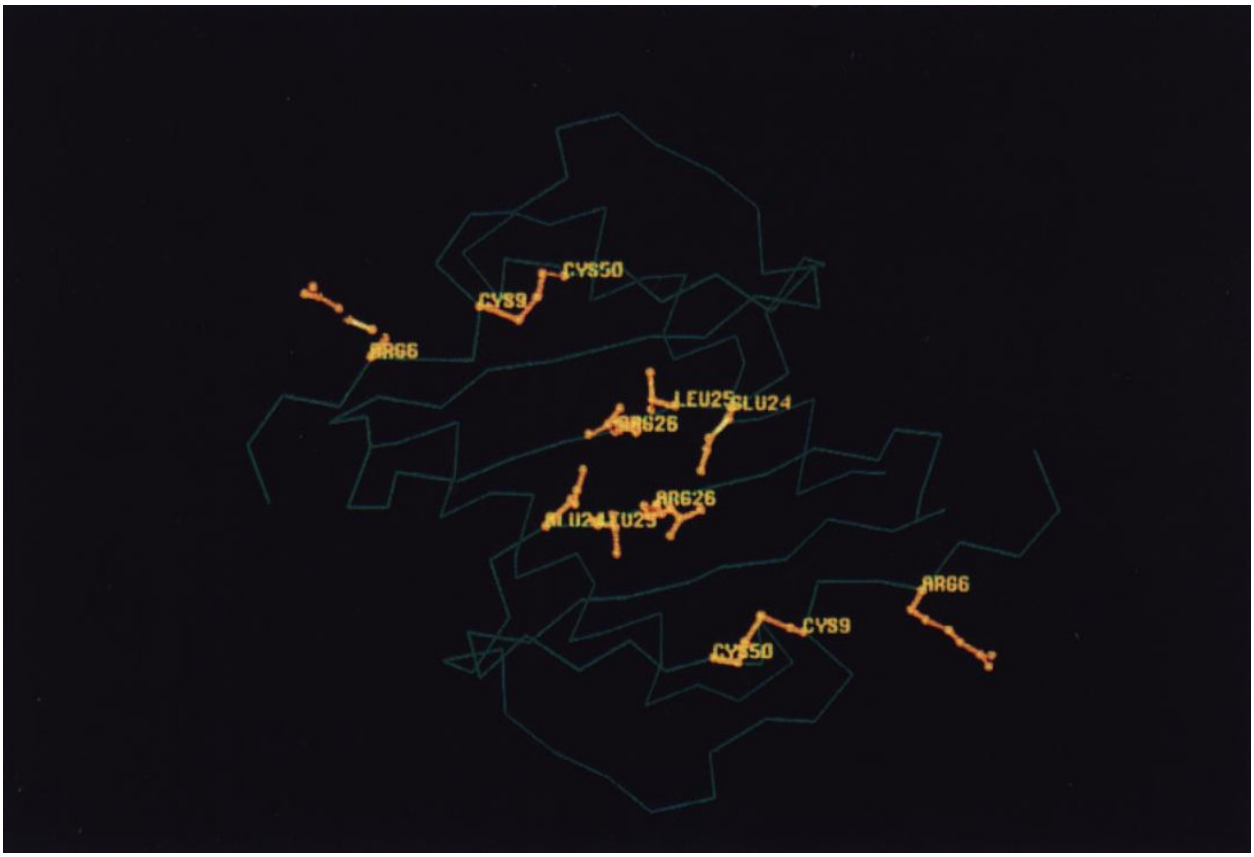


Figure 2.3 The X-ray crystal structure of Interlukin-8 and the homology model of the *nef-fs* sequence based upon it. Panel A shows IL-8 3D structure. Panel B shows the *nef-fs* model result based on IL-8.


CYS50
CYS9
ARG6
LEU25
GLU24
ARG626
ARG626
ARG6
CYS9
CYS50

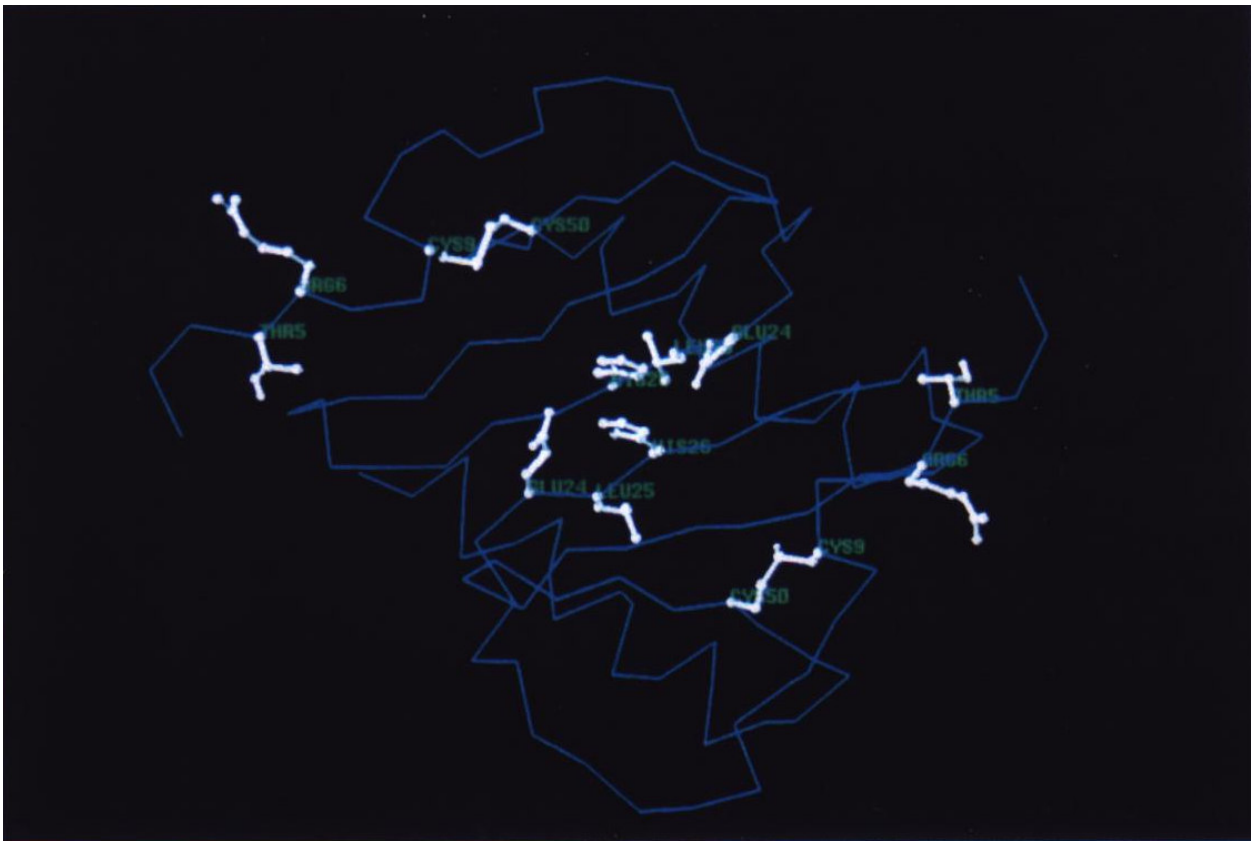

CYS50
CYS9
ARG6
THR5
GLU24
THR5
ARG6
CYS9
CYS50

Figure 2.4 The important residues in IL-8 and conserved as in the *nef-fs* sequence. Panel A shows important residues in IL-8. Panel B shows the corresponding residues in *nef-fs* model.

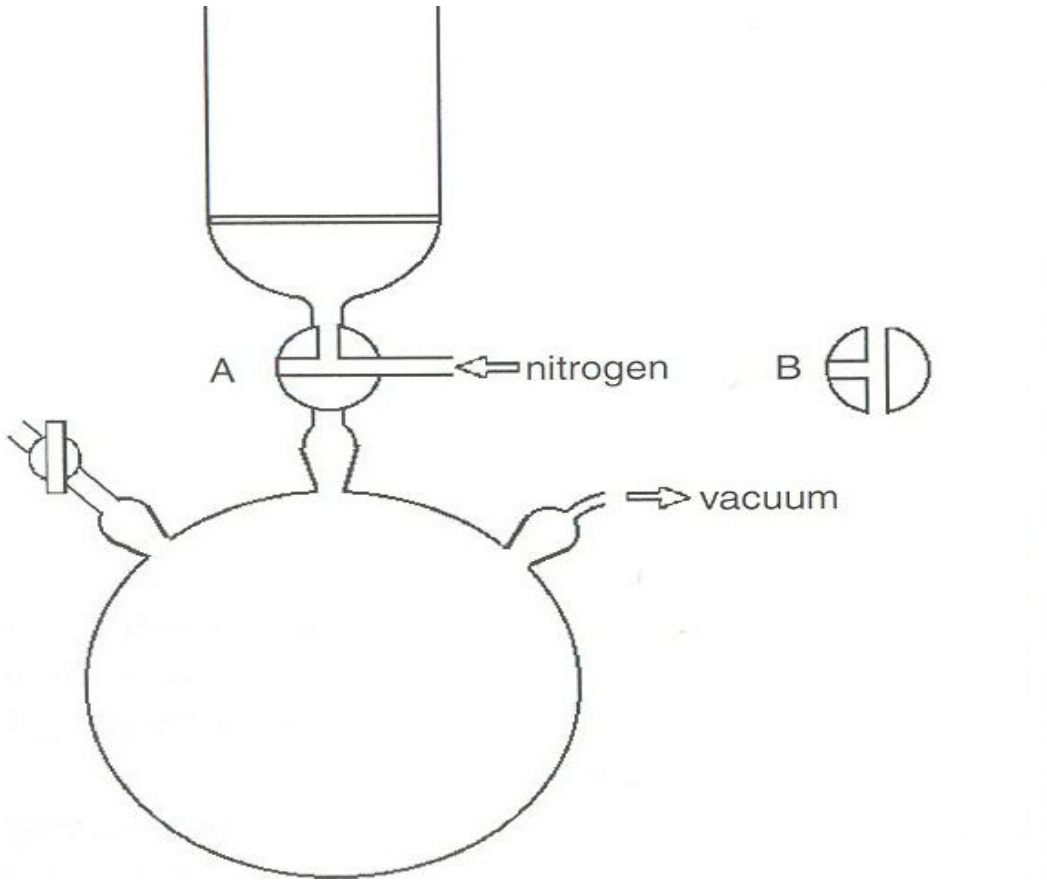
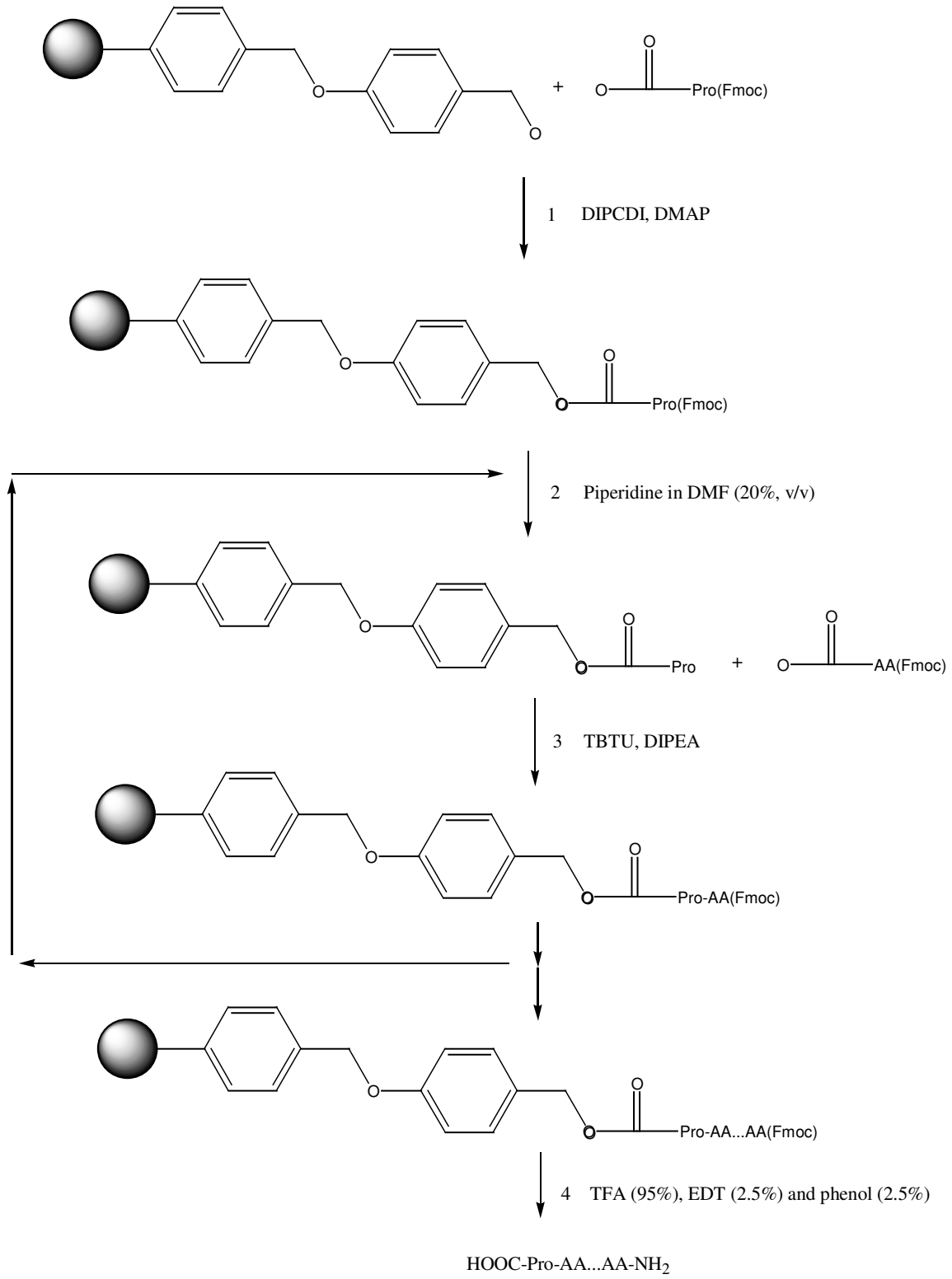


Figure 2.5 Diagram of manually operated nitrogen-stirred solid-phase peptide synthesis reactor



Scheme 2.1 General scheme of the Fmoc solid-phase peptide synthesis.

Table 2.1 Alignement of HIV-1 *nef* chemokine homologue with the selected fingerprint IL-8 sequence aftr 25 randomizations (GAP WT=2.0; GAP WT-0.2)

#	FDB CODE	Structure	SD SCORE
1	lil8.A	IL-8	-5.031
2	lser.A	Serl-tRNA synthetase	4.330
3	lrro	Rat oncomodulin	-3.592
4	lapl.C	Mat alpha2 homeodomain complex	-3.259
5	lgof	Galactose oxidase	-3.514
6	lmgs.A	Human melanoma growth stimulating factor	-3.500
7	lplq	Proliferating cell nuclear antigen	-3.468
8	lavd.A	Avidin complex with biotin	-3.436
9	lmyt	Myoglobin (MET)	-3.064
10	llea	Lexa repressor DNA binding domain	-3.034

Table 2.2 Sequences of synthesized *nef-fs* peptides and their MASS

Nef-fs fragment	Sequence	Length	Molecular mass (calculated)	Molecular mass (experimental)
Peptide 102-110	SLPTKTRYP	9	1062	1063
Peptide 103-110	LPTKTRYP	8	975	976
Peptide 104-110	PTKTRYP	7	862	864
Peptide 105-110	TKTRYP	6	765	766
Peptide 106-110	KTRYP	5	664	665

CHAPTER 3

CALCIUM MOBILIZATION PROPERTY OF *NEF-FS* PEPTIDES

Introduction

Chemokines constitute a large family of small chemotactic cytokines. Typically, each chemokine molecule is composed of 70-400 amino acids with either two or four conserved cysteines linked by one or two disulfide bonds [216]. Four subfamilies have been designated based on the positions of the amino-terminal cysteines: CC, CXC, C, and CX_3C. The only exception to this rule is lymphotactin, which has one-cysteine residue[217, 218].

The chemokine-mediated movements of immune cells from peripheral blood into tissues is essential for both innate and adaptive immunity in higher organisms [219]. In addition, it is now apparent that the role of chemokines is not restricted to cell attraction and mobility; they also play crucial roles in controlling the development, proliferation and functional encode chemokine aspects of leukocytes [62, 220-222]. Modulation of chemokine activity may also allow regulation of other physiological functions, as these mediators also play roles in hematopoiesis [223], angiogenesis [224], and differentiation [180, 225-227]. Recent *in vitro* and *in vivo* results also support the contribution of the chemokine system in the resolution of viral infections. However, the chemokine response to other viral infections can be detrimental to the host because of aberrant cellular activity. The central role of chemokines in antiviral defense is

highlighted by the vast number of viruses that -like molecules or chemokine-binding proteins to modulate the host chemokine system.

It is well known that HIV uses chemokines and their receptors to facilitate entry and transmission. The chemokine receptors CCR5 and CXCR4 are required for HIV to infect its target cells [92-96, 116]. The entry mechanism as currently understood is an ordered process in which the viral envelope protein, gp120, following interaction with the host cell-surface protein CD4, undergoes a conformational change enabling it to bind to the appropriate chemokine receptor, CCR5 for M-tropic or R5 strains, and CXCR4 for T-tropic or X4 strains. This second interaction produces a further conformational change in gp120, activating gp41 and thereby initiating fusion with the cell membrane and viral entry [228-234]. Beyond the fundamental issue of viral entry, the balance between $CCR5^+$ versus $CXCR4^+$ T-cells has been posited as a factor in disease progression *via* the emergence of X4 viral strains [235]. Central to this question is the role of the HIV-1 *Tat* protein [236, 237].

Tat is an HIV-encoded transcription factor which has been detected in the sera of infected patients [162, 238]. *Tat* also behaves as a chemokine mimic with agonist activities found for neutrophils, basophils, mast cells (via CCR2) and monocytes (via CCR3) [159, 160]. During specific studies of *tat* and monocytes/macrophages, Albini *et al*. found parallels with CC chemokine function (for example, intracellular calcium flux) [158]. *Tat* was also found to share receptors with CCL2, CCL7 and CCL11; they concluded that CC chemokine features are mimicked by HIV to facilitate infection via the attraction of monocytes and macrophages. Although the attraction of suitable cells is important, the modulation of chemokine receptors is possibly also of immense relevance to HIV infection.

Another HIV protein related to chemokine activity is HIV-*nef*. The *nef* gene from the HIV-1 encodes a myristylated 206-amino acid length protein, which is an auxiliary and non-structural protein produced early during the viral life cycle in the host.

One important property of the chemokines is their ability to induce calcium mobilization in cells. Several signal pathways could be involved in this phenomenon as shown in Figure 3.1.

Chemokine/chemokine receptor can regulate phosphoinositide-specific phospholipase C (PLC) which catalyses the hydrolysis of phosphatidylinositol to inositol-trisphosphates (IP3) and diacylglycerol (DAG). These second messengers trigger the mobilization of Ca^{2+} from intracellular stores [44]. Two groups of Ca^{2+} channels, voltage-dependent Ca^{2+} channels (VDCCs) and second messenger-operated Ca^{2+} channels (SMOCs), are regulated by chemokine/chemokine receptor pathways[45, 46]. In addition, chemokines are able to promote Ca^{2+} fluxes via the cyclic adenosine diphosphate-ribose (cADPR)/ryanodine receptor pathway and the sphingosine kinase/sphingosine 1-phosphate cascade[49].

Usually, a rapid transient rise in the free cytosolic Ca^{2+} concentration can be induced by a chemokine. To measure this rise in Ca^{2+} concentration, the most popular assay protocol was developed by *V. Tscharner* [239]. In this assay, the cells are first loaded with the Ca^{2+} binding fluorescence indicator quin-2 [240] or fura-2 [241]. After washing off the excess fluorescence indicator, the cells are challenged with the chemokine and the time courses of change in Ca^{2+} concentration were measured by following the fluorescence change, which is highly sensitive. Today, many fluorescence indicators have been developed for different purposes, but the protocol remains unchanged. In this chapter, we used this calcium mobilization assay to test the chemically synthesized *nef-fs* peptides and identified the peptides that can induce calcium mobilization in *Jurkat* cells.

Experimental Section

Reagents

Fura-2-AM was obtained from SIGMA, SDF-1 was obtained from UPSTATE (At 10nM, this lot of SDF-1 stimulated MAP Kinase activity in human *Jurkat* T-cells)

Cells

All experiments were performed on a *Jurkat* cell line (A cell line derived from human T-cell leukaemia).

Buffer

Roswell Park Memorial Institute's medium (RPMI 1540, obtained from SIGMA-ALDRICH) with 10% NCS and 5 μg/ml GEN.

Instrument

Perkin Elmer LS 55 Luminescence Spectrometer, Fisher Scientific Isotemp Plus, Jouan CT422, Nikon TMS.

Cell culture

The *Jurkat* cells were used. Cell cultures were maintained in Roswell Park Memorial Institute's medium (RPMI1540) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90% (Sigma), supplemented with 10% Newborn Calf Serum (NCS) at 37 °C with 5% CO_2.

Measurement of Cytosolic free Calcium Concentration ($[Ca^{2+}]I$)

Before use, the *Jurkat* cells were counted and stained by trypan blue. Cells were not used in experiment if the culture contained more than 3% dead cells. The harvested cells were centrifuged at 4000 rpm for 10 minutes, washed with fresh medium and suspended in new

medium. The final suspension was adjusted to 4×10^6 cells per ml and kept at room temperature until use. The prepared *Jurkat* cells were incubated for 40 minutes at 37°C in the presence of Fura-2 (0.4 nmol Fura-2 acetoxymethyl ester/10^6 cells). During the treatment, fura-2-AM was incorporated into the cytoplasm and converted to fura-2 by esterases, resulting in an average final intracellular accumulation of 100-150 μM of fura-2, based on the assumption that cell volume is 1.1 μl/10^6 cells. After loading, the cells were centrifuged at 4000 rpm for 10 minutes and washed with medium, excess fura-2-AM was removed by repeating the procedure three times[242]. The cells were resuspended to 4×10^6 cells per ml. Fluorescence measurements were made in a 1cm quartz cuvette using a Perkin Elmer LS 55 luminescence spectrometer with stirring at 37°C. During fluorescence monitoring, cells were stimulated by applying peptide synthesized or SDF-1. For all experiments, fluorescence ratio data were collected, Channel 1: Excitation 340 nm, Emission 500 nm; Channel 2: Excitation 380 nm, Emission 500 nm, integrated time: 0.1 sec, data interval: 1.9 sec. Usually, the observation of the fluorescence change was stopped after 100 seconds because the calcium mobilization is always short and did not last more than 15 seconds. Every assay was repeated twice for consistency. All of the peptides and the SDF-1 were assayed at two concentrations, 1 μg/ml and 10 μg/ml.

Results and Discussion

As shown in Figure 3.2, a rapid increase in $[Ca^{2+}]I$ occurred in response to the SDF-1. In this experiment we used fura-2 as an indicator of intracellular free calcium in *Jurkat* cells. When fura-2 was complexed with Ca^{2+}, the maximum wavelength for excitation shifted from 380 to 340 nm. Therefore, we quantitated $[Ca^{2+}]I$ from the ratio of fluorescence intensity excited at 340 nm and 380 nm by changing the excitation wavelength every 2s. The fluorescence intensity

excited at 340 nm increased after peptide stimulation. On the other hand, the fluorescence intensity excited at 380 nm had a tendency to decrease after peptide stimulation (data not shown). The 340/380 nm fluorescence ratio increase rapidly immediately after peptide stimulation, reached its maximum several seconds later, and remained there for some time.

As shown in Fig 3.2A, 3.2D, 3.2E and 3.2F, $[Ca^{2+}]I$ in *Jurkat* cells increased rapidly after peptides stimulation for several seconds followed by a gradual decrease or sustained for some time.

The pattern of $[Ca^{2+}]I$ change after peptide stimulation (Figure 3.3) in the presence of 10 μg/ml of peptide was essentially the same as that of Figure 3.2. As shown in Figure 3.2 peptides 102-110, 105-110 and 106-110 showed similar pattern to SDF-1 with peptide 102-110 demostrating a larger increase in $[Ca^{2+}]I$ and peptides 105-110, 106-110 a smaller increase. Peptides 104-110 and 103-110 have no effect on $[Ca^{2+}]I$ in *Jurkat* cells at this concentration. After increasing the peptide concentration similar results were obtained, but peptide 105-110 lost its effect. The result indicates that peptides 102-110 and 106-110 can induce $[Ca^{2+}]I$ increase at 1 μg/ml or 10 μg/ml, peptide 105-110 only can induce $[Ca^{2+}]I$ increase at 1 μg/ml and peptide 104-110, 105-110 has no effect at both concentrations.

The results presented here describe the role of the COOH-terminal regions of the *nef-fs* internal signal peptide in determining biological activity. We choose *Jurkat* cells to carry out the biological activity evaluation since T cells are the primary target for HIV infection and express most of the interested chemokine receptors. We demonstrated here that two peptides 102-110 and 106-110 from the *nef-fs* internal signal peptide COOH-terminal regions can induce significant calcium mobilization in *Jurkat* cells. Another peptide 105-110 showed very mild calcium mobilization ability while peptide 104-110 and 103-110 shows no activity at all.

Comparing the results in Figure 3.2 and Figure 3.3, we also can observe some kind of correlation between the calcium mobilization and the concentration of peptides. This result strongly supports our hypothesis that these *nef-fs* peptides could be chemotactic. The interesting finds are the positions of the active peptides. The two active peptides represented the longest and the shortest sequence of the potential *nef-fs* internal signal peptide sequence. There are two possible explanations for this phenomenon, the other peptides also can induce calcium mobilization but need a different concentration or the functional binding site needs correct conformation, which the inactive peptides cannot offer. The first possibility will be tested in the following chemotaxis assay. There is a report that *nef* and *Tat* together represent the lower molecular mass chemoattractant released by HIV-induced syncytia and the chemotactic response of T-helper cells to *Tat* and *nef* requires parallel gradients, which provie\de some evidence supporting the second possibility[208]. The author proved some of the *nef*'s chemotactic property but also pointed out that the *nef* in the HIV-induced T-cell syncytia needs HIV *tat* protein to show its chemotactic ability. The peptide 105-110 could have some small chemotactic ability since it shows mild calcium mobilization ability at one concentration.

Conclusion

We tested the HIV *nef-fs* COOH-terminal internal signal peptides that synthesized by solid-phase peptide synthesis in previous chapter. *Jurkat* cells were chosen for the assay. Two out of five peptides, peptide 102-110 and peptide 106-110 can induce significant calcium mobilization at different concentrations while the rest of the peptides show little activity. This result supported our hypothesis that *nef-fs* was a chemokine analog. Additional experiments are required to determine the details of the mechanism of the calcium mobilization induced by these

nef-fs peptides. To determine a possible explanation for the difference exhibited by these peptides and confirm their chemotactic ability, chemotaxis assay were carried out and explained in next chapter.

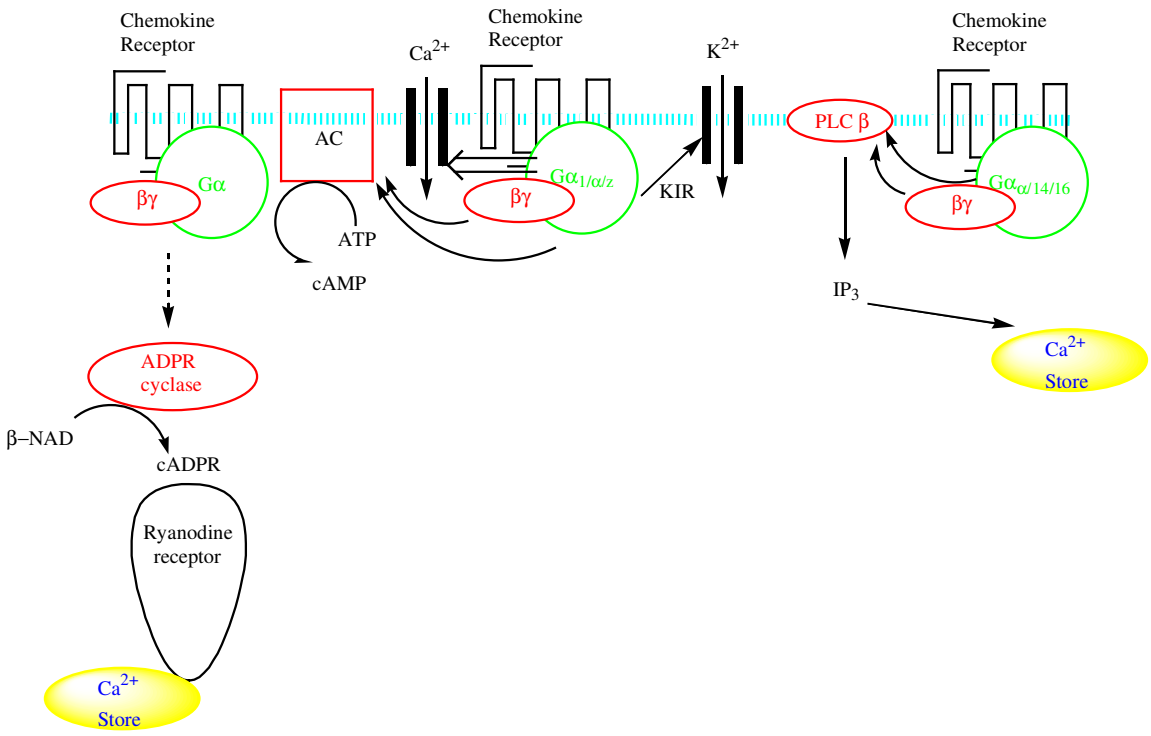


Figure 3.1 Intracellular signaling pathways activated by chemokine receptors involved in Calcium mobilization.

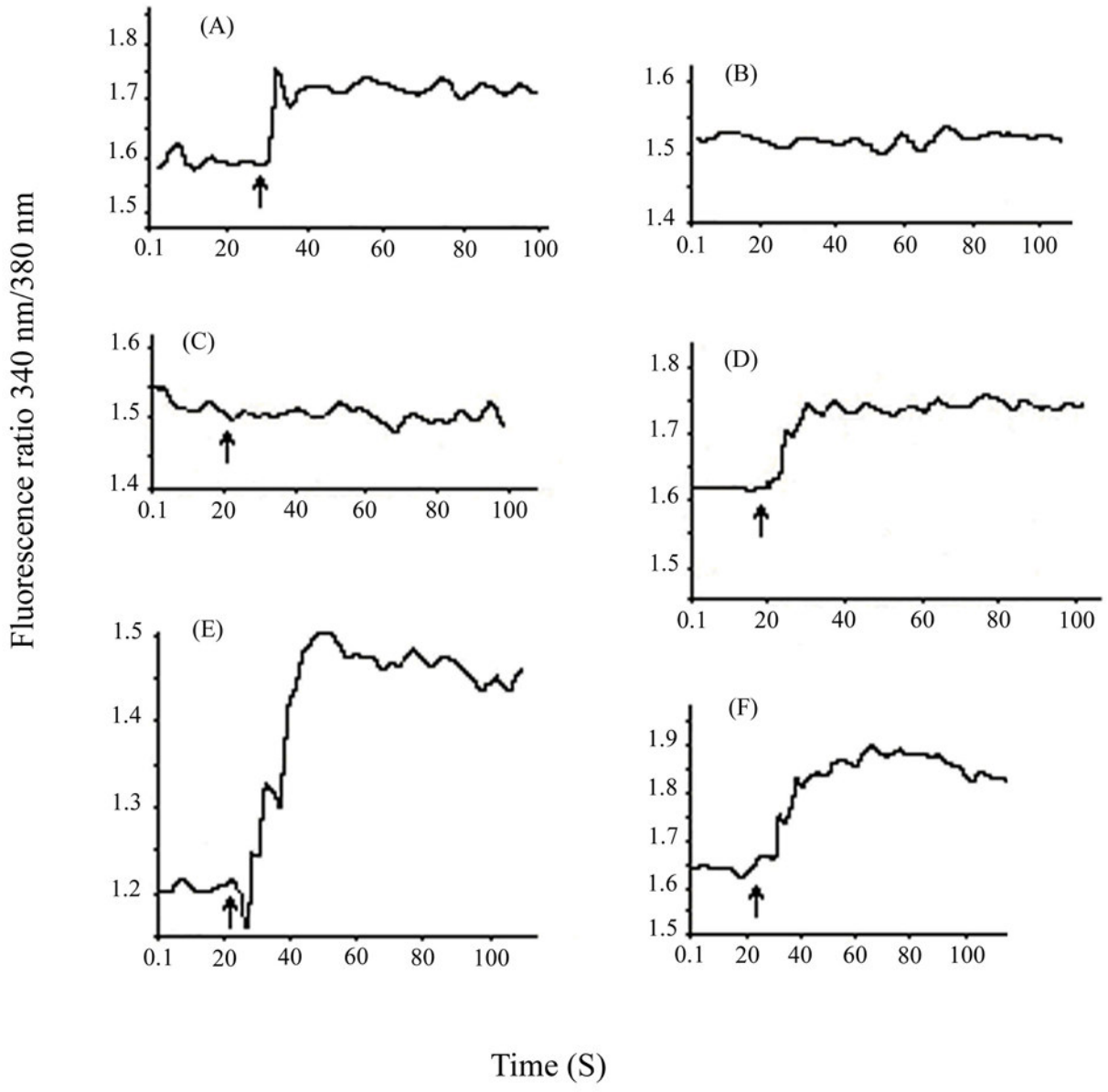


Figure 3.2 Ca^{2+} mobilization in Fura-2 loaded *Jurkat* cells

Fura-2 loaded *Jurkat* cells (final 2×10^6 cells/ml) were mixed with peptides or SDF-1 (final 1 μg/ml) at 37°C. The fluorescence measurements (excitation, 340 or 380 nm; emission, 500nm) were performed on fura-2 loaded cells. All figures were time course of fluorescence ratio of 340/380 nm. The results presented are representative of three experiments. (A) Peptide 102-110

(1 μg/ml). (B) Peptide 103-110 (1 μg/ml). (C) Peptide 104-110 (1 μg/ml). (D) Peptide 105-110 (1 μg/ml). (E) Peptide 106-110 (1 μg/ml). (F) SDF-1 (1 μg/ml).

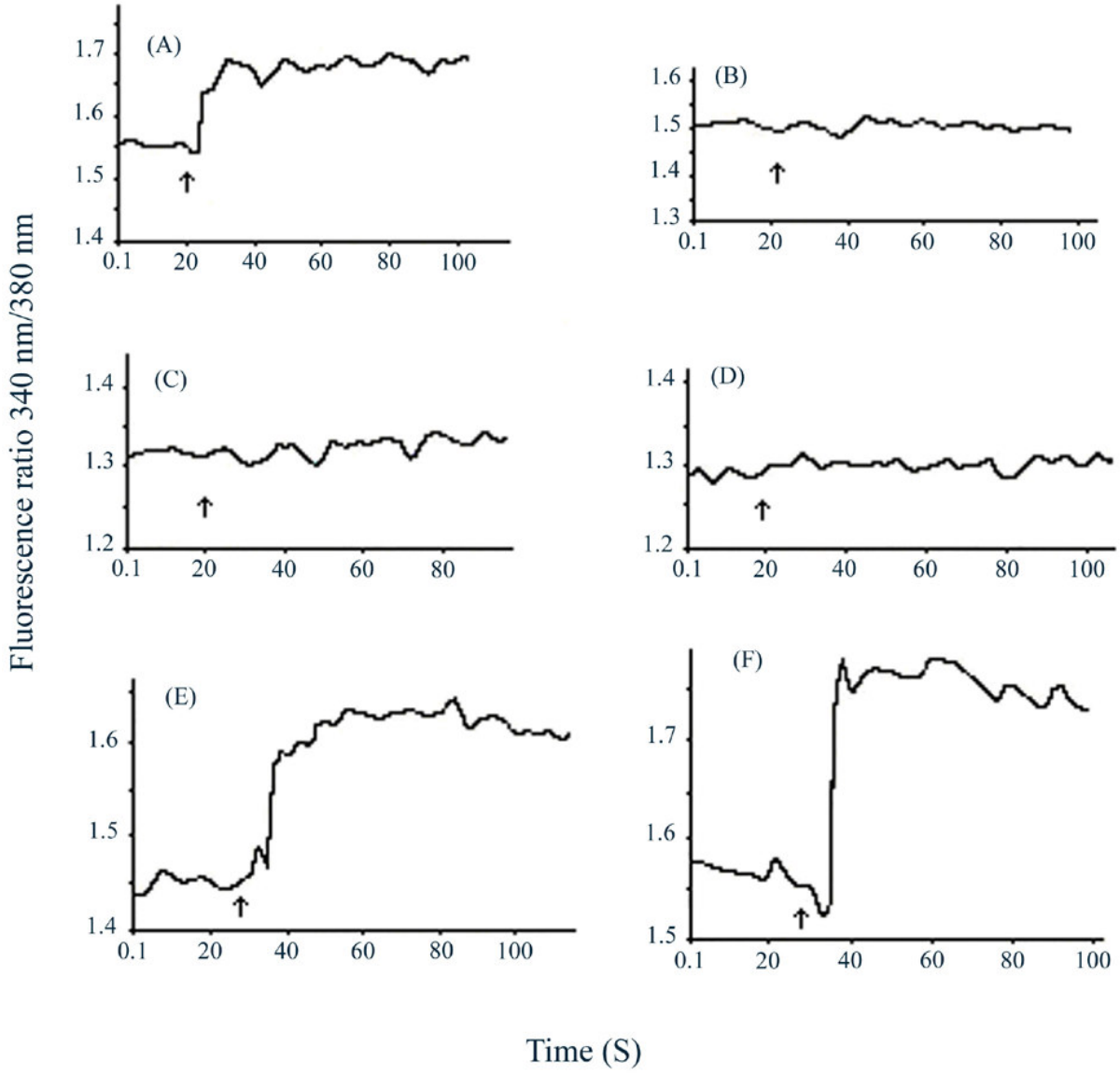


Figure 3.3 Ca^{2+} mobilization in Fura-2 loaded *Jurkat* cells

Fura-2 loaded *Jurkat* cells (final 2×10^6 cells/ml) were mixed with peptides or SDF-1 (final 1 μg/ml) at 37°C. The fluorescence measurements (excitation, 340 or 380 nm; emission, 500nm) were performed on fura-2 loaded cells. All figures were time course of fluorescence ratio of 340/380 nm. The results presented are representative of three experiments. (A) Peptide 102-110 (10 μg/ml). (B) Peptide 103-110 (10 μg/ml). (C) Peptide 104-110 (10 μg/ml). (D) Peptide 105-110 (10 μg/ml). (E) Peptide 106-110 (10 μg/ml). (F) SDF-1 (10 μg/ml).

CHAPTER 4

JURKAT CELL MIGRATION INDUCED BY *NEF-FS* PEPTIDES

Introduction

The chemokine-mediated movements of leukocytes play crucial roles in controlling the development of HIV infection. One of the cornerstones of immune system function is cell motility. When a virus has entered the body, chemical signals tell lymphocytes to proliferate and travel to the site of infection. Efforts to combat HIV have focused on understanding how the virus disrupts this immune response, in the hopes of developing drugs to block its replication, as well as vaccines to control the virus itself. Toward this end, scientists are investigating how each of the virus's nine genes—which all appear to have multiple functions—contribute to HIV infection. Naturally, it is not a benefit to the virus that its host develops disease but as suggested [235], by delaying the onset of disease, which in the case of AIDS can be for many years, the chance of transmission to another host is greatly enhanced. Experimental evidence supporting this view includes findings that gp120 peptides induced desensitization of cell responses to other chemoattractant proteins (including decreased intracellular calcium mobilization), which resulted in impaired immune cell migration. This was found to occur via the reduced expression of CCR5 and CXCR4 and might help explain HIV-associated immune suppression [243].

HIV replicates in T-lymphocytes and macrophages. HIV would, therefore, attract leukocytes because additional naïve cells are required for expanded infection [158, 235]. At the same time, the migration of other hostile immune cells' would be suppressed to decrease virus' exposure to immune response as shown in Figure 4.1. HIV successfully achieves this by mimicing human chemokines through its pleotropic genes and manipulation of the human chemokine/chemokine receptor system.

When HIV infects a cell, viral enzymes copy its RNA genes into DNA, which can then invade the infected cell's chromosomes. The viral DNA might lay dormant or it might use the cell to reproduce more viruses which go on to infect other cells. The course of infection is controlled by interactions between circulating T cells and antigen-presenting cells (cells that present evidence of infection), like macrophages, which may unwittingly aid the virus by transferring it to the T cells. Macrophages, for example, produce proteins that direct T cells to investigate an infection. More investigation showed that macrophages start to express and secrete several chemokines, such as SDF-1, which can induce T cells migration [86] as shown in figure 4.1.

A viral protein called *nef* sparked intensive research after observations that patients with a rare strain of HIV lacking *nef* took a very long time to develop AIDS symptoms [244]. Somehow, *nef* induces T cells to migrate to the site of infection [163, 166] and inhibits T cells from migrating to lymphatic tissues as shown in figure 4.1 which slows the antiviral immune response [245]. The detailed mechanism involved in these conflicting functions remains unclear. *Nef* also has been linked to molecules involved in macrophage- and other antigen-signaling pathways and may use the molecules to appropriate these pathways for its own ends—enhancing virulence by facilitating viral replication. How *nef* does this is not entirely clear. Skowronski

and coworkers have identified the key molecules that *nef* enlists to co-opt the signaling machinery of immune cells.

To understand how *nef* could successfully affect cell migration, Skowronski's group first tried to determine which molecules *nef* associates with. As an adaptor protein, *nef* does not directly catalyze reactions, but binds to enzymes that do. The researchers identified two proteins, DOCK2 and ELMO1, which form a complex with *nef*. DOCK2 regulates enzymes, called Rac1 and Rac2, which are required for normal lymphocyte migration and antigen-specific responses [246]. ELMO1 has also been shown to help DOCK2 activate Rac [247]. Because DOCK2 activates Rac as part of two different signaling pathways—one activated by the T cell receptor, which mediates T cell activation, and one by a chemokine receptor, which controls T cell migration—the researchers investigated whether *nef* could affect these important pathways by modulating Rac activity. They found that *nef* in fact activates Rac by binding to the DOCK2–ELMO1 complex. They went on to show that HIV uses these components of the chemokine receptor pathway to disrupt T cell migration [246]. To generate an effective immune response, it is crucial that T cells travel to sites within lymphatic tissues where they interact with other lymphocytes. By inhibiting T cell migration, the researchers propose, *nef* prevents these critical interactions, thereby providing a mechanism for stifling the immune response.

These results, the authors argue, provide the biochemical evidence that *nef* targets a protein “switch” that can interfere with important aspects of T cell function. In this way, *nef* subverts the immune response pathways controlled by receptors on the surface of T cells to effectively disarm the immune system and turn T cells into viral replication factories.

Tat was found to share receptors with CCL2, CCL7 and CCL11 [158]. This findings lead to the conclusion that CC chemokine features are mimicked by HIV to facilitate infection via the

attraction of monocytes and macrophages as shown in figure 4.1. Although the attraction of suitable cells is important, the modulation of chemokine receptors is possibly also of immense relevance to HIV infection.

HIV-1 *tat* also reprograms immature dendritic cells to express chemoattractants for activated T cells and macrophages [248] as shown in Figure 4.1. Immature dendritic cells are among the first cells infected by retroviruses after mucosal exposure. Using DNA microarray analysis and functional assays, the effects of human immunodeficiency virus-1 (HIV-1) and its *tat* transactivator on these primary antigen-presenting cells were carried out by E. Izmailova *et. al*. The result showed that HIV-1 infection or *tat* expression induces interferon (IFN)-responsive gene expression in immature human dendritic cells without inducing maturation [248]. Among the induced gene products are chemokines that recruit activated T cells and macrophages, the ultimate target cells for the virus. Dendritic cells in the lymph nodes of macaques infected with simian immunodeficiency virus (SIV) have elevated levels of monocyte chemoattractant protein 2 (MCP-2), demonstrating that chemokine induction also occurs during retroviral infection in vivo [248]. These results show that HIV-1 *tat* reprograms host dendritic cell gene expression to facilitate expansion of HIV-1 infection.

Natural killer (NK) cells are an heterogeneous population of cells that by definition are $CD32^{+}$ large, granular lymphocytes expressing CD56 (NK-cell-associated antigen NKH-1) and/or CD16 (FcgRIII receptor essential for antibody-dependent cell cytotoxicity) [249, 250]. These cells mediate cytotoxicity against target cells with an impaired expression of major histocompatibility Complex I and play an important role in the first line of defense against acute and chronic viral infections and malignant cells [249]. Human immunodeficiency virus (HIV) infection is associated with progressive and universal immune suppression [251] and several

reports have described impaired natural immunity mediated by NK cells as an early manifestation of HIV infection [252-254], as shown in figure 4.1. Furthermore, NK-cell-mediated natural immunity has been shown to be of prognostic value for progression of HIV disease [255]. Therefore, HIV benefits from the inhibition of the NK cell migration [256]. However, the mechanism behind this inhibition remains unknown.

Tat can induce profound proinflammatory effects in the brain, leading to monocyte migration into brain, as shown in Figure 4.1. Induction of inflammatory proteins on both endothelial cells and astrocytes which are structural elements of the blood–brain barrier can directly stimulate the adhesion and migration of HIV-infected monocytes and thus provide virus entry into the brain [257-260]. Migration of these cells is a major factor for CNS infection and related pathogeneses in AIDS development.

Evidence indicates that the HIV infection affects neutrophil response to bacteria and bacterial products *in vitro* [261] as shown in figure 4.1. *In vivo* neutrophil adhesion and emigration out of the vasculature were severely reduced, and in vitro neutrophil chemotaxis from HIV-infected patients was significantly impaired in response to fMLP or IL-8 [261]. In summary, *in vivo* HIV infection leads to a severe impairment in neutrophil rolling, adhesion, and emigration in response to bacterial stimulants potentially involving both endothelial and neutrophil dysfunction.

The mechanisms underlying chemotactic signal generation, detection and transduction through G protein coupled serpentine receptors, which resulted in cell movement, have been extensively investigated. Many different mechanisms are involved in different cell migrations [262]. For example, the mechanism involved in neutrophil chemotactic movement has been partially identified. Neutrophils protect our bodies from more than 90% of the pathogens that we

encounter every day. This continuous quest of the neutrophil to inactivate pathogens has led to its denotation as a ‘‘professional’’ killing machine. The cell senses the presence of pathogens, moves toward the sites of acute inflammation in a process called chemotaxis, and destroys the pathogens by either phagocytosis or release of peroxide products and/or proteolytic enzymes. The crawling of the neutrophil shows some fundamental similarities with the crawling of other vertebrate motile cells, such as fibroblasts and microphages. In all cases, the crawling cell protrudes surface filopodia and microspikes [263, 264]. The filopodia support thin veils of membrane protected cytoplasm, called lamellipodia. The lamellipodia are filled with actin filaments coupled with myosin II and numerous structural and regulatory proteins forming a meshwork. It is now widely accepted that the actin–myosin meshwork is the ‘‘engine’’ of neutrophil motility [265]. This locomotory ‘‘engine’’ works against the cell-to-substratum adhesion facilitated by adhesion receptors members of the integrin family [266-268]. The synchronization of lamellipodia extension with adhesion to substratum and cell body contraction results in cell movement or crawling. During crawling the cell encounters a complex chemical environment, where it is capable of detecting shallow gradients in chemoattractant concentration ~in many cases less than 1% concentration difference across the cell length [269]. The ability of the neutrophil to detect small concentration gradients allows it to ‘‘navigate’’ through tissue and to the sites of inflammation. The ‘‘navigation’’ process is controlled by chemoattractant receptors, members of the G-protein-coupled receptor superfamily [270-272], which bind to chemoattractants produced by the invading bacteria and/or chemokines produced by the cells from the injured tissue. The control of motility by the chemoattractant receptors is mediated by a variety of signaling processes that involve a variety of molecules grouped in signaling pathways. In addition to the signaling from the activated chemoattractant receptors, the motility of the

neutrophil is affected by the signaling from the bound adhesion receptors. The precise mechanism of coupling of the signals from the different membrane receptors and the processes controlling the rearrangement of the cytoskeleton are not well understood.

Recently, many of the components involved in the signaling of motility responses, such as actin polymerization, adhesion receptors priming, and adhesion receptor detachment, have been identified. Therefore, a unified model of signaling of motility was built as shown in figure 4.2. Signaling of actin dynamics via PI3K [273] activation starts with the binding of chemoattractant receptors to their ligands. The binding of the receptors triggers the release of the G βG γ subunit of the receptor associated G protein. The released G β G γ activates phospholipase C-β, PLC-β, phosphoinositide 3-kinase-γ (PI3K γ) and adenylyl cyclase (AC). PLC- β and AC are not directly involved in the signaling of motility, however, they may modulate the motility responses through protein kinase C (PKC) and protein kinase A (PKA) activation [274]. PI3K γ is a major signaling molecule in motility [275, 276]. It produces prolactin-inducible protein 3 (PIP3), which has multiple targets among the pleckstrin homology (PH) domains proteins, such as the Wiskott-Aldrich syndrome protein (WASp) family proteins WASp and WASP family Verprolin-homologous protein (WAVE), and the guanine nucleotide exchange factors (GEFs), such as the Vav family of proteins. PH domain proteins also bind PIP2, which modulates their activation by PIP3. The activated GEFs activate the GTPases Cdc42 and Rac2 [277]. Cdc42 forms a complex with WASp, PIP2, profilin, and Arp2/3 and initiates the nucleation of free barbed ends for actin polymerization [278]. Similarly, Rac2 binds WAVE, profilin, and Arp2/3 and generates free barbed ends for polymerization. The polymerization of F-actin is coupled with actin disassembly, which is also controlled by Cdc42 and Rac2 via p21 activated kinase 1 (Pak 1) and downstream by LIM (named from the Lin-11,

Isl-1 and Mec-3 genes) kinase and actin-depolymerizing factor (ADF)/cofilin [279]. The efficiency of cofilin to disassemble F-actin filaments is modulated by PIP2 and profilin. Actin disassembly is signaled also by the GTPase RhoA, which in the human neutrophil modulates integrin detachment via Rho-associated coiled-coil containing protein kinase (ROCK) [280, 281]. RhoA and Rac2 activate PI(4)P 5-kinase, which produces PIP2. The activation of chemoattractant receptors also primes the adhesion receptors to bind to their ligands and provide traction for crawling. The distant signaling of integrin priming by the chemoattractant receptors may depend on PKC, however, this signaling is unclear. Priming of the integrins is directly dependent on the phosphorylation of L-plastin by PKA [282]. The bound adhesion receptors may contribute to the overall neutrophil activation by inducing the phosporilation of guanine nucleotide exchange factors such as Vav, by tyrosine kinase. There are still signaling relations that are unclear. These include the activation of Cdc42 by PI3K γ and the signaling of ζ PKC activation.

The passive neutrophil is round and uniform. It behaves as a Newtonian liquid droplet when deformed into a pipet [283] due to the contraction of the membrane bound F-actin cortex [283, 284]. Immediately after the exposure of the cell to a gradient of chemoattractant its shape changes in a process called polarization. The polarized cell has well defined leading lamella, which extends toward the direction of increasing chemoattractant concentration, and a trailing ‘‘tail’’ called uropod, extending in the opposite direction [285]. After polarization the neutrophil starts to crawl. The crawling cell continuously creates new extensions at the leading edge of the lamella region, while the main cell body remains adherent to the supporting substrate. The crawling of the cell is coupled with body contraction, which prevents the cell from being

overextended along its crawling path. The maintenance of the spatial and functional asymmetry of the polarized cell is essential for motility.

Our modeling result shows a potential chemotactic peptide encoded by the HIV-1 *nef* gene. This nef isoform (*nef-fs*) could induce T cell migration. This *nef-fs* also might also compete with host derived chemokines to inhibit the migration of certain leukocytes. All of these changes in cell migration might be able to benefit the virus. Our calcium mobilization assay supports a close association between *nef-fs* and chemotaxis. To test this potential function of *nef-fs*, we need to assay its chemotactic activity by testing a series of *nef-fs* peptides.

All of the chemotaxis assays employed the same principle as shown in Figure 4.3. Applying a membrane filter to separate the cells and chemoattractant solution generates the chemoattractant gradient which are critical for determine the chemoattractant induced cell migration. The membrane will stop the passive cells from dispersing and allowed the activated cells to migrate. After the equilibrium is reached, the cells that have migrated towards the chemoattractant are counted and the chemotactic property is determined.

EXPERIMENT SECTION

Reagents:

Positive control SDF-1 was obtained from UPSTATE (At 10nM, this lot of SDF-1 stimulated MAP Kinase activity in human *Jurkat* T-cells)

Cells

All experiments were performed on a *Jurkat* cell line (A cell line derived from human T-cell leukaemia).

Buffer

Roswell Park Memorial Institute's medium (RPMI 1540, obtained from SIGMA-ALDRICH) with 10% NCS and 5 μg/ml GEN.

Wells & films

Blind well chamber BW200L (400 μl/200 μl) obtained from Neuro Probe Inc were used in all experiments. Filters were Isopore Membrane Filters (polycarbonate membrane, 5.0 μm diameter pores) provided by Millipore.

Instrument

Perkin Elmer LS 55 Luminescence Spectrometer, Fisher Scientific Isotemp Plus, Jouan CT422, Nikon TMS

Cell culture.

The *Jurkat* cells were used. Cell culture was maintained in Roswell Park Memorial Institute's medium (RPMI1540) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90% (Sigma), supplemented with 10% Newborn Calf Serum (NCS) at 37 °C with 5% CO_2.

Chemotaxis Assay

The *Jurkat* cells were counted and stained by trypan blue, the cells were not to be used in experiment if the culture contained more than 3% dead cells. The harvested cells were centrifuged at 4000 rpm for 10 minutes, washed with fresh medium and suspended in new medium. The final suspension was adjusted to 2×10^6 cells per ml and kept at room temperature until use. The blind single wells were thoroughly cleaned. Chemoattractants were diluted in RPMI 1640 supplemented with 10% NCS and 20 mM Hepes, pH 7.4 to desired concentration and kept at 37°C until use. The prepared solutions were sonicated to remove the excess gas

before the experiment started. These solutions were carefully added to fill the lower chamber of the well (200 μl) without contaminating the upper chamber. With a small forceps, the isopore polycarbonate membrane filter was placed over the filled well, making sure that no air bubbles were trapped inside. The filter retainer was screwed in so that the filter was fixed. The upper well in the retainer was filled with *Jurkat* cell suspension (2×10^6 cells/ml) in RPMI 1640 supplemented with 10% NCS and 20 mM Hepes, pH7.4 (400 μl). The prepared chambers were incubated 3 hours at 37°C. After incubation, the fluid was carefully removed from above the filter and the retainer was unscrewed without contaminating the lower chamber. The membrane was washed on the upper side with PBS and removed. The cells in the lower well were counted [286]. All assays were done in triplicate. Spontaneous migration was determined in the absence of chemoattractant and repeated three times. All of the peptides were assayed at four different concentrations, 10 μg/ml, 1.0 μg/ml, 2.0 μg/ml, 0.2 μg/ml. Standard errors were calculated. The experiment diagram is shown in Figure 4.3.

Result and discussion

Chemotaxis can be quantified *in vitro* on the basis of the maximum number of migrated cells (efficacy) and the concentration at which this maximum is reached (potency). To increase the accuracy of our comparison, the chemokines were tested in parallel with each cell preparation. As shown in Figure 4.4, two peptides, the shortest 106-110 and the longest 102-110, elicited a migration response with bimodal concentration dependence. The highest numbers of migrating cells were observed with these two peptides. Comparing with SDF-1, the shortest peptide 106-110 showed somewhat higher efficacy, and a little lower efficacy was obtained with the longest peptide 102-110. However, when the potencies are compared, both peptides are

weaker. Peptides 102-110 induced significant migration at a concentration of 1 μg/ml, and peptides 106-110 induced highest migration at a concentration of 0.2 μg/ml, while SDF-1 induced strongest migration at 0.02 μg/ml. Peptide 103-110 showed mild chemotactic activity at 1 μg/ml.

As shown in figure 4.4, various concentrations of peptide or SDF-1 were placed in the lower wells of the blind well chambers and *Jurkat* cells (2×10^6 cells/ml) were added in the upper wells of the chambers. These were incubated for 3 hours at 37°C, the contents were removed from the upper wells and the cells that migrated to the lower well were counted. The results presented are the average of three experiments +/- Standard Error.

Our results presented here describe the role of an internal region of *nef-fs* that align with the chemokine active site region, and therefore might be expected to have chemotactic activity. The strategy used was to chemically synthesize a series of *nef-fs* peptide analogs by solid phase methods, purifying and characterizing them, and then examining their possible biological activity by Ca^{2+} mobilization assay and chemotaxis assay in *Jurkat* cells. We choose *Jurkat* cells to carry out the biological activity evaluation, since T cells are the primary targets for HIV infection. The study differs from conventional protein structure-function studies in that genetic methods are usually used to engineer the whole protein or modified forms. However the synthesis of small peptides is much easier and also can yiled good results[287].

We demonstrated here that two internal *nef-fs* peptides, 102-110 and 106-110, have significant chemoattractant activity. Both of these peptides can induce an increase in Ca^{2+} concentration and chemotactic movement of *Jurkat* cells. Another peptide 103-110 showed very mild chemotactic activity and peptides 104-110 and 105-110 shows no activity at all.

There are many reports related HIV *nef* to the chemotaxis activity. Pfister *et al.* first showed that extracellularly soluble *nef* protein of HIV-1 provoked leukocyte recruitment into CNS in a rat model[163]. They also suggested that HIV-infected cells can release *nef* protein and extracellular *nef* can bind to a cellular surface receptor although HIV-1 *nef* protein is predominantly localized inside infected cells. They did not identify the sequence with chemotactic activity after assay of the analogs from 141-205 in COOH-terminal. Our findings could explain this since our chemotactic peptide is located in the middle of the protein.

The role of *nef* in T cell activation and migration is also controversial. David. R.S *et al.* found two HIV-induced T-cell syncytia release two separable chemoattractants into the medium with approximate molecular masses of 30 and 120 kDa. The 120 kDa chemoattractant is proved to be gp120 and they thought the lower molecular mass chemotactic activity released by HIV-induced syncytia could come from Rev, p24, *nef* and *tat*. Their experiment proved that *nef* and *tat* together represent the lower molecular mass chemoattractant released by HIV-induced syncytia and the chemotactic response of T-helper cells to *tat* and *nef* requires parallel gradients and do not function through the CD4 receptor[208]. But later experiments showed that HIV *nef* inhibited $CD4^+$ T lymphocyte chemotaxis and altered critical downstream molecules in the CXCR4 pathway, including cytoskeletal regulatory proteins[245]. The author provided two possible reasons for this observation, that HIV *nef* may blunt the T cell response to SDF-1α via intracellular disruption of CXCR4 receptor signaling or T cell receptor activation by *nef*. We think our findings could offer some explanations to these conflicting reports, the *tat* may be involved in helping the chemotactic sequence keep the right conformation and show chemotactic activity (some of our peptides show no activity at all although they are basically very similar to the active one).

Given the importance of chemokine regulation in HIV infection, it is reasonable for the virus to try to encode its homologues of chemokine related molecules. Together with the known controversial reports, our results may help to provide a possible mechanism of how *nef* affects the T cell trafficing, as shown in figure 4.5. *Nef-fs* may induce the chemotactic migration of T cell to the site of infection. At the same time, *nef* activates Rac by binding to the DOCK2–ELMO1 complex and result in inhibiting T cells to migrate to lymphatic tissues induced by SDF-1, which slowed the immune system to act against virus.

It must be pointed out that, at this stage, *nef-fs* is still a hypothetical protein. There are several reasons why *nef-fs* has escaped detection before now. Due to the inefficiency of ribosomal frameshifting, which only occurs during by 1-10% of translational events, *nef-fs* only has 10% or less chance to be expressed as compared to HIV *nef*. This would result in the very low abundance of *nef-fs* in HIV-infected cells. In addition, HIV only intends to make necessary enzyme proteins when necessary, it makes hard to detect the presence of *nef-fs*.

The peptide 106-110 shows the largest chemotactic activity and this suggests that the106-110 residue is the most important for the protein's chemotactic activity. Peptides 104-110 and 105-110 could not induce any chemotaxis migration in *Jurkat* cells while peptides 102-110 induced strong chemotaxis movement of *Jurkat* cells. One possible explanation for this finding is that by introducing an "unnatural" charged amino terminal at certain positions in the peptide, there could be varying effects on either the peptide conformation, or the interaction with the chemokine receptor, i.e., in certain positions, introduction of a charged terminal amino group might have an unfavorable electrostatic interaction with the receptor, reducing binding affinity. Alternatively, the peptides of intermediate length might still be binding to the receptor, but without producing an agonist effect. Further studies are needed to investigate the possibility that

these apparently non-chemotactic *nef-fs* peptides of intermediate length may actually be chemokine antagonists. Nonetheless, the activity demonstrated by several of the *nef-fs* peptide anlogs strongly supports the hypothesis that a chemotactic peptide sequence is encoded in the HIV-1 *nef-fs* gene.

Conclusion

We successfully synthesized a series of the proposed *nef-fs* COOH-terminal internal signal peptides by Fmoc solid phase peptide synthesis. The peptides were tested by calcium mobilization assay and chemotaxis assay. The results showed that the shortest and the longest peptide analog have significant chemotactic property and can induce calcium mobilization and chemotactic migration in *Jurkat* cell. Our study strongly supported a previous modeling result that the *nef-fs* could be a chemokine analog. This evidence also could help to explain many of controversial facts about *nef*'s effect on T cell traffic.

Given the complexity of the chemokine/chemokine receptor system involved, the responsive chemokine receptor and related signaling pathway should be further explored and identified. Due to the low abundance of *nef-fs*, the entire protein should be expressed and characterized in live mammalian cells. Finally, this *nef-fs* should be identified in live HIV virus and its expression pattern determined in a different virus tropism.

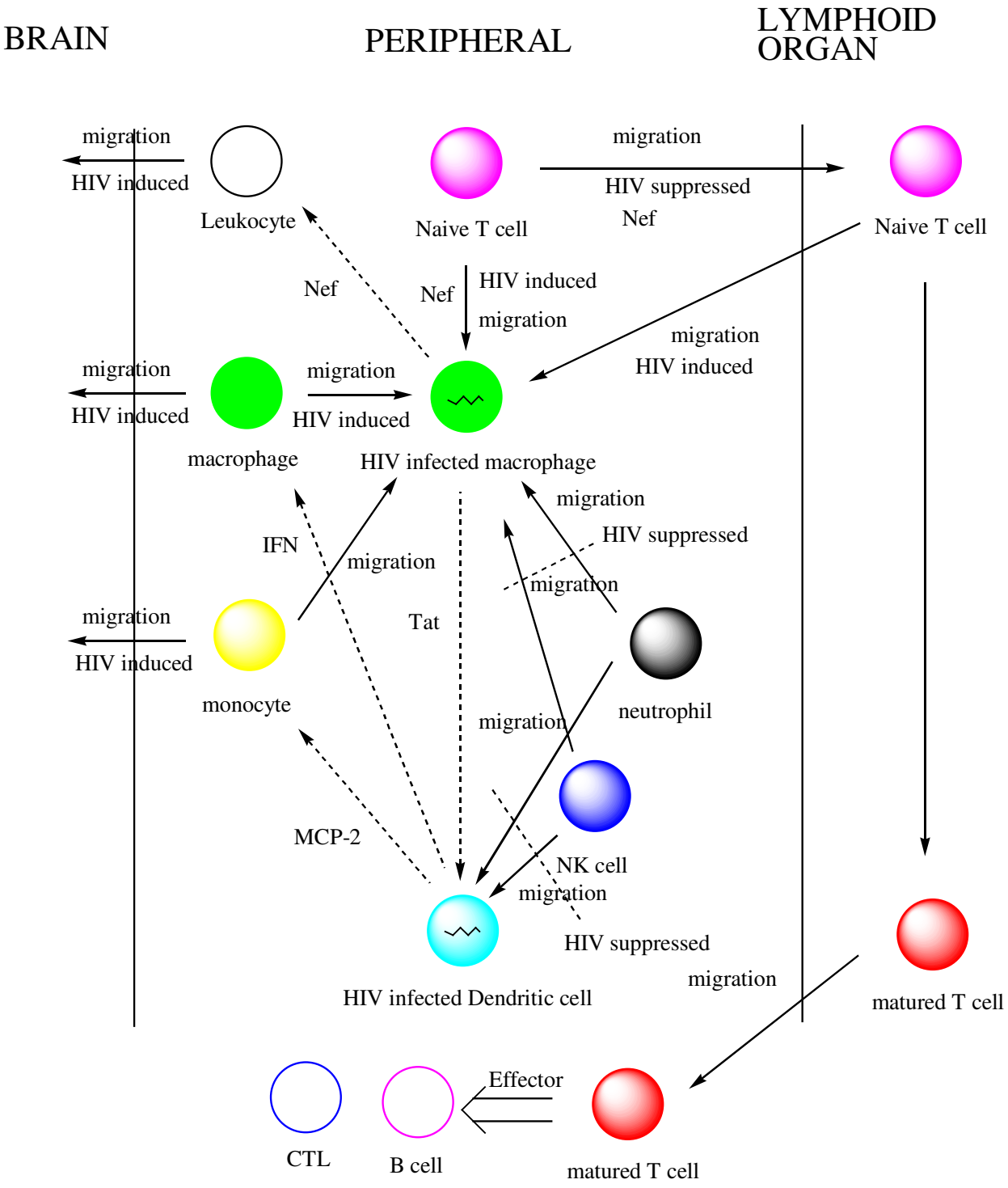


Figure 4.1 HIV affected Immune cell migration

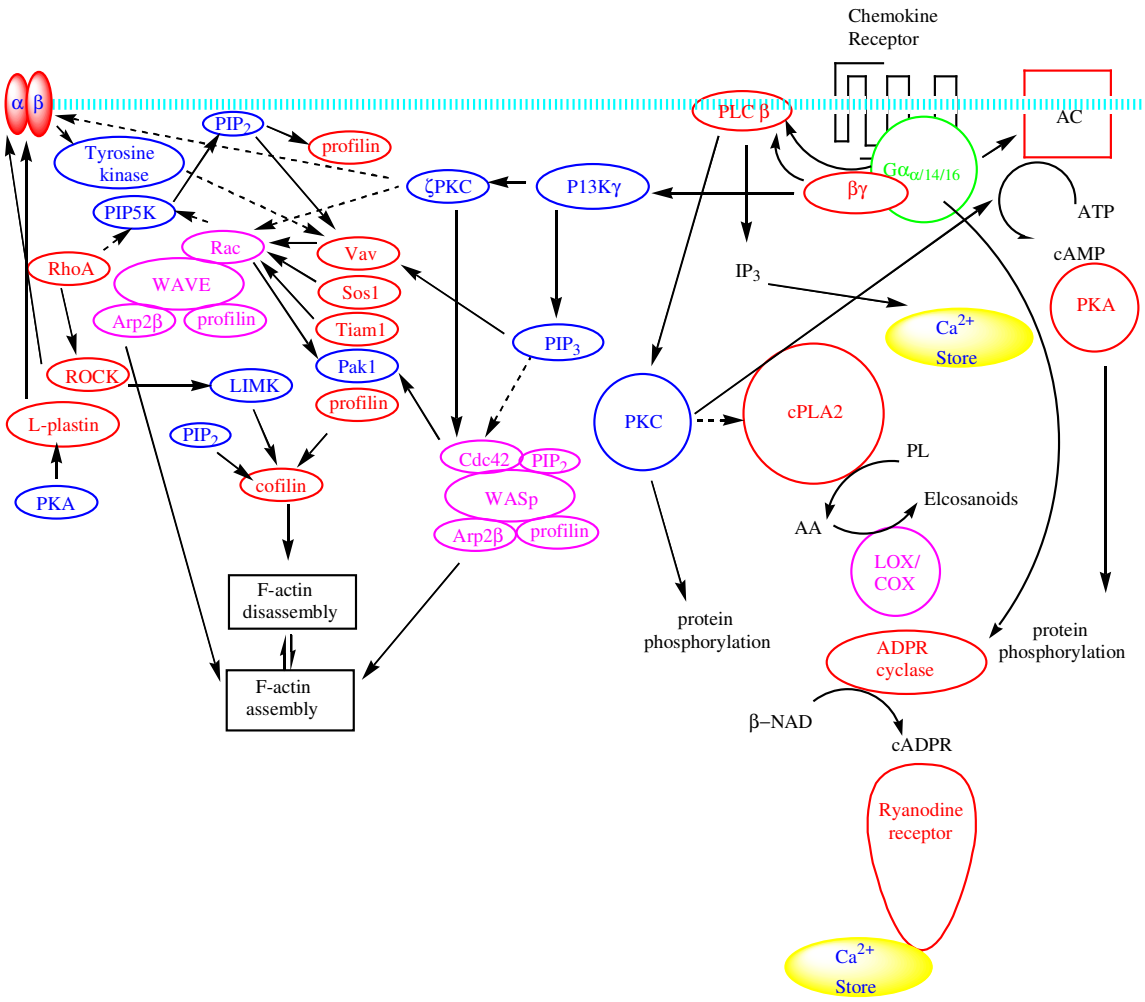


Figure 4.2 Signaling in motility response.

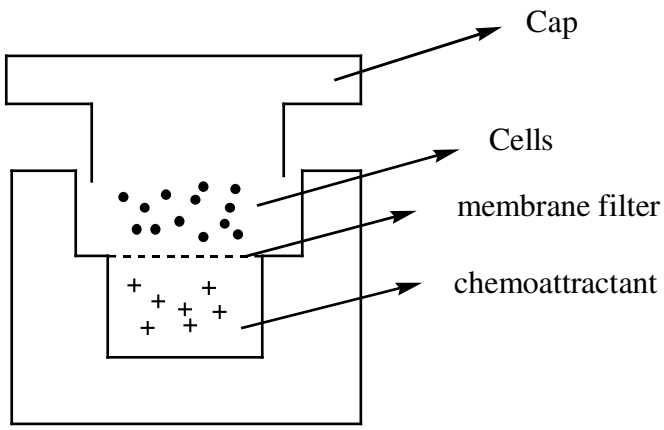


Figure 4.3 Chemotaxis assay setup.

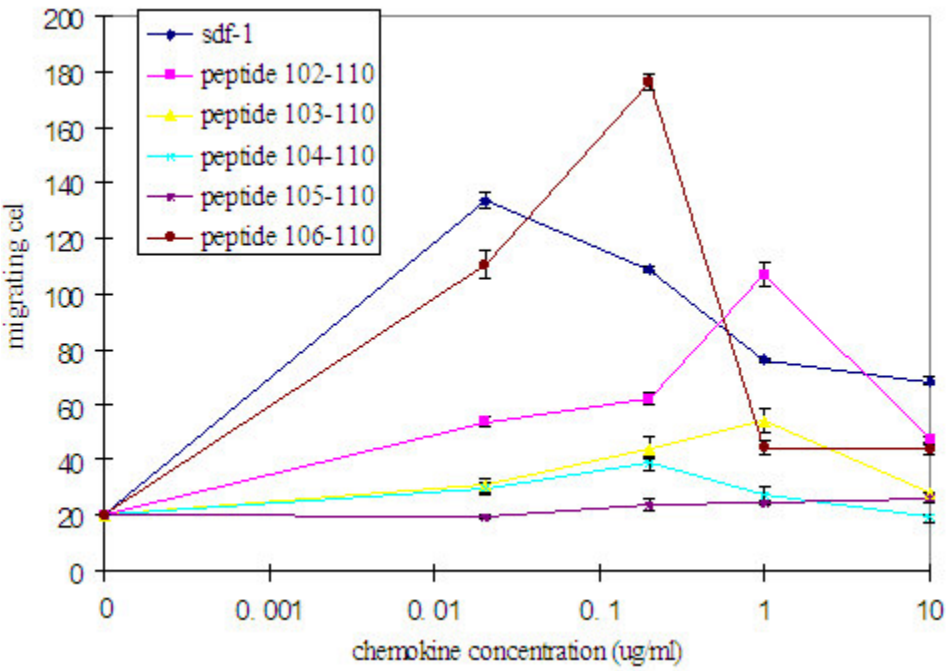


Figure 4.4 *Jurkat* cells migration induced by *Nef-fs* peptides.

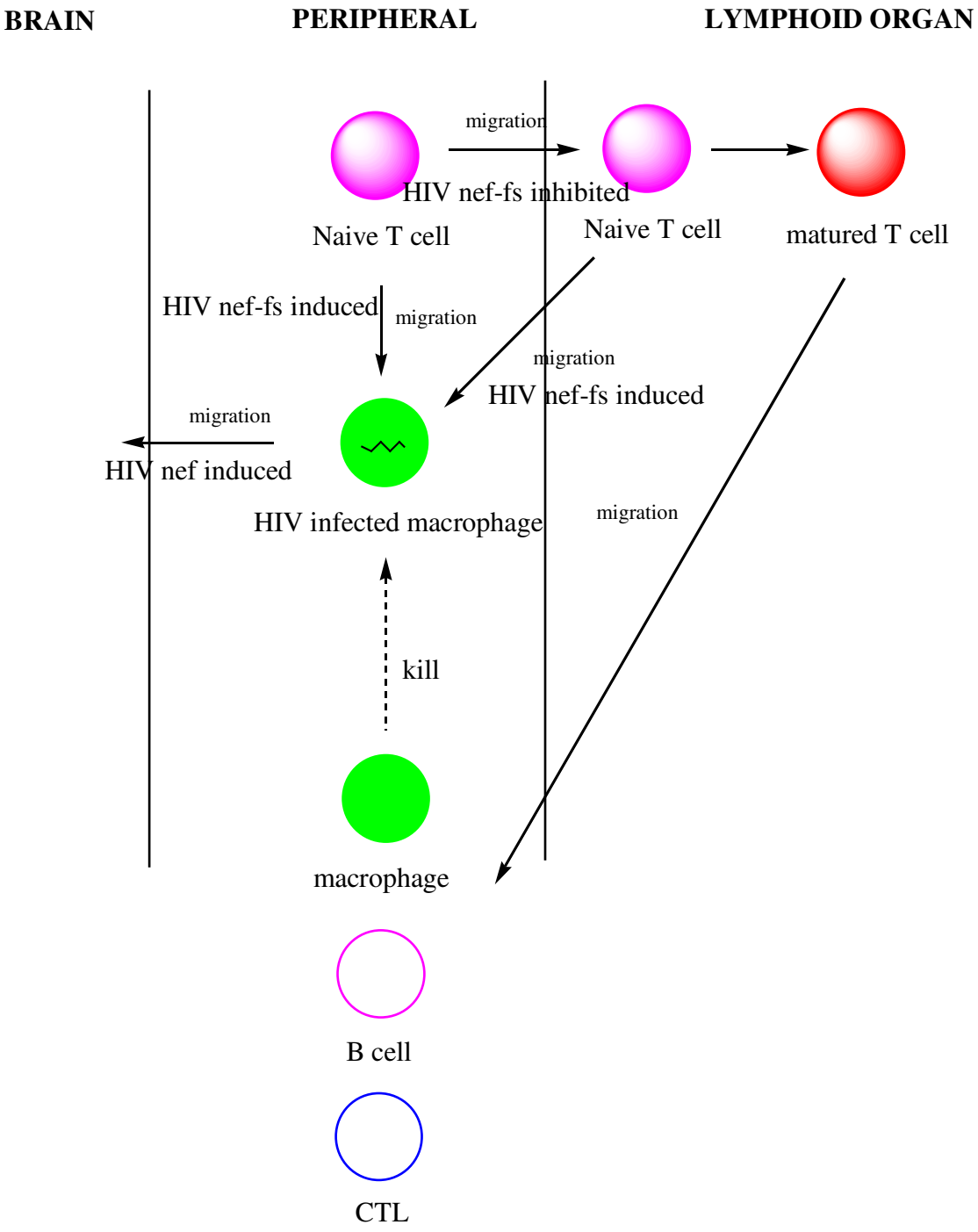


Figure 4.5 Possible HIV *nef* affected cell migration

REFERENCE

1. Garcia-Zepeda, E.A., et al., *Genomic organization, complete sequence, and chromosomal location of the gene for human eotaxin (SCYA11), an eosinophil-specific CC chemokine.* Genomics, 1997. **41**(3): p. 471-6.
2. Guan, P., et al., *Genomic organization and biological characterization of the novel human CC chemokine DC-CK-1/PARC/MIP-4/SCYA18.* Genomics, 1999. **56**(3): p. 296-302.
3. Mayo, K.H., et al., *NMR solution structure of the 32-kDa platelet factor 4 ELR-motif N-terminal chimera: a symmetric tetramer.* Biochemistry, 1995. **34**(36): p. 11399-409.
4. Baldwin, E.T., et al., *Crystal structure of interleukin 8: symbiosis of NMR and crystallography.* Proc Natl Acad Sci U S A, 1991. **88**(2): p. 502-6.
5. Clore, G.M. and A.M. Gronenborn, *NMR and X-ray analysis of the three-dimensional structure of interleukin-8.* Cytokines, 1992. **4**: p. 18-40.
6. Mikhailov, D., et al., *Heparin dodecasaccharide binding to platelet factor-4 and growth-related protein-alpha. Induction of a partially folded state and implications for heparin-induced thrombocytopenia.* J Biol Chem, 1999. **274**(36): p. 25317-29.
7. Katancik, J.A., A. Sharma, and E. de Nardin, *Interleukin 8, neutrophil-activating peptide-2 and GRO-alpha bind to and elicit cell activation via specific and different amino acid residues of CXCR2.* Cytokine, 2000. **12**(10): p. 1480-8.

8. Lodi, P.J., et al., *High-resolution solution structure of the beta chemokine hMIP-1 beta by multidimensional NMR.* Science, 1994. **263**(5154): p. 1762-7.

9. Crump, M.P., et al., *Solution structure of eotaxin, a chemokine that selectively recruits eosinophils in allergic inflammation.* J Biol Chem, 1998. **273**(35): p. 22471-9.

10. Elisseeva, E.L., et al., *NMR studies of active N-terminal peptides of stromal cell-derived factor-1. Structural basis for receptor binding.* J Biol Chem, 2000. **275**(35): p. 26799-805.

11. Kuloglu, E.S., et al., *Monomeric solution structure of the prototypical 'C' chemokine lymphotactin.* Biochemistry, 2001. **40**(42): p. 12486-96.

12. Baggiolini, M., B. Dewald, and B. Moser, *Human chemokines: an update.* Annu Rev Immunol, 1997. **15**: p. 675-705.

13. Murphy, P.M., et al., *International union of pharmacology. XXII. Nomenclature for chemokine receptors.* Pharmacol Rev, 2000. **52**(1): p. 145-76.

14. Fernandez, E.J. and E. Lolis, *Structure, function, and inhibition of chemokines.* Annu Rev Pharmacol Toxicol, 2002. **42**: p. 469-99.

15. Horuk, R., *Chemokine receptors.* Cytokine Growth Factor Rev, 2001. **12**(4): p. 313-35.

16. Chou, C.C., et al., *Pharmacological characterization of the chemokine receptor, hCCR1 in a stable transfectant and differentiated HL-60 cells: antagonism of hCCR1 activation by MIP-1beta.* Br J Pharmacol, 2002. **137**(5): p. 663-75.

17. Gao, J.L., et al., *Structure and functional expression of the human macrophage inflammatory protein 1 alpha/RANTES receptor.* J Exp Med, 1993. **177**(5): p. 1421-7.

18. Gong, X., et al., *Monocyte chemotactic protein-2 (MCP-2) uses CCR1 and CCR2B as its functional receptors.* J Biol Chem, 1997. **272**(18): p. 11682-5.

19. Chung, I.Y., et al., *Eotaxin and monocyte chemotactic protein-3 use different modes of action.* Biochem Biophys Res Commun, 2004. **314**(2): p. 646-53.

20. Votta, B.J., et al., *CKbeta-8 [CCL23], a novel CC chemokine, is chemotactic for human osteoclast precursors and is expressed in bone tissues.* J Cell Physiol, 2000. **183**(2): p. 196-207.

21. Ko, J., et al., *Human LZIP binds to CCR1 and differentially affects the chemotactic activities of CCR1-dependent chemokines.* Faseb J, 2004.

22. Daugherty, B.L., et al., *Cloning, expression, and characterization of the human eosinophil eotaxin receptor.* J Exp Med, 1996. **183**(5): p. 2349-54.

23. Power, C.A., et al., *Molecular cloning and functional expression of a novel CC chemokine receptor cDNA from a human basophilic cell line.* J Biol Chem, 1995. **270**(33): p. 19495-500.

24. Baba, M., et al., *Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC.* J Biol Chem, 1997. **272**(23): p. 14893-8.

25. Yoshida, R., et al., *Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7.* J Biol Chem, 1997. **272**(21): p. 13803-9.

26. Haque, N.S., et al., *Chemokine receptor-8 (CCR8) mediates human vascular smooth muscle cell chemotaxis and metalloproteinase-2 secretion.* Blood, 2004. **103**(4): p. 1296-304.

27. Pabst, O., et al., *Chemokine Receptor CCR9 Contributes to the Localization of Plasma Cells to the Small Intestine.* J Exp Med, 2004. **199**(3): p. 411-6.

28. Murakami, T., et al., *Immune evasion by murine melanoma mediated through CC chemokine receptor-10.* J Exp Med, 2003. **198**(9): p. 1337-47.

29. Holmes, W.E., et al., *Structure and functional expression of a human interleukin-8 receptor.* Science, 1991. **253**(5025): p. 1278-80.

30. LaRosa, G.J., et al., *Amino terminus of the interleukin-8 receptor is a major determinant of receptor subtype specificity.* J Biol Chem, 1992. **267**(35): p. 25402-6.

31. Agostini, C., et al., *CXC chemokines IP-10 and mig expression and direct migration of pulmonary CD8+/CXCR3+ T cells in the lungs of patients with HIV infection and T-cell alveolitis.* Am J Respir Crit Care Med, 2000. **162**(4 Pt 1): p. 1466-73.

32. Loetscher, M., et al., *Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes.* J Exp Med, 1996. **184**(3): p. 963-9.

33. Federsppiel, B., et al., *Molecular cloning of the cDNA and chromosomal localization of the gene for a putative seven-transmembrane segment (7-TMS) receptor isolated from human spleen.* Genomics, 1993. **16**(3): p. 707-12.

34. Bleul, C.C., et al., *The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry.* Nature, 1996. **382**(6594): p. 829-33.

35. Aust, G., et al., *The role of CXCR5 and its ligand CXCL13 in the compartmentalization of lymphocytes in thyroids affected by autoimmune thyroid diseases.* Eur J Endocrinol, 2004. **150**(2): p. 225-34.

36. Wuttge, D.M., et al., *CXCL16/SR-PSOX Is an Interferon-{gamma}-Regulated Chemokine and Scavenger Receptor Expressed in Atherosclerotic Lesions.* Arterioscler Thromb Vasc Biol, 2004.

37. Birkenbach, M., et al., *Epstein-Barr virus-induced genes: first lymphocyte-specific G protein-coupled peptide receptors.* J Virol, 1993. **67**(4): p. 2209-20.

38. Combadiere, C., S.K. Ahuja, and P.M. Murphy, *Cloning, chromosomal localization, and RNA expression of a human beta chemokine receptor-like gene.* DNA Cell Biol, 1995. **14**(8): p. 673-80.

39. Gao, J.L. and P.M. Murphy, *Human cytomegalovirus open reading frame US28 encodes a functional beta chemokine receptor.* J Biol Chem, 1994. **269**(46): p. 28539-42.

40. Ahuja, S.K., et al., *Comparison of the genomic organization and promoter function for human interleukin-8 receptors A and B.* J Biol Chem, 1994. **269**(42): p. 26381-9.

41. Taussig, R. and G. Zimmermann, *Type-specific regulation of mammalian adenylyl cyclases by G protein pathways.* Adv Second Messenger Phosphoprotein Res, 1998. **32**: p. 81-98.

42. Tsu, R.C. and Y.H. Wong, *Gi-mediated stimulation of type II adenylyl cyclase is augmented by Gq-coupled receptor activation and phorbol ester treatment.* J Neurosci, 1996. **16**(4): p. 1317-23.

43. Wu, D., C.K. Huang, and H. Jiang, *Roles of phospholipid signaling in chemoattractant-induced responses.* J Cell Sci, 2000. **113 (Pt 17)**: p. 2935-40.

44. Kuang, Y., et al., *Selective G protein coupling by C-C chemokine receptors.* J Biol Chem, 1996. **271**(8): p. 3975-8.

45. Wickman, K. and D.E. Clapham, *Ion channel regulation by G proteins.* Physiol Rev, 1995. **75**(4): p. 865-85.

46. Campbell, V., N. Berrow, and A.C. Dolphin, *GABAB receptor modulation of Ca2+ currents in rat sensory neurones by the G protein G(0): antisense oligonucleotide studies.* J Physiol, 1993. **470**: p. 1-11.

47. Kyriakis, J.M. and J. Avruch, *Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation.* Physiol Rev, 2001. **81**(2): p. 807-69.

48. von Tscharner, V., D.A. Deranleau, and M. Baggiolini, *Calcium fluxes and calcium buffering in human neutrophils.* J Biol Chem, 1986. **261**(22): p. 10163-8.

49. Maghazachi, A.A., *Intracellular signaling events at the leading edge of migrating cells.* Int J Biochem Cell Biol, 2000. **32**(9): p. 931-43.

50. Van Damme, J., et al., *Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family.* J Exp Med, 1992. **176**(1): p. 59-65.

51. Meunier, S., et al., *Determination of the three-dimensional structure of CC chemokine monocyte chemoattractant protein 3 by 1H two-dimensional NMR spectroscopy.* Biochemistry, 1997. **36**(15): p. 4412-22.

52. Carr, M.W., et al., *Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3652-6.

53. Loetscher, P., et al., *Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes.* Faseb J, 1994. **8**(13): p. 1055-60.

54. Roth, S.J., M.W. Carr, and T.A. Springer, *C-C chemokines, but not the C-X-C chemokines interleukin-8 and interferon-gamma inducible protein-10, stimulate transendothelial chemotaxis of T lymphocytes.* Eur J Immunol, 1995. **25**(12): p. 3482-8.

55. Taub, D.D., et al., *Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis.* J Immunol, 1995. **155**(8): p. 3877-88.

56. Jose, P.J., et al., *Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation.* J Exp Med, 1994. **179**(3): p. 881-7.

57. Luster, A.D. and J.V. Ravetch, *Biochemical characterization of a gamma interferon-inducible cytokine (IP-10).* J Exp Med, 1987. **166**(4): p. 1084-97.

58. Taub, D.D., et al., *Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells.* J Exp Med, 1993. **177**(6): p. 1809-14.

59. Farber, J.M., *HuMig: a new human member of the chemokine family of cytokines.* Biochem Biophys Res Commun, 1993. **192**(1): p. 223-30.

60. Liao, F., et al., *Human Mig chemokine: biochemical and functional characterization.* J Exp Med, 1995. **182**(5): p. 1301-14.

61. Oberlin, E., et al., *The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1.* Nature, 1996. **382**(6594): p. 833-5.

62. Nagasawa, T., et al., *Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1.* Nature, 1996. **382**(6592): p. 635-8.

63. Mavilio, D., et al., *Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 15011-6.

64. Zajac, A.J., et al., *Natural killer cell activity in lymphocytic choriomeningitis virus-infected beta 2-microglobulin-deficient mice.* Int Immunol, 1995. **7**(10): p. 1545-56.

65. Malnati, M.S., et al., *Recognition of virus-infected cells by natural killer cell clones is controlled by polymorphic target cell elements.* J Exp Med, 1993. **178**(3): p. 961-9.

66. Oliva, A., et al., *Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro.* J Clin Invest, 1998. **102**(1): p. 223-31.

67. Fehniger, T.A., et al., *Natural killer cells from HIV-1+ patients produce C-C chemokines and inhibit HIV-1 infection.* J Immunol, 1998. **161**(11): p. 6433-8.

68. Kottilil, S., et al., *Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function.* J Infect Dis, 2003. **187**(7): p. 1038-45.

69. Persson, T., et al., *Expression of the neutrophil-activating CXC chemokine ENA-78/CXCL5 by human eosinophils.* Clin Exp Allergy, 2003. **33**(4): p. 531-7.

70. Rosenberg, H.F. and J.B. Domachowske, *Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens.* J Leukoc Biol, 2001. **70**(5): p. 691-8.

71. Youle, R.J., et al., *RNase inhibition of human immunodeficiency virus infection of H9 cells.* Proc Natl Acad Sci U S A, 1994. **91**(13): p. 6012-6.

72. Rugeles, M.T., et al., *Ribonuclease is partly responsible for the HIV-1 inhibitory effect activated by HLA alloantigen recognition.* Aids, 2003. **17**(4): p. 481-6.

73. Walker, C.M., et al., *CD8+ T lymphocyte control of HIV replication in cultured CD4+ cells varies among infected individuals.* Cell Immunol, 1989. **119**(2): p. 470-5.

74. Yang, O.O., et al., *Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms.* J Virol, 1997. **71**(4): p. 3120-8.

75. McKinney, D.M., et al., *The antiviral activity of HIV-specific CD8+ CTL clones is limited by elimination due to encounter with HIV-infected targets.* J Immunol, 1999. **163**(2): p. 861-7.

76. McMichael, A.J. and S.L. Rowland-Jones, *Cellular immune responses to HIV.* Nature, 2001. **410**(6831): p. 980-7.

77. Andersson, J., et al., *Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection.* Aids, 1999. **13**(11): p. 1295-303.

78. Cocchi, F., et al., *Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells.* Science, 1995. **270**(5243): p. 1811-5.

79. Hadida, F., et al., *Cutting edge: RANTES regulates Fas ligand expression and killing by HIV-specific CD8 cytotoxic T cells.* J Immunol, 1999. **163**(3): p. 1105-9.

80. Hadida, F., et al., *HIV-specific T cell cytotoxicity mediated by RANTES via the chemokine receptor CCR3.* J Exp Med, 1998. **188**(3): p. 609-14.

81. Wagner, L., et al., *Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans.* Nature, 1998. **391**(6670): p. 908-11.

82. Colamussi, M.L., et al., *Stromal derived factor-1 alpha (SDF-1 alpha) induces CD4+ T cell apoptosis via the functional up-regulation of the Fas (CD95)/Fas ligand (CD95L) pathway.* J Leukoc Biol, 2001. **69**(2): p. 263-70.

83. Xiong, H., et al., *Inhibition of long-term potentiation by interleukin-8: implications for human immunodeficiency virus-1-associated dementia.* J Neurosci Res, 2003. **71**(4): p. 600-7.

84. Lane, B.R., et al., *Interleukin-8 stimulates human immunodeficiency virus type 1 replication and is a potential new target for antiretroviral therapy.* J Virol, 2001. **75**(17): p. 8195-202.

85. Richardson, R.M., et al., *Interleukin-8-mediated heterologous receptor internalization provides resistance to HIV-1 infectivity. Role of signal strength and receptor desensitization.* J Biol Chem, 2003. **278**(18): p. 15867-73.

86. Gross, E., et al., *Macrophages and lymphocytes differentially modulate the ability of RANTES to inhibit HIV-1 infection.* J Leukoc Biol, 2003. **74**(5): p. 781-90.

87. Pal, R., et al., *Inhibition of HIV-1 infection by the beta-chemokine MDC.* Science, 1997. **278**(5338): p. 695-8.

88. Struyf, S., et al., *Enhanced anti-HIV-1 activity and altered chemotactic potency of NH2-terminally processed macrophage-derived chemokine (MDC) imply an additional MDC receptor.* J Immunol, 1998. **161**(6): p. 2672-5.

89. Cota, M., et al., *Selective inhibition of HIV replication in primary macrophages but not T lymphocytes by macrophage-derived chemokine.* Proc Natl Acad Sci U S A, 2000. **97**(16): p. 9162-7.

90. Greco, G., C. Mackewicz, and J.A. Levy, *Sensitivity of human immunodeficiency virus infection to various alpha, beta and gamma chemokines.* J Gen Virol, 1999. **80 (Pt 9)**: p. 2369-73.

91. Kim, B.O., et al., *Induction of C chemokine XCL1 (lymphotactin/single C motif-1alpha/activation-induced, T cell-derived and chemokine-related cytokine) expression by HIV-1 Tat protein.* J Immunol, 2004. **172**(3): p. 1888-95.

92. Alkhatib, G., et al., *CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1.* Science, 1996. **272**(5270): p. 1955-8.

93. Choe, H., et al., *The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates.* Cell, 1996. **85**(7): p. 1135-48.

94. Deng, H., et al., *Identification of a major co-receptor for primary isolates of HIV-1.* Nature, 1996. **381**(6584): p. 661-6.

95. Doranz, B.J., et al., *A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors.* Cell, 1996. **85**(7): p. 1149-58.

96. Dragic, T., et al., *HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5.* Nature, 1996. **381**(6584): p. 667-73.

97. Berson, J.F., et al., *A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains.* J Virol, 1996. **70**(9): p. 6288-95.

98. Rucker, J., et al., *Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses.* J Virol, 1997. **71**(12): p. 8999-9007.

99. Horuk, R., et al., *The CC chemokine I-309 inhibits CCR8-dependent infection by diverse HIV-1 strains.* J Biol Chem, 1998. **273**(1): p. 386-91.

100. Choe, H., et al., *The orphan seven-transmembrane receptor apj supports the entry of primary T-cell-line-tropic and dualtropic human immunodeficiency virus type 1.* J Virol, 1998. **72**(7): p. 6113-8.

101. Liao, F., et al., *STRL33, A novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1.* J Exp Med, 1997. **185**(11): p. 2015-23.

102. Deng, H.K., et al., *Expression cloning of new receptors used by simian and human immunodeficiency viruses.* Nature, 1997. **388**(6639): p. 296-300.

103. Farzan, M., et al., *Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection.* J Exp Med, 1997. **186**(3): p. 405-11.

104. Edinger, A.L., et al., *An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus.* J Virol, 1998. **72**(10): p. 7934-40.

105. Doms, R.W. and S.C. Peiper, *Unwelcomed guests with master keys: how HIV uses chemokine receptors for cellular entry.* Virology, 1997. **235**(2): p. 179-90.

106. Doranz, B.J., et al., *Two distinct CCR5 domains can mediate coreceptor usage by human immunodeficiency virus type 1.* J Virol, 1997. **71**(9): p. 6305-14.

107. Liu, R., et al., *Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection.* Cell, 1996. **86**(3): p. 367-77.

108. Samson, M., et al., *Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene.* Nature, 1996. **382**(6593): p. 722-5.

109. Dean, M., et al., *Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study.* Science, 1996. **273**(5283): p. 1856-62.

110. Smith, M.W., et al., *Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study.* Science, 1997. **277**(5328): p. 959-65.

111. Schuitemaker, H., et al., *Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population.* J Virol, 1992. **66**(3): p. 1354-60.

112. Connor, R.I., et al., *Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals.* J Virol, 1993. **67**(4): p. 1772-7.

113. Roos, M.T., et al., *Viral phenotype and immune response in primary human immunodeficiency virus type 1 infection.* J Infect Dis, 1992. **165**(3): p. 427-32.

114. Tersmette, M., et al., *Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex.* J Virol, 1988. **62**(6): p. 2026-32.

115. Tersmette, M., et al., *Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates.* J Virol, 1989. **63**(5): p. 2118-25.

116. Feng, Y., et al., *HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.* Science, 1996. **272**(5263): p. 872-7.

117. Bleul, C.C., et al., *The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes.* Proc Natl Acad Sci U S A, 1997. **94**(5): p. 1925-30.

118. Gupta, S.K., et al., *Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines.* J Biol Chem, 1998. **273**(7): p. 4282-7.

119. Hesselgesser, J., et al., *CD4-independent association between HIV-1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons.* Curr Biol, 1997. **7**(2): p. 112-21.

120. He, J., et al., *CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia.* Nature, 1997. **385**(6617): p. 645-9.

121. Hesselgesser, J., et al., *Neuronal apoptosis induced by HIV-1 gp120 and the chemokine SDF-1 alpha is mediated by the chemokine receptor CXCR4.* Curr Biol, 1998. **8**(10): p. 595-8.

122. Rubbert, A., et al., *Dendritic cells express multiple chemokine receptors used as coreceptors for HIV entry.* J Immunol, 1998. **160**(8): p. 3933-41.

123. Boshoff, C., et al., *Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines.* Science, 1997. **278**(5336): p. 290-4.

124. Nagira, M., et al., *Enhanced HIV-1 replication by chemokines constitutively expressed in secondary lymphoid tissues.* Virology, 1999. **264**(2): p. 422-6.

125. Combadiere, C., et al., *Identification of CX3CR1. A chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1.* J Biol Chem, 1998. **273**(37): p. 23799-804.

126. Lee, C., et al., *Macrophage activation through CCR5- and CXCR4-mediated gp120-elicited signaling pathways.* J Leukoc Biol, 2003. **74**(5): p. 676-82.

127. Marschner, S., et al., *Ligation of human CD4 interferes with antigen-induced activation of primary T cells.* Immunol Lett, 2002. **82**(1-2): p. 131-9.

128. Selliah, N. and T.H. Finkel, *HIV-1 NL4-3, but not IIIB, inhibits JAK3/STAT5 activation in CD4(+) T cells.* Virology, 2001. **286**(2): p. 412-21.

129. Liu, Q.H., et al., *HIV-1 gp120 and chemokines activate ion channels in primary macrophages through CCR5 and CXCR4 stimulation.* Proc Natl Acad Sci U S A, 2000. **97**(9): p. 4832-7.

130. Del Corno, M., et al., *HIV-1 gp120 and chemokine activation of Pyk2 and mitogen-activated protein kinases in primary macrophages mediated by calcium-dependent, pertussis toxin-insensitive chemokine receptor signaling.* Blood, 2001. **98**(10): p. 2909-16.

131. Cicala, C., et al., *Induction of phosphorylation and intracellular association of CC chemokine receptor 5 and focal adhesion kinase in primary human CD4+ T cells by macrophage-tropic HIV envelope.* J Immunol, 1999. **163**(1): p. 420-6.

132. Misse, D., et al., *HIV-1 glycoprotein 120 induces the MMP-9 cytopathogenic factor production that is abolished by inhibition of the p38 mitogen-activated protein kinase signaling pathway.* Blood, 2001. **98**(3): p. 541-7.

133. Misse, D., et al., *A CD4-independent interaction of human immunodeficiency virus-1 gp120 with CXCR4 induces their cointernalization, cell signaling, and T-cell chemotaxis.* Blood, 1999. **93**(8): p. 2454-62.

134. Berger, E.A., P.M. Murphy, and J.M. Farber, *Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease.* Annu Rev Immunol, 1999. **17**: p. 657-700.

135. Blumenthal, R., et al., *Membrane fusion.* Chem Rev, 2003. **103**(1): p. 53-69.

136. Colman, P.M. and M.C. Lawrence, *The structural biology of type I viral membrane fusion.* Nat Rev Mol Cell Biol, 2003. **4**(4): p. 309-19.

137. Jahn, R., T. Lang, and T.C. Sudhof, *Membrane fusion.* Cell, 2003. **112**(4): p. 519-33.

138. Dubay, J.W., et al., *Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity.* J Virol, 1992. **66**(11): p. 6616-25.

139. Gabuzda, D.H., et al., *Effects of deletions in the cytoplasmic domain on biological functions of human immunodeficiency virus type 1 envelope glycoproteins.* J Virol, 1992. **66**(6): p. 3306-15.

140. Wilk, T., et al., *Glycoprotein incorporation and HIV-1 infectivity despite exchange of the gp160 membrane-spanning domain.* Virology, 1996. **218**(1): p. 269-74.

141. Skehel, J.J. and D.C. Wiley, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.* Annu Rev Biochem, 2000. **69**: p. 531-69.

142. Weissenhorn, W., et al., *Crystal structure of the Ebola virus membrane fusion subunit, GP2, from the envelope glycoprotein ectodomain.* Mol Cell, 1998. **2**(5): p. 605-16.

143. Baker, K.A., et al., *Structural basis for paramyxovirus-mediated membrane fusion.* Mol Cell, 1999. **3**(3): p. 309-19.

144. Lu, M., S.C. Blacklow, and P.S. Kim, *A trimeric structural domain of the HIV-1 transmembrane glycoprotein.* Nat Struct Biol, 1995. **2**(12): p. 1075-82.

145. Chan, D.C., et al., *Core structure of gp41 from the HIV envelope glycoprotein.* Cell, 1997. **89**(2): p. 263-73.

146. Salzwedel, K., J.T. West, and E. Hunter, *A conserved tryptophan-rich motif in the membrane-proximal region of the human immunodeficiency virus type 1 gp41 ectodomain is important for Env-mediated fusion and virus infectivity.* J Virol, 1999. **73**(3): p. 2469-80.

147. Weissenhorn, W., et al., *Atomic structure of the ectodomain from HIV-1 gp41.* Nature, 1997. **387**(6631): p. 426-30.

148. Caffrey, M., et al., *Monomer-trimer equilibrium of the ectodomain of SIV gp41: insight into the mechanism of peptide inhibition of HIV infection.* Protein Sci, 1999. **8**(9): p. 1904-7.

149. Malashkevich, V.N., et al., *Crystal structure of the simian immunodeficiency virus (SIV) gp41 core: conserved helical interactions underlie the broad inhibitory activity of gp41 peptides.* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9134-9.

150. Kobe, B., et al., *Crystal structure of human T cell leukemia virus type 1 gp21 ectodomain crystallized as a maltose-binding protein chimera reveals structural evolution of retroviral transmembrane proteins.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4319-24.

151. Fass, D., S.C. Harrison, and P.S. Kim, *Retrovirus envelope domain at 1.7 angstrom resolution.* Nat Struct Biol, 1996. **3**(5): p. 465-9.

152. Gallaher, W.R., et al., *A general model for the transmembrane proteins of HIV and other retroviruses.* AIDS Res Hum Retroviruses, 1989. **5**(4): p. 431-40.

153. Helseth, E., et al., *Human immunodeficiency virus type 1 gp120 envelope glycoprotein regions important for association with the gp41 transmembrane glycoprotein.* J Virol, 1991. **65**(4): p. 2119-23.

154. Maerz, A.L., et al., *Functional analysis of the disulfide-bonded loop/chain reversal region of human immunodeficiency virus type 1 gp41 reveals a critical role in gp120-gp41 association.* J Virol, 2001. **75**(14): p. 6635-44.

155. Chong, Y.H., et al., *Analysis of equine humoral immune responses to the transmembrane envelope glycoprotein (gp45) of equine infectious anemia virus.* J Virol, 1991. **65**(2): p. 1013-8.

156. Gnann, J.W., Jr., J.A. Nelson, and M.B. Oldstone, *Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus.* J Virol, 1987. **61**(8): p. 2639-41.

157. Albini, A., et al., *Identification of a novel domain of HIV tat involved in monocyte chemotaxis.* J Biol Chem, 1998. **273**(26): p. 15895-900.

158. Albini, A., et al., *HIV-1 Tat protein mimicry of chemokines.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13153-8.

159. de Paulis, A., et al., *Tat protein is an HIV-1-encoded beta-chemokine homolog that promotes migration and up-regulates CCR3 expression on human Fc epsilon RI+ cells.* J Immunol, 2000. **165**(12): p. 7171-9.

160. Benelli, R., et al., *Human immunodeficiency virus transactivator protein (Tat) stimulates chemotaxis, calcium mobilization, and activation of human polymorphonuclear*

leukocytes: implications for Tat-mediated pathogenesis. J Infect Dis, 2000. **182**(6): p. 1643-51.

161. Ghezzi, S., et al., *Inhibition of CXCR4-dependent HIV-1 infection by extracellular HIV-1 Tat.* Biochem Biophys Res Commun, 2000. **270**(3): p. 992-6.

162. Xiao, H., et al., *Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11466-71.

163. Koedel, U., et al., *HIV type 1 Nef protein is a viral factor for leukocyte recruitment into the central nervous system.* J Immunol, 1999. **163**(3): p. 1237-45.

164. Cullen, B.R., *HIV-1 Nef protein: An invitation to a kill.* Nat Med, 1999. **5**(9): p. 985-6.

165. Du, Z., et al., *Requirements for lymphocyte activation by unusual strains of simian immunodeficiency virus.* J Virol, 1996. **70**(6): p. 4157-61.

166. Swingler, S., et al., *HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages.* Nat Med, 1999. **5**(9): p. 997-103.

167. Schrager, J.A. and J.W. Marsh, *HIV-1 Nef increases T cell activation in a stimulus-dependent manner.* Proc Natl Acad Sci U S A, 1999. **96**(14): p. 8167-72.

168. Pohlmann, S., et al., *DC-SIGNR, a DC-SIGN homologue expressed in endothelial cells, binds to human and simian immunodeficiency viruses and activates infection in trans.* Proc Natl Acad Sci U S A, 2001. **98**(5): p. 2670-5.

169. Su, S.V., et al., *DC-SIGN binds to HIV-1 gp120 in a distinct but overlapping fashion compared to ICAM-2 and ICAM-3.* J Biol Chem, 2004.

170. Sanejouand, Y.H., *[Role of the conformation changement of CD4 in the HIV-cell fusion].* C R Acad Sci III, 1997. **320**(2): p. 163-70.

171. Furuta, R.A., et al., *Capture of an early fusion-active conformation of HIV-1 gp41.* Nat Struct Biol, 1998. **5**(4): p. 276-9.

172. Baba, M., et al., *A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5698-703.

173. Strizki, J.M., et al., *SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo.* Proc Natl Acad Sci U S A, 2001. **98**(22): p. 12718-23.

174. Kim, D., et al., *Discovery of human CCR5 antagonists containing hydantoins for the treatment of HIV-1 infection.* Bioorg Med Chem Lett, 2001. **11**(24): p. 3099-102.

175. Donzella, G.A., et al., *AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor.* Nat Med, 1998. **4**(1): p. 72-7.

176. De Clercq, E., *The bicyclam AMD3100 story.* Nat Rev Drug Discov, 2003. **2**(7): p. 581-7.

177. Xu, Y., et al., *Marked increase in anti-HIV activity, as well as inhibitory activity against HIV entry mediated by CXCR4, linked to enhancement of the binding ability of tachyplesin analogs to CXCR4.* AIDS Res Hum Retroviruses, 1999. **15**(5): p. 419-27.

178. Murakami, T., et al., *Inhibitory mechanism of the CXCR4 antagonist T22 against human immunodeficiency virus type 1 infection.* J Virol, 1999. **73**(9): p. 7489-96.

179. Ma, Q., D. Jones, and T.A. Springer, *The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment.* Immunity, 1999. **10**(4): p. 463-71.

180. Ma, Q., et al., *Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice.* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9448-53.

181. Kawabata, K., et al., *A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5663-7.

182. Schols, D., et al., *Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4.* Antiviral Res, 1997. **35**(3): p. 147-56.

183. Datema, R., et al., *Antiviral efficacy in vivo of the anti-human immunodeficiency virus bicyclam SDZ SID 791 (JM 3100), an inhibitor of infectious cell entry.* Antimicrob Agents Chemother, 1996. **40**(3): p. 750-4.

184. Hendrix, C.W., et al., *Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers.* Antimicrob Agents Chemother, 2000. **44**(6): p. 1667-73.

185. Allaway, G.P., et al., *Expression and characterization of CD4-IgG2, a novel heterotetramer that neutralizes primary HIV type 1 isolates.* AIDS Res Hum Retroviruses, 1995. **11**(5): p. 533-9.

186. Trkola, A., et al., *Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG.* J Virol, 1995. **69**(11): p. 6609-17.

187. Gauduin, M.C., et al., *Effective ex vivo neutralization of human immunodeficiency virus type 1 in plasma by recombinant immunoglobulin molecules.* J Virol, 1996. **70**(4): p. 2586-92.

188. Wild, C., et al., *A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition.* Proc Natl Acad Sci U S A, 1992. **89**(21): p. 10537-41.

189. Wild, C.T., et al., *Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection.* Proc Natl Acad Sci U S A, 1994. **91**(21): p. 9770-4.

190. Chan, D.C. and P.S. Kim, *HIV entry and its inhibition.* Cell, 1998. **93**(5): p. 681-4.

191. Eron, J.J., et al., *Short-Term Safety and Antiretroviral Activity of T-1249, a Second-Generation Fusion Inhibitor of HIV.* J Infect Dis, 2004. **189**(6): p. 1075-83.

192. Lehner, T., et al., *Protective mucosal immunity elicited by targeted lymph node immunization with a subunit SIV envelope and core vaccine in macaques.* Dev Biol Stand, 1998. **92**: p. 225-35.

193. Lehner, T., et al., *Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques.* Nat Med, 1996. **2**(7): p. 767-75.

194. Lusso, P., *Chemokines and viruses: the dearest enemies.* Virology, 2000. **273**(2): p. 228-40.

195. Ahlers, J.D., I.M. Belyakov, and J.A. Berzofsky, *Cytokine, chemokine, and costimulatory molecule modulation to enhance efficacy of HIV vaccines.* Curr Mol Med, 2003. **3**(3): p. 285-301.

196. Lu, Y., et al., *Macrophage inflammatory protein-1alpha (MIP-1alpha) expression plasmid enhances DNA vaccine-induced immune response against HIV-1.* Clin Exp Immunol, 1999. **115**(2): p. 335-41.

197. Kim, J.J., et al., *Intracellular adhesion molecule-1 modulates beta-chemokines and directly costimulates T cells in vivo.* J Clin Invest, 1999. **103**(6): p. 869-77.

198. Shugars, D.C., et al., *Analysis of human immunodeficiency virus type 1 nef gene sequences present in vivo.* J Virol, 1993. **67**(8): p. 4639-50.

199. Greenway, A.L., et al., *Nef 27, but not the Nef 25 isoform of human immunodeficiency virus-type 1 pNL4.3 down-regulates surface CD4 and IL-2R expression in peripheral blood mononuclear cells and transformed T cells.* Virology, 1994. **198**(1): p. 245-56.

200. Ross, T.M., A.E. Oran, and B.R. Cullen, *Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein.* Curr Biol, 1999. **9**(12): p. 613-21.

201. Jacks, T., et al., *Two efficient ribosomal frameshifting events are required for synthesis of mouse mammary tumor virus gag-related polyproteins.* Proc Natl Acad Sci U S A, 1987. **84**(12): p. 4298-302.

202. Jacks, T., *Translational suppression in gene expression in retroviruses and retrotransposons.* Curr Top Microbiol Immunol, 1990. **157**: p. 93-124.

203. Hatfield, D. and S. Oroszlan, *The where, what and how of ribosomal frameshifting in retroviral protein synthesis.* Trends Biochem Sci, 1990. **15**(5): p. 186-90.

204. Jacks, T., et al., *Signals for ribosomal frameshifting in the Rous sarcoma virus gag-pol region.* Cell, 1988. **55**(3): p. 447-58.

205. Parkin, N.T., et al., *Mutational analysis of the 5' non-coding region of human immunodeficiency virus type 1: effects of secondary structure on translation.* Embo J, 1988. **7**(9): p. 2831-7.

206. Jacks, T., et al., *Characterization of ribosomal frameshifting in HIV-1 gag-pol expression.* Nature, 1988. **331**(6153): p. 280-3.

207. Bidou, L., et al., *In vivo HIV-1 frameshifting efficiency is directly related to the stability of the stem-loop stimulatory signal.* Rna, 1997. **3**(10): p. 1153-8.

208. Shutt, D.C. and D.R. Soll, *HIV-induced T-cell syncytia release a two component T-helper cell chemoattractant composed of Nef and Tat.* J Cell Sci, 1999. **112 (Pt 22)**: p. 3931-41.

209. Chang, C.D. and J. Meienhofer, *Solid-phase peptide synthesis using mild base cleavage of N alpha-fluorenylmethyloxycarbonylamino acids, exemplified by a synthesis of dihydrosomatostatin.* Int J Pept Protein Res, 1978. **11**(3): p. 246-9.

210. Atherton, K.T. and D.C. Burke, *The effects of some different metabolic inhibitors on interferon superinduction.* J Gen Virol, 1978. **41**(2): p. 229-37.

211. Bedford, J., et al., *Amino acid structure and "difficult sequences" in solid phase peptide synthesis.* Int J Pept Protein Res, 1992. **40**(3-4): p. 300-7.

212. Kates, S.A., et al., *Optimized preparation of deca(L-alanyl)-L-valinamide by 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase synthesis on polyethylene glycol-polystyrene (PEG-PS) graft supports, with 1,8-diazobicyclo [5.4.0]-undec-7-ene (DBU) deprotection.* Pept Res, 1996. **9**(3): p. 106-13.

213. Atherton, E., L.R. Cameron, and R.C. Sheppard, *Peptide-Synthesis .10. Use of Pentafluorophenyl Esters of Fluorenyl Methoxycarbonylamino Acids in Solid-Phase Peptide-Synthesis.* Tetrahedron, 1988. **44**(3): p. 843-857.

214. Knorr, R., et al., *New Coupling Reagents in Peptide Chemistry.* Tetrahedron Letters, 1989. **30**(15): p. 1927-1930.

215. Kaiser, E., et al., *Color Test for Detection of Free Terminal Amino Groups in Solid-Phase Synthesis of Peptides.* Analytical Biochemistry, 1970. **34**(2): p. 595-&.

216. Lavi, E., et al., *Chemokine receptors in the human brain and their relationship to HIV infection.* J Neurovirol, 1998. **4**(3): p. 301-11.

217. Mahalingam, S., et al., *Antiviral potential of chemokines.* Bioessays, 2001. **23**(5): p. 428-35.

218. Baggiolini, M., *Chemokines and leukocyte traffic.* Nature, 1998. **392**(6676): p. 565-8.

219. Mahalingam, S., et al., *Chemokines and viruses: friends or foes?* Trends Microbiol, 2003. **11**(8): p. 383-91.

220. Gao, J.L., et al., *Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1.* J Exp Med, 1997. **185**(11): p. 1959-68.

221. Strieter, R.M., et al., *The role of CXC chemokines as regulators of angiogenesis.* Shock, 1995. **4**(3): p. 155-60.

222. Forster, R., et al., *A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen.* Cell, 1996. **87**(6): p. 1037-47.

223. Broxmeyer, H.E., et al., *Dominant myelopoietic effector functions mediated by chemokine receptor CCR1.* J Exp Med, 1999. **189**(12): p. 1987-92.

224. Keane, M.P. and R.M. Strieter, *The role of CXC chemokines in the regulation of angiogenesis.* Chem Immunol, 1999. **72**: p. 86-101.

225. Quackenbush, E.J., et al., *Eotaxin influences the development of embryonic hematopoietic progenitors in the mouse.* J Leukoc Biol, 1997. **62**(5): p. 661-6.

226. Tachibana, K., et al., *The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract.* Nature, 1998. **393**(6685): p. 591-4.

227. Zou, Y.R., et al., *Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development.* Nature, 1998. **393**(6685): p. 595-9.

228. Thali, M., et al., *Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding.* J Virol, 1993. **67**(7): p. 3978-88.

229. Wyatt, R., et al., *The antigenic structure of the HIV gp120 envelope glycoprotein.* Nature, 1998. **393**(6686): p. 705-11.

230. Sattentau, Q.J., et al., *Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding.* J Virol, 1993. **67**(12): p. 7383-93.

231. Trkola, A., et al., *CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5.* Nature, 1996. **384**(6605): p. 184-7.

232. Wu, L., et al., *CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5.* Nature, 1996. **384**(6605): p. 179-83.

233. Rizzuto, C.D., et al., *A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding.* Science, 1998. **280**(5371): p. 1949-53.

234. Lapham, C.K., et al., *Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines.* Science, 1996. **274**(5287): p. 602-5.

235. Murphy, P.M., *Viral exploitation and subversion of the immune system through chemokine mimicry.* Nat Immunol, 2001. **2**(2): p. 116-22.

236. Huang, L., et al., *Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T-lymphotropic HIV-1 strains.* J Virol, 1998. **72**(11): p. 8952-60.

237. Secchiero, P., et al., *Extracellular HIV-1 tat protein up-regulates the expression of surface CXC-chemokine receptor 4 in resting CD4+ T cells.* J Immunol, 1999. **162**(4): p. 2427-31.

238. Gallo, R.C., *Tat as one key to HIV-induced immune pathogenesis and Tat (correction of Pat) toxoid as an important component of a vaccine.* Proc Natl Acad Sci U S A, 1999. **96**(15): p. 8324-6.

239. von Tscharner, V., et al., *Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration.* Nature, 1986. **324**(6095): p. 369-72.

240. Slonczewski, J.L., M.W. Wilde, and S.H. Zigmond, *Phosphorylase a activity as an indicator of neutrophil activation by chemotactic peptide.* J Cell Biol, 1985. **101**(4): p. 1191-7.

241. Marks, P.W. and F.R. Maxfield, *Local and global changes in cytosolic free calcium in neutrophils during chemotaxis and phagocytosis.* Cell Calcium, 1990. **11**(2-3): p. 181-90.

242. Teshima, R., et al., *The effect of staurosporine on Ca2+ signals in rat basophilic leukemia (RBL-2H3) cells.* Chem Pharm Bull (Tokyo), 1991. **39**(3): p. 747-51.

243. Deng, X., et al., *A synthetic peptide derived from human immunodeficiency virus type 1 gp120 downregulates the expression and function of chemokine receptors CCR5 and CXCR4 in monocytes by activating the 7-transmembrane G-protein-coupled receptor FPRL1/LXA4R.* Blood, 1999. **94**(4): p. 1165-73.

244. Baker, R., *Defective HIV may hold clue to effective vaccine.* Beta, 1995: p. 9.

245. Choe, E.Y., et al., *HIV Nef inhibits T cell migration.* J Biol Chem, 2002. **277**(48): p. 46079-84.

246. Janardhan, A., et al., *HIV-1 Nef Binds the DOCK2-ELMO1 Complex to Activate Rac and Inhibit Lymphocyte Chemotaxis.* PLoS Biol, 2004. **2**(1): p. E6.

247. Nishihara, H., et al., *Non-adherent cell-specific expression of DOCK2, a member of the human CDM-family proteins.* Biochim Biophys Acta, 1999. **1452**(2): p. 179-87.

248. Izmailova, E., et al., *HIV-1 Tat reprograms immature dendritic cells to express chemoattractants for activated T cells and macrophages.* Nat Med, 2003. **9**(2): p. 191-7.

249. Robertson, M.J., et al., *Human natural killer cell adhesion molecules. Differential expression after activation and participation in cytolysis.* J Immunol, 1990. **145**(10): p. 3194-201.

250. Moretta, A., et al., *Human natural killer cell receptors for HLA-class I molecules. Evidence that the Kp43 (CD94) molecule functions as receptor for HLA-B alleles.* J Exp Med, 1994. **180**(2): p. 545-55.

251. Miedema, F., *Immunological abnormalities in the natural history of HIV infection: mechanisms and clinical relevance.* Immunodefic Rev, 1992. **3**(3): p. 173-93.

252. Ullum, H., et al., *Defective natural immunity: an early manifestation of human immunodeficiency virus infection.* J Exp Med, 1995. **182**(3): p. 789-99.

253. Hu, P.F., et al., *Natural killer cell immunodeficiency in HIV disease is manifest by profoundly decreased numbers of CD16+CD56+ cells and expansion of a population of CD16dimCD56- cells with low lytic activity.* J Acquir Immune Defic Syndr Hum Retrovirol, 1995. **10**(3): p. 331-40.

254. Jewett, A., M. Cavalcanti, and B. Bonavida, *Pivotal role of endogenous TNF-alpha in the induction of functional inactivation and apoptosis in NK cells.* J Immunol, 1997. **159**(10): p. 4815-22.

255. Ullum, H., et al., *Natural immunity and HIV disease progression.* Aids, 1999. **13**(5): p. 557-63.

256. Sondergaard, S.R., et al., *Epinephrine-induced mobilization of natural killer (NK) cells and NK-like T cells in HIV-infected patients.* Cell Immunol, 1999. **197**(2): p. 91-8.

257. Pu, H., et al., *HIV-1 Tat protein upregulates inflammatory mediators and induces monocyte invasion into the brain.* Mol Cell Neurosci, 2003. **24**(1): p. 224-37.

258. Wu, D.T., et al., *Mechanisms of leukocyte trafficking into the CNS.* J Neurovirol, 2000. **6 Suppl 1**: p. S82-5.

259. Hofman, F.M., et al., *HIV-1 tat protein induces the production of interleukin-8 by human brain-derived endothelial cells.* J Neuroimmunol, 1999. **94**(1-2): p. 28-39.

260. Lokensgard, J.R., et al., *Diazepam inhibits HIV-1 Tat-induced migration of human microglia.* J Neurovirol, 2001. **7**(5): p. 481-6.

261. Kubes, P., et al., *In vivo impairment of neutrophil recruitment during lentivirus infection.* J Immunol, 2003. **171**(9): p. 4801-8.

262. Zhelev, D.V. and A. Alteraifi, *Signaling in the motility responses of the human neutrophil.* Ann Biomed Eng, 2002. **30**(3): p. 356-70.

263. Adams, J.C. and M.A. Schwartz, *Stimulation of fascin spikes by thrombospondin-1 is mediated by the GTPases Rac and Cdc42.* J Cell Biol, 2000. **150**(4): p. 807-22.

264. Holt, M.R. and A. Koffer, *Cell motility: proline-rich proteins promote protrusions.* Trends Cell Biol, 2001. **11**(1): p. 38-46.

265. Mitchison, T.J. and L.P. Cramer, *Actin-based cell motility and cell locomotion.* Cell, 1996. **84**(3): p. 371-9.

266. Mackay, C.R. and B.A. Imhof, *Cell adhesion in the immune system.* Immunol Today, 1993. **14**(3): p. 99-102.

267. Hughes, P.E. and M. Pfaff, *Integrin affinity modulation.* Trends Cell Biol, 1998. **8**(9): p. 359-64.

268. Springer, T.A., *Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration.* Annu Rev Physiol, 1995. **57**: p. 827-72.

269. Zigmond, S.H., *Mechanisms of sensing chemical gradients by polymorphonuclear leukocytes.* Nature, 1974. **249**(456): p. 450-2.

270. Schroder, J.M., *Chemoattractants as mediators of neutrophilic tissue recruitment.* Clin Dermatol, 2000. **18**(3): p. 245-63.

271. Murphy, P.M., *The molecular biology of leukocyte chemoattractant receptors.* Annu Rev Immunol, 1994. **12**: p. 593-633.

272. Ben-Baruch, A., D.F. Michiel, and J.J. Oppenheim, *Signals and receptors involved in recruitment of inflammatory cells.* J Biol Chem, 1995. **270**(20): p. 11703-6.

273. Jimenez, C., et al., *Role of the PI3K regulatory subunit in the control of actin organization and cell migration.* J Cell Biol, 2000. **151**(2): p. 249-62.

274. Blume-Jensen, P., et al., *Modulation of Kit/stem cell factor receptor-induced signaling by protein kinase C.* J Biol Chem, 1994. **269**(34): p. 21793-802.

275. Zhou, K., et al., *Disruption of Dictyostelium PI3K genes reduces [32P]phosphatidylinositol 3,4 bisphosphate and [32P]phosphatidylinositol trisphosphate levels, alters F-actin distribution and impairs pinocytosis.* J Cell Sci, 1998. **111 (Pt 2)**: p. 283-94.

276. Hirsch, E., et al., *Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation.* Science, 2000. **287**(5455): p. 1049-53.

277. Movilla, N., et al., *How Vav proteins discriminate the GTPases Rac1 and RhoA from Cdc42.* Oncogene, 2001. **20**(56): p. 8057-65.

278. Rohatgi, R., et al., *The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly.* Cell, 1999. **97**(2): p. 221-31.

279. Bamburg, J.R., *Proteins of the ADF/cofilin family: essential regulators of actin dynamics.* Annu Rev Cell Dev Biol, 1999. **15**: p. 185-230.

280. Nakano, K., et al., *Distinct actions and cooperative roles of ROCK and mDia in Rho small G protein-induced reorganization of the actin cytoskeleton in Madin-Darby canine kidney cells.* Mol Biol Cell, 1999. **10**(8): p. 2481-91.

281. Sullivan, R., L.S. Price, and A. Koffer, *Rho controls cortical F-actin disassembly in addition to, but independently of, secretion in mast cells.* J Biol Chem, 1999. **274**(53): p. 38140-6.

282. Wang, J. and E.J. Brown, *Immune complex-induced integrin activation and L-plastin phosphorylation require protein kinase A.* J Biol Chem, 1999. **274**(34): p. 24349-56.

283. Evans, E. and A. Yeung, *Apparent viscosity and cortical tension of blood granulocytes determined by micropipet aspiration.* Biophys J, 1989. **56**(1): p. 151-60.

284. Zhelev, D.V., D. Needham, and R.M. Hochmuth, *Role of the membrane cortex in neutrophil deformation in small pipets.* Biophys J, 1994. **67**(2): p. 696-705.

285. Stossel, T.P., *On the crawling of animal cells.* Science, 1993. **260**(5111): p. 1086-94.

286. Gong, J.H. and I. Clark-Lewis, *Antagonists of monocyte chemoattractant protein 1 identified by modification of functionally critical NH2-terminal residues.* J Exp Med, 1995. **181**(2): p. 631-40.

287. Suzuki, K., et al., *Localization of chemotactic activity and 64 kD protein phosphorylation for human polymorphonuclear leukocytes in N-terminus of the chemotactic protein LUCT/IL-8.* Biochem Biophys Res Commun, 1989. **163**(3): p. 1298-305.