INTRACELLULAR INHIBITION OF IMMUNE DYSFUNCTION INDUCED BY HIV-1 NEF PROTEIN

by

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ABSTRACT

Current ‘cocktail-therapy’ toward HIV-1 infection using reverse transcriptase and protease inhibitors have been successful in controlling the viral growth, but not very effective in eradicating the reservoir of HIV-1 infected cells. It is a new challenge for HIV therapy to find ways to remove the virus reservoir that is composed of latently infected CD4+ T cells carrying integrated provirus. A potential new therapeutic target is Nef, a HIV-1 viral protein that downregulates class I MHC and by doing so it enables infected cells to elude killing by cytotoxic T lymphocytes.

In this thesis research, intracellular inhibition of Nef-mediated downregulation of CD4 and MHC-1 molecules was studied using recombinant single-chain antibodies (ScFvs) and a dominant-negative Hck. Several anti-Nef single-chain antibodies were first constructed. All retained the binding activity of their corresponding parental monoclonal antibodies when expressed intracellularly. However, ScFv expression was unable to inhibit CD4 or MHC-1 downregulation induced by Nef. This indicated that the intracellular binding of ScFv with Nef and the following Nef sequestration may not be sufficient to prevent the receptor downregulation events induced by Nef. The expression of molecules capable of binding to epitopes in Nef, that are implicated specifically in receptor modulation, may be required for these effects.
A dominant-negative form of Hck protein-tyrosine kinase, DN-Hck, composed of the Hck amino terminal region and its SH3 and SH2 domains, was then studied as a potential candidate for preventing MHC-1 downregulation; it is known that the Hck SH3 domain binds Nef with a very high affinity (Kd=0.25 μM). In addition, the SH3-binding motif in Nef, PXXP78 is also a major determinant in downregulation of MHC-1. It was demonstrated that DN-Hck was able to block Nef-induced downregulation of class I MHC surface expression in human cells. This effect required a functional SH3 domain, as it was not evident in cells that expressed DN-Hck-W93F, an SH3 domain mutation that results in diminished binding affinity for Nef. The results in this thesis research thus support a model that DN-Hck prevents Nef-induced class I downregulation by blocking the interaction between Nef and an as yet unidentified SH3-containing cellular protein that is capable of coupling Nef to the MHC-1 molecule. Upon binding with Nef, this cellular protein might recruit class I MHC molecules via a specific interaction with their cytoplasmic motifs, which in turn routes these molecules towards an intracellular degradation pathway. The SH3-binding region of Nef therefore represents a new target for therapeutic intervention in individuals infected with HIV-1.
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LIST OF ABBREVIATIONS

AIDS------------------acquired immunodeficiency syndrome
AP-1------------------adaptor protein complex-1
AP-2------------------adaptor protein complex-2
β-COP-I----------------β subunit of coat protein-I
CA------------------capsid protein (p24 in HIV)
CD4------------------clusters of differentiation-4
CDR------------------complementarity determining region
CTL------------------cytotoxic T-lymphocyte
DN-Hck----------------dominant-negative Hck
EF-1α promoter----------------Elongation factor-1α promoter
ELISA------------------enzyme-labeled immunosorbant assay
ER------------------endoplasmic reticulum
Env------------------proteins encoded by envelope orf
Gag------------------protein encoded by group-specific antigen open reading frame
GFP------------------green fluorescent protein
GST------------------glutathione S-transferase
HAART------------------highly active anti-retroviral therapy
Hck------------------hematopoietic cell kinase
HLA------------------human leukocyte antigen
HIV-1------------------human immunodeficiency virus-1
HIV-2------------------human immunodeficiency virus-2
HTLV------------------human T-cell leukemia virus
IN-------------------------integrase
LAV------------------------lymphoadenopathy-associated virus
LTNP------------------------long term non-progressor
LTR-------------------------long terminal repeat
MA--------------------------matrix protein (p17 in HIV)
Mab-------------------------monoclonal antibody
MHC-------------------------major histocompatibility complex
NC--------------------------nuclear capsid protein (p7 in HIV)
NK--------------------------natural killer cells
Nef-------------------------negative factor
NMR-------------------------nuclear magnetic resonance
orf--------------------------open reading frame
PBMC------------------------peripheral blood mononuclear cells
PBS--------------------------phosphate buffered saline
PCR--------------------------polymerase chain reaction
PHA-------------------------phytohemagglutinin
Pol--------------------------protein encoded by polymerase open reading frame
PR--------------------------protease
Rev-------------------------regulator of viral expression
RRE-------------------------Rev responsive element
RT--------------------------reverse transcriptase
RT loop---------------------a region of the SH3 domain which has functionally important arginine and threonine residues.
ScFv------------------------single-chain (variable fragment) antibody.
SCID------------------------severe combined immunodeficiency.
SH2 —------------------------Src homology domain 2
SH3 —------------------------Src homology domain 3
SIV —------------------------simian immunodeficiency virus
Src —------------------------src oncogene of Rous sarcoma virus
SU —------------------------surface protein (gp120 in HIV)
TAR —------------------------trans-activating response element
Tat —------------------------trans-activator of viral transcription
TCR —------------------------T cell receptor
TGN —------------------------trans-Golgi network
TM —------------------------transmembrane protein (gp41 in HIV)
Vif —------------------------viral infectivity factor
VH —------------------------variable region of the heavy chain
VK —------------------------variable region of the kappa light chain
Vpr —------------------------viral protein R
Vpu —------------------------viral protein U
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DEDICATION

To my parents, who are not here to see this day,
I hope that I can make you proud.

And

To my wife, Yanbo,
Thanks for your patience, I promise that we will have a normal life from now on.

And

To my two boys, Arthur and Eddie,
You make me feel young and energized everyday.
CHAPTER 1
INTRODUCTION

1.1 Foreword

Nef is an important regulatory protein of the primate lentiviruses, human immunodeficiency viruses (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV). Although the exact role of Nef in the life cycle of lentiviruses remains to be fully elucidated, it is known that Nef is essential for high viral load and increased disease progression in rhesus monkey animal models infected with SIV, as well as individuals infected with HIV-1 (Deacon et al., 1995; Kirchhoff et al., 1995). Several distinct functions of Nef have been characterized in vitro. First, Nef downregulates the cell surface expression of CD4 and MHC-1 receptors (Piguet et al., 1999b). Second, Nef increases viral infectivity at a stage after entry of the virus into the cell (Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al., 1995). Nef also alters cellular signal transduction and activation pathways (Baur et al., 1994; Hanna et al., 1998; Iafrate et al., 1997; Skowronska et al., 1993).

Nef, in concert with Env and Vpu, downregulates CD4 receptors. This function may serve to inhibit superinfection and increase the infectivity of the viruses (Piguet et al., 1999b). Nef-induced MHC-1 downregulation may play an important role in the pathogenesis of HIV-1 infection through immune escape by protecting infected cells from recognition of cytotoxic T lymphocytes (Collins et al., 1998).
The mechanisms used by Nef in CD4 and MHC-1 downregulation are different. Moreover, Nef uses distinctive determinants for the two activities. Nef downregulates CD4 by acting as a connector between the receptor and intracellular trafficking pathways causing CD4 internalization (Mangasarian et al., 1997). Nef associates with the CD4 cytoplasmic tail by recognizing the dileucine-based motif that also functions as an endocytosis signal (Aiken et al., 1994; Hua and Cullen, 1997). The immediate downstream partner of Nef for CD4 downregulation is the clathrin-associated adaptor protein complex-2 (AP-2) (Piguet et al., 1999b). The internalized CD4 molecules are then targeted for lysosomal degradation through an interaction with β subunit of coat protein-I (β-COP-I) in endosomes (Piguet et al., 1999a).

The pathway through which MHC-1 molecules are modulated by Nef is yet to be elucidated. No direct association has been found between Nef and MHC-1 (Le Gall et al., 1998). A cryptic tyrosine-based sorting signal motif in the cytoplasmic domain of HLA-A and -B heavy chains was revealed, in connection with MHC-1 downregulation in the presence of Nef (Le Gall et al., 1998). The HLA-C molecules, which do not bear the tyrosine-sorting signal motif, are not affected by Nef (Le Gall et al., 1998). The adaptor protein complex-1 (AP-1) may be involved in the MHC-1 downregulation by Nef, since AP-1 co-localizes with MHC-1 in the trans-Golgi network (Greenberg et al., 1998b; Le Gall et al., 1998) and binds with Nef in a yeast two-hybrid system as well as in cell-free assays (Le Gall et al., 1998).
The ability of Nef to downregulate MHC-1 receptor expression requires a well-conserved Src homology 3 (SH3) domain-binding PXXP motif in the Nef core (Greenberg et al., 1998b), which is dispensable for the down-modulation of CD4 receptors by Nef (Goldsmith et al., 1995; Saksela et al., 1995). This indicates that a cellular protein with an SH3 domain capable of interaction with Nef may be involved in the MHC-1 modulation activity. Several tyrosine kinases of the Src family bind to the polyproline (PXXP78) motif in Nef via their SH3 domains (Lee et al., 1996; Saksela et al., 1995). Full-length Nef binds to the Hck SH3 domain with the highest affinity reported for an SH3-mediated interaction (Kd = 0.25 µM) (Lee et al., 1995).

The research described in this thesis uses two approaches to inhibit the effects of Nef expression in the host cells as indicated by CD4 and MHC-1 expression. One approach is to use recombinant intracellular single-chain antibody (ScFv), which has been successfully employed to study the function of cytosolic proteins by either blocking the function or sequestrating the protein of interest. ScFvs, which have been shown to have specific binding affinities equivalent to those of the parent monoclonal antibodies (Whitlow, 1991b; Winter and Milstein, 1991), can be stably expressed intracellularly where they are capable of inactivating specific cellular gene products (Biocca et al., 1990; Carlson, 1988). Intracellular ScFv proteins with specificity for virally-encoded proteins thus provide a unique way of studying the role of these viral proteins in HIV-1 infection (Marasco et al., 1993), as well as offering a potential gene therapy strategy for inhibiting the development of AIDS.
Another approach is to use a dominant-negative form of Hck, a protein tyrosine kinase that was shown to have high binding affinity with Nef through its SH3 domain. Dominant-negative Hck (DN-Hck) consisting of the Hck amino-terminal domain, together with its SH3 and SH2 domains has been co-expressed with proviral DNA in HIV-1 producer cells and the virus so produced has been shown to have reduced viral infectivity (Tokunaga et al., 1998). In addition to the proposed mechanism that DN-Hck may bind to the Src family kinases and hence inhibit their enzymatic activity (Tokunaga et al., 1998), it may also reduce viral infectivity through binding with the polyproline motif in Nef and thereby blocking the downstream interaction of cellular proteins with Nef. The latter mechanism raises the possibility that the SH3-binding motif in Nef may be a good therapeutic target, since this motif is involved in class I molecule downregulation and several other detrimental effects of Nef on the host cells in HIV-1 infection (Arold et al., 1997; Briggs et al., 1997; Collette et al., 1996; Goldsmith et al., 1995; Greenberg et al., 1998b; Mangasarian et al., 1999).

1.2 Discovery of the AIDS virus

AIDS (acquired immunodeficiency syndrome) was first found to be caused by a retrovirus in 1983, when scientists at the Pasteur Institute recovered a reverse-transcriptase-containing virus from the lymph node of a patient with persistent lymphadenopathy; accordingly, this virus was designated lymphadenopathy-associated virus (LAV) (Barre-Sinoussi et al., 1983; Wain-Hobson et al., 1991). Lymphadenopathy-associated virus was shown to replicate and cause cytopathology in cultures of human peripheral blood lymphocytes. At the same time, human T-cell leukemia virus (HTLV) was reportedly isolated from
individuals with AIDS and was suggested to be the causative agent (Gallo et al., 1983). Further studies in 1983 by Montagnier and coworkers refuted the notion that HTLV is the virus responsible for AIDS (Chermann et al., 1983). Their results indicated that this retrovirus, although similar to HTLV in infecting CD4+ lymphocytes, had quite distinct properties. The virus they isolated grew to substantial titer in CD4+ cells and killed them instead of transforming the cells in culture as HTLV does. In early 1984, Gallo and associates reported another HTLV that has the characteristics of a human retrovirus with lymphotropic and cytopathic properties. Levy and coworkers also reported, in 1984, the identification of retroviruses recovered from AIDS patients from different known risk groups (Levy et al., 1984). These viruses were later confirmed to be members of the same group of retroviruses from lentivirinae that had many properties distinguishing them from HTLV (Table 1) (Levy, 1994). In 1986 the International Committee on Taxonomy of Viruses recommended giving the AIDS virus a separate name, the human immunodeficiency virus (Levy, 1994).

### Table 1: Comparison of HIV and HTLV (Table reproduced from Levy, 1994)

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<th>HTLV</th>
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<td>Lentivirus</td>
<td>HTLV/BLV</td>
</tr>
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<td>Genome size (kb)</td>
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<td>9.0</td>
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<tr>
<td>Core morphology</td>
<td>Cone</td>
<td>Cuboid</td>
</tr>
<tr>
<td>Accessory genes</td>
<td>6</td>
<td>2</td>
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<tr>
<td>Cytotoxic</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cell transformation</td>
<td>-</td>
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HIV isolates were subsequently recovered from the blood of many patients with AIDS, as well as from the PBMC of clinically healthy individuals (Levy et al., 1985; Salahuddin et al., 1985). Soon after the discovery of HIV-1, a separate virus type, HIV-2, was identified in western Africa (Clavel et al., 1986). Although both of these viruses cause AIDS, individuals infected with HIV-2 exhibit a longer period of clinical latency and lower morbidity (Essex M, 1994).

The discovery of additional distinct lentiviruses in non-human primates as well as in humans has provided important insights into the biologic significance and evolutionary relationships of these viruses. In 1985, a lentivirus was isolated from captive Asian Macaques with an AIDS-like disease (Daniel et al., 1985) and was later designated as simian immunodeficiency virus (SIV) because of its morphologic similarity and serologic cross-reactivity. Additional lentiviruses have since been isolated from several monkey species in the wild throughout several regions in Africa (Hirsch VM, 1993) in the indigenous or natural host, these viruses do not produce disease (Gardner MB, 1994). SIV is the closest known animal-virus relative of HIV, although it is only about 50% related on the basis of sequence analysis. Interestingly, several HIV-2 isolates of West African origin are almost indistinguishable at the nucleotide sequence level from certain strains of SIV (Gao et al., 1992), indicating that the primate and human viruses share evolutionary roots and that there might have been interspecies infection (Essex and Kanki, 1988).
1.3 The immunopathogenesis of HIV infection

The typical course of HIV infection is characterized by multiple phases that occur over a period of eight to ten year (Abrams et al., 1984; Buchbinder et al., 1994; Fauci, 1991; Lifson et al., 1991). It generally includes three phases 1) primary infection, which is characterized in approximately 50-70% of infected individuals by nonspecific symptoms such as fever, lethargy, sore throat, myalgias, lymphadenopathy, and macupapular rash (Clark et al., 1991; Daar et al., 1991; Tindall and Cooper, 1991); 2) clinical latency, which varies in length, with an average of 10 years, during which there is lack of clinical symptoms; 3) AIDS disease phase, which is characterized by low peripheral blood CD4+ T cell counts (<200 per μl), severe and persistent constitutional symptoms and increased susceptibility to neoplasms and opportunistic infections.

Based on the clinical course of HIV infection, several subgroups of patients have been identified (Borrow et al., 1994; Collins and Baltimore, 1999; Koup et al., 1994; Pantaleo and Fauci, 1996). These include typical progressors who represent the majority of HIV-infected individuals (Figure 1). The median time from initial infection to progression to AIDS in typical progressors is eight to ten years (Buchbinder et al., 1994; Fauci, 1991; Lifson et al., 1991). Subjects who have an unusually rapid progression of disease are called rapid progressors. Those who do not experience progressive disease for several years (eight to ten) following primary infection are called long-term nonprogressors. Finally, those who progress to AIDS within a time frame similar to typical progressors but with stable clinical and laboratory parameters for unusually long period of time once disease progression has occurred are named long-term survivors.
Figure 1 Typical clinical course of HIV infection on the basis of the changes of CD4+ T-cell counts and viremia over time. Following primary infection, typical progressors experience a long period of clinical latency. Progression to AIDS generally occurs within eight to ten years. ■ CD4 T cell count, ● Culturable plasma viremia, ▲ HIV RNA Copies per ml plasma.
(Figure reproduced from Pantaleo et al., 1994).
HIV infection occurs at the sites of bloodstream or mucosa. Following the initial peak of viremia, HIV-specific humoral and cell-mediated immune responses are readily detected (Borrow et al., 1994; Graziosi et al., 1993; Koup et al., 1994; Moore et al., 1994; Pantaleo et al., 1994a; Reimann et al., 1994). A variety of anti-HIV antibodies and cytotoxic T lymphocytes (CTLs) specific for different HIV proteins are detected very early during primary infection. High titers of antibodies specific for a variety of HIV proteins are a major component of the primary immune response to the virus (Clark et al., 1991; Daar et al., 1991; Moore et al., 1994; Tindall and Cooper, 1991). However, the HIV antibodies produced during primary infection lack neutralizing activity against HIV-1 infection (Koup et al., 1994; Moore et al., 1994). Therefore, these antibodies may have little effect on initial virus dissemination and the primary HIV-specific antibody response is generally considered nonprotective. However, this antibody response may contribute significantly to the dramatic reduction of viremia by trapping the circulating virus particles in the lymph tissue. This notion was supported by the SIV animal model of acute infection, which demonstrated that the decline of HIV viremia coincides with a rise in HIV-specific antibody titers, and with the intensity of virus trapping in lymph nodes (Pantaleo and Fauci, 1995; Pantaleo et al., 1994b).

HIV-specific CTLs are considered to play a major role in the initial suppression of virus replication. Virus-specific cytotoxic CD8+ T cells can be detected as early as five days following infection, as indicated by experiments performed in the SIV model of acute infection (Reimann et al., 1994). Similarly, in most subjects with primary HIV infection, virus-specific CTLs are consistently and readily detected
in PBMC during the acute viral infection, and their appearance correlates with the reduction of viremia. In general, HIV-specific cytotoxic activity may be detected against both structural (Env and Gag) and regulatory (Tat and Rev) proteins of HIV. However, during the primary infection HIV specific cytotoxic activity is detected predominantly against structural proteins. In addition to mediating cytotoxic activity, it is also demonstrated that CD8+ T cells from subjects with primary HIV infection release a number of soluble factors (chemokines, such as RANTES, MIP-1α, MIP-1β and IL-16) with suppressing activity on virus replication (Baier et al., 1995; Cocchi et al., 1995). Therefore, CD8+ T lymphocytes may contribute to the suppression of viremia either by elimination of virus-infected cells via a cytotoxic mechanism or by soluble factor-mediated suppression of virus replication.

Despite the early appearance of a vigorous immune response, this immune reaction ultimately fails to eliminate the virus since transition to the chronic phase of infection occurs in most individuals (Pantaleo et al., 1993b). Furthermore, although a dramatic suppression of viremia is observed in infected individuals as progression to the chronic phase of infection occurs, virus replication is never completely curtailed. It has been well established in recent studies that HIV actively and continuously replicates even during the period of clinical latency, and that lymphoid organs serve as the primary site of virus replication (Embretson et al., 1993; Pantaleo et al., 1991; Pantaleo et al., 1993a; Piatak et al., 1993). These observations indicated that HIV disease is active and progressive throughout the course of infection and that clinical latency is not equivalent to silence in disease. This persistent virus replication and the chronic
immune activation associated with it have been proposed to be responsible for the progressive destruction of lymphoid tissue, and hence the deterioration of the immune system (Fauci, 1993; Pantaleo and Fauci, 1994).

In favor of this hypothesis, recent studies have demonstrated that virus replication and turnover are extremely high, and that there is a direct correlation between the level of viremia and changes in CD4+ T cell counts (Ho et al., 1995; Wei et al., 1995). It has been suggested that there is an extremely high turnover of CD4+ T cells and that a large number of CD4+ T cells are depleted and replaced daily. However, the mechanisms responsible for this rapid increase of CD4+ T cells following virus suppression remain to be elucidated.

Taken together, this knowledge and observations have greatly contributed to a better understanding and delineation of the pathogenic mechanisms leading to progression of HIV disease, and they have provided great insights for the current 'cocktail' antiviral therapy and for the future development of therapeutic strategies.

1.4 The challenge of HIV therapy
Despite the decline of deaths from AIDS in industrial countries, that stemmed mainly from the introduction of powerful therapies able to retard the activity of HIV in 1996, the international pandemic of HIV infection and AIDS is still expanding rapidly in the developing nations where the vast majority of people reside (Mann and Tarantola, 1998). According to new estimates from the joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health
Organization (WHO) (AIDS epidemic update: December 1999, UNAIDS Joint United Nations Program on HIV/AIDS), 32.4 million adults and 1.2 million children were living with HIV by the end of 1999, and almost 16.3 million people have died since the beginning of the epidemic in early 1980s. In 1999 alone, some 5.6 million people, close to 15,000 people a day, acquired HIV, and some 2.6 million perished from it, including 470,000 children. Control of the pandemic will require extensive prevention programs and more effective and economic therapies.

HIV infection presented a new challenge to modern medicine: the retroviruses can integrate into the genome of host cells, where they can lie dormant for as long as the life of the host cells. Without effective therapeutic intervention, HIV infection is almost invariably a progressive, lethal disease that completely destroys the patient's immune system and causes death of the infected individual from opportunistic infections.

Better understanding of how HIV behaves in the body and more choice of drugs in the anti-HIV arsenal have led to several break-throughs in medical history that provide much brighter prospects for most patients who receive treatment today (Bartlett and Moore, 1998). With the advance of highly sensitive viral detection technology, it is clear now that HIV replicates prolifically from the onset of the infection. HIV levels remain fairly stable for several years only because the body responds to the infection by manufacturing extraordinary numbers of CD4+ T cells. Patients who display cellular immunity usually have lower viral load and their disease course usually progress more slowly than
individuals who mount a weaker immune response. In addition, viral levels seem to correlate with prognosis. Such findings indicate that the amount of virus or the viral load in the host immune system plays a major role in determining disease progress. Therapy must therefore aim to suppress viral replication immediately following infection and throughout the disease course.

So far, all approved anti-HIV drugs attempt to block viral replication within cells by inhibiting either reverse transcriptase or the HIV protease. There are two classes of drugs that inhibit reverse transcriptase and thus prevent viral RNA copying itself into cDNA for viral integration. The nucleoside analogues resemble the natural substances that become building blocks of HIV DNA and terminate the reverse transcription process when added to a developing strand of HIV DNA. This group includes the first anti-HIV drug-zidovudine (AZT) that was introduced in 1987, and its close chemical relatives. Non-nucleoside reverse transcriptase inhibitors such as nevirapine bind close to the polymerase active site and thereby keep the RT inactive. A relatively new class of drugs, protease inhibitors, block the active catalytic site of the HIV protease and thereby preventing it from cleaving newly translated HIV proteins and hereby inhibit the subsequent viral assembly.

The current optimal therapy to achieve maximum viral suppression is the 'cocktail-therapy' or HAART (highly active antiretroviral therapy) (Figure 2). At the moment, HAART usually consists of triple therapy, including two nucleoside analogues and a protease inhibitor (Bartlett and Moore, 1998). Treatment with these potent antiretroviral regimens can produce sustained suppression of HIV-
1 replication, with reduction of HIV RNA in infected individuals to below the limits of detection in blood for two or more years (Gulick et al., 1997). Patients receiving HAART have seen their CD4 + T cells rebound significantly as a sign of the immune system in recovery (Autran et al., 1997).

While the powerful combination therapies can suppress HIV in the blood stream of infected patients to a level below the limits of detection using the most sensitive detection methods, the virus continues to persist in a dormant form in a small number of CD4+ T lymphocytes. This reservoir of latently infected cells does not die out and take the virus with them into eradication, as many AIDS researchers had predicted (Perelson et al., 1997). Clinical evidence has shown that HIV continues to replicate at a low level even in patients with undetectable levels of virus in their blood (Ho, 1997). Moreover, the progeny viruses are wild-type rather than drug-resistant strains, indicating that even viruses sensitive to the drugs are not completely suppressed.

The key issue for virus eradication seems to be removing the virus reservoir that is composed of latently-infected memory CD4+ T cells carrying integrated provirus (Chun et al., 1997; Chun et al., 1995; Coffin, 1995). Post-integration latency appears to result from the reversion of productively infected CD4+ T lymphoblast to a resting memory state in which there is minimal transcription of viral genes. Because memory CD4+ T cells can persist for months to years (McLean and Michie, 1995; Michie et al., 1992), resting memory CD4+ T cells carrying replication-competent viral genomes may represent an important long-term viral reservoir in patients on HAART. More research is required to
Figure 2 Current HIV therapy. Current HIV drugs aim to stop viral replication by inhibiting reverse transcriptase or protease. Other drugs under investigation are also shown. (Reprint with permission, from Tomo Narashima, Medical & Science Illustrator and Scientific American).
overcome this problem as current therapy is not effective in eradicating provirus integrated in resting T lymphocytes.

In this thesis research, Nef is examined as a potential drug target, since one of its detrimental effects in the infected cells is to downregulate MHC-1 and in doing so it enables virus infected cells to elude killing by cytotoxic T-lymphocytes. Interfering with Nef-induced class I MHC downregulation may therefore represent a novel strategy for increasing HIV-1-specific cytotoxic cell activity against infected cells. Therapies designed to inhibit MHC-1 downregulation-induced by Nef will be potentially beneficial to patients in all stages of HIV infection, since its main function is to make the infected cells more ‘visible’ to the CTLs-cellular immune system that is responsible to clear virus infected cells from the immune system. It is hopeful that together with other powerful anti-HIV treatment which inhibits viral replication, they may eventually reach the goal of curing HIV infection.

1.5 An overview of Human immunodeficiency virus type-1

Human immunodeficiency virus type 1 (HIV-1) has been the subject of intense investigation for almost two decades, and a great deal has been learned about how the retrovirus infects cells, replicates, and causes disease (reviewed in (Frankel and Young, 1998)).

The HIV-1 genome encodes nine open reading frames (Figure 3). Three of these encode the Gag, Pol, and Env polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses. The four Gag proteins,
Figure 3 Organization of the HIV-1 genome and virion. The HIV-1 genome is around 9 kb and encodes nine open reading frames. Three of these encode the Gag, Pol, and Env polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses. The four Gag proteins, matrix (MA), capsid (CA), nucleocapsid (NC), and p6, and the two Env proteins, gp120 or surface protein (SU) and gp41 or transmembrane protein (TM), are structural components that make up the core of the virion and outer membrane envelope. The HIV-1 genome is around 9 kb and encodes nine open reading frames. Three of these encode the Gag, Pol, and Env polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses. The four Gag proteins, matrix (MA), capsid (CA), nucleocapsid (NC), and p6, and the two Env proteins, gp120 or surface protein (SU) and gp41 or transmembrane protein (TM), are structural components that make up the core of the virion and outer membrane envelope. The three Pol proteins, protease (PR), reverse transcriptase (RT), and integrase (IN), provide essential enzymatic functions and are also encapsulated within the particle. HIV-1 also encodes six regulatory proteins, three of which (Vif, Vpr and Nef) are found in the viral particle. Two other regulatory proteins, Tat and Rev, provide essential gene regulatory functions, and the last regulatory protein, Vpu, assists in assembly of the virion by releasing Env from endoplasmic reticulum.

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The cycle of HIV-1 infection and replication is involved with the following distinct steps (reviewed in (Frankel and Young, 1998)) (Figure 4). Viral entry (Step 1 and 2, Figure 4) is initiated by the binding of the SU glycoprotein, located on the viral membrane surface, to specific cell surface receptors. The major receptor for HIV-1 is CD4, an immunoglobulin-like protein expressed on the surface of a subset of T cells and primary macrophages. The SU-CD4 interaction alone is not sufficient for HIV-1 entry. Instead a group of chemokine receptors (a family of seven transmembrane G-coupled proteins, e.g. CXCR4, CCR5, CCR3, etc.) serve as essential viral coreceptors (Berger et al., 1999). Following binding, the transmembrane protein (TM), gp41, undergoes a conformational change that promotes virus-cell membrane fusion, thereby allowing entry of the viral core into the cell.
Figure 4 HIV-1 replication cycle.

HIV-1 infection begins with virus binding to a susceptible target cell via a specific interaction between the viral gp120 envelope glycoprotein and the CD4 cell-surface receptor (step 1) (Young, 1997). Following binding, a process of membrane fusion, facilitated by the viral gp41 envelope glycoprotein and by the HIV coreceptors, CXCR4 OR CCR5, results in the introduction of the HIV-1 core particle into the cell cytoplasm (step 2). In activated and proliferating T-lymphocytes, reverse transcription of the viral RNA (step 3) and the integration of resulting DNA copy into the host-cell chromosome ensues (step 4). In resting cells, however, these events proceed inefficiently, if at all. Once integrated in the chromosome, the transcriptional activity of the HIV-1 provirus is regulated by constitutive host-cell transcription factors (i.e., Sp1 and the TATA-binding factors), the activation-inducible members of the NF-kB family of host transcription factors (i.e., p50 and p65), as well as the virally encoded tat protein (step 5). Following synthesis of a full-length viral RNA, a complex array of alternatively spliced viral mRNAs can be produced. The differential expression of distinct species of viral mRNAs is controlled by the HIV-1 Rev protein. The level of Rev present in an infected cell determines the preferential production of either the unspliced or singly spliced RNAs that provide viral RNA genomes or encode essential structural or enzymatic proteins (i.e., Gag, Pol, and Env), or the multiply spliced mRNAs that encode the viral regulatory gene products (i.e., Tat, Rev, and Nef). In circumstances in which the amount of Rev present in an infected cell is limiting, such as the early stages of viral infection or when the overall level of transcription is low, only the multiply spliced mRNA transcripts are available in the cytoplasm for the translation of viral proteins (step 6). Once a sufficient level of rev accumulates, the singly spliced and unspliced HIV-1 RNAs appear in the cytoplasm, and the synthesis of viral structural proteins can proceed (step 7). HIV-1 particles assemble at the host-cell surface (step 8), and they acquire viral env proteins as they bud through the host-cell membrane. The viral Gag and Gag-Pol polyproteins are cleaved by viral protease during or shortly after budding, generating mature infectious virions (step 9).

HIV Env-CD4 Binding

CD4

HIV-1

Membrane Fusion and Entry

Virion budding and maturation

Cyttoplasm

Single stranded RNA

Reverse Transcription

Linear double stranded DNA

Virion Assembly

Translation

Multiply spliced RNAs (Tat, Rev, Nef)

u5 u3 r

u5 u3 r gag-pol mRNA

Rev-Independent Cytoplasmic Expression

Rev-Dependent Cytoplasmic Expression

Integration

Proviral Transcription

NUCLEUS

Rev

u5 u3 r env mRNA

U3 R U5

U3 R U5

U3 R U5

K B Sp 1 TATA

NF-xB Sp 1

Inducible and Constitutive Host Factors

5' LTR
The virion core is then uncoated to expose a viral nucleoprotein complex, which contains MA, RT, IN, Vpr, and RNA. Following entry the viral RT and RNase H contained within the complex convert the viral RNA genome to double-stranded DNA. Vpr facilitates the nuclear localization of the viral nucleoprotein complex and is especially important for nuclear localization in nondividing cells with an intact nuclear envelope membrane. RT is responsible for the reverse transcription initiated from the 3' end of the host tRNA<sub>Lys</sub> primer annealed to the primer binding site near the 5' end of the genomic RNA (Figure 5). RT contains an RNase H domain that cleaves the RNA portion of RNA-DNA hybrids generated during the reaction. Following tRNA-primed initiation, reverse transcription involves two DNA strand transfer reactions that are catalyzed by RT and are important for the synthesis of both minus and plus strands of the viral DNA. RT has been a major target for drug design. Two classes of RT inhibitors are already in clinical use: nucleoside analogs such as AZT (3'-azido-2',3'-dideoxythymidine) and ddI (didanosine) that are presumed to bind to the polymerase active sites, and non-nucleoside inhibitors such as nevirapine that binds close to the polymerase active site and thereby keeps RT inactive. Following reverse transcription, IN catalyzes integration of the viral DNA into a host chromosome and the DNA is then repaired.

Upon cell activation, viral transcripts are expressed from the promoter located in the 5' long terminal repeats (LTR) (Step 5, Figure 4), with Tat greatly enhancing the rate of transcription. Tat binds to an RNA hairpin known as the trans-activating response element (TAR) located at the 5' end of the nascent viral
Figure 5 HIV-1 reverse transcription.

The viral RNA genome has plus strand polarity and is shown as a thin solid line, designated (+) (Luciw, 1996). Synthesis of the first strand of DNA [negative polarity, represented as a heavy solid line and designated (-)] is primed by a tRNA^{lys} molecule hydrogen bonded to the PBS of the template RNA. Synthesis of the second DNA strand is primed by a short viral RNA fragment generated by Rnase H activity at the PPT that borders U3 and a central PPT (cPPT) located in viral sequences near the 3' end of the pol gene. The second DNA strand has plus strand polarity and is represented as a heavy gray line. A CTS, which functions to terminate plus strand viral DNA in step F, is located near the 3' end of the pol gene and is shown as a solid circle; about 100 nucleotides of plus strand DNA is displaced to allow for termination at the CTS. To simplify the pictorial representation of lentivirus reverse transcription, important cis-acting signals (PBS, CTS, cPPT, and PPT) are shown only in key steps. PBS: primer binding site; PPT: polypurine track; cPPT: central polypurine track; CTS: central terminal signal. (Figure reproduced from Luciw, 1996).
A. (-) strand priming

B. first strand-transfer

C. (-) strand elongation

D. (+) strand priming

E. second strand-transfer

F. (+) strand elongation

G. strand completion
transcripts through its arginine-rich domain, and enhances the efficiency of the transcription polymerase in the production of viral mRNAs up to 100 fold. Tat is therefore essential for viral replication.

Spliced and genomic-length RNAs are then selectively transported by Rev (Figure 6), from the nucleus to the cytoplasm, where viral mRNAs are translated, viral genomic RNAs are packaged and new virons are assembled. When viral mRNAs are first produced, most are multiply spliced and encode the Tat, Rev, and Nef proteins. Afterwards, singly spliced and unspliced transcripts are transported to the cytoplasm, where viral proteins are translated and viral genomic RNAs are packaged as infectious virions. Rev is critical for this switch of transporting singly spliced and unspliced transcripts instead of multiply spliced viral mRNA to the cytoplasm. This is achieved by a binding interaction between Rev and the Rev responsive element (RRE) site located in the env coding region.

Vpu promotes degradation of CD4 which holds newly synthesized Env glycoprotein (gp160) in the endoplasmic reticulum, thus allowing Env transport to the cell surface for assembly into viral particles. In addition to its role in CD4 degradation, Vpu can also stimulate virion release. Nef, an important regulatory HIV-1 viral protein which will be discussed in more detail later in this chapter, downregulates CD4 from the cell surface. By doing so, it may enhance Env incorporation into virions, promote particle release, and possibly affect CD4+ T-cell signaling pathways. Nef can also downregulate the cell surface expression of MHC class I molecules, which may help protect infected cells from killing by cytotoxic T cells. In addition, Nef has been shown to enhance viral infectivity in vitro and to be responsible for disease progression in patients and animal
Figure 6 Splicing patterns of HIV-1 transcripts. The gag and gag-pol mRNAs are unspliced; mRNAs for the early genes tat, rev, and nef are doubly spliced; mRNAs for the late genes vpu, env, vif, and vpr are singly spliced. Also shown are two mRNA species which encode novel regulatory genes: tev/tnv and the first exon of tat. Shown in parentheses are additional transcripts generated by alternative splicing (vpr, miniexons 2 and 3). (Figure reproduced from Luciw, 1996).
Vif is important for the production of highly infectious virions and may also play a role in viral assembly and maturation.

MA is the N-terminal component of the Gag polyprotein and is important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane prior to viral assembly. In the mature viral particle, the MA protein lines the inner surface of the virion membrane. CA is the second component of the Gag polyprotein and forms the core of the virus particle, with around 2000 molecules per virion. NC is the third component of the Gag polyprotein and coats the genomic RNA inside the virion core. The primary function of NC is to bind specifically to the packaging signal and deliver full-length viral RNAs into the assembling virion. P6 comprises the C-terminal 51 amino acids of Gag and is important for incorporation of Vpr during viral assembly. The immature virion is composed of cell membrane embedded with Env and Gag viral proteins, and a viral core assembled from the Gag and Gag-Pol polyproteins, accessory proteins, Vif, Vpr Nef, and two identical strands of the genomic RNA. As the particle buds and is released from the cell surface, virion maturation occurs through the proteolytic processing of the Gag and Gag-Pol polyproteins by PR. PR cleaves at several polyprotein sites to produce the final MA, CA, NC and p6 proteins from Gag, as well as PR, RT, and IN proteins from Pol. Because viral assembly and maturation must be highly coordinated, factors that influence PR activity can have dramatic effects on virus production. PR has been a prime target for drug design, and several PR inhibitors are already in clinical use (Hammer, 1996). After the virion maturation step, which involves proteolytic processing of the Gag and Gag-Pol polyproteins by PR and a less well defined function of Vif, the
viral replication cycle is completed and the mature virion is then ready to infect the next cell.

1.6 An overview of HIV-1 Nef protein

The *nef* gene is located at the 3' end of the viral genome, partially overlapping the U3 region of the 3' LTR (Figure 3). Nef is expressed early in the HIV replication cycle from multiply spliced mRNA transcripts that encode a 27 kD myristoylated protein that is largely membrane associated (Trono, 1995).

Considerable controversy has surrounded the biological function of the HIV-1 Nef viral protein. Initial studies suggested that this early nonstructural viral protein functioned as a negative regulatory factor and acted to suppress both viral replication and transcription activity of the HIV-1 LTR (Harris, 1996). It was therefore proposed to play a role in establishing or maintaining viral latency. However, other investigations failed to confirm these negative effects of Nef (Harris, 1996). It is clear now that Nef expression is critical for efficient virus replication under conditions similar to virus replication in vivo.

Several distinct functions of Nef have been well characterized in vitro. First, Nef, in concert with Env and Vpu, downregulates the cell surface expression of CD4 receptors (Piguet *et al.*, 1999b). Such a function may serve to inhibit superinfection and increase the infectivity of the viruses. Nef also downregulates cell surface MHC-1 molecules and this may play an important role in the pathogenesis of HIV-1 infection through immune escape by protecting infected cells from recognition of cytotoxic T lymphocytes (Piguet *et al.*, 1999b). Third, Nef
increases viral infectivity at the stage of post-entry of the virus into the cell (Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al., 1995), which may be responsible for the high viral load in animal models infected with Nef+ HIV-1. Nef also alters cellular signal transduction and activation pathways which may be linked to the pathogenesis of HIV-1 infection (Baur et al., 1994; Hanna et al., 1998; Iafrate et al., 1997; Skowronski et al., 1993).

The biological importance of Nef has been demonstrated in several animal models. Transgenic mice expressing Nef developed severe AIDS-like symptoms, thus indicating that nef harbors a major disease determinant (Hanna et al., 1998). Expression of the Nef protein in transgenic mice perturbs development of CD4+ T cells, elicits depletion of peripheral CD4+ T cells, and alters T cells activation responses (Hanna et al., 1998; Skowronski et al., 1993). Nef has also been shown to be functional in enhancing both virus replication and pathogenicity of HIV-1 in SCID-Hu mice (Aldrovandi and Zack, 1996; Jamieson et al., 1994). In Rhesus monkey models infected with simian immunodeficiency virus (SIV), a close relative of HIV, Nef was found to be critical for high viral load and disease progression (Kestler et al., 1991).

Further evidence for the involvement of Nef in HIV-1 pathogenicity in vivo comes from the observation of Nef deletions in several case studies of long-term non-progressor (LTNP) patients (Deacon et al., 1995; Kirchhoff et al., 1995). Although these individuals have been infected for more than 10 years, they remain free from HIV-related disease, and have normal CD4+ lymphocyte counts and low viral load.
1.7 Structure of Nef protein

The structure of Nef has been determined by two recent studies, which examined the nuclear magnetic resonance (NMR) structure of the core of Nef (residues 40-206, with a 150-170 deletion) in solution (Grzesiek et al., 1996a), and the X-ray crystal structure of the Nef core (residues 54-205) bound to the high affinity R96I mutant of the Fyn SH3 domain (Lee et al., 1996). Only the crystal structure of Nef protein will be reviewed in this section, since the general features of the crystal structure of the Nef protein are consistent with the demonstration by NMR of the presence of two anti-parallel α helices and the anti-parallel nature of the β strands (Lee et al., 1996).

The structure of Nef consists of three layers (Figure 7 and 8) (Lee et al., 1996). The N-terminal region forms an outer layer that consists of α polyproline-II (PP-II) helix (Arg71 to Arg77), which contains the PXXP motif, followed by two anti-parallel α helices that pack against a middle layer of four anti-parallel β strands. The C-terminal region consists of two short α helices and extends the other side of the β strands.

Nef contains a highly conserved PXXP motif that is responsible for the tight binding interaction with Src family SH3 domains (Kd = 0.25 μM for Hck and 0.38 μM for Fyn mutant [R96I]) (Lee et al., 1995). The Src family kinases have a common modular architecture composed of a conserved catalytic domain and two Src homology domains, SH3 and SH2, that bind to proline-containing sequences and phosphotyrosine, respectively (Cohen et al., 1995; Pawson, 1995).
Figure 7 The crystal structure of the conserved core of HIV-1 Nef complexed with the SH3 domain of Fyn (R96I mutant). The SH3 domain is shown in white; the Nef protein is in gray. The RT loop is so named because of critical arginine and threonine residues in Src. Nef interacts with the SH3 via a polyproline helix (PxxP) and a hydrophobic pocket formed by the two anti-parallel $\alpha$-helices that follow the PxxP motif.

The amino acid sequences of the conserved core region of Nef protein from different strains of HIV-1, HIV-2, and SIV are shown. Residues numbers above the sequences correspond to HIV-1 NL4-3. The secondary structural elements of the Nef core crystal structure are indicated by arrows for strands (labeled βA-βD) and boxes for a helices (labeled αA-αD); the PP-II helix is indicated. A loop connecting βC and βD is disordered in the structure and is indicated as a broken line. Residues involved in the interaction with SH3 are labeled with an asterisk above the sequence. (Reprint with permission from Author, Lee et al., 1996.)
Figure 9 Sequence alignment of SH3 domains. Several SH3 domains are shown, and the common secondary structural elements are boxed. The RT loop and n-Src loop are so named because of critical arginine and threonine residues in Src (the RT loop) and a long insertion in the neuronal form of Src (n-Src loop).

SH3 target peptides adopt a polyproline type II helical conformation, characterized by the sequence PXXP (Kuriyan and Cowburn, 1997). The Nef PP-II helix (R71-R77) that spans the PXXP motif interacts with the SH3 domain of Fyn (R96I) through its RT loop (between the first and second strands of SH3 domain) and n-Src loop (between the second and third strands of SH3 domain) (Figure 7, 8 and 9) (Lee et al., 1996). The R96I mutation in the RT loop of the Fyn SH3 domain greatly increases its interaction with Nef from low affinity (Kd >20 μM) to high affinity (Kd = 0.38 μM), which makes it comparable with that of the wild-type Hck SH3 binding with Nef (Kd = 0.25 μM). Trp-119 of SH3 was found at the binding interface between Nef PP-II helix and Fyn (R96I) SH3 (Figure 7 and 9). Although the physiological relevance of the interaction of Nef with SH3 domains is unknown, mutagenesis of Nef showed that the PXXP motif is essential for MHC-1 downregulation (Le Gall et al., 1997; Mangasarian et al., 1999; Schwartz et al., 1996) and optimal spread of HIV-1 in primary cell cultures (Saksela et al., 1995), suggesting that the virus has evolved to exploit SH3-mediated interactions with cellular proteins to evade the host cellular defence system and increase viral replication.

1.8 Nef downregulation of CD4 and its role in HIV-1 infection
CD4 is a membrane glycoprotein that is expressed in T helper lymphocytes and cells of the macrophage and monocyte lineage (Maddon et al., 1986; Maddon et al., 1985). It comprises a large extracellular component made up of four immunoglobulin-like domains, a transmembrane region, and a short cytoplasmic tail.
CD4 plays a key role in the maturation and proper function of helper T lymphocytes (reviewed in (Weiss and Littman, 1994)). CD4 stabilizes the interaction between the antigen-specific T cell receptor (TCR) on helper T cells with the antigen-MHC-II complex on antigen presenting cells (Doyle and Strominger, 1987). Following the antigen activation of T cells, CD4 recruits the p56Lck protein tyrosine kinase to the vicinity of the TCR, through a cysteine-based binding site for Lck, resulting in cell proliferation and interleukin-2 production (Acres et al., 1986; Shaw et al., 1989; Turner et al., 1990; Veillette et al., 1988; Veillette et al., 1989). CD4 activation also exerts TCR-independent influences, including phospholipase C stimulation, Ca\(^{2+}\) signaling and apoptosis (reviewed in (Foti et al., 1995)).

During HIV-1 infection, CD4 serves as the primary receptor for the envelope glycoprotein (Env), allowing for the docking of these viruses onto the surface of target cells (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986). This initial event allows an interaction between Env and specific members of the chemokine receptor family, such as CXCR4 or CCR5 for HIV-1 (reviewed in (Littman, 1998)), which triggers the fusion of the viral and cellular membrane, and results in delivering the virion inner components into the cytoplasm.

In HIV-1 infection, Nef downregulates CD4 in concert with two additional HIV-1 gene products, Env and Vpu (Chen et al., 1996). However, the mechanisms of CD4 downregulation induced by the three viral proteins are different. Nef acts on CD4 molecules that have already reached the cell surface. In contrast, Env and
Vpu modulate CD4 along the biosynthetic pathway in the endoplasmic reticulum (ER).

The CD4 downregulation in HIV-1 infected cells might serve to inhibit superinfection and to reduce the envelope-induced cytopathic effect. This would allow a more efficient production of viral particles in infected cells and contribute to the maintenance of high viral loads. Alternatively, downregulating this viral receptor may contribute to the infectivity of outgoing virions by preventing the interference of high CD4 levels on the surface of HIV-producing cells with the particle incorporation and function of the viral envelope (reviewed in (Piguet et al., 1999b)). The presence of high CD4 levels on the surface of HIV-producing cells can dramatically interfere with the particle incorporation and function of the viral envelope (Piguet et al., 1999b). By downregulating the viral receptor, Nef together with Vpu and Env efficiently counteract this inhibition, thus preserving the infectiousness of outgoing virions.

Nef downregulates CD4 molecules by acting as a connector between the receptor and intracellular trafficking pathways (Mangasarian et al., 1997) (Figure 10). Nef associates with the CD4 cytoplasmic tail by recognizing the dileucine-based motif that also functions as an endocytosis signal (Aiken et al., 1994; Grzesiek et al., 1996a; Hua and Cullen, 1997). Residues in Nef involved in contacting CD4 are clustered in a proximal flexible loop (WLE57-59) and in the core domain of the viral protein (G95, G96, L97, R106 and L110) (Grzesiek et al., 1996a; Grzesiek et al., 1996b) (Figure 11). The immediate downstream partner of Nef for CD4 downregulation is the clathrin-associated adaptor protein complex-2
Figure 10. The mechanism of CD4 downregulation induced by Nef. Nef downregulates CD4 molecules by acting as a connector between the receptor and intracellular trafficking pathway. Nef associates with the CD4 cytoplasmic tail by recognizing the dileucine-based motif. The immediate downstream partner of Nef for CD4 downregulation is the clathrin-associated adaptor protein complex-2 (AP-2). AP-2 interacts with Nef through its C-terminal dileucine-based motif (LL165). In the presence of Nef, internalized CD4 molecules are targeted to lysosomal degradation. This is achieved by a lysosomal targeting signal in Nef (EE155) that mediates the binding of Nef to the β subunit of coat protein-I (β-COP-1) in early endosome. (Reprint with permission, from the Immunological Reviews Volume 168, 1999 by Munksgaard International Publishers Ltd., Copenhagen, Denmark).
Figure 11 Domains of Nef implicated in receptor downregulation. The determinants important for CD4 or MHC-1 downregulation are indicated with black and open arrows, respectively. The important regions in Nef structure are boxed. The epitopes that are recognized by monoclonal antibodies (Mab) are also denoted.
(AP-2), a heterotetramer mediates the formation of clathrin-coated pits and targets plasma membrane proteins to early endosomes (Le Gall et al., 1998; Piguet et al., 1998). AP-2 interacts with Nef through its C-terminal dileucine-based motif (LL165) (Bresnahan et al., 1998; Greenberg et al., 1998a). In the presence of Nef, internalized CD4 molecules are targeted to lysosomal degradation. This is achieved by a lysosomal targeting signal in Nef (EE155) that mediates the binding of Nef to the β subunit of coat protein-I (β-COP-I) in early endosome (Piguet et al., 1999a).

1.9 Nef downregulation of MHC-I and its role in HIV evasion of the cellular immune response

After exposure to HIV an infected individual becomes acutely viremic. This phase subsequently resolves coincident with the appearance of anti-HIV cytotoxic T lymphocytes (CTLs) which play an important role in the control of virus load (Borrow et al., 1994; Koup et al., 1994). CTLs detect infected cells through the T-cell receptor and CD8 molecules which recognize foreign peptides assembled in the groove of MHC class I proteins expressed on the surface of almost all mammalian cells. CTLs are well known for their ability to lyse cells expressing foreign antigens by the release of granzymes and perforins and by the activation of Fas-mediated killing (Berke, 1995). In addition, activated anti-HIV CTLs secrete soluble substances including chemokines that potently inhibit infection by HIV (Wagner et al., 1998; Walker et al., 1986; Yang et al., 1996). Recently, the crucial role of CD8+ T cells have been demonstrated in controlling simian immunodeficiency virus (SIV) replication in vivo in monkeys by two laboratories (Jin et al., 1999; Schmitz et al., 1999).
During HIV-1 infection, the cell surface expression of MHC-1 is downregulated (Kerkau et al., 1989; Scheppler et al., 1989). MHC-1 consists of a highly polymorphic, membrane-anchored heavy chain non-covalently associated with β2-microglobulin (β2m) (Ploegh, 1998). MHC-1 molecules are stably expressed at the cell surface, with only a minor fraction of the molecules being internalized spontaneously in T cells and in monocytes/macrophages (Neefjes et al., 1990; Reid and Watts, 1990).

So far, Nef is the only HIV-1 viral protein that has been found to downregulate MHC-1 in primary CD4+ T cells (Collins and Baltimore, 1999). In Nef producing cells, MHC-1 molecules are rapidly endocytosed and directed to lysosomes for degradation (Schwartz et al., 1996). Nef does not affect MHC-1 synthesis and transport through the ER and cis-Golgi, but rather misroutes the antigen presenting receptor from both the tran-Golgi network (TGN) and the cell surface towards the endosomal pathway (Le Gall et al., 1998; Schwartz et al., 1996).

With the exception of the N-terminal myristoylation signal sequence which is required for its membrane localization and important for both CD4 and MHC-1 downregulation, other determinants of HIV-1 Nef which are distinct are responsible for MHC-1 downregulation (reviewed in (Piguet et al., 1999b) (Figure 11). They include a conserved α-helix near the N-terminus of the protein (Mangasarian et al., 1999), an acidic stretch in the proximal region of HIV-1 Nef EEEEE65 (Greenberg et al., 1998a), and the polyproline motif forming the SH3-binding surface, PXXP78. Nef appears to induce MHC-1 endocytosis using a not-
well characterized cryptic motif in the cytoplasmic domain of the MHC-1 molecule. The LL165 dileucine motif which mediates the interaction of HIV-1 Nef with adaptor complexes and is required for accelerated CD4 endocytosis, is dispensable for MHC-1 downregulation (Mangasarian et al., 1999).

Nef is able to downregulate exogenously expressed MHC-1 HLA-A and HLA-B molecules, but not HLA-C (Le Gall et al., 1998) or HLA-E (Collins and Baltimore, 1999). The sequence YSQA323 in the cytoplasmic tail of MHC-1 HLA-A and HLA-B found to be responsible for Nef action is reminiscent of tyrosine-based motifs that mediate endocytosis and sorting of a number of surface molecules (Marks et al., 1996; Sandoval et al., 1994; Trowbridge et al., 1993). HLA-C, which is unaffected by the viral protein (Le Gall et al., 1998), carries a cysteine instead of tyrosine at position 320 of this cryptic endocytosis motif. The adaptor protein complex-1 (AP-1) may be involved in MHC-1 downregulation by Nef, since HLA-A and -B molecules accumulate in a region containing clathrin-coated vesicles budding from the Golgi that contain AP-1 in the presence of Nef (Le Gall et al., 1998). In addition, Nef was shown to bind with the μ1 subunit of AP-1 and the complete AP-1 complex in yeast two-hybrid and cell-free assays, respectively (Le Gall et al., 1998).

Nef-induced MHC-1 modulation protects HIV-1 infected cells against lysis by virus-specific CTLs in vitro (Collins et al., 1998). The HIV-infected cells escape CTL recognition by reducing the density of antigenic peptide complexed to MHC class I antigen on the cell surface. However, unless HLA-C is expressed, downregulating MHC-1 normally renders target cells more sensitive to
destruction by natural killer (NK) cells (Brutkiewicz and Welsh, 1995; Colonna et al., 1993). The Nef-dependent downregulation of MHC-1 was demonstrated to be selective on HLA-A and –B antigens but not on HLA-C or –E antigens (Collins et al., 1998). A rationale for the specificity of MHC class I downregulation is that HLA-A and –B are the major MHC class I encoded proteins known to present antigens to CTLs, while HLA-C and –E can interact with various inhibitory receptors on NK cells and can protect cells against these killer cells (Collins et al., 1998). The selective downregulation of HLA-A and –B, but not –C and -E, induced by Nef might therefore permit infected cells to display limited recognition for CTLs without being exposed to the attack of NK cells.

Based on these findings that Nef-expressing HIV infected cells with low levels of MHC-1 class I antigens have a survival advantage in the presence of CTLs, it is to be expected that these cells will survive longer in vivo, produce more infectious viral particles, and eventually make the immune system more vulnerable to viral infection leading to more rapid disease progression.

In addition to the effects of Nef, other mechanisms that diminish the anti-HIV CTL response have been established. A growing number of studies suggest that antigenic variation is an important element of HIV immune evasion. Individual patients with immunodominant CTL responses can escape the CTL response by generating antigenic variants not recognized by CTLs (Borrow et al., 1997; Goulder et al., 1997; Phillips et al., 1991). In addition, some individuals who mount a CTL response to the immunodominant strain appear to lack the ability to respond to immunogenic variants that arise later (Klenerman et al., 1995;
Klenerman and Zinkernagel, 1998; McAdam et al., 1995). Furthermore, it has been reported that certain types of peptides that are sequence variants of the cognate peptide antigens recognized by antigen-specific T cell receptors can act as antagonists (Klenerman et al., 1994; Meier et al., 1995). It is not surprising that HIV employs multiple mechanisms to evade the host’s immune system and lead to the almost universal lethality of virus infection in untreated individuals. Therefore, a greater understanding of precisely how the immune system is weakened by Nef and other factors, as well as how to prevent such damage to the host’s immune system will provide ways of designing more effective therapeutics to counter HIV infection.

1.10 The application of intracellular single-chain antibody in HIV research

An immunoglobulin G (IgG) antibody is a bivalent molecule composed of four chains, two heavy chains and two light chains. The heavy chains have four domains, one variable domain ($V_H$) and three constant domains ($C_H1$-$C_H3$); the light chains have one variable domain ($V_L$) and one constant domain ($V_H$). The region that is responsible for antigen-specific binding in an antibody is composed of $V_H$ and $V_L$ domains. The recombinant ScFv is commonly composed of the basic antigen recognizing $V_H$ and $V_L$ domains that are joined by a flexible polypeptide linker (Bird et al., 1988; Huston et al., 1988). Intracellular ScFvs have been constructed and fused to signal peptide domains that target several different cellular compartments, such as endoplasmic reticulum, nucleus, and mitochondria (Persic et al., 1997), in order to direct target proteins to different cellular compartments.
Single-chain antibodies (ScFvs) were studied for their ability to block Nef functions intracellularly in this thesis research. ScFv can be constructed from the variable domain sequences of an antibody cDNA by molecular biology techniques. There are two common sources: hybridomas and antibody libraries. Hybridomas producing either murine monoclonal (Biocca et al., 1993; Duan et al., 1994a; Richardson et al., 1995), which are most commonly used for constructing ScFv, or human monoclonal antibodies (Marasco et al., 1993) have been the source of the $V_h$ and $V_L$ cDNA for that particular antibody. Antibody libraries have been built using cDNA from mouse spleens (Clackson et al., 1991), human peripheral blood lymphocytes (Marks et al., 1991), and bone marrow from the immunized host (Burton et al., 1991). These antibody fragments are expressed on the surface of bacterial phages, which are able to infect and multiply in *E. coli*. Antigen-specific binding phages can be isolated and characterized after several rounds of selection and enrichment steps, making use of the character that an antibody expressing phage carries the particular genotype of that antibody. The ScFvs used in this thesis were derived from murine hybridomas obtained from our collaborators.

Early studies showed that the heavy and light chain cDNAs of an antibody against alcohol dehydrogenase I (ADH I) can be expressed in the cytoplasm of yeast (*Saccharomyces cerevisiae*), and the heavy and light chain polypeptides so produced could neutralize the enzyme intracellularly (Carlson, 1988). Further studies verified that the assembly of an antibody can take place in the reducing environment of a mammalian cytoplasm (Biocca et al., 1990). The feasibility of joining heavy chain and light chain variable domains through a synthetic linker
and maintaining binding specificity and affinity of the parent antibody was first demonstrated in *Escherichia coli* (Bird *et al.*, 1988; Huston *et al.*, 1988). This kind of recombinant antibody is termed single-chain antibody (ScFv). Following these discoveries and advances in the field, intracellular ScFvs were shown to bind the target protein and inhibit its functions in mammalian cells (Marasco *et al.*, 1993).

In addition to the potential gene therapy application of intracellular antibodies for treatment of HIV-1 infection, single-chain antibodies can also provide a powerful research tool for studying gene products intracellularly. In this thesis, ScFvs are used to study the inhibitory effect on the cell surface CD4 and MHC-1 downregulation-induced by Nef functions. If successful, it can provide an additional tool to the intracellular antibody arsenal against HIV-1 which could potentially be used in combination gene therapy for the treatment of HIV-1 infection. This study can also provide important information on the use of Nef as a new drug target for the treatment of HIV-1 infection.

### 1.11 The interaction of Hck SH3 domain with HIV-1 Nef protein

A novel strategy for blocking Nef functions explored in this research was the use of a dominant negative form of a tyrosine kinase-Hck, a natural intracellular ligand of Nef viral protein, to block Nef from downregulation of host cell surface receptors. Hck belongs to the Src family non-receptor-type tyrosine kinases that consists of nine members (Src, Lck, Hck, Fyn, Fgr, Yes, Blk, Lyn, and Yrk) (Brown and Cooper, 1996) (Figure 12). They share a common structural and regulatory mechanism, but differ in cellular expression and localization. Each of these kinases has a N-terminal signal region for myristoylation. The post-
translational modification of these kinases allows them to attach to the cell plasma membrane. Adjacent to the N-terminal region is a Src homology domain (SH3) which binds to target proteins via specific proline-rich sequences that adopt a polyproline type II helical conformation (Cohen et al., 1995; Pawson, 1995). C-terminal to the SH3 domain is the SH2 domain which binds tightly to specific tyrosine-phosphorylated sequences. The SH2 domain is followed by the kinase domain and a C-terminal tail with a highly conserved tyrosine residue essential to kinase regulation. SH2 and SH3 domains cooperate in the negative regulation of Src family kinase activity. The dominant-negative Hck (DN-Hck) used in this work contains only the N-terminal myristoylation signal region, and the SH3 and SH2 domain, without the C-terminal kinase domain.

**Figure 12 Structure of Src family tyrosine kinases.**

The functional domains of Src family tyrosine kinases were shown (SH4, SH3, SH2 and the catalytic domain). Phosphorylation at Y527 in the C-terminal regulatory tail inactivates the kinase activity, and phosphorylation at Y416 activates the kinase activity. The intramolecular binding site of the SH3 domain is denoted by the PXXp consensus sequence (the second proline residue is conserved in only four of the nine family members, including Hck). (Figure reproduced frm Sicheri and Kuriyan, 1997).
Hck is expressed primarily in granulocytes, monocytes, and macrophage (Holtzman et al., 1987; Quintrell et al., 1987; Ziegler et al., 1987). Several lines of evidence suggest that Hck regulates phagocyte differentiation and function. Hck expression is strongly induced by agents that promote macrophage differentiation (Boulet et al., 1992; Lichtenberg et al., 1992). Hck also associates with the Fc receptor and is activated following receptor engagement (Durden et al., 1995; Ghazizadeh et al., 1994; Wang et al., 1994) which may serve to activate the respiratory burst. In addition, Hck has been implicated in hematopoietic cytokine signal transduction. In this regard, interleukin-3, granulocyte-macrophage colony-stimulating factor, and leukemia inhibitory factor have all been shown to induce Hck kinase activation (Anderson and Jorgensen, 1995; Ernst et al., 1994; Linnekin et al., 1994).

Nef contains a polyproline SH3-binding motif, PXXP78, that directs specific binding to Hck, Lyn, and Lck (Collette et al., 1996; Saksela et al., 1995) and is strictly conserved among the different HIV-1, HIV-2 and SIV isolates (Figure 8). Full-length Nef binds the Hck SH3 domain with the highest affinity reported for an SH3-mediated interaction (Kd = 0.25 μM). The significance of the interaction of Nef with non-receptor-type tyrosine kinases has been supported by the finding that disruption of the Nef proline-rich motif impairs the replicative potential of HIV-1 in cultured peripheral blood mononuclear cells (PBMCs) (Saksela et al., 1995).

A dominant-negative mutant of a cellular protein kinase has been widely used for dissecting signal transduction pathways (reviewed in (Perlmutter and
Alberola-Ila, 1996). An advantage of using the dominant-negative mutant to study signal transduction pathways is that there is no need to know the exact pathways to which it contributes. In this work, a strategy for blocking Nef functions using a dominant-negative Hck mutant (DN-Hck) was adopted. The rationale for this is that Hck was shown to bind Nef with high affinity through the Nef SH3-binding motif that has been involved in MHC-1 downregulation. By using the DN-Hck approach, it is possible that the Nef-SH3-binding domain-dependent effect can be blocked.

In addition, the same Hck mutant devoid of the kinase domain has been studied for its effect on HIV-1 infectivity in target cells (Tokunaga et al., 1998). Interestingly enough, viral particles assembled in the presence of DN-Hck demonstrated reduced infectivity, suggesting that DN-Hck was inhibiting Nef-induced activation of specific Src kinases at an early post-viral entry stage (Tokunaga et al., 1998). In these studies, it is hypothesized that DN-Hck might also be capable of interfering with Nef-mediated effects within cells that express this viral protein. In keeping with this notion, we found that DN-Hck prevented Nef-induced class I MHC downregulation, and that this effect was dependent on the presence of a functional Hck SH3 domain. The ability to block Nef function using this approach is of potential therapeutic importance, given that an intact Nef SH3-domain binding site is required for the downregulation of MHC-1, and the expression of MHC-1 molecules is essential for CTLs to recognize and eliminate virus infected cells. In addition, since the SH3-binding site has also been shown to play a role in other viral activities, such as regulation of infectivity, cell
activation and abnormalities of cell signal transduction pathways, therapies directed against this Nef site could have additional benefits for the patients.

1.12 Thesis objectives and hypotheses

The specific aims of this thesis were to: 1) genetically engineer single-chain antibodies against Nef viral protein; 2) evaluate the effect of expressing intracellular single-chain antibodies against Nef, monitored by cell surface expression of CD4 and MHC-1; 3) evaluate the effect of expressing a dominant-negative Hck mutant in cells transfected with Nef, represented by the cell surface expression of CD4 and MHC-1.

The hypotheses addressed in this thesis are: 1) Nef-induced MHC-1 downregulation enables HIV-infected cells to escape killing by cytotoxic T-lymphocytes, which may be responsible for the failure of the host immune system to control HIV infection and disease progression, 2) intracellular expression of Nef-specific single-chain antibodies or the expression of a dominant-negative form of Hck, a Src-family tyrosine kinase which has been shown to have high binding affinity with Nef, could potentially inhibit the immune dysfunction induced by Nef in HIV-infected cells, 3) interfering with Nef-induced class I MHC-1 downregulation may therefore represent a novel strategy for controlling HIV infection by increasing HIV-specific cytotoxic cell activity against infected cells.

The rationale supporting these proposals are: 1) HIV-specific cytotoxic T lymphocytes (CTL) have been shown to be an important host cellular defence
factor against viral infection, since the large increases in the number of CD8+ T lymphocytes and the appearance of virus-specific CTLs correlates with the decrease of viral load and the resolving of the acute viremia phase of HIV infection into the subsequent period of clinical latency that is variable in length and proportional to the amount of circulating virus (Borrow et al., 1994; Collins and Baltimore, 1999; Koup et al., 1994; Pantaleo and Fauci, 1996). An inverse correlation between the quantity of anti-HIV CTLs and viral load has also been found, in support of the importance of CTLs in the host defence against HIV infection (Daar et al., 1991). In addition, HIV-specific CTL activity is also correlated to disease progression and strong HIV-specific CTL activity is associated with long-term nonprogression of disease in a small group of HIV-infected individuals who have controlled their viremia in the absence of antiviral therapy (Harrer et al., 1996).

2) The critical role of Nef in the establishment of high viral loads is best demonstrated by the requirement of an intact nef gene for the development of AIDS in humans and monkeys (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). Nef is a 27 kD myristoylated protein that mediates CD4 and MHC-1 downregulations through different domains. Recent studies have implicated the acidic and proline-rich regions of Nef as being important for MHC-1 downregulation (Greenberg et al., 1998b). Expression of Nef in an infected cell was shown to protect it from anti-HIV CTL recognition. This is achieved by downregulation of MHC-1 in infected cells and hence to prevent CTL recognition by reducing the density of antigenic peptide complexed to MHC class I antigen on the cell surface (Chen et al., 1996; Collins et al., 1998). It was demonstrated that
Nef-mediated MHC class I downregulation can be profound and that Nef-expressing cells with low levels of MHC class I antigens have a survival advantage in the presence of CTLs (Collins et al., 1998). Thus the activity of Nef is likely to be synergistic with other reported mechanisms of HIV escape from CTLs that rely on antigenic variation (Borrow et al., 1997; Goulder et al., 1997).

3) Intracellular expression of ScFvs can either block function or sequester the protein of interest. ScFvs, which have been shown to have specific binding affinities equivalent to those of the parent monoclonal antibodies (Whitlow, 1991a; Winter and Milstein, 1991), can be stably expressed intracellularly where they are capable of inactivating specific cellular gene products (Carlson, 1988). Intracellular ScFv proteins with specificity for Nef thus provide a unique way of studying the mechanism of Nef in virus evasion of the host immune system, as well as offering a potential gene therapy strategy for inhibiting the disease progression in HIV patients.

Similarly, other Nef-ligands that bind with Nef intracellularly could also potentially block the function of Nef or sequester the Nef protein. DN-Hck, consisting of the Hck amino-terminal domain, and the SH3 and SH2 domains, is a good candidate. Src family protein-tyrosine kinases, including Hck, Lck and Lyn, are able to bind the polyproline (PXXP78) motif in Nef with varying affinities by virtue of their SH3 domain (Lee et al., 1996; Saksela et al., 1995), with Hck SH3 domain has the highest affinity yet reported for an SH3-polyproline tract interaction with Nef protein (Kd = 0.25 μM) (Lee et al., 1995). In addition to the high affinity of the interaction between Hck SH3 domain and Nef, the
polyproline binding motif in Nef has been shown to be critical for class I MHC downregulation induced by Nef expression (Greenberg et al., 1998b). This raises the possibility that DN-Hck expression may inhibit MHC-1 downregulation induced by Nef.

Current combination therapies aimed to inhibit HIV replication can suppress HIV in the blood stream of infected patients to such a level that is below the limits of detection using the most sensitive detection method. But they are ineffective in eradicating infected reservoir cells that persist in the host immune system and continue to produce low level of virus in the blood (Balter, 1997; Ho, 1997). Therefore, removing the virus reservoir cells is critical for virus eradication from an infected individual. In this regard, Nef may be a potential drug target for reducing virus infected cells, since one of its detrimental effects enables the infected cells to escape killing by CTLs through downregulation of class I MHC molecules. Thus, therapies directed against Nef could increase the probability of infected cells being recognized and subsequently killed by CTL.

The results presented in this thesis suggest that intracellular binding to Nef alone, as demonstrated by single-chain antibodies, is not sufficient to inhibit the downregulation of MHC-1 molecules induced by Nef. Specific binding interaction to the SH3-binding surface in Nef, such as that of the DN-Hck with Nef, is required for such an action. Chapter 3 addresses the hypothesis that Nef-specific intracellular single-chain antibody (ScFv) expression could potentially inhibit the CD4 and MHC-1 downregulation effect induced by Nef. Using cDNA reverse transcribed from mRNA of hybridoma clones, several anti-Nef ScFvs...
were constructed from the variable regions of monoclonal antibodies that recognized the C-terminus and a central domain (Nef83-88) in Nef. All of them retained the binding activity of their corresponding parental monoclonal antibodies when expressed intracellularly as demonstrated by ‘pull-down’ assays using recombinant Nef protein. However, none of the ScFvs was able to inhibit CD4 or MHC-1 downregulation when co-expressed with Nef in the in vitro transient transfection system developed in this thesis. It is concluded that the intracellular binding interaction of ScFv with Nef and the following Nef sequestration may not be sufficient to block the receptor downregulation events induced by Nef. The expression of an intracellular Nef-ligand capable of binding to domains in Nef that are implicated in CD4 or MHC-1 modulation may be required for this effect.

In Chapter 4, the question of whether expression of a dominant-negative Hck mutant (DN-Hck) can inhibit class I MHC downregulation is addressed. DN-Hck, consisting of the Hck amino-terminal domain, and the SH3 and SH2 domains was co-expressed with Nef in the transient transfection system as described above. The results suggest that DN-Hck prevented Nef-induced class I MHC downregulation, and this effect was dependent on the presence of a functional Hck SH3 domain. It is concluded that the SH3-binding surface on Nef represents a target for therapeutic intervention in individuals infected with HIV-1. Therefore, interfering with Nef SH3 binding site function, as we have done with DN-Hck, represents a potential therapeutic strategy for assisting the host immune system to eliminate HIV-1-infected cells. The results also suggested a model in which DN-Hck prevents Nef-induced class I MHC downregulation by
blocking the interaction between Nef and an as yet unidentified SH3-containing cellular protein that is able to couple Nef to the MHC molecule. Upon binding with Nef, this cellular protein might recruit class I MHC molecules via an interaction with their cytoplasmic tyrosine-based sorting motifs, which in turn routes these molecules towards an intracellular degradation pathway (Greenberg et al., 1998b; Le Gall et al., 1998; Mangasarian et al., 1999).
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Anti-Nef hybridoma clones and monoclonal antibodies AG11, AE6 and EH1 were obtained from Dr. James Hoxie, University of Pennsylvania. The clones AG11 and AE6 were raised against the recombinant Nef protein of HIV-1_{LAI} strain and were derived from the same fusion. They both produce IgG1 monoclonal antibodies that recognize Nef of the HIV-1_{LAI} but not SF2 strain, and are specific for the C-terminus of Nef (epitope: VARELHPEYFKNC). Clone EH1, raised against the Nef protein of the HIV-1_{SF2} strain, is an IgG1 monoclonal antibody that reacts with Nef from both HIV-1 LAI and SF2 strains. It was also mapped to the C-terminus of Nef protein (epitope: MARELHPEYYKDC). A monoclonal antibody clone F14.11 (a gift from Dr. Rita De Santis, Menarini Ricerche Sud, Rome, Italy) generated against a different epitope of Nef (Nef 83-88: AAVDLS), was also used (De Santis et al., 1991).

P4.2, a HeLa cell line expressing the human CD4 molecule, was obtained from Dr. Oliver Schwartz, Institut Pasteur. This cell line has been successfully used for studying CD4 and MHC-1 receptor downregulation by transient expression of Nef (Le Gall et al., 1998; Schwartz et al., 1996).

Nef expression vectors: The CMV-driven Nef-FT vector (pCMV-Nef-FT) carrying the nef LAI gene and the control Nef plasmid carries the nef gene in an antisense orientation (pCMV-AS) are both from Dr. Oliver Schwartz, Institut Pasteur (Le Gall et al., 1998).
Vectors expressing single-chain antibodies: Single-chain antibody constructs derived from EH1 and F14.11 were tagged with myc at their C-termini by subcloning into a mammalian expression vector, pDEF-myc, constructed from pDEF3 (Kozak, 1987). Further modifications were done to fuse the Nef myristoylation signal sequence (MGGKWSKRVSWSWPAVRER) to the N-termini of the ScFv constructs by PCR. The resulting pDEF-ScFv-myc and pDEF-myrScFv-myc vectors have a EF-1α promoter and a myc tag at the C-terminus of the expression cassette (Figure 20).

Expression of dominant-negative Hck and mutants: Expression vectors for the dominant Hck and its mutants (Figure 23) (Tokunaga et al., 1998) are gifts from Dr. Michiyuki Matsuda, International Medical Center of Japan, Japan. The dominant-negative Hck expression vector, pCAGGS-DN-Hck, consists solely of the amino-terminal regulatory domains, SH3 and SH2. The DN-Hck-W93F vector has substituted Trp93 with Phe, and the DN-Hck-R151S vector has a Arg151 to Ser substitution. These two residues are essential for the functions of SH3 and SH2, respectively (Tokunaga et al., 1998). CrkII adaptor protein, which consists mostly of the SH2 and SH3 domains, was used as a control for DN-Hck (Tokunaga et al., 1998).

2.2 Inhibition ELISA assay
Recombinant Nef-GST produced from E. coli transformed with pGEX-BH10Nef (the plasmid is a gift from Dr. Mark Harris, University of Glasgow, UK) (Harris et al., 1992) was used as the coating antigen. This recombinant Nef protein from HIV-
1BH10 shares 96% identity with the amino acid sequence of HIV-1LAI. Microtiter wells were coated with 50 µl of Nef-GST per well (12.5 µg/ml in PBS buffer) overnight at 4°C. The wells were blocked with 1% BSA/PBS for 1 hr at 37°C. Mab from clone EH1 was biotinylated using the ImmunoPure Sulfo NHS-LC-Biotinylation Kit (Pierce). Serially-diluted antibodies from clones AG11 and AE6, as well as a Mab (F14.11) generated against a different epitope of Nef (Nef 83-88: AAVDLS), were mixed with 5 µg/ml biotinylated EH1 Mab in the wells and incubated for 1 hr at 37°C. After three washes with 0.05% Tween 20/PBS, avidin-alkaline phosphatase (Pierce) was added at a dilution of 1:1000 and incubated at 37°C for 1 hr and washed as above. Immune complexes were detected by the enzyme-substrate reaction with p-nitrophenyl phosphate (Sigma), with the reactions being read at 405 nm after 30 min.

2.3 RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from 10⁷ hybridoma cells, using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). The variable regions of the light chain and heavy chain were amplified by RT-PCR, using the following primers: forward primers: VκForward (5' AAGCTTCCATGGA[CT][AG]T[TC][TG][TA]GATGAC[CA][CA][GA][TA]CTCC 3'), VHForward (5' GGATCCGGTGTTCTGGTGGG[AG]GGT[CG]CA[AG]CT[GT][GC][TA][G][GC]AGT C[AT]GG 3'); and reverse primers: VκReverse (5' GGATCCACCACCACCACTGTTCCAGCTTGGTGCCAGCAGAACG 3'), VHReverse (5' AAGCTTCTATGAGTAGACGGTGACCGTGGTCCCGGGCCCCAG 3') (Figure 12a). Primers VκForward and VHForward are degenerate primers with alternative bases.
indicated in the brackets. The synthesis of cDNA was performed using Superscript Moloney leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL/Life Technologies) according to manufacturer’s instructions. The reverse primers were used to prime the reverse transcription reactions. Polymerase chain reactions (PCR) were then performed using Taq DNA polymerase (Perkin-Elmer) in a GeneAmp PCR System 9600 (Perkin Elmer), for 35 cycles under the following conditions: denaturation at 96°C for 30 sec, annealing at 58°C for 45 sec, and extension at 72°C for 1 min; finishing with 72°C for 10 min.

2.4 Cloning and sequencing of amplified products
Amplified DNA fragments were digested with BamHI and HindIII and gel purified by electrophoresis on 1.5% agarose gels, using the QIAquick gel extraction kit (QIAGEN). The purified products were ligated into the BamHI/HindIII restriction sites of a cloning vector, pGEM-4Z (Promega), and transformed into competent E. coli DH10B (GIBCO/BRL). Several recombinant clones were selected and sequenced in both directions using dye-terminator cycle sequencing kit (Applied Biosystems), and T7/SP6 promoter primers (University Core DNA Services, University of Calgary, Canada) on a 373 automated sequencer (Applied Biosystems). Alignment of the antibody sequences was performed using Clustal V (Chomczynski and Sacchi, 1987). Sequences of the recombinant clones were also compared with the non-redundant database at National Center for Biotechnology Information (NCBI) using the Blast program (Altschul et al., 1990).
Figure 12a Single-chain antibody construction.

Following RNA isolation from hybridoma cells, RT-PCR was performed and the variable regions of the light and heavy chain were cloned and the sequences were analyzed. Single-chain antibodies were constructed by ligating the \( V_k \) and \( V_H \) DNA fragments via the BamHI site at the 3' end of \( V_k \) and 5' end of \( V_H \). PCR was used to add the Kozak sequence (GCCACC), the start codon, and the restriction sites, EcoRI and NheI, to the 5' and 3' ends of the ScFv constructs, respectively. Alternatively, overlapping PCR was performed to introduce the Nef myristoylation signal sequence to the N-termini of ScFv constructs, together with Kozak sequence, the start codon, and restriction sites as above. The resulting ScFv or myristoylation modified-ScFv (myr-ScFv) cassettes were subsequently cloned into a mammalian expression vector that has an EF-1\( \alpha \) (elongation factor) promoter and either a GFP reporter or a myc tag at the C-terminus of the expression cassette. The predicted molecular weight of expressed proteins are: ScFv-GFP, 54.2 kD; ScFv-myc, 31.1 kD; GFP alone, 27.0 kD; myc alone, 3.8 kD. (Compute pI/Mw tool, ExPASy Molecular Biology Server, Swiss Institute of Bioinformatics, www.expasy.ch/tools/pi_tool.html).
Hybridoma cells ($10^7$)

\[ \downarrow \]

Isolation of RNA

\[ \downarrow \]

Total RNA

\[ \downarrow \]

RT-PCR

\[ \downarrow \]

RNA template

\[ \downarrow \]

V\textsubscript{k}Reverse

V\textsubscript{k}Forward

V\textsubscript{k}Reverse (BamHI)

V\textsubscript{H}Reverse

V\textsubscript{H}Forward (BamHI)

PCR product

\[ \downarrow \]

Restriction digestion and ligation

\[ \downarrow \]

BamHI

ScFv cassette

\( \text{VK (GGGGS)}_3 \text{VH} \)

linker

\[ \downarrow \]

PCR and cloning into mammalian expression vectors

\[ \downarrow \]

myristoylation sequence-fused ScFv cassette (myr-ScFv)

\[ \downarrow \]

Forward (EcoRI, Kozak sequence, start codon)

\[ \downarrow \]

Reverse (Nhel)

Start codon

Stop codon

\( \text{EF-1}\alpha \)

ScFv or myr-ScFv cassette

GFP reporter or myc tag

\[ \downarrow \]

pDEF-ScFv-GFP/pDEF-ScFv-myc/pDEF-myr-ScFc-myc vector
2.5 Construction and expression of ScFv

The ScFvs were constructed by ligating the $V_K$ and $V_H$ DNA fragments via the BamHI site at the 3' end of $V_K$ and 5' end of $V_H$. PCR was used to add the Kozak translation consensus sequence, GCCACC (Kozak, 1987), the start codon, and the restriction sites, EcoR I and NheI I, to the 5' and 3' ends of the ScFv constructs, respectively. The primers used were, Forward primer, GGAATTCCTGCCACCATGGACATTTTGATGACCCAGTCT; Reverse primer, CGCCTAGCTAGCTGAGGAGACGGTGACCG. PCR products were subsequently cloned into a mammalian expression vector, pDEF-GFP, constructed from pDEF3 (Kozak, 1987) and pQBI25 (Quantum). The resulting pDEF-ScFv-GFP vector has a EF-1α promoter and GFP reporter gene at the C-terminus of the expression cassette (Figure 12a and Figure 16). HEK 293 cells were transfected with pDEF-ScFv-GFP using Superfect (QIAGEN).

For co-transfection studies using both Nef and ScFv vectors, the ScFv cassettes were subcloned into pDEF-myc, a vector with the same promoter, restriction sites and plasmid backbone, but with a myc tag instead of GFP reporter gene (Figure 12a and Figure 20).

For the addition of Nef myristoylation signal sequence to the N-termini of ScFv constructs, the following primers were used: Nef-MF, ACGAATTCGCCACCATGGGTAAGTGCTCA; Nef-MR, AGACTGGGTCATCAAAATGTCTCTTTCCCTACAGCAGG; ScFv-MF, CCTGCTGTAAGGGAAAGAGACATTTTGATGACCCAGTCT; ScFv-MR, TCCACCGCGTGGCGGCGCCCGCTTAGACTAGCC. Overlapping PCR was
performed using Nef-MF/Nef-MR as primers and pGEX-BH10Nef as template; as well as ScFv-MF/ScFv-MR as primers and pDEF-ScFv-myc as template, respectively. The two PCR products were annealed and used as a template for primers, Nef-MF/ScFv-MR. PCR conditions were similar as section 2.3. The overlapping PCR products were subsequently cloned into the mammalian expression vector, pDEF-myc (Figure 12a and Figure 20).

2.6 Fluorescent microscopy
HEK 293 cells were transfected and cultured overnight in chamber slides (Lab-Tek, NUNC). The slides were then washed three times with PBS and fixed for 10 min in 4% paraformaldehyde at room temperature. After three PBS washes, the slides were mounted with Gel/Tol Aqueous Mounting Medium (Immunon, Fisher), and sealed with nail polish. A fluorescent microscope (Zeiss) and CCD camera were used to monitor the GFP fusion protein expression.

2.7 Characterization of expressed ScFv by immunoprecipitation
HEK 293 cells were transfected and cultured overnight in 6-well tissue culture plates (Nunclon Surface, NUNC), and washed once with cold PBS and lysed in 1 ml of lysis buffer (0.5% NP-40, 100 mM NaCl, 25 mM Tris, pH7.5, 2 mM EDTA, 10% glycerol, 50 mM NaF, and 10 µg/ml of each of the protease inhibitors: leupeptin, aprotinin, soybean trypsin inhibitor). The supernatant was first cleared of cell debris by centrifugation and subsequently pre-cleared by GST cross-linked to Sepharose beads (CNBr-activated Sepharose 4B, Pharmacia). The recombinant Nef-GST fusion protein cross-linked to Sepharose beads was used to immunoprecipitate ScFv by incubating for 1 hr with rotation at 4°C. The precipitated products were
resolved by SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in TBS-T buffer (10 mM Tris, 50 mM NaCl, and 0.5% Tween 20) and probed with anti-GFP antibody (1:4000 dilution, Boehringer Manheim). Immunodetection was accomplished using goat anti-mouse antibody conjugated to horseradish peroxidase, washed with TBS-T and followed by ECL detection (Amersham). The membrane was subsequently exposed to X-ray film (X-OMAT, Kodak).

2.8 Co-transfection of Nef and ScFv or DN-Hck
P4.2 cells were co-electroporated with 15 μg of recombinant plasmid expressing Nef (pCMV-Nef-FT) or the antisense control plasmid (pCMV-AS), and 15 μg of the ScFv plasmids (pDEF-ScFv-myc or pDEF-mryScFv-myc), or dominant-negative Hck plasmid (pCAGGS-DN-Hck) or its mutants (pCAGGS-DN-Hck-W93F, DN-Hck-R151S), in association with 4 μg of the pDEF-GFP reporter plasmid.

Electroporations were performed at 200 V, 960 μF using 4 mm-gap cuvettes in a Bio-Rad Gene Pulser. Approximately at 20 h post-transfection, the cells were harvested by trypsinization and washed with PBS for further analysis.

2.9 Flow cytometry analysis of CD4 and MHC-1 expression
The cells were stained with either anti-CD4-PE (RPA-T4, Pharmingen) or anti-MHC-1-PE (G46-2.6, Pharmingen) for 30 min on ice in FACS buffer (PBS with 2% FBS). The cells were then washed for three times with FACS buffer. GFP positive
cells were gated and analyzed on FACSort flow cytometer (Becton-Dickenson) using CellQuest software.

2.10 SDS-PAGE and Western blot analysis of expressed protein

The expression of ScFv, dominant-negative Hck and its mutant, the control CrkII, Nef and GFP were monitored by SDS-PAGE and Western blot of the total cell lysate. SDS-PAGE was performed on 12.5% polyacrylamide gels under reducing conditions. After protein transfer onto nitrocellular membranes, the blots were probed with individual primary antibodies: anti-c-myc-peroxidase (9E10, Boehringer Mannheim), anti-Hck (N-30, Santa Cruz), anti-Crk (Transduction labs), Rabbit anti-Nef serum (331, NIH AIDS Research and Reference Reagent Program) or anti-GFP Mab (Boehringer Mannheim), separately, followed by a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, or anti-mouse, DAKO) and ECL detection (Amersham).

2.11 Immunoprecipitation of DN-Hck by immobilized Nef-GST fusion protein

For immunoprecipitation studies, cells were lysed in the lysis buffer (0.5% NP-40, 100 mM NaCl, 25 mM Tris, pH 7.5, 2 mM EDTA, 10% glycerol, 50 mM NaF, and 10 µg/ml of each of the protease inhibitors: leupeptin, aprotinin, soybean trypsin inhibitor). The supernatant was first cleared of cell debris by centrifugation and subsequently pre-cleared by GST cross-linked to Sepharose beads. The Nef-GST fusion protein cross-linked beads were used to immunoprecipitate dominant-negative Hck and its mutants. The precipitated products were resolved by SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane. The
membrane was then blocked with 2% skim milk in TBS-T buffer and probed with anti-Hck antibody (1:4000 dilution, Santa Cruz) or anti-Crk (1:8000 dilution, Transduction Labs) followed by immunodetection as described before.
CHAPTER 3
INTRACELLULAR SINGLE-CHAIN ANTIBODIES TARGETING NEF HAVE NO EFFECT ON NEF-INDUCED CD4 AND MHC-1 DOWNREGULATION

3.1 Introduction
It is well established that Nef is essential for rapid lentivirus replication in vitro and efficient viral growth in SIV animal models and human HIV-1 infection (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). In general Nef has been shown in vitro to: downregulate cell surface CD4 and MHC-1 expression (Piguet et al., 1999b); increase viral infectivity at an early post-entry stage (Aiken and Trono, 1995); and disturb cell signaling and activation pathways (Baur et al., 1994; Hanna et al., 1998; Iafrate et al., 1997; Skowronski et al., 1993).

The major interest of this thesis research is to study how to prevent or inhibit the detrimental effects Nef has on host cells. More specifically, the studies in this chapter examine the possibility of expressing intracellular single-chain antibodies (ScFvs) to block the downregulation of CD4 and MHC-1 molecules-induced by Nef. Both of these receptor down-modulation effects may contribute to the HIV pathogenesis. Nef downregulates CD4 with two other viral proteins, Env and Vpu. This may serve to dysregulate CD4+ T cell function, inhibit viral superinfection, and increase viral infectivity (Piguet et al., 1999b). The function of Nef-induced MHC-1 downregulation may be to prevent cytotoxic T-lymphocyte recognition and the subsequent killing of virally infected cells (Collins et al., 1998).
The emphasis of current anti-HIV therapy has been on the inhibition of HIV viral replication by administration of inhibitors for HIV reverse transcriptase and protease. The problem that arises is that, despite efficient viral growth inhibition by the powerful multi-drug “cocktail-therapy”, the HIV infection in a patient cannot be eradicated even after several years of treatment. Therefore, therapeutic strategies toward other viral targets need to be studied for their effects on inhibition of HIV infection, as well as on the enhancement of the host immunity to eliminate HIV infection.

The studies in this chapter examine the effect of Nef-specific single-chain antibody expression on the CD4 and MHC-1 downregulation induced by Nef. The rationale for these studies is based on the observation that ScFvs have specific binding affinities equivalent to those of the parent monoclonal antibodies (Whitlow, 1991b; Winter and Milstein, 1991), and can be stably expressed intracellularly where they are capable of inactivating specific cellular gene products (Biocca et al., 1990; Carlson, 1988). Intracellular ScFv proteins with specificity for Nef thus provide a unique way of studying this viral protein as a potential therapeutic target (Marasco et al., 1993), as well as offering a gene therapy strategy for inhibiting the development of AIDS.

3.2 Results

3.2.1 Monoclonal antibodies AG11 and AE6 bind to an overlapping epitope with EH1.

Using the binding inhibition ELISA assay, it was shown that Mabs from clones AG11 and AE6 inhibited the binding of the biotinylated EH1 Mab to immobilized Nef protein (Chang et al., 1998). This binding inhibition was specific, since
another Mab, F14.11 that was mapped to a different epitope failed to inhibit the binding of biotinylated EH1 Mab to immobilized Nef (Figure 13).

3.2.2 Cloning and sequencing of mouse IgG variable regions

The variable regions of light and heavy chains were cloned from the mouse anti-Nef monoclonal antibody producing hybridoma clones, AG11, AE6 and EH1. At least three clones from two independent RT-PCR reactions were sequenced to minimize the possibility of errors introduced during the amplification step.

An aberrant Vk transcript was identified in all three hybridoma cell lines as described (Carroll et al., 1988; Duan and Pomerantz, 1994). Using primers specific for the CDR-1 and CDR-3 regions of the Sp2/0 endogenous kappa chain variable region, it was possible to identify plasmids containing the aberrant kappa chain cDNA using the colony PCR method (Duan and Pomerantz, 1994). An internal HindIII site was found in the PCR amplified kappa chain (between CDR-2 and CDR-3, at position 206, Figure 14-A.) from clone EH1. Therefore, the TA cloning vector (Invitrogen), instead of pGEM-4Z, was used to subclone this cDNA.

Sequences from the three hybridoma cell lines were then analyzed. The aligned DNA and predicted amino acid sequence of the light chain and heavy chain variable regions are shown in Figure 14 and 15, respectively. The complementarity determining regions (CDRs) were defined according to the Kabat-Wu numbering scheme (Kabat, 1991).
Figure 13 Binding inhibition of biotinylated EH1 Mab by unlabeled AG11 (♦) and AE6 (▲) Mabs. F14.11 (○) was used as a control Mab. Each Data set is representative of three independent experiment.
Figure 14 Alignment of the cDNA sequences of variable regions derived from clones AG11, AE6 and EH1.

A: Light chain variable regions. B: Heavy chain variable regions. ‘-’ denotes identical residues; ‘/’ denotes gaps. On the light chain variable region of clone EH1, there is an internal HindIII restriction site (position 206) (underlined). The complementarity determining regions are indicated.
Figure 15 Deduced amino acid sequence alignment of the variable regions of clones AG11, AE6 and EH1. A: Light chain variable regions. B: Heavy Chain variable regions. ‘-’ denotes identical residues; ‘/’ denotes gaps.
Table 2 Comparison of the variable region sequences of clones AE6 and EH1 with AG11.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>% similarity&lt;sup&gt;1&lt;/sup&gt; relative to clone AG11</th>
<th>% similarity&lt;sup&gt;1&lt;/sup&gt; relative to clone AG11</th>
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<tr>
<td></td>
<td>Nucleic acid sequence</td>
<td>Amino acid sequence</td>
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<tr>
<td></td>
<td>$V_k$  $V_H$  Total CDRs&lt;sup&gt;2&lt;/sup&gt;</td>
<td>$V_k$  $V_H$  Total CDRs</td>
</tr>
<tr>
<td>AE6</td>
<td>96.5%  98.6%  95.1%</td>
<td>93.8%  97.5%  91.2%</td>
</tr>
<tr>
<td>EH1</td>
<td>73.8%  76.6%  57.9%</td>
<td>61.7%  63.2%  36.8%</td>
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</table>

<sup>1</sup>The similarity study was done according to the overlapping sequences.
<sup>2</sup>Total CDRs: The total complementarity determining regions of both $V_k$ and $V_H$.

All of the sequences of the variable regions from the three hybridomas contained open reading frames. At the cDNA level, the total CDRs of clone AG11 was 95.1% similar to that of clone AE6 (Table 2). Both antibodies recognize the C-terminus of Nef from LAI strain. The total CDRs of clone AG11, in contrast, was only 57.9% similar to clone EH1, which recognizes an overlapping epitope at the C-terminus of Nef (Table 2). A low percentage of sequence similarity was also found at the amino acid level when clones AG11 and EH1 were compared (Table 2). Thus, while there was 91.2% similarity when the amino acids of the total CDRs of clones AG11 and AE6 were compared, there was only 36.8% identity when clone AG11 was compared with EH1.

3.2.3 Construction and expression of intracellular ScFv tagged with a GFP reporter

Single-chain antibodies (ScFvs) were constructed for clones AG11 and EH1 as described in the Materials and Methods section. The cDNA of the light chain
variable region was tethered to the heavy chain variable region through a linker DNA sequence encoding (GGGGS)_3. The ScFv cDNA constructs were then ligated to the EcoRI and NheI sites of the pDEF-GFP expression vector (Figure 16).

To assess the intracellularly expressed single-chain antibodies, HEK 293 cells were transfected and cultured overnight. The transfected cells were then examined using a fluorescent microscope and attached CCD camera. Comparable levels of expression were achieved using all ScFv-GFP constructs, as well as the control vector expressing only GFP (Figure 17).

The ability of the intracellularly expressed ScFv to bind with Nef protein was assessed by immunoprecipitation of ScFv-GFP, performed using recombinant Nef protein-immobilized on Sepharose beads. The results demonstrated that the immobilized Nef was able to precipitate expressed ScFv-GFP (Figure 18, panel A), but not GFP alone (Figure 18, panels B and C). The ScFv constructs were used in the subsequent intracellular gene expression assays against Nef protein.
Figure 16 The single chain antibody assembly in pDEF-GFP expression vector. A: pDEF-GFP vector which express GFP. B: pDEF-ScFv-GFP vector which express single chain antibody-GFP fusion protein. The predicted molecular weight of expressed proteins are: ScFv-GFP, 54.2 kD; GFP alone, 27.0 kD; (Compute pI/Mw tool, ExPASy Molecular Biology Server, Swiss Institute of Bioinformatics, www.expasy.ch/tools/pi_tool.html).
Figure 17 HEK 293 cells transfected with pDEF-GFP or pDEF-ScFv-GFP.
A: GFP (phase); B: GFP (fluorescence); C: AG11 ScFv tagged with GFP (phase); D: AG11 ScFv tagged with GFP (fluorescence); E: EH1 ScFv tagged with GFP (phase); F: EH1 ScFv tagged with GFP (fluorescence); G: F14.11 ScFv tagged with GFP (phase); H: F14.11 ScFv tagged with GFP (fluorescence). Magnification, x36.
Figure 18 Intracellularly expressed ScFv is immunoprecipitated by immobilized recombinant Nef-GST protein. A, Immunoprecipitation of ScFv with Nef-GST cross-linked to Sepharose beads from cells transfected with the control vector (1), pDEF-GFP (2), pDEF-(AG11)ScFv-GFP (3), pDEF-(EH1)ScFv-GFP (4), pDEF-(F14.11)ScFv-GFP (5). B, Immunoblot of the total cell lysate of the cells transfected with control vector (1) and pDEF-GFP (2). C, Immunoprecipitation of cells expressing the control vector (1) and pDEF-GFP (2) corresponding to B, by Nef-GST cross-linked with Sepharose beads. SDS-PAGE was performed in 12.5% polyacrylamide gels under reducing conditions.
3.2.4 CD4 and MHC-1 downregulation induced by Nef expression in P4.2 cells

To verify that HIV-1 Nef protein is capable of downregulating CD4 and MHC-1 receptors (Le Gall et al., 1998), as well as to identify an optimal dose of Nef-expressing plasmids for the subsequent transfection studies, P4.2, a Hela cell line expressing CD4, was transfected with various doses (2-15 μg) of pCMV-Nef-FT (expressing Nef), or 12 μg of control vector-pCMV-AS (carrying anti-sense Nef sequence), and co-transfected with 4 μg of pDEF-GFP (expressing green fluorescence protein) as an expression reporter. Flow cytometry was performed for the analysis of CD4 and MHC-1 receptors on cells gated for GFP expression 20 hr post-transfection. The extent of receptor downregulation was examined for various levels of Nef expression (Figure 19).

Maximum modulation of MHC-1 (24-26%) was reached with transfection of 12-15 μg Nef-FT plasmid. More dramatic downregulation of CD4 (46-49%) was observed with transfection of Nef-FT plasmid even at low doses (2-4 μg). Western blot analysis showed that levels of Nef expression were proportional to the amount of transfected Nef-FT plasmid (Figure 19 B). The optimal dose of Nef plasmid DNA was determined to be at 12-15 μg for transfection assays in order to accommodate the moderate effect of Nef transfection on MHC-1 downregulation in P4.2 cells.

3.2.5 Intracellular expression of ScFv EH1 and F14.11 did not affect receptor modulation induced by Nef

Intracellular expression of ScFv against Nef was achieved using two ScFv constructs, EH1 and F14.11 (Figure 20). The ScFv F14.11 that was raised against a central domain of Nef (AAVDSLSS) was constructed and its Nef binding capability
Figure 19 CD4 and MHC-1 modulation in response to Nef.

P4.2 cells were co-electroporated with 4 μg of pDEF-GFP, and the indicated amounts of pCMV-Nef-FT. A. Cells were stained 20 hr later with the anti-HLA-A, -B, -C Mab or anti-CD4 Mab labeled with PE and analysed by flow cytometry. The contour plots represent CD4 or MHC-1 fluorescence levels in GFP+ cells when the Nef-FT vector used for transfection was 0 (a and g), 2 μg (b and h), 4 μg (c and i), 8 μg (d and j), 12 μg (e and k), and 15 μg (f and l). The percentage of cells located in the lower left quadrant of each plot was indicated. B. Western blot analysis. Lysates from transfected cells were analyzed with a rabbit polyclonal anti-Nef antibody. NT, nontransfected control cells. AS, cells were transfected with 12 μg of the antisense Nef vector. SDS-PAGE was performed in 12.5% polyacrylamide gel under reducing conditions. Each data set is the representative of three independent experiments.
A. 

CD4-PE fluorescence intensity

<table>
<thead>
<tr>
<th>Graph</th>
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<tr>
<td>a</td>
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<td>b</td>
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<td>c</td>
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<tr>
<td>k</td>
<td>72.2%</td>
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<tr>
<td>l</td>
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High angle scatter (90° light scatter)

B. 

Nef (27kD)

<table>
<thead>
<tr>
<th>Sample</th>
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<tbody>
<tr>
<td>NT</td>
<td></td>
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<tr>
<td>AS</td>
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Figure 20 The single-chain antibody assembly in pDEF-myc expression vector. A. pDEF-myc vector which contain the myc tag. B. pDEF-ScFv-myc which express single-chain antibody tagged with myc. C. pDEF-myr-ScFv-myc which express single-chain antibody fused with Nef myristoylation signal sequence at N-terminus and tagged with myc at C-terminus. The predicted molecular weight of expressed proteins are: ScFv-myc, 31.1 kD; myr-ScFv-myc, 33.5 kD; myc alone, 3.8 kD. (Compute pi/Mw tool, ExPASy Molecular Biology Server, Swiss Institute of Bioinformatics, www.expasy.ch/tools/pi_tool.html).
confirmed by immunoprecipitation as described previously. To improve the efficiency of ScFv expression, both ScFv constructs were subcloned into another pDEF vector with a myc tag. Further modification was done to fuse the myristoylation signal sequence of Nef to the N-termini of the two ScFv constructs by PCR as described in Materials and Methods (Figure 12a and Figure 20). These modifications could potentially serve to increase the efficiency of binding association of the ScFv proteins with Nef since both have membrane anchoring myristoylation signals.

To study the effects of expressing these intracellular antibodies on class I MHC and CD4 expression in Nef expressing cells, P4.2 cells were transfected with control vectors, Nef alone, or Nef plus each of the ScFv constructs. Comparable expression levels of EH1, F14.11 and their myristoylation modified counterparts were obtained in the transient expression experiments (Figure 21). Although Nef expression levels were similar in the transient transfection of Nef plasmid alone, and Nef plus ScFv EH1 or ScFv F14.11, the transfection of the two myristoylated ScFv plasmids tended to be associated with more significant reduction in the levels of Nef expression compared to the transfection of their corresponding non-myristoylated ScFv constructs (Figure 21). This might indicate that the addition of the myristoylation signal sequence to the ScFvs made them more efficient in reducing the Nef expression level, probably by increasing protein sequestration due to more efficient intracellular binding interaction. As an expression control, GFP expression levels were also monitored, they were similar in all transfections with various vectors (Figure 21).
Figure 21 Co-expression of single-chain antibodies and Nef in P4.2 cells. Cells were electroporated with 15 ug of pCMV-AS and pDEF-myc (Control), 15 ug pCMV-NEF-FT together with pDEF-myc vector (Nef), orpCMV-NEF-FT with pDEF-EH1-myc (Nef+EH1 lane), pDEF-myr-EH1-myc (Nef+myr-EH1), pDEF-F14.11-myc (Nef+F14.11), pDEF-myr-F14.11-myc (Nef+myr-F14.11), along with 4 ug of the pDEF-GFP reporter plasmid in each case. Cell lysates were collected 20 hr post-transfection and analyzed by immunoblotting with a monoclonal anti-myc antibody (9E10), rabbit anti-Nef antibody and monoclonal antibodies against GFP, after stripping of each previous blot. SDS-PAGE was performed in 12.5% polyacrylamide gel under reducing conditions.
Flow cytometry of P4.2 cells at 20 hr post-cotransfection with ScFv and Nef expression plasmids demonstrated that the intracellular antibodies expression did not prevent CD4 or MHC-1 downregulation (Figure 22). The myristoylation modified ScFvs that were shown to reduce Nef expression levels did not have significant effect on Nef-induced CD4 or MHC-1 downregulation, either (Figure 22). This indicated that the intracellular binding of ScFv with Nef and the Nef sequestration by the transfection of ScFv constructs may not be sufficient to prevent the receptor downregulation induced by Nef. The expression of a molecule capable of binding to the domains of Nef that are implicated in receptor downregulation may be required for this effect.

3.3 Discussion

In this chapter, intracellular single-chain antibody approach was studied to inhibit the cell surface CD4 and MHC-1 molecules downregulation induced by Nef, in order to restore T cell function and to enhance the host cellular immunity to better recognize and clear the infected cells. The knowledge obtained from these studies can be potentially used for the discovery of new drug targets and for the development of alternative gene therapy against HIV infection.

To interfere with Nef function intracellularly, single-chain antibodies against Nef were first constructed and used in the subsequent studies to evaluate their effect on the CD4 and MHC-1 receptors downregulation induced by Nef. During the process of constructing single-chain antibodies, the sequences of the variable regions from a group of three monoclonal antibodies (AG11, AE6 and EH1) that recognize an overlapping epitope of Nef protein were cloned and analyzed. It
Figure 22 Flow cytometry analysis of CD4 and MHC-1 receptor expression in P4.2 cells co-transfected with Nef and ScFv constructs.

Surface CD4 (a-e) and MHC-1 receptor (f-j) levels were assessed by FACSort 20 hr post transfection of P4.2 cells with: Nef and pDEF-myc control vector (a and f), Nef with pDEF-EH1-myc (b and g), Nef with pDEF-myr-EH1-myc (c and h), Nef with pDEF-F14.11-myc (d and i), Nef and pDEF-myr-F14.11-myc (e and j) (open histograms). The filled histograms in the top two panels (a and f) represent the Nef-AS and pDEF-myc control vector-transfected cells. The filled histograms in others (b-d and g-j) represent the Nef-FT and pDEF-myc vector-transfected cells. These results were representative of three independent experiments.
has been shown that clones AG11 and AE6, but not EH1, were highly related. Although the variable region sequences of EH1 clone were significantly different from those of the other two Mabs, EH1 recognizes an overlapping epitope within the binding site of the AG11 and AE6 Mabs. This is supported by the binding inhibition assay which demonstrated that the antibodies from clones AG11 and AE6 inhibited the binding of the biotin-labeled EH1 Mab to Nef protein (Figure 13). This study confirmed observations made by others that the antigen recognition capability of an antibody is determined by the protein structure rather than the primary sequence of the antibody variable regions (Mariuzza et al., 1987). More detailed analysis using mutation studies or comparison of crystal structures of the antibody-antigen complex would be required to elucidate this further.

A strategy for efficient construction and evaluation of single-chain antibodies was developed. The single-chain antibody cassettes composed of cDNA of the variable regions of heavy and light chains that were linked by a DNA sequence encoding a 15 amino acid peptide (GGGGS)_3 were cloned into a plasmid vector with EFα promoter and green fluorescent protein (GFP) fusion at the C-terminus of the ScFv constructs. A transient transfection system using HEK293 cell line was established. GFP was used as a reporter for monitoring ScFv expression in eukaryotic cells. The advantage of GFP is that the ScFv expression can be readily monitored in either live or fixed cells. Several anti-Nef single chain antibodies were constructed from the variable regions of monoclonal antibodies that recognize the C-terminus and a central domain (AAVDLS88) in Nef (Figure 16 and Figure 20). All retained the binding activity of their corresponding parental
monoclonal antibodies when expressed intracellularly as demonstrated by 'pull-down' assays using recombinant Nef protein (Figure 18).

P4.2, a Hela cell line stably expressing CD4 receptor, was used for studying intracellular single-chain antibody expression against Nef. The effect of intracellular ScFv expression against Nef was studied using two ScFv constructs, EH1 and F14.11, that recognized the C-terminus and the central domain of Nef (AAVDLS88), respectively. Both were confirmed for Nef binding ability by immunoprecipitation as introduced previously.

When the ScFvs and Nef are co-expressed in P4.2 cells after transient transfection, it was somewhat surprising to find that expression of the intracellular antibody did not significantly inhibit CD4 or MHC-1 downregulation induced by Nef. This was unexpected as the ScFvs were shown to bind with Nef, and one of the possible modes of action by these intracellular antibodies is to facilitate the sequestration of the Nef protein upon binding. To rule out the possibility of inefficient binding interaction between these two ScFvs and Nef due to post-translational myristoylation of Nef, which makes Nef a membrane-anchoring protein, the two ScFv constructs were further modified to fuse with the Nef-myristoylation signal sequence at the N-terminus. Transient transfection of P4.2 cells with the corresponding N-terminus myristoylation-modified ScFv vectors showed more significant reduction in the levels of Nef expression compared to the transfection of their corresponding non-myristoylated ScFv clones (Figure 21). This indicated that the addition of the myristoylation signal sequence to the ScFv clones made them more efficient in
reducing Nef expression level, possibly due to more efficient intracellular interaction with their target protein. However, regardless of the N-terminal myristoylation status, none of the expressed ScFv was able to disrupt CD4 or MHC-1 downregulation induced by Nef (Figure 22). This indicated that the intracellular binding of ScFv with Nef and the subsequent Nef sequestration may not be sufficient to prevent receptor downregulation events induced by Nef. The expression of molecules capable of binding to domains in Nef that are implicated specifically in receptor modulation may be required for this effect. This confirmed the observation made by others that binding activity of ScFv with a target viral protein can not be used to predict its intracellular activity, and the targeting epitope of an intracellular antibody plays a pivotal role in disrupting the function of that viral protein (Rondon and Marasco, 1997).

In summary, anti-Nef single chain antibodies were successfully constructed. All retained the binding activity of their corresponding parental monoclonal antibodies when expressed intracellularly. The incorporation of GFP as a reporter enabled intracellular ScFv expression to be readily evaluated. A C-terminal specific and a central domain specific anti-Nef ScFvs were studied for their capability for inhibition of the CD4 and MHC-1 downregulation effects induced by Nef. Neither one blocked the CD4 and MHC-1 down modulation induced by Nef when co-expressed in P4.2 cells. Modification of these ScFvs with N-terminal Nef myristoylation signal sequence fusion reduced the level of Nef expression significantly, probably due to more efficient binding and the subsequent sequestration of Nef. However, the myristoylation modification of these ScFvs did not make them effective inhibitors of CD4 or MHC-1
downregulation induced by Nef. It is concluded therefore that the binding interaction between ScFv with Nef and the subsequent sequestration may not be sufficient to interfere with its intracellular function. A ScFv or other intracellular ligands that could recognize and block epitopes in Nef that are important for these functions would be required to have these specific effects.
CHAPTER 4
HCK SH3 DOMAIN-DEPENDENT ABROGATION OF NEF-INDUCED CLASS I MHC DOWNREGULATION

4.1 Introduction
As stated in Chapter 3, while current anti-HIV therapy is successful in keeping viral replication suppressed, it fails to eradicate the viral infection because of its inability to eliminate the reservoir of infected cell (Balter, 1997). Therefore, therapeutic strategies toward the goal of eliminating the remaining HIV infected cells, in combination with current anti-HIV therapies that suppress viral replication, are desirable in the treatment of HIV infection. In this regards, Nef may be a potential therapeutic target for HIV infection, because of its role in immune escape of HIV infected cells by downregulation of antigen presentation MHC-1 molecules on cell surface (Collins and Baltimore, 1999).

Among the many detrimental effects of Nef on host cells, the MHC-1 downregulation effect deserves special attention. Nef is the only HIV-1 viral protein that has been found to downregulate MHC-1 in primary CD4+ T cells (Collins and Baltimore, 1999). This is in contrast with its role in downregulation of CD4 receptors which overlaps with Env and Vpu. Furthermore, the determinants of HIV-1 Nef which is responsible for MHC-1 downregulation are distinct from that of the CD4 downregulation. As reviewed in Chapter 1, these determinants include a conserved α-helix near the N-terminus of the protein, an acidic stretch in the proximal region of HIV-1 Nef, EEEE65, and the polyproline motif forming the SH3-binding surface, PXXP78 (Greenberg et al., 1998b;
Mangasarian et al., 1999). The mechanism of downregulation of MHC-1 induced by Nef remains to be elucidated; the LL165 dileucine motif which mediates the interaction of HIV-1 Nef with adaptor complexes and is required for accelerated CD4 endocytosis, is dispensable for MHC-1 downregulation (Mangasarian et al., 1999).

Nef-induced MHC-1 modulation was shown to protect HIV-1 infected cells against lysis by virus-specific CTLs in vitro (Collins et al., 1998). However, unless HLA-C or -E is expressed, downregulating MHC-1 normally renders target cells more sensitive to destruction by NK cells (Brutkiewicz and Welsh, 1995; Colonna et al., 1993). Since these molecules are required for the negative control of NK mediated cytotoxicity. In this regards, Nef was shown to selectively downregulate MHC-1 HLA-A, and HLA-B molecules, but not HLA-C or HLA-E (Collins and Baltimore, 1999; Le Gall et al., 1998). This selective downregulation of HLA-A and -B, but not -C, induced by Nef might therefore permit infected cells to display limited recognition for CTLs without being exposed to the attacks of NK cells.

In Chapter 3, the effect of Nef-specific single-chain antibody expression in an attempt to block the CD4 and MHC-1 downregulation induced by Nef was examined. It was shown that the anti-Nef single-chain antibodies constructed retained the antigen-specificity of their corresponding parent antibodies. However, these ScFvs failed to inhibit the CD4 and MHC-1 downregulation induced by Nef. It is therefore concluded that the binding interaction and the following sequestration of Nef is not sufficient for blocking Nef functions, and an intracellular ligand that
could recognize the Nef determinants directly implicated in these receptor modulation effects are required to have maximum inhibition effect. As a natural extension of the Chapter 3, the thesis research in this chapter examines the possibility of inhibition of Nef functions by a dominant-negative Hck, a Src family of tyrosine kinases devoid of the kinase domain.

Several Src family tyrosine kinase are able to bind the polyproline (PXXP78) motif in Nef by their corresponding SH3 domains, with the Hck SH3 domain being able to bind Nef with the highest affinity yet reported for an SH3-polyproline tract interaction (Kd = 0.25 μM) (Lee et al., 1995; Saksela et al., 1995). Taking advantage of this property, a dominant-negative Hck mutant, DN-Hck, consisting of the Hck amino-terminal domain, as well as the SH3 and SH2 domains was expressed in HIV-1 producing cells (Tokunaga et al., 1998). Interestingly, viral particles assembled in the presence of DN-Hck demonstrated reduced infectivity suggesting that DN-Hck might be inhibiting an unknown Src family kinase activated by Nef at an early post-viral entry stage (Tokunaga et al., 1998).

In this thesis, it is hypothesized that DN-Hck might also be capable of interfering with Nef-mediated effects through direct interaction with Nef and hence blocking the downstream events induced by Nef. It is further hypothesized that the Nef-mediated effect that DN-Hck interferes with, is very likely the MHC-1 downregulation effect, since the SH3-binding surface in Nef was implicated in this effect as determined by mutation studies. In keeping with this notion, this research revealed that DN-Hck prevented Nef-induced class I MHC downregulation, and this effect was dependent on the presence of a functional
Hck SH3 domain. Inhibition of the Nef-mediated effect demonstrated in this work is of potential therapeutic importance, given that an intact Nef SH3-domain binding site is not only required for the MHC-1 downregulation, it also has a number of other detrimental effects attributed to Nef during HIV-1 infection (Arolf et al., 1997; Briggs et al., 1997; Collette et al., 1996; Goldsmith et al., 1995; Greenberg et al., 1998b; Iafrate et al., 1997; Mangasarian et al., 1999; Moarefi et al., 1997).

4.2 Results

4.2.1 Dominant-negative Hck binds Nef through its SH3 domain

Expression of the dominant-negative Hck (DN-Hck), its SH3 and SH2 mutants, as well as a CrkII control vector were evaluated in transient transfection of P4.2 cells (Figure 23) (Tokunaga et al., 1998). The cells overexpressing these proteins were lysed and the cell lysates were examined for their ability to bind Nef cross-linked with Sepharose beads in immuno-precipitation assays.

In addition to the wild type DN-Hck construct, two DN-Hck mutants were also used. The amino acid, Trp93, which was shown at the binding interface between Nef PP-II helix and Fyn (R96I) SH3 in the crystal structure of Nef-SH3 (corresponding to Trp119 of SH3 described in Literature Reviews) (Lee et al., 1996), was substituted with Phe in DN-Hck-W93F construct (Tokunaga et al., 1998). Arg151, which is essential for the function of SH2, was substituted with Ser in DN-Hck-R151S construct (Tokunaga et al., 1998). CrkII adaptor protein, which consists mostly of the SH2 and SH3 domains, was used as a control for DN-Hck (Tokunaga et al., 1998).
Figure 23 Structures of the wild-type Hck(A), the dominant-negative mutant form of Hck(B), and the two DN-Hck mutants (C). (Reproduced from Tokunaga et al., 1998)
Figure 24 A functional Hck SH3 domain is required for efficient binding of Nef to DN-Hck. A, Expression of DN-Hck in the P4.2 cells. Cells were electroporated with 15 µg of PCAGGS vector (control lane), pCAGGS-DN-Hck, pCAGGS-DN-Hck-W93F, pCAGGS-DN-Hck-R151S, or pCAGGS-CrkII, along with 4 µg of the pDEF-GFP reporter plasmid. Cell lysates were collected 20 h later and analyzed by immunoblotting with rabbit anti-Hck antibodies or with a monoclonal antibody against CrkII. The blot was stripped and reprobed with anti-GFP antibody. B, Immunoprecipitation of DN-Hck by recombinant Nef protein. P4.2 cell lysates were pre-cleared by GST cross-linked to Sepharose beads, then the Nef-GST fusion protein cross-linked beads were used to immunoprecipitate DN-Hck, its mutants and the control CrkII. The precipitated products were analyzed by immunoblot with anti-Hck antibody and the anti-Crk antibodies. SDS-PAGE was performed in 12.5% polyacrylamide gels under reducing conditions.
DN-Hck, its mutants and the CrkII control were expressed at comparable levels in P4.2 cells (Figure 24 Panel A). Only DN-Hck and its SH2 mutant, DN-Hck-R151S, were efficiently immunoprecipitated by Nef-GST cross-linked on Sepharose beads (Figure 24 Panel B). The binding interaction between DN-Hck with Nef is specific, since there was no binding between Nef and CrkII, an adaptor protein with SH2 and SH3 domains. The binding between DN-Hck-R151S with Nef was not affected by the Arg151 to Ser mutation, indicating that the SH2 domain is not directly involved in binding to Nef. However, the SH3 domain mutant-DN-Hck-W93F was not efficiently immunoprecipitated by Nef cross-linked on Sepharose beads, indicating that W93 residue is critical for the efficient binding interaction between these two molecules and that the Hck SH3 domain is responsible for the binding interaction with Nef.

To study the effects of DN-Hck and the two point mutants of this molecule on class I MHC expression in Nef expressing cells, P4.2 cells were transfected with Nef alone, or Nef plus each of the DN-Hck mutants (a representative experiment is shown in Figure 25). Equivalent expression levels of all three DN-Hck molecules were obtained in the transient expression experiments, and although expression of the DN-Hck molecules tended to be associated with a modest reduction in the levels of Nef expression (not seen with the CrkII control transfection), Nef (and GFP control) expression levels were similar in the presence of vectors expressing DN-Hck, DN-Hck-W93F, and DN-Hck-R151S (Figure 25).
Figure 25 Co-expression of DN-Hck mutant proteins and Nef in P4.2 cells. Cells were electroporated with 15 ug of AS and pCAGGS vector (control lane), 15 ug Nef vector together with pCAGGS (Nef lane), or Nef vector with HckN pCAGGS-DN-Hck (Nef+HckN lane), pCAGGS-DN-Hck-W93F (Nef+HckN-W93F lane), pCAGGS-DN-Hck-R151S (Nef+HckN-R151S lane), or pCAGGS-CrkII (Nef+CrkII lane), along with 4 ug of the pDEF-GFP reporter plasmid in each case. Cell lysates were collected 20h post-transfection and analyzed by immunoblotting with rabbit anti-Hck antibodies and a monoclonal antibody that recognized CrkII (top row). After stripping, the immunoblot was first probed with anti-Nef antibody (middle row), and after re-stripping, with the anti-GFP antibody (bottom row). SDS-PAGE was performed in 12.5% polyacrylamide gels under reducing conditions.
4.2.2 Differential rate of CD4 and MHC-1 downregulation induced by Nef

Nef is well known to downregulate both CD4 and MHC-1 receptors (Piguet et al., 1999b). In keeping with this, surface expression of CD4 and MHC-1 receptors were significantly downregulated within 20 hrs after electroporation of P4.2 cells with the Nef-expressing vector (Figure 26 a and f). MHC-1 downregulation was less profound than that of CD4, likely due in part to the observed differences in turnover rates of these two molecules in different cell types (Collette, 1997; York and Rock, 1996), and also to the observation that not all class I MHC molecules are subject to downregulation by Nef (Cohen et al., 1999; Le Gall et al., 1998).

4.2.3 DN-Hck inhibits class I MHC downregulation induced by Nef

Flow cytometry analysis of P4.2 cells at 24 hr post-cotransfection with plasmids of DN-Hck and Nef demonstrated that DN-Hck completely blocked Nef-induced class I MHC downregulation (Figure 26 g and Figure 27a). DN-Hck-R151S was equally effective in blocking this Nef effect (Figure 26i; Figure 27a). However, expression of DN-Hck-W93F, a mutant predicted to have impaired SH3 function, failed to interfere with Nef-induced class I downregulation (Figure 26h, Figure 27a). Likewise, CrkII expression had no appreciable effect on class I MHC expression levels (Figure 26j, Figure 27a). Transfection of P4.2 cells with increasing concentrations of the vector expressing DN-Hck revealed a dose-dependent inhibition of Nef-induced class I downregulation (Figure 29). To control for the possibility that DN-Hck expression might itself be able to alter class I MHC cell surface expression, DN-Hck, DN-Hck-W93F, Hck-R151S, and CrkII were transfected into P4.2 cells in the absence of Nef, and MHC class I (and
CD4) levels were monitored by flow cytometry. No significant change in the cell surface expression of either molecule was observed as compared with controls (Figure 28). Taken together, these results suggested that an interaction between the SH3 domain of DN-Hck and the SH3-binding surface of Nef might be the mechanism responsible for preventing the down-modulation of class I MHC downregulation caused by this viral protein.

These results demonstrate that DN-Hck, upon binding to the SH3-binding site of Nef, blocks the downstream interaction of Nef with cellular proteins responsible for recruiting and targeting MHC-1 molecules towards degradation pathway. The region on DN-Hck that is required for this action is the SH3 domain of the DN-Hck, since the SH2 domain mutant did not affect this blocking interaction.

4.2.4 DN-Hck partially affects CD4 receptor downregulation by Nef

The CD4 receptor downregulation by Nef was partially blocked by co-expressing DN-Hck (Figure 26b and Figure 27a). The same effect was observed in cells co-expressing Nef and the SH2 mutant-DN-Hck-R151S (Figure 26d and Figure 27a). The SH3 mutant, DN-Hck-W93F, as well as the control vector expressing CrkII failed to affect the CD4 receptor downregulation in the presence of Nef (Figure 26c, 26e and Figure 27a).

It is well established that distinct determinants on Nef are used for CD4 and MHC-1 downregulation (Piguet et al., 1999b). Except for the amino-terminal myristoylation signal sequence of Nef, which is required for the membrane localization and critical for Nef to interact with both CD4 and MHC-1 molecules,
Figure 26 The dominant-negative Hck blocks the CD4 and MHC-1 cell surface molecule downregulation effect induced by Nef.

Surface CD4 (a-e) and MHC-1 receptor (f-j) levels in P4.2 cells after transient transfection of Nef with control vector (a and f), Nef with DN-Hck (b and g), Nef with DN-Hck-W93F (c and h), Nef with DN-Hck-R151S (d and i), as well as Nef and CrkII (e and j) (open histograms). Control vector transfected cells: filled histograms.
Figure 27 Summary of CD4 and MHC-1 receptors downregulation-blocking effect by dominant negative Hck. The x axes represent geometric mean values of MHC-1 (a) and CD4 (b) fluorescence from cells transiently transfected with Mock, Nef, Nef and DN-Hck, Nef and DN-Hck-W93F, Nef and DN-Hck-R151S, as well as Nef and Crkll. Error bars represent the standard deviation from three independent experiments.
other motifs required for CD4 downregulation are dispensable for the MHC-1 downregulation, and *vice versa* (Piguet et al., 1999b). Furthermore, the SH3-binding surface PXXP78 on Nef which binds with Hck was implicated only in MHC-1 downregulation, but not in CD4 downregulation. The reason for the partial blocking effect of DN-Hck on Nef-mediated CD4 downregulation can be explained by the steric hindrance effect of DN-Hck when it binds the SH3-binding surface in Nef, rather than the direct blocking of Nef interaction with downstream cellular proteins or Nef binding to CD4. The fact that both wild type and R151S mutants of DN-Hck, which bind with Nef, could partially block the CD4 receptor modulation induced by Nef, but neither the SH3 mutant, DN-Hck-W93F, nor the CrkII control which do not bind with Nef, interfere with the CD4 receptor downregulation in the presence of Nef, supports the notion that binding of DN-Hck with Nef on the SH3-binding surface is required for this steric hindrance effect.

4.2.5 *DN-Hck does not modulate CD4 or MHC-1 receptor expression by itself*

DN-Hck may also interact with cellular proteins directly and upregulate MHC-1 and CD4 receptors by activation of cellular signaling pathways. To rule out this possibility, P4.2 cells were transfected with DN-Hck alone and surface expression of receptors were evaluated by flow cytometry. No significant cell surface CD4 or MHC-1 molecule modulation was observed (Figure 28a and 28e). Similarly, DN-Hck-W93F, Hck-R151S, and the control CrkII do not have significant effect on cell surface expression of CD4 and MHC-1 molecules (Figure 28).
This indicates that the effect of the DN-Hck on CD4 and MHC-1 receptor downregulation by Nef is mainly through the direct interaction between DN-Hck and Nef. The DN-Hck, upon binding with the SH3-binding surface of Nef through its SH3 domain, effectively blocks the interaction of Nef with the downstream cellular protein(s) responsible for MHC-1 downregulation.

4.2.6 Dose response of dominant-negative Hck in Nef expressing cells
Electroporation of P4.2 cells with increasing amounts of plasmid expressing DN-Hck demonstrated a saturable dose-dependent increase of MHC-1 receptor in the presence of Nef (Figure 29, upper panel). This further supports that the binding interaction, most likely between the SH3 domain of DN-Hck and the SH3-binding surface in Nef, plays a central role in the prevention of MHC-1 receptor downregulation by Nef. There is also a slight dose-dependent increase of CD4 receptors on P4.2 cell when DN-Hck was co-expressed with Nef (Figure 29, lower panel). The magnitude of this increase is much smaller than that of the MHC-1. The mechanism of the latter effect is different from that of the MHC-1 modulation and most likely because of inefficient CD4 downregulation due to the increasing amount of Nef-complexed with DN-Hck.

4.3 Discussion
The main hypothesis in this thesis research is that during HIV infection class I MHC molecules are downregulated by Nef and without sufficient viral peptide-class I MHC antigen complexes on the cell surface, the HIV-infected cells escape the recognition and subsequent killing by CTL-the major host immunity against
Figure 28 The dominant-negative Hck alone does not modulate CD4 and MHC-1 cell surface molecule expression.

Surface CD4 (a-d) and MHC-1 receptor (e-h) levels in P4.2 cells after transient transfection of DN-Hck (a and e), DN-Hck-W93F (b and f), DN-Hck-R151S (c and g), as well as CrkII (d and h) (open histograms). Cells transfected by control vectors: closed histograms.
Figure 29 The dominant-negative Hck blocks the MHC-1 cell surface molecule downregulation induced by Nef in a dose-dependent manner. The geometric mean of MHC-1 (a) and CD4 (b) fluorescence in cells transiently transfected with Nef (15 ug) and increasing doses of vector expressing DN-Hck (2 to 30 ug) was shown on the ordinate, where 100% values correspond to the fluorescence level of cells transfected with the control vectors (pCMV-AS and pCAGGS). Error bars represent the standard deviation from three independent experiments.
HIV-1 infection (Collins and Baltimore, 1999). It is conceivable that by correcting this problem, the virus infected cells which could not be reached by conventional HIV reverse transcriptase and protease inhibitors could be eliminated. Nef may therefore represent a new target for therapeutic intervention in individuals infected with HIV-1.

The studies summarized in this chapter were initiated because of the concerns that in chapter 3, the single-chain antibodies targeting Nef failed to prevent CD4 and MHC-1 downregulation due to their 'non-essential' epitope binding characteristics, since none of the parent antibodies from the corresponding ScFvs bind to the determinants that are responsible for the CD4 or MHC-1 downregulation induced by Nef. In addition, it has been demonstrated previously that the targeting epitope of an intracellular antibody, rather than its binding activity, plays a pivotal role in disrupting the function of that viral protein (Rondon and Marasco, 1997).

The rationale for using a dominant-negative Hck to block the MHC-1 downregulation induced by Nef is that the Hck SH3 domain was known to bind the polyproline (PXXP78) motif in Nef with the highest affinity ever reported for an SH3-polyproline tract interaction (Kd = 0.25 μM). Furthermore, the Nef SH3 domain-binding PXXP78 motif is required for the MHC-1 downregulation effect.

The results demonstrated that the dominant-negative Hck mutant, DN-Hck, is able to prevent Nef-induced MHC-1 downregulation. The effect is Hck SH3 domain dependent as suggested by the inability of the DN-Hck-W93F mutant to
inhibit this effect. As W93 is a key residue within the SH3 domain binding surface (Lee et al., 1995), it suggested that DN-Hck interferes with Nef function by directly binding to the PXXP site of this molecule. The critical importance of W93 in the Nef-DN-Hck interaction was confirmed by an in vitro ‘pull-down’ experiment (Figure 24). This thesis also suggests that DN-Hck prevents Nef-induced class I MHC down-modulation by blocking the interaction between Nef and an as-yet unidentified SH3-containing cellular protein that is able to couple Nef to the MHC-1 molecule. Upon binding with Nef, this cellular protein might recruit class I MHC molecules via an interaction with their cytoplasmic tyrosine-based sorting motifs, which in turn routes these molecules towards an intracellular degradation pathway (Greenberg et al., 1998b; Le Gall et al., 1998; Mangasarian et al., 1999).

As the SH3-binding sites in Nef are dispensable for CD4 downregulation (Greenberg et al., 1998b; Mangasarian et al., 1999), the modest inhibition of Nef-induced CD4 downregulation observed may have been due to either a weak steric hindrance effect, or possibly an allosteric effect subsequent to DN-Hck binding to the Nef SH3 domain binding sites. Since it has been established that distinct determinants on Nef are used to bring about CD4 and MHC-1 downregulation (Piguet et al., 1999b). With the exception of the amino-terminal myristoylation sequence required for membrane localization and which is therefore critical for the interaction of Nef with both CD4 and class I MHC molecules, the specific motifs required for CD4 downregulation are dispensable for MHC-1 molecule downregulation, and vice versa (Piguet et al., 1999b). Indeed, site-directed mutagenesis of the SH3-binding PXXP site of Nef has been shown
to inhibit Nef-induced class I MHC downregulation, but not CD4
downregulation (Piguet et al., 1999b). However, in contrast to experiments that
rely on introducing mutations into the sequence of Nef, the direct binding of the
DN-Hck protein to Nef may also be capable of interfering, albeit inefficiently,
with Nef and CD4 interactions. This may provide an explanation for the
relatively modest effect of DN-Hck on Nef-induced CD4 downregulation
observed in this thesis.

In conclusion, the research in this chapter demonstrated that a dominant-
negative form of Hck protein-tyrosine kinase was able to block Nef-induced
downregulation of class I MHC surface expression in human cells. This effect
required a functional SH3 domain, as it was not evident in cells that express DN-
Hck-W93F, an SH3 domain mutation that results in diminished binding affinity
for Nef. The results also suggest that this Nef-mediated effect requires an
interaction between Nef and an as yet unidentified polyproline site-binding
molecule. The SH3-region of Nef therefore represents a target for therapeutic
intervention in individuals infected with HIV-1.
CHAPTER 5
DISCUSSION

5.1 Summary of results
The objective of the studies presented in this thesis was to examine the possibility of preventing Nef-induced immune dysfunction by intracellular expression of protein ligands. Two important markers of immune dysfunction induced by Nef were studied, i.e. CD4 and MHC-1 expression. Expression of single-chain antibodies (ScFvs) against Nef were evaluated in blocking CD4 and MHC-1 molecule downregulation induced by Nef. This was then extended to studies evaluating the effect of expressing dominant-negative Hck in interfering with these Nef-mediated receptor downregulation effects.

In chapter 3, the effect of Nef-specific single-chain antibody expression in an attempt to block the CD4 and MHC-1 downregulation induced by Nef was examined. It was shown that the anti-Nef single-chain antibodies constructed retained the antigen-specificity of their corresponding parent antibodies. However, these ScFvs failed to inhibit the CD4 and MHC-1 downregulation induced by Nef. It is therefore concluded that the binding interaction and the following sequestration of Nef is not sufficient for blocking Nef functions, and an intracellular ligand that could recognize the determinants in Nef that is directly implicated in these receptor modulation effects may be required to have the maximum inhibition effect.
In chapter 4, the possibility of inhibition of Nef functions by a dominant-negative Hck (DN-Hck), a Src tyrosine kinase devoid of the kinase domain, was examined. *In vitro* studies demonstrated that DN-Hck is able to prevent Nef-induced MHC-1 downregulation. This effect is Hck SH3 domain-dependent as suggested by the inability of a DN-Hck-W93F mutant to inhibit this effect. As W93 is a key residue within the SH3 domain binding surface (Lee *et al.*, 1995), it suggested that DN-Hck was interfering with Nef function by directly binding to the PXXP site of this molecule. The critical importance of W93 in the Nef-DN-Hck binding interaction was confirmed by an *in vitro* 'pull-down' immunoprecipitation experiment (Figure 24).

5.2 Discussion and conclusion

Advances in HIV research have made it possible to halt viral replication indefinitely, something inconceivable just several years ago (Bartlett and Moore, 1998). This is achieved by ‘cocktail-therapy’, in which the patients have to take several drugs twice or more a day according to strict guidelines. All of the approved anti-HIV drugs are designed to block viral replication within cells by inhibiting either reverse transcriptase or the HIV protease, enzymes required for reverse transcription of viral RNA upon entry or viral protein post-translational processing during viral maturation, respectively.

Nevertheless, both scientific understanding and treatment remains far from perfect. None of the current therapies can eradicate the virus from patients once infected with HIV and it is still unknown whether the impressive therapeutic responses from the ‘cocktail-therapy’ can be sustained.
A major new challenge for HIV therapy is finding ways to eliminate HIV from infected resting CD4+ T cells, which people used to believe that they did not actively produce viral particles but harbor the proviral DNA for doing so in the future. To develop such therapies, new drug targets and alternative treatments such as gene therapy are under extensive research for their potential role in the eradication of viruses from the infected resting cells (Bartlett and Moore, 1998; Rondon and Marasco, 1997).

A potential new drug target is Nef, a HIV viral protein which is implicated in virus immune escape by protecting infected cells from recognition of cytotoxic T lymphocytes (Collins and Baltimore, 1999). Among many other detrimental effects upon host cells, Nef is capable of downregulating cell surface CD4 and MHC-1 receptors which occur through distinctive determinants in Nef (reviewed in (Piguet et al., 1999b)). The later activity deserves special attention, because it is linked to Nef’s role in protecting HIV-1 infected cells against lysis by virus-specific cytotoxic lymphocytes (Collins and Baltimore, 1999), which is the major host defence during HIV infection.

In this thesis, several anti-Nef single chain antibodies were first constructed from the variable regions of monoclonal antibodies that recognize the C-terminus and a central domain (Nef83-88) in Nef (Figure 16 and Figure 20). All retained the binding activity of their corresponding parental monoclonal antibodies when expressed intracellularly as demonstrated by ‘pull-down’ assays using recombinant Nef protein (Figure 18).
Two ScFv clones targeting the C-terminus and the central domain of Nef were studied for their potential inhibition of Nef-mediated effects. Neither had any significant effect on CD4 and MHC-1 downregulation induced by Nef. In comparison, transient transfection of P4.2 cells with the corresponding N-terminus myristoylation-modified ScFv vectors showed significant reductions in the levels of Nef expression (Figure 21), which indicated that the addition of the myristoylation signal sequence to the ScFv clones made them more efficient in reducing Nef expression level, possibly due to more efficient intracellular interaction with their target protein. However, regardless of the N-terminal myristoylation status, none of the expressed ScFv was able to prevent CD4 or MHC-1 downregulation induced by Nef (Figure 22). This indicated that the intracellular binding of ScFv with Nef and the resulting Nef sequestration may not be sufficient to prevent the receptor downregulation events induced by Nef. The expression of molecules capable of binding to epitopes in Nef that are implicated specifically in receptor modulation may be required for this effect. Others (Rondon and Marasco, 1997), have demonstrated that the affinity with which a ScFv binds to the activation domain of HIV-1 Rev maybe significantly lower than if a different ScFv binds to a nonactivation region. However, the first ScFv demonstrated more potent activity in inhibiting virus production in human T cell lines and PBMCs than did the later. Their results indicate that binding affinities of an ScFv for a target viral protein can not be used to predict its intracellular activity, and that the targeting epitope of the ScFv might play a more pivotal role in disrupting the function of that viral protein (Rondon and Marasco, 1997).
A Hck mutant with SH3 domain was then examined as a potential candidate for preventing MHC-1 downregulation. Since the Hck SH3 domain binds Nef with the highest affinity known for an SH3-mediated interaction ($K_d = 0.25 \mu M$) (Lee et al., 1995). In addition, the SH3-binding motif (PXXP78) in Nef is also one of the major determinants in downregulation of MHC-1 (Greenberg et al., 1998b). The implication of intracellular expression of dominant-negative Hck was therefore studied for receptor modulation effect in Nef-expressing P4.2 cells.

This research demonstrated that DN-Hck prevents Nef-induced MHC-1 downregulation. This likely occurs via an interaction between the SH3 domain of DN-Hck and the PXXP motif in Nef. The SH3-dependence of this effect was suggested by the inability of DN-Hck-W93F to inhibit this effect. As W93 is a key residue forming the binding surface of Hck SH3 Domain (Lee et al., 1995), it indicated that DN-Hck was interfering with Nef function by direct binding to the PXXP motif of Nef. The critical role of W93 in the Nef-DN-Hck binding interaction was confirmed by the in vitro 'pull-down' immunoprecipitation experiment (Figure 24).

Several models have been proposed for the mechanism of Nef-induced class I MHC downregulation (Greenberg et al., 1998b; Le Gall et al., 1998; Mangasarian et al., 1999). It has been proposed that Nef recruits clathrin-adaptor protein complexes, which in turn associate with the cytoplasmic domain tyrosine-based sorting motifs within HLA-A and HLA-B molecules that have been shown essential for the Nef effect (Cohen et al., 1999; Greenberg et al., 1998b; Le Gall et
al., 1998; Mangasarian et al., 1999). Alternatively, Nef could act as a connector between the class I MHC cytoplasmic domains and an adaptor protein complex (Greenberg et al., 1998b; Le Gall et al., 1998; Mangasarian et al., 1999). However, the adaptor protein complex and Nef have not been demonstrated to bind to class I MHC directly (Greenberg et al., 1998b; Le Gall et al., 1998; Mangasarian et al., 1999). The involvement of a Src family kinase, or an SH3-containing protein lacking protein-tyrosine kinase activity in Nef-induced class I downregulation has also been proposed (Greenberg et al., 1998b; Le Gall et al., 1998; Mangasarian et al., 1999).

The results of this thesis strongly support a model that DN-Hck inhibits Nef-induced class I downregulation by preventing an interaction between Nef with an as-yet unidentified SH3-containing cellular protein that couples Nef to the MHC molecule (Figure 30). Upon binding with Nef, this cellular protein might recruit class I MHC molecules via an interaction with their cytoplasmic tyrosine-based sorting motifs thus routing these molecules towards the intracellular degradation pathway (Greenberg et al., 1998b; Le Gall et al., 1998; Mangasarian et al., 1999) (Figure 30).

As the SH3-binding sites in Nef appear to be dispensable for CD4 downregulation (Greenberg et al., 1998b; Mangasarian et al., 1999), the modest inhibition of Nef-induced CD4 downregulation observed in this thesis research may have been due to either a weak steric hindrance effect, or an allosteric effect, resulting from DN-Hck binding to the Nef SH3 domain-binding sites. It has been established that distinct determinants on Nef are used to bring about CD4
and MHC-1 downregulation (Piguet et al., 1999b). With the exception of the amino-terminal myristoylation sequence that is required for membrane localization and is thus critical for interaction of Nef with both CD4 and class I MHC molecules, the motifs required for CD4 downregulation are dispensable for MHC molecule downregulation, and vice versa (Piguet et al., 1999b). Thus, by site-directed mutagenesis of the SH3-binding PXXP site of Nef, this motif has been shown to inhibit Nef-induced class I MHC downregulation, but not CD4 downregulation. However, in contrast to such experiments that rely on mutating the sequence of Nef, the binding of the DN-Hck protein to Nef may be capable of interfering, albeit inefficiently, with the interaction that occurs between Nef and CD4. This may provide an explanation for the modest effect of DN-Hck on Nef-induced CD4 downregulation observed in this thesis research.

The SH3-binding region of Nef represents a new target for therapeutic intervention in individuals infected with HIV-1. Using a model system it has been shown that co-expression of Nef with a molecule, DN-Hck, that is capable of binding the Nef PXXP SH3-binding site, can reverse Nef-induced class I downregulation, despite the fact that Nef was likely being expressed at supra-physiological levels. While the effect of DN-Hck on class I expression in cells infected with HIV-1 needs to be further determined, it is conceivable that DN-Hck will also prevent MHC-1 down-modulation in these cells. In vivo such an effect would be predicted to increase the probability of infected cells being recognized and subsequently killed by CTL. In addition, since the Nef SH3-binding site has been shown to play a role in other viral activities, such as regulation of infectivity, cell activation and abnormalities of cell signal
Figure 30 Model for the dominant-negative Hck inhibition of MHC-1 receptor downregulation induced by Nef. A, Nef downregulates cell surface MHC-1 molecules through interaction with an unidentified SH3-containing cellular protein that couples Nef to the MHC molecule. Upon binding with Nef, this cellular protein might recruit class I MHC molecules via an interaction with their cytoplasmic tyrosine-based sorting motifs thus routing these molecules towards the intracellular degradation pathway. B, DN-Hck inhibits Nef-induced class I downregulation by preventing this interaction between Nef and that unknown cellular protein.
transduction pathways (Arold et al., 1997; Briggs et al., 1997; Collette et al., 1996; Goldsmith et al., 1995; Iafrate et al., 1997; Moarefi et al., 1997), therapies directed against this Nef site could have additional benefits for the host.

There are several potential limitations in this thesis research. First, due to limited resources, only mRNA from hybridomas raised against 'non-essential' regions of Nef were obtained. Therefore, ScFv constructed from the cDNA of these hybridomas were limited by their corresponding binding specificities. Ideally, ScFv could be generated from hybridomas producing antibodies targeting epitopes on Nef that are implicated in CD4 or MHC-1 downregulation induced by Nef. Such ScFvs are more likely to have a suppressive effect on CD4 or MHC-1 downregulation induced by Nef. Second, the P4.2 cell line transient transfection system used to evaluate potential intracellular anti-Nef reagents is an in vitro assay in a research laboratory setting. The effect of DN-Hck in preventing MHC-1 downregulation induced by Nef suggests, but does not directly prove, the potential clinical benefit using such a therapeutic strategy. Only after extensive in vitro and in vivo testing in HIV-1 infection models, the therapeutic strategy suggested from this thesis research can be established.

It is worth to emphasize that cytotoxic T-lymphocyte (CTL) responses arise early after HIV or SIV infection and are important in controlling viral replication throughout the course of infection (Borrow et al., 1994; Koup and Ho 1994; Yasutomi et al., 1993; Reimann et al., 1998; Ogg et al., 1998; Schmitz et al., 1999; Jin et al., 1999; Matano et al., 1998). A number of clinical and experimental observations have implicated virus-specific CTLs in this process. CD8+
lymphocytes from infected individuals have been shown to inhibit HIV-1 replication in vitro (Walker et al., 1986). Control of the surge of viral replication in primary HIV-1 infection coincides with the appearance of virus-specific CTLs (Koup et al., 1994). Potent virus-specific CTL responses have been observed in infected individuals with low viral loads and persistent, nonprogressive infections (Rinaldo et al., 1995; Ogg et al., 1998). In addition, the importance of CTLs in controlling SIV has been demonstrated by deleting this cell population using anti-CD8 monoclonal antibody in macaques infected with SIV, which resulted in a rapid and marked increase in viremia that was again suppressed coincident with the reappearance of SIV-specific CD8+ T cells (Jin et al., 1999; Schmitz et al., 1999). These results confirmed the crucial role of cell-mediated immunity in controlling HIV-1 infection.

One future application from this thesis research will be to develop anti-Nef therapy aimed at preventing the MHC-1 downregulation effect induced by Nef during HIV-1 infection. This therapeutic strategy, once developed, could be used for assisting the host immune system to eliminate HIV-1-infected cells. It is hopeful that together with the powerful “cocktail-therapy” that suppresses viral replication, HIV infection can one day be cured. But there is one potential limitation for the anti-Nef approach: it only works if there is HIV protein expression in the infected cells. It will not enhance viral antigen presentation by MHC-1, in order for CTLs to better recognize and kill viral infected cells, in a truly latent viral state; since in such a state there is no viral protein expression. Current evidence supports that the dormant reservoir of HIV which is established early during primary infection (Chun et al., 1998), consists of latently
infected, resting CD4+ T cells carrying replication-competent HIV (Chun et al.,
1999). This pool of infected cells can persist even in individuals who are receiving
highly active antiretroviral therapy (HAART) (Chun et al., 1997; Chung et al.,
1997; Finzi et al., 1999; Zhang et al., 1999), and viral replication rapidly rebounds
from this reservoir within weeks of discontinuing HAART treatment (Chun et al.,
1999). Clinical evidence has also shown that HIV continues to replicate at a low
level even in patients with undetectable levels of virus in their blood (Ho, 1997).
Although these studies did not demonstrate directly the viral protein expression
status in those latently infected cells, they indicated that there might not be a
truly latent viral state, viral protein production in those infected cell might be on
all the time. The only limiting factor for the viral protein production are the
drugs inhibiting the reverse transcriptase and protease of HIV-1. Even if the
latent infection of HIV means complete silence of viral replication, anti-Nef
therapy can still be effective against the re-activation of HIV in the latently
infected cells or prevent the infection from the latent state to replication
competent.

It should be emphasized that there is still a long way to go before translating the
results generated from this thesis into HIV therapy. In future studies, it will be
necessary to prove that the inhibition effect on the Nef-induced MHC-1
downregulation affects the ability of the CTLs to kill virus infected cell in an in
vitro model of HIV-1 infection. This can be achieved by the method used for
studying the protective effect of the expression of Nef in infected cells against
anti-HIV CTL recognition (Collins et al., 1998). Basically, primary CD4+ T cells
are infected with an HIV that can place a reporting marker, such as GFP or
placental alkaline phosphatase (PLAP) in the infected cells. Then, CTL clones restricted to MHC class I HLA-A2 antigens and the Gag epitope (SLYNTIAVL (SL9)) could be used (Collins et al., 1998). DN-Hck or other Nef PXXP ligands, such as ScFv binds to the PXXP domain in Nef, can be introduced \textit{in cis} within the HIV vector. This will allow DN-Hck or other Nef-binding agents to be co-expressed intracellularly with Nef in cells infected by this modified HIV vector. Alternatively, cells used for infection can also be pre-transfected with vectors carrying the cDNAs of these ligands. The cytotoxic activity of these CTLs towards HIV-infected cells can be evaluated and the protective role of therapies targeting the SH3-binding surface of Nef can then be established \textit{in vitro}.

Second, the \textit{in vitro} benefit of inhibition of Nef-induced MHC-1 downregulation has to be further tested in an HIV infection animal model. Ideally, this animal model can be SIV infection of chimpanzee or macaques, since they are the closest animal models to HIV infection in human. The clinical benefit of such a therapeutic strategy can be monitored by measuring viral loads RT-PCR, or examination of infected cells from local lymph node or peripheral blood. Small molecules that can be selected by rational drug design according to the structure and conformation of Nef protein, or peptides that can be selected according to their binding affinity to Nef protein, could be developed as drugs targeting this Nef SH3-binding domain. The advantage of small molecules is that they can be administered by oral and intravenous administration. It is expected that the combined HIV therapeutic scheme using drugs targeting the SH3-binding surface on Nef, together with inhibitors suppressing HIV replication, will be
more effective in controlling HIV infection and may eventually reach the goal of eradicating HIV infection.

One potential application suggested by this research is gene therapy. Gene therapy is a new form of molecular medicine that has gained special interest among AIDS researchers, since conventional therapies have shown limited success. Alteration of the host cell could potentially confer permanent suppression of viral replication after infection, or could provide protection against viral infection. Several gene therapy strategies for HIV infection are currently being studied, including immune reconstitution which involves \textit{ex vivo} expansion of selected and sometimes genetically modified T cells, followed by their reinfusion into the infected patients (Roberts \textit{et al.}, 1994); nucleic acid-based therapeutic vaccines involve direct delivery of HIV-1 genes to mimic viral infection wherein the expression of viral proteins encoded by these nucleic acids elicits both cellular and humoral response (Wang \textit{et al.}, 1993); lastly, intracellular immunization transfers a therapeutic gene into target cells to render them resistant to viral infection, the resistant cells will then limit the spread of the virus in the patient (Baltimore, 1988).

Several research groups have successfully constructed and characterized ScFvs against different HIV-1 structural and regulatory proteins. ScFvs targeting HIV-1 envelope protein gp120 have been shown to inhibit the envelope protein processing in ER and reduce infectivity of HIV-1 particles released by ScFv expressing cells (Marasco \textit{et al.}, 1993). Currently, a clinical trial is in progress using conventional gene therapy vectors containing these ScFvs to transduce and
reinfuse autologous CD4+ T cells in asymptomatic HIV-1 infected patients (Rondon and Marasco, 1997). This gene therapy approach may provide a therapeutic benefit for HIV-1 infected patients. ScFvs targeting other HIV-1 viral regulatory and structural proteins, such as Tat, Rev, reverse transcriptase, integrase, matrix and nucleocapsid, have also been generated. Each of them exhibited various degrees of inhibition of viral infection and cell protection (Duan et al., 1994a; Duan et al., 1994b; Levin et al., 1997; Levy-Mintz et al., 1996; Maciejewski et al., 1995; Mhashilkar et al., 1995; Shaheen et al., 1996; Wu et al., 1996). In summary, a variety of HIV-1 proteins are sensitive to neutralization by intracellular antibodies, and HIV inhibition can be achieved at different stages of the viral life cycle. These intracellular antibodies may be useful for the gene therapy of HIV-1 infection.

The knowledge obtained from this thesis can be used to develop a novel gene therapy that targets HIV infected cells. Retroviral vectors pseudotyped with CD4 and different chemokine receptors can be used to carry genes encoding ligands targeting the PXXP region in Nef and transduce the reservoir of HIV-infected cells. The entry of HIV-1 is mediated by interactions between the viral glycoprotein and a cellular receptor complex, which consists of CD4 and one of the CC or CXC chemokine receptor family proteins; and it has been demonstrated that these viral receptors can be used to target HIV-infected cells (Endres et al., 1997). The ligands can be DN-Hck, single-chain antibody or peptide specific for the PXXP region in Nef.
Basically, a retroviral vector coated with functional HIV viral receptors can be made by co-transfection the viral packaging cells with plasmids encoding CD4, a chemokine receptor (CXCR4 or CCR5), and an envelope-deficient HIV-1 vector that encodes a protein ligand for the PXXP region in HIV-1 Nef. These receptor-pseudotyped virions can be examined for the ability to enter HIV-infected cells, as well as the ability to interfere with Nef functions intracellularly. Such vectors will be useful to prevent MHC-1 downregulation induced by Nef, and in doing so, might let the host HIV-1 specific CTLs better recognize and remove the infected cell.

In conclusion, a strategy was first established to construct and evaluate Nef-specific single-chain antibodies; however, intracellular single-chain antibody expression against ‘non-essential’ regions on Nef did not prevent CD4 or class I MHC downregulation induced by Nef. The dominant-negative Hck was then evaluated and it was demonstrated that the dominant-negative Hck expression inhibited MHC-1 downregulation induced by Nef, and this effect is Hck SH3 domain dependent. A model was proposed that DN-Hck inhibits Nef-induced class I downregulation by preventing an interaction between Nef with an unknown SH3-containing cellular protein that links Nef to the MHC molecule. Therefore, interfering with Nef SH3-binding site function represents a potential therapeutic strategy for assisting the host immune system to eliminate HIV-1-infected cells. The knowledge obtained from these studies can be potentially used for the discovery of new drug targets and for the development of gene therapy against HIV infection.
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