CHAPTER II

LITERATURE REVIEW

HIV-1 Biology[55-57]

Human immunodeficiency virus type1 (HIV-1) is a retrovirus of the lentivirus subfamily. Particles of HIV are made up of 2 copies of the single-stranded RNA genome packaged inside a protein core, or capsid. The core particle is surrounded by a lipid bilayer containing the viral surface (gp120) and transmembrane (gp41) envelope proteins. The core particle also contains viral proteins that are essential for the early steps of the virus life cycle, such as reverse transcription and integration.[58]

Infectious virions of HIV contain two identical copies of single-stranded RNA, about 9.2 kilobase (kb) long that have positive polarity with respect to translation. The integrated form of HIV-1, also known as the provirus, is approximately 9.8 kb in length. Both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs). The genes of HIV are located in the central region of the proviral DNA and encode at least 9 proteins. These proteins are divided into three classes.

1. The major structural proteins, Gag (group-specific antigen), Pol (polymerase), and Env (envelope)
2. The regulatory proteins, Tat (trans-activator of transcription) and Rev (regulator of expression of virion protein)
3. The accessory proteins, Vpu (viral protein U), Vpr (viral protein R), Vif (viral infectivity factor), and Nef (negative factor)

Structural proteins

Gag (group-specific antigen) protein

The *gag* gene gives rise to the 55-kilodalton (kD). Gag precursor protein, also called p55, which is expressed from the unspliced viral mRNA. The Gag precursor forms the core viral particle and interacts with other viral and cellular components

to facilitate their incorporation into the budding viral particle. After budding, p55 is cleaved into 4 smaller proteins designated matrix (p17), capsid (p24), nucleocapsid (p9), and p6

Matrix (MA) domain

Matrix protein is located in the matrix between the virion capsid and envelope.[59] A nuclear localization signal has been proposed within the MA protein. In addition, MA plays a role in the transport of the preintegration complex to the nucleus.[60] This phenomenon allows HIV to infect nondividing cells, an unusual property for a retrovirus.

Capsid (CA) domain

The p24 (CA) protein forms the conical core of viral particles. CA also interacts with the cellular protein, which is incorporated into HIV particles and required for efficient viral replication and particles production.

Nucleocapsid (NC) domain

NC is a basic protein that binds to and is responsible for the packaging of the viral genomic RNA.

P6 ^{gag} protein

The only established function of $p6^{gag}$ is the interaction with Vpr, resulting in virion incorporation of this accessory protein.[61]

The viral enzymes

The viral enzymes protease (PR), reverse transcriptase (RT), ribonuclease H (RNase H), and integrase (IN) are produced by cleavage of the Gag-Pol polyprotein $Pr160^{gag\text{-}pol}$, which is generated by a ribosomal frame shifting event during translation of the viral mRNA. The virally encoded protease cleaves the Pol polypeptide away from Gag and further digests it to separate the protease (p10), RT (p50), RNase H (p15), and integrase (p31).

Protease (PR)

Protease activity is required for cleavage of the Gag and Gag-Pol polyprotein precursors during virion maturation. Inhibition of HIV protease results in noninfectious particles.[62]

Reverse transcriptase/ Ribonuclease H (RT/ RNase H)

Reverse transcriptase possesses 3 enzymatic activities: RNA-dependent DNA polymerase, DNA-dependent RNA polymerase, and ribonuclease H (RNase H). RNase H functions in reverse transcription by degrading the RNA moiety of RNA/DNA hybrids and thereby uncovering the template for viral DNA synthesis. Reverse transcriptase plays a major role in the generation of diversity of HIV because RT does not contain a proofreading activity. As a result, RT cannot correct any errors it makes during DNA synthesis and introduces several point mutations into each new copy of the viral genome.

Integrase (IN)

Integrase is the viral protein responsible for the integration of the proviral DNA into the host nuclear DNA.

The envelope glycoprotein

The HIV-1 envelope glycoproteins are the product of the *env* gene. The 160-kD Env (gp160) is expressed from singly spliced mRNA. A cellular protease cleaves gp160 to generate gp41 (transmembrane glycoprotein, TM) and gp120 (surface glycoprotein, SU). Gp41 contains the transmembrane domain of Env, while gp120 is located on the surface of the infected cell and of the virion through noncovalent interactions with gp41.

Regulatory proteins

The 2 essential regulatory proteins of HIV-1 are Tat and Rev. These are the 2 gene products for which there is direct proof of involvement in different steps of gene expression: transcription for Tat and posttranscriptional regulation for Rev.

Tat (transactivator of transcription)

Tat is a transcriptional transactivator and is essential a regulatory protein for HIV-1 replication.[63-64] The 16-kD, two exon Tat protein is encoded from two separate exons of multiply spliced mRNA.[65-67] Tat binds to a short-stem loop structure, known as the transactivation response element (TAR). Threr are various mechanisms for transcription initiation including an increase in elongation through increased processivity of the polymerase complex and increased elongation through suppression of specific termination events.[68-72]

Rev (regulator of expression of virion protein)

Rev is a 13-kD sequence-specific RNA binding protein. Rev is only required for the posttranscriptional regulation of the unspliced and partial spliced mRNA.[73]

Accessory proteins

In addition to the *gag*, *pol,* and *env* genes contained in all retroviruses, and the *tat* and *rev* regulatory genes, HIV-1 contains four additional genes: *vif*, *vpr*, *vpu,* and *nef* which encode the so-called accessory proteins. The accessory genes were dispensable for virus replication in many cultured cells in vitro, although their conservation in all virus isolates demonstrates that they are essential during the virus life cycle in the host.

Nef (negative factor)

Nef has been shown to have multiple activities, including the down regulation of the cell surface expression of CD4 and the stimulation of HIV infectivity.

Vif (viral infectivity factor)

The viral infectivity factor, Vif has been shown to influence the infectivity but not the production of virus particles. Vif is essential for the replication of HIV in peripheral blood lymphocytes, macrophages, and certain cell lines.

Vpr (viral protein R)

Vpr is incorporated into viral particles. Vpr plays a role in the ability of HIV to infect non-dividing cells by facilitating nuclear localization of the preintegration

complex. Proposed functions for Vpr include cell growth arrest, transactivation of cellular genes, and induction of cellular differentiation.

Vpu (viral protein U)

Vpu is expressed from the bicistronic mRNA that also encodes *env*. Vpu has at least two different biologic functions: degradation of CD4 in the endoplasmic reticulum and enhancement of virion release from the plasma membrane of HIV-1-infected cells.

Classification of HIV

According to estimates from the Joint United Nations Programme on HIV/AIDS and the World Heath Organization (WHO), 40 million adults and 2.7 million children were living with HIV at the end of 2001.[74] The AIDS is caused by two different human immunodeficiency viruses, HIV-1 and HIV-2. Phylogenetic analyses of the nucleotide sequences of the *env* and *gag* genes of a large number of HIV-1 isolates, HIV-1 can be classified into group M, O, and N. Group M (major) comprises the great majority of HIV-1 isolates and can be further assigned to 9 subtypes, which are subtypes A, B, C, D, F, G, H, J, and K including 14 different circulating recombinant forms that are CRF01_AE CM240, CRF02_AG IbNG, CRF03_AB Kal153, CRF04_cpx 94CY032, CRF05_DF VI1310, CRF06_cpx BFP90, CRF07_BC 97CN54, CRF08_BC 97CNGX-6F, CRF09_cpx P2911 (not yet published), CRF10_CD TZBF061, CRF11_cpx GR17, CRF12_BF ARMA 159, CRF13_cpx, and CRF14_BG. A second group of HIV-1 isolates has been identified in patients from Cameroon, and designated as group O (outlier) virus.[75] Recently, a new subgroup named N (non-M and non-O) has been identified.[76]

Geographical Distribution of HIV

Distinct subtypes are now known to be the predominant pathogens in different parts of the world.[77-80] Subtype B viruses are more prevalent in North America, Latin America and the Caribbean, Europe, Japan, and Australia. Almost every subtype is

present in sub-Saharan Africa, with subtypes A and D predominating in central and eastern Africa, and subtype C in southern Africa.

There are 2 major strains of HIV-1 found in Thailand. Almost all HIV-1 infected Thais carried CRF01_AE; the rest carried subtype B.[4]

Name: CRF01_AE Reference strain: CM240 Subtypes: A, E

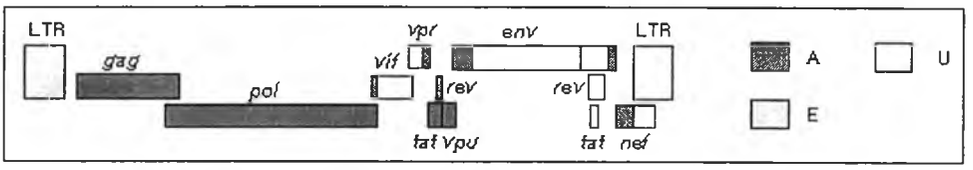

Figure 1: CRF01_AE (reference strain CM240) represents a putative subtype A/E recombinant form of HIV-1 which is spreading epidemically in Asia, but originated from Central Africa. In the future, putative recombinants with only one full-length "parental" subtype representative would be designated as being comprised of this subtype and unclassified regions (U). Under the new nomenclature system CRF01_AE would be referred to as CRF01_AU because the putative "parental" non-recombinant E strain has not been found. But, as the "E" designation for the env region of these strains is very commonly used, renaming it would lead to confusion. Thus, the "E" designation will be retained.

Cellular Immunity in HIV

HIV-1-specific CTL and T-helper responses are thought to be important components of the immune response in the course and control of HIV-1 infection. HIV-1-specific CTL are considered to play a central role in the immune response against HIV-1.[16-21] CTL recognize viral proteins in the form of short peptides comprising 8-12 amino acids presented in association with major histocompatibility (MHC) class I molecules on the surface of the infected cells.[81] Viral proteins within infected cells are degraded into peptides and transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP). Some of these peptides can bind to MHC class I molecules and are transported to the cell surface (figure 2).

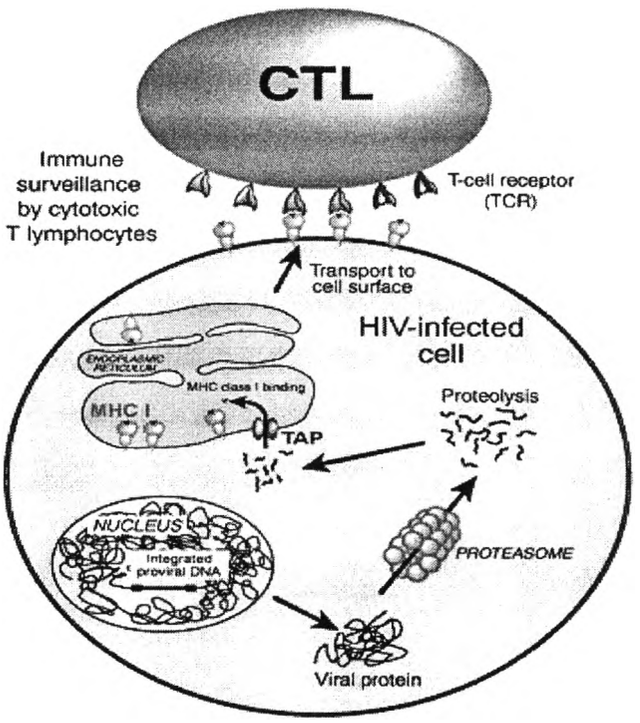

Figure 2: The MHC class I antigen presentation pathway.

Cells expressing foreign peptide-MHC complexes can be recognized and lysed by CTL through very specific interaction. Recognition of viral peptide-MHC class I complexes in the surface of infected cells is a function of the T-cell receptor (TCR), which in conjunction with the CD8 coreceptor, can bind specifically to a particular MHC-peptide complex. [82-84]

Both peptide and MHC molecule restrict this binding. Even single amino acid alterations in these residues potentially can completely abrogate binding.[85-86] Differences in the effectiveness of HIV-1-specific CTL responses of different specificity are increasingly apparent. Certain HLA class I molecules have been associated with more rapid or slower progression of HIV-1 disease.[87-89] The possible mechanism by which HLA class I molecules might influence the speed of disease progression is unclear. A better understanding of these interactions depends upon the precise fine mapping of the optimal CTL epitopes and the definition of HLA class I restriction of these responses.

HIV-1-specific CTL responses

CTLs kill the infected target cell through both lytic and apoptotic pathways.[16-17] CTLs contain cytolytic granules composed of proteoglycans to which chemokines are complexed. These granules hold pore forming called perforins and proteolytic enzymes called granzymes in a protected state. When the TCR and CD8 of the CTL binds to the MHC class I/ epitope on the surface of the virus-infected cell, this sends a signal through a CD3 molecule which triggers the release of the perforins, granzymes, and chemokines.[90] They also release soluble factors, including proinflammatory cytokines such as IFN-γ and CC-chemokines.[19]

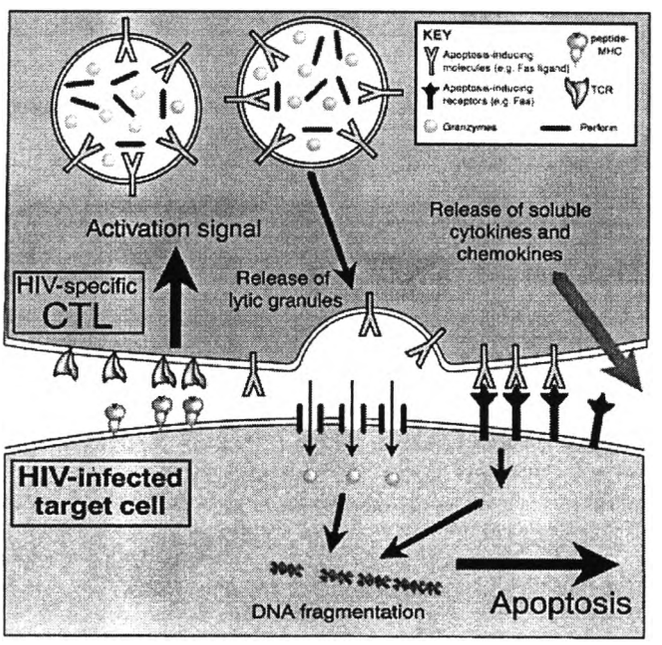

Figure 3: CTL recognition. Recognition of viral peptide-MHC complexes on the infected cell surface leads to an activation signal, which turn initiates the effector functions of the CTL

The perforin molecules form pores in the membrane of the infected cell which allow granzymes to enter. The granzymes can then activate the proteases, which lead to apoptosis of the infected cell. The proteases involved in apoptosis destroy the protein structure scaffolding of the cell and lead to destruction of the cell's DNA. In addition, the granzymes can interact with the calcium ions entering through the pores and cause cell lysis. CTLs can also trigger apoptosis of the infected cells through FasL/Fas interactions and with the cytokine tumor necrosis factor-beta (TNF-β). CTLs have a receptor called FasL that can interac with Fas molecules found on the surface of most cell types. This interaction triggers an intracellular transduction that

leads to chromosomal degradation and destruction of structural proteins in the infected cell.[91-93] CTLs produce TNF-β, a cytokine that can bind to TNF receptors on target cells causing the same sequence of events. Since CTLs are not destroyed in these reactions, they can function over and over again to destroy more virus-infected cells.

Control of HIV-1 infection by CTL

In primary infection, generation of HIV-specific CTL responses is temporally correlated with a drop in viremia, suggesting that CTL inhibit viral replication in *vivo*.[27] In addition, CTL responses have been correlated with disease progression; the data indicate an inverse correlation of virus-specific CTL activity with plasma viral load.[94] Furthermore, such CTLs are commonly found in high numbers during the asymptomatic phase of infection and decline with progression to AIDS.[26-29] HIV-specific CTLs have been documented in HIV-exposed but uninfected infants, the uninfected heterosexual partners of HIV seropositive individuals, and in repeatedly exposed but persistently seronegative (HEPS) female prostitutes in West Africa. These studies suggest that CTLs may have the capacity to prevent transmission of HIV-1.[30-34]

There is a strong evidence that HIV evolves to escape CTL recognition by epitope-specific mutations.[35] In addition, a recent study in SIV-infected macaques elegantly demonstrated the selection of viral escape mutants under immune pressure.[96] These mean CTLs exert a significant antiretroviral force in *vivo*. The evidence that supporting a role of CTL has come from CD8 depletion monkeys depleted of CD8+ lymphocytes. Similarly, dramatic viruses in plasma viremia were demonstrated following depletion of this lymphocyte subset in established infection.[97-98] These studies are supported by observations that the emergence of SIV-specific CTL in primary infection correlate with suppression of viremia, and that CTL activity is inversely correlated with virus load and disease progression.[98-100]

Much of the evidence supporting the proposal that protective immunity in general is mediated primarily by CTL has necessarily originated from studies of animal models of infection. Multiple experimental approaches, including the adoptive transfer of virus-specific CTL and vaccination strategies designed to induce specific cellular immune reactivity in the absence of humoral responses, have confirmed the importance of these effector cells in antiviral immunity.

Trans-activator of transcription

Tat protein is a transactivator essential not only for effective transcription and replication of the virus within cells but also for the prodigious output of virus that follows initial infection, because this is largely due to released Tat protein, which passes extracellular to other cells and renders them susceptible to productive viral infection. Tat is an unusual activator in that it binds to a Tat-responsive RNA element, termed TAR, located 40 base pairs downstream from the transcription initiation site. Tat protein is released from infected cells by a process that does not involve cell death and appears to involve nonclassical pathways, since Tat contains no amino acid-terminal hydrophobic leader sequence that could directed secretion into the endoplasmic reticulum. Tat binds to and is avidly taken up by cells, with eventual translocation to the nucleus. Amino acids 49-57 of Tat, containing the basic domain, is essential for uptake, and this sequence also contains a nuclear localization signal (GRKKR)[101]. Tat uptake not only enhances HIV-1 transcription in infected cells, it also affects a range of host cell genes in both infected and uninfected cells.[12]

Tat-specific CTL responses

The analysis of HIV-1-specific CTL responses has been largely dominated by the study of structural HIV-1 proteins Gag, Pol, and Env, expressed later in the viral life cycle.[10] The extent to which the earlier expressed regulatory proteins HIV-1 Tat and Rev remains to be defined. Rev- and Tat-specific CTL may influence viral load more efficiently than CTL against structural proteins in different ways. Firstly, in the asymptomatic stage, many infected cells, both in circulation and in lymph nodes, do

not actively produce virus, but do express multiple spliced mRNAs that encode the regulatory proteins. This renders these cells targets for CTL against regulatory, but not against structural proteins. Secondly, in the replicative cycle, Rev and Tat are expressed earlier than structure proteins. This may allow specific CTL to kill infected cells well before release of progeny virus. It has been suggested that CTL responses against early expressed epitopes may be more efficacious than those against late expressed epitopes. [42]

Responses directed against these early proteins may be particularly important in viral containment during AIDS-virus infection, because HIV-1 Tat and Rev are the dominant viral proteins produced before Nef down-regulation MHC class I molecules on the cell surface. There is an evidence shows that HIV-1 Rev- and Tat-specific CTL frequencies inversely correlate with rapid progression to AIDS and no such correlation was found for detection of CTLp against Gag, RT, or Nef. [10]

HIV-1 Tat protein as a potential AIDS vaccine

Most AIDS vaccine strategies aimed at blocking virus entry have failed to induce sterilizing immunity and protection against heterologous virus challenge.[5-7] Therefore, control of virus infection and block of disease onset are now accepted as more achievable goals of AIDS vaccine development. Early regulatory HIV-1 proteins Tat and Rev were described as potential candidates for AIDS vaccines.

It has recently been shown that a *tat-rev* vaccine has been shown to protect rhesus macaques from pathogenic SIV challenge. Similarly, a subunit vaccine consisting of a biologically active HIV-1 Tat protein can control replication of the highly pathogenic SHIV89.6P to undetectable levels in most (5 out of 7) of the vaccinated macaques, preventing the CD4+ T cell decline and disease onset for prolonged periods of time.[13-15]

Furthermore, the researchers found that most of the SIV-specific CTL, which the monkeys produced in response to the infection, recognized peptides from either the Tat or Gag proteins of the virus. After 8 weeks, the entire original virus was gone

showing the effect of the immune response. On the other hand, the original virus had been replaced by SIV with small changes, usually in Tat. For these reasons, Tat alone or combined with other viral products may represent an optimal target for AIDS vaccine development for both preventive and therapeutic applications.

Technique for Measurement of CTL

A reliable procedure to measure antigen specific T cell responses is required to determine the efficacy of vaccines. There are several approaches available to monitor cell-mediated immune responses, including classical chromium release CTL assay and Limiting Dilution Analysis (LDA) as well as more recently developed Enzyme-linked immunospot assay (Elispot), Fluorescence-activated cell sorter (FACS) based MHC tetramer, and intracellular cytokine staining assays (ICC).[52-54]

Classical ^{51}Cr-release CTL assay

This assay measures the ability of cells removed from whole blood to kill target cells expressing a specific antigen. The assay is carried out after culturing PBMC for roughly two weeks, together with cells expressing the specific antigen. Killing is then measured as the amount of radioactivity released when the cultured PBMCs are mixed with radioactively labelled target cells. The advantage of this assay is that it measures a clear, important T-cell function. However, it is cumbersome, relatively insensitive, difficult to quantitate reproducibly, and not very quantitative.

Limiting Dilution Analysis

This method quantitates the bulk lytic assay described above by culturing dilutions of PBMC. While this method does yield quantitative data, it is even more cumbersome than the CTL assay and therefore it is not suitable for scale-up.

Enzyme-linked immunospot assay

The most widely used of the new generation of assays. Elispot measures the number of T cells secreting a specific cytokine, such as IFN-γ or TNF-β, that serves as a marker of T cell effectors. Its advantages are many. Elispot is highly sensitive, quantitative, easy to perform, even in low-technique setting, and it can be scaled up for large numbers of samples. The remaining disadvantages include a high background, which makes it harder to distinguish low responses from background with confidence.

Intracellular cytokine staining

Like Elispot, this assay also measures cytokine-producing cells following antigen stimulation, in this case detection the intracellular form. Detection is based on fluorescent labeling of cells, which are then counted using a FACS. Sensitivity is similar to Elispot, although this still varies from lab to lab.

Tetramer Staining

This assay uses flow cytometry to measure CD8+ T-cells that recognize a specific HIV epitope. It works by mixing the cell sample with four molecules of a single epitope joined to a class I HLA molecule. Key advantages are that it directly measures cells with receptors for a specific epitope without prior antigen stimulation, it is highly quantitative and sensitive. On the contrary, tetramer assays require knowing the HLA type of each person being sampled and having a specific epitope known to be recognized by that HLA type. It is very useful for in-depth analysis of responses in specific populations where this information is available.

The Elispot assay is increasingly being used for the monitoring of the induction of antigen-reactive T cells. It has recently been shown that Elispot is a reliable tool to map optimal CTL epitopes,[46-47] correlating well with other methods, such as intracellular staining, tetramer staining, and the classical chromium release assay. This assay allowed testing of a wide spectrum of synthetic peptides and screening of a large number of HIV-infected individuals rapidly and effectively.

Then, Elispot assay is often a method of choice in the preliminary screening of dominant CTL responses in population studies.

HIV-1-specific CTL is thought to be an important component of the immune response in the course and control of HIV-1 infection. Most CTL epitope studies performed have been based in HIV-1 B and HLA types that are most often found in the Caucasian population. A limited number of CTL studies have targeted non-B subtypes and identified CTL epitopes restricted by HLA subtypes common in non-Caucasian ethnic groups. The characteristics of the predominant virus that causes the HIV-1 epidemic in a certain geographic area and also the genetic background of the population, through the distribution of common HLA class I alleles, might impact dominant CTL responses in the vaccinee and in the general population.

The identified and characterized immunodominant regions within HIV-1 CRF01_AE Tat might then represent components for further consideration a multi-component vaccine design. In this study, HIV-1 CRF01_AE Tat protein were targeted for the screening of potential response by IFN-γ Elispot, an assay that was successful applied to identify CTL response.