CHAPTER III

LITERATURE REVIEW

Acquired Immunodeficiency Syndrome

Human immunodeficiency virus (HIV) is the cause of the acquired immunodeficiency syndrome (AIDS) (17). The first cases of AIDS in humans were recognised in 1981 based on an appearance of diseases including Kaposi's sarcoma and *Pneumocystis carinii* pneumonia in young homosexual men (18-20). In 1983, HIV was isolated, visualised and established as the cause of AIDS (21). AIDS has now become a worldwide public health problem.

Human immunodeficiency virus (HIV) type-1

HIV type-1 is a retrovirus, a member of the Lentivirinae subfamily. By electron microscopy, the HIV-1 virion measures approximately 100 to 150 nm in diameter. The virion core contains two copies of single-stranded viral genomic RNA in length of 9.2 kilo bases (kb). The viral genomic RNA has a positive polarity with respect to translation. In the early stages of infection, the virion RNA genome is converted as double-strand linear DNA by the process of reverse transcription. This process involves two strand-transfer steps to synthesise linear viral DNA with long terminal repeats (LTRs) flanking viral genes. This linear viral DNA is integrated into the host cell genome to produce the provirus in the next step.

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 2 classes comprising the structural proteins and regulatory proteins.

1. The major structural proteins include the three genes required for a replicating retrovirus such as the *gag*, which encodes the virion structural components, the *pol*, which encodes several viral enzymes, and the *env*, which encodes the envelope glycoproteins.

2.   The regulatory proteins, the HIV-1 genome also contains at least six additional genes such as *tat, rev, vpu, vpr, vif* and *nef* which regulate viral expression and are important in disease pathogenesis *in vivo*. (Figure 1)
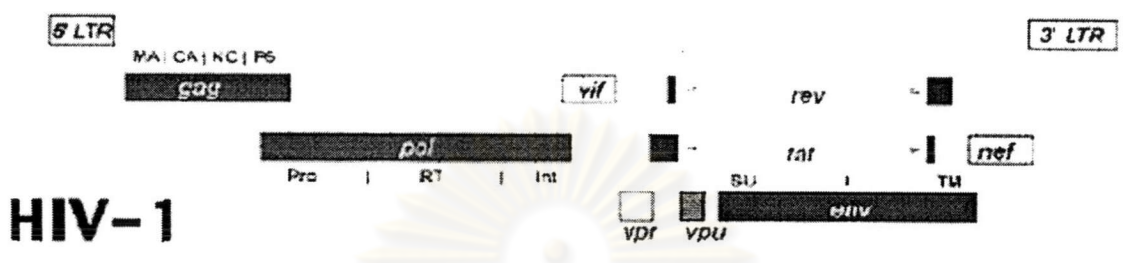

Figure 1 Genomic organisation of HIV-1. Three structural genes: *gag, pol, env*; 6 regulatory genes: *tat, rev, nef, vif, vpu*, and *vpr* (www.aids.harvard.edu/research/discoveries.html).

Structural proteins

For HIV structure, precursor polyproteins Gag-Pol (Pr160), Gag (Pr55), and Env (gp160) are encoded and processed by enzyme to produce mature virion proteins. Gag-Pol and Gag undergo several cleavage steps by the viral protease to produce several functional proteins.

Gag (Group-specific antigen) proteins

The major core proteins of HIV are mainly synthesised from *gag*. The *gag* encodes a precursor protein of the 55-kilodalton (KD), myristoylated precursor protein (pr55), which is expressed from the unspliced viral mRNA. Cleavage of the Gag precursor polyprotein Pr55 by the viral protease produces the mature capsid protein consisting matrix protein (p17), capsid protein (p24), nucleocapsid protein (p7/p9), and p6 (22).

Matrix (MA) domain

Matrix protein is encoded by sequences at the $5'$ end of the *gag* and is cleaved from the N-terminus of Pr55 by viral protease. MA protein is located in the matrix between the

virion capsid and envelope (23). MA plays a role in the preintegration complex to the nucleus (22, 24).

Capsid (CA) domain

Capsid protein is cleaved from the central of Gag polyprotein by the viral protease which capsid protein is the major subunit of the capsid shell. Specific interactions of CA with the viral RNA genome, other Gag proteins, as well as posttranslational modifications are presumed to play important roles in CA function during capsid assembly (25). Furthermore, early step of viral replication, CA protein is required for efficient viral replication.

Nucleocapsid (NC) protein

Cleavage of C-terminus of Gag precursor protein by the viral protease produces NC protein. NC is an intrinsic component of the nucleocapsid. This protein plays a role in virion uncoating during entry and may influence in reverse transcription process that takes place in a nucleoprotein complex containing viral RNA as well as capsid protein (26-28).

Pol (Polymerase proteins)

The viral protease (PR), reverse transcription (RT), ribonuclease H (RNase H), and integrase (IN) are produced by cleavage of the Gag-Pol polyprotein Pr 160 $^{gag-pol}$. These viral enzymes are located in the viral core of HIV virions.

Protease (PR)

PR is translated as a Gag-Pol fusion product that is produced from a ribosomal frameshift, and is released from the Gag-Pol precursor protein by an autocatalytic mechanism (29, 30) in the infected cell. The mature PR is released by an autocatalytic cleavage from the precursor protein. PR is required for replication. Indeed, inactivation of HIV protease results in noninfectious particles because precursor protein could not be cleaved to produce the functional proteins (31, 32).

Reverse transcriptase/Ribonuclease H (RT/ RNase H)

RT is an RNA-dependent DNA polymerase. RT plays a role in the generation of diversity of retroviruses since RT does not have $3'$-$5'$ exonuclease activity for proof-reading (33, 34), RNase H functions in reverse transcription by degrading the RNA moiety of RNA/DNA hybrids and thereby uncovering the template for viral DNA synthesis.

Integrase (IN)

Integrase is essential for incorporation of the viral DNA into the chromosomal DNA of the target cell (35). Integrase is a viral enzyme possessing both DNA cleavage and joining activities of linear double-stranded viral DNA into the host cell genome.

Env (Envelope protein)

The *env* encoded a glycosylated polypeptide precursor (gp160) which is processed to generate gp 41 and gp 120. The gp 120 envelope component binds to CD4 on target cells and undergoes conformational changes that allow gp120 to interact with certain G-protein-coupled receptors (GRCRs) on the same target membranes. The GPCRs that function as HIV coreptors were found to be chemokine receptors. The primary coreptors are CCR5 and CXCR4, but several other chemokine receptors were identified as "minor coreceptors", indicating their ability support entry of some HIV strains in tissue cultures (36).

Regulatory proteins

In addition to the *gag*, *pol*, and *env* contained in the retrovirus, HIV-1 contains six additional genes: *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef*. Proteins encoded by these genes function both early and late in the replication cycle to regulate transcription and translation of viral proteins and to enhance assembly and release of particles from infected cells.

Tat (transactivator of transcription)

Tat is transactivator protein to upregulate transcription by binding at the transactivation response element (TAR). This binding enhances the stable of RNA polymerase II and elongation of RNA transcription.

Rev (regulator of expression of virion protein)

Rev plays a role in the regulation of viral mRNA production by binding at Rev-responsive element (RRE) and facilitates initiation and elongation of viral transcription.

Nef (negative factor)

Nef is expressed in high concentrations shortly after viral infection (Goldsmith 1995 4112-4121 69) and is important for achieving and maintaining high viral loads. Nef is multifunctional and exerts pleiotropic effect such as enhancement of virion infectivity, activation of T cell, and CD4 and MHC downregulation on surface of the infected cells (37) (38, 39). One important determinant of viral pathogenicity is the *nef* of primate lentivirus. In Rhesus macaques infected with a mutant form of a pathogenic molecular clone of simian immunodeficiency virus (SIV) containing a deletion in *nef* maintained very low viral burdens with normal CD4+T cell counts (40, 41). There is some evidence that Nef also plays an important role in disease progression in HIV-1-infected humans. In long-term nonprogressors (LTNP) in who only *nef* deletion forms of HIV-1 could be detected shown remained healthy (42-44). Therefore, Nef is the important protein in HIV pathogenesis (40, 45, 46).

Vif (viral infectivity factor)

The viral infectivity factor, Vif is required for virion infectivity. This protein is essential for the replication of HIV in peripheral blood lymphocytes, macrophages, and certain cell lines. Vif prevents the encapsidation of APOBEC3G and APOBEC3F, two potent antiretroviral cytidine deaminases. APOBEC3G and APOBEC3F inhibit the replication of Δvif HIV-1 by deaminating the minus-strand of the viral reverse transcripts, introducing numerous G to A mutations. These two APOBEC3 family members appear to be the major contributors to G to A hypermutations in HIV-1 in vivo (47).

Vpr (viral protein R)

Vpr play a role in nuclear localization of the viral preintregration complex in the ability of HIV to infect non-dividing cells and arrest cellular growth in G2 phase to increase long term survival of infected cells.

Vpu (viral protein U)

Vpu is essential for degradation of CD4 in the endoplasmic reticulum and enhance the viral release from HIV-1 infected cells (48, 49).

Classification of HIV

The AIDS is caused by two different human immunodeficiency viruses, HIV-1 and HIV-2. HIV-1 can be classified into three broad group such as group M, O, and N by phylogenetic analyses of the nucleotide sequences of the *env* and *gag* (50). Group M (Major) comprises the great majority of HIV-1 isolates and can be assigned to a subtypes or genotypes or clades designated A through K (A, B, C, D, F, G, H, J, K), excluding I and 16 circulating recombinant forms (CRFs) which are CRF01_AE CM240, CRF02_AG IbNG, CRF03_AB Kal153, CRF04_cpx 94CY032, CRF05_DF VII310, CRF06_cpx BFP90, CRF07_BC 97CN54, CRF08_BC 97CNGX-6F, CRF09_cpx P2911, CRF10_CD TZBF061, CRF11_cpx GR17, CRF12_BF ARMA 159, CRF13_cpx, CRF14_BG, CRF15_01B, and CRF16_A2D (http://hiv-web.lanl.gov). Sequence variation of Env amino acid within group M ranges from 3 to 23 % among members of the same subtype and from 23 to 35 % among members of different subtypes. Viruses in group O "Outlier" has been identified in patients from Equatorial Guinea which are highly divergent from group M (50). Viruses between group M and O differ up to 47 % within the nucleotide sequence of *env*. Recently, new subgroup named "N" (non-M and non-O) which can be isolated from Cameroon (51). In Southeast Asia especially in Thailand, subtype E, CRF01_AE, is most common subtype. It appears to be mosaics, with the *gag* from subtype A and the *env* from subtype E (52). In recent study, they found CRF15_01B which is the new circulating recombinant form in Thailand which was the recombinant virus of subtype A/E and subtype B. CRF15_01B had a recombinant structure with three segment comprising subtype B from the beginning of *gag* until mid-*vpu*, where the subtype shifts to subtype E. It then changes back to subtype B in the C5 region of gp120 and remains subtype B through gp41 and *nef* (53).

The course of infection

The course of infection generally includes the three phases or stages such as primary infection, asymptomatic infection, and AIDS (54-58).

1. Primary infection

In HIV-1 infected patients, primary or acute viral infection results within a few weeks. In primary infection, the virus persisted in the blood and lymphoid tissues are able to replicate at high levels of several week. In contrast, the number of CD4+ T cells in the blood drop perhaps because of HIV-dependent destruction.

2. Asymptomatic phase

Within a few weeks, a specific immune response to HIV is increased, and viral replication is greatly reduced allowing the number of CD4 + T cell to rebound to near-normal levels. The patients remain asymptomatic throughout this phase with relatively stable HIV virus load. On the other hand, CD4+ T cells slowly decrease at the rate of 50-100 cells/mm^3 per year.

3. AIDS

The end stage of disease, the viral load is increased to the high level concomitant with destruction of immune system. On the other hand, the CD4 count drops below a level that is compatible with effective immune function leading to development of opportunistic infection and the infected individual develops symptoms of AIDS.

The immune response

The immune responses are 2 levels of defense against invasion by external agent such as innate immunity and adaptive immunity (acquired immunity).

Innate immunity

The innate immune system, has acquired during evolutionary development the deployment of a rapid response in protection against microbial infection. Indeed, innate immune responses may play a critical role in the early stages of HIV transmission. The various cells such as natural killer (NK) cells, γδ+ T cells and macrophages able to secrete a number of chemokines and cytokines which may block HIV transmission and replication. NK cells function as effector cells in the early innate immune response and may exert a direct anti-viral effect. In HIV infection, HIV selectively down-regulates surface HLA class I expression and antigen presentation to T lymphocytes. Thus, NK cells might play an important part in the pathogenesis of HIV infection. For γδ+ T cells, they also generate anti-viral suppressor factors, RANTES, MIP-1α and MIP-1β that can prevent SIV infection by binding to and down-modulating the CCR5 co-receptors. The CC chemokines RANTES, MIP-1α and MIP-1β are produced not only by activation of CD8+ T cells, but also macrophages, DC, T, NK, and γδ T cells. There is a great deal of evidence that the three CC chemokines can block the CCR5 co-receptors and prevent HIV infection in vitro or SIV infection in vivo (59-62).

Adaptive immunity

If the defenses provided by the innate immunity fail to prevent infection, the adaptive immunity is required for protection of host cells. The adaptive immunity comprises humoral immunity and cell-mediated immunity

The key cells in humoral immune system are B lymphocytes (B cells) that interact with antigen through their membrane-bound immunoglobulin (antibody). After the recognition of antigen, lymphocytes proliferate and differentiated to produce the antibody-secreting cells. Antibody would bind to the antigen and help eliminate it.

To mediate cellular immunity, T lymphocytes (T cells) are important cells in controlling of infection. The T cells are composed of two major populations which are T helper cells and cytotoxic T cells (CTL). The T helper cells express the membrane glycoprotein called CD4. When T helper cells are activated, these cells secrete several

cytokines which have various effects on other cells of the immune system. For most cytotoxic T cells, they express the membrane glycoprotein called CD8. When CTL are activated, these cells differentiate into effector cells that can destroy virus-infected and tumour cells (63).

Cellular immunity in HIV

Any immune response involves the interaction of many different cell types and unable to separate the relation of cell-mediated responses and antibody-mediated responses. However, HIV-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 (64, 65). The great majority of cytotoxic lymphocytes are CD8+ cells which have general role in limiting or suppressing viral replication (57).

Cytotoxic T lymphocyte

CD8+ cytotoxic T lymphocytes (CTL) lyse target cells expressing antigen. CTL recognise viral proteins in the form of short peptides approximated 8-12 amino acids in length which is presented in association with major histocompatibility (MHC) class I molecules on the surface of infected cells (66). The endogenous pathway machinery processes proteins which are denovo synthesised within antigen presenting cells (APC). These viral peptides are derived from nascent proteins which are cleaved by cytosolic proteases of infected cells. Viral proteins within infected cells are degraded as fragment at the C-termini by proteasomal processing and transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the ER, trimming of the peptides at the N-termini is occurring (67). After that, the transported peptides then stabilise the folding of class I molecules, associated with β2-microglobuin. Epitopes express certain residues that serve as anchors which these peptides can bind to MHC class I molecules. After the peptide-MHC complexes are formed, these complexes are transported to the cell surface for immune surveillance by the TCR of CTL (68) (Figure 2).

Recognition of viral peptide-MHC class I complexes on the surface of infected cells is a function of the T-cell receptor (TCR) which can bind specifically to a particular MHC-peptide complex. This binding is restricted by both peptide and MHC molecule. Engagement of the TCR triggers a signal of function activation of the CTL through a complex signaling cascade (69-71).

Majority of TCR is a heterodimer consisting of α and β chains that are formed by the rearrangement of noncontiguous V, D, and J joining. In genetic rearrangement of the TCR genes during T cell development, a tremendous diversity of CTL is generated. In response to new foreign antigens, naïve CTL that recognize the new antigen are selectively activated through the TCR. CTL then proliferate and become the activated killer cells. Most of these differentiated CTL die after clearance of cells expressing antigen, whereas, a small number become memory cells during resting phase which able of rapid development on rechallenge with the same antigen (72).
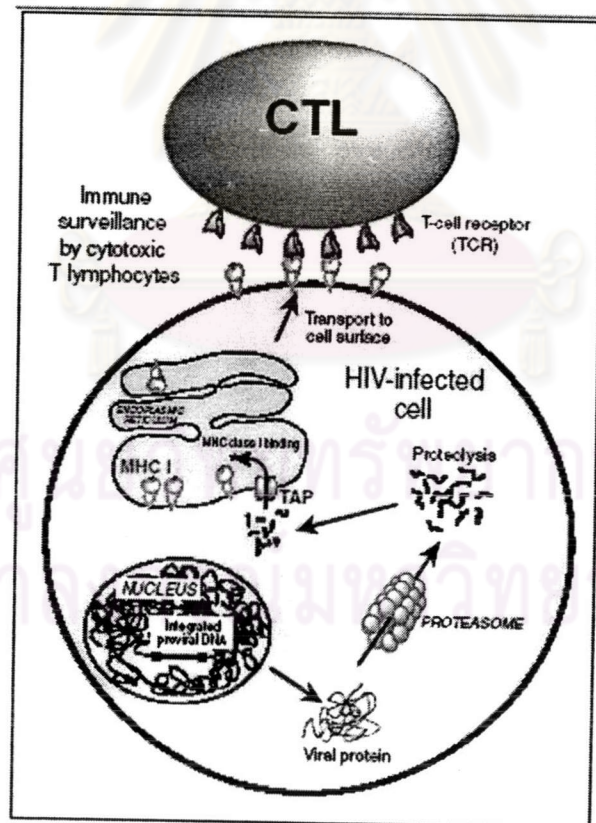

Figure 2   The HLA class I antigen presentation pathway (73).

Multiple mechanisms of CTL functions are utilised to control viral replication (74, 75). Target cells destruction of CTL can be divided into 2 groups by calcium dependent and calcium-independent process. In a calcium-dependent process, direct lysis of infected cells is caused by the release of perforin from lytic granules which perforin is pore-forming protein. In the presence of Ca2+, perforin form a cylindrical structure to insert into lipid bilayer and polymerise structural and functional pores. Target cell death through osmotic lysis or high concentration of Ca2+ may induce apoptosis. Granzymes are protease that cleave and activate caspases apoptosis. Both perforin and granzymes are required for active cells lysis. After antigen recognition, perforin make pores and granzymes move into target cells and activate enzymatic cascade to induce apoptosis of target cells (76).

Calcium-independent cytotoxicity is mediated through specific ligands such as Fas-Ligand (FasL). Fas (CD95) is a member of the tumour necrosis factor receptor superfamily expressing on target cells. Fas L which cross link with Fas is expressed on activated CTL. This interaction lead to apoptosis of target cells (76-79). In addition CTL also act by releasing soluble factors including interferon-γ (IFNγ), tumour necrosis factor-γ (TNF-γ), and chemokines which have diverse antiviral and immunological effects (80-84). Non-lytic control of HIV-1 infection by CTL involves CC chemokine-mediated blockade of virus entry through legend competition with the V3 domain of the gp120 envelope glycoprotein for coreceptor binding sites (85, 86), and suppression of replication in CD4+ cells at step of transcription by soluble agent called CD8+ T cell antiviral factor (CAF) (87, 88).

Human leukocyte antigen (HLA)

A cluster of genes coding for major histocompatibility antigens is called the major histocompatibility complex (MHC). In human, MHC is calls human leukocyte antigen (HLA). The HLA complex is mapped on the short arm of the sixth chromosome. HLA is a region of highly polymorphic. There are three different classes of HLA molecules which are class I, class II, and class III.

Class I HLA genes encode glycoproteins expressed on the surface of nearly all nucleated cells. The major function of the class I gene products is associated with

presentation of peptide antigens to CTL. Class II HLA genes encode glycoproteins expressed primarily on antigen-presenting cells. The class II HLA genes are important for presenting peptide to CD4+ T cells. Class III HLA genes generally encode various secreted proteins that have immune functions including components of the complement system and molecules involved in inflammation.

Structure of class I molecules contains a large α chain associated noncovalently with the smaller β2-microglobulin (β_2m) molecule. The α chain is a polymorphic transmembrane glycoprotein of about 45 KD, while β2-microglobulin is a protein of about 12 KD. Association of the α chain with β_2m is required for expression of class I molecules on cell membranes. The α chain includes three external domains (α1, α2, and α3). The α1 and α2 domains interact to form a platform of eight antiparallel β strands spanned by two long α-helical regions. The structure forms a deep groove, approximately 25 Å ×10 Å× 11 Å, with the long α helices as sides and the β strands of the β sheet as the bottom. β2m interacts extensively with the α3 domain and also interacts with amino acids of the α1 and α2 domains. The interaction of β2m and peptide with the class I α chain is essential for the class I molecule to reach its fully folded conformation. Peptide-binding groove is located on the top surface of the class I HLA molecule which it is large enough to bind a peptide of 8-12 amino acid (66).

CD8+ cytotoxic T lymphocytes recognise foreign antigens which bound to class I HLA molecules. The class I HLA molecules have groove for binding to specific anchor residue of peptide (the amino acid of peptide is important for binding to HLA molecule). The primary anchor residue of peptide is at the position 2 (P2) and C-terminus (89). Each CTL expresses a surface receptor, TCR, which can bind specifically to a particular HLA-peptide complex. This binding is restricted by both peptide and HLA molecule. Whereas the anchor residues of peptide (P2 and C-terminus) bind to HLA molecule, the amino acids protrude from cleft of HLA molecule and contact TCR during engagement. Engagement of TCR triggers a signal to induce cell death of target cells (90). For the HLA molecule in Southeast Asia and Papua NewGuinea, HLA-A11 is one of the most common molecules. In Thailand, HLA-A11 is the most frequently molecule (32.5%), followed by HLA-A2 (25.5%), HLA-Cw3 (15%), and HLA-A24 (14.6) (http://hiv-web.lanl.gov). In particular, populations of Northern Thailand have HLA-A11 approximate 56%. This HLA molecule have been

divided to 20 alleles including A*1101, A*1102, A*1103, A*1104, A*1105, A*1106, A*1107, A*1108, A*1109, A*1110, A*1111, A*1112, A*1113, A*1114, A*1115, A*1116, A*1117, A*1118, A*1119, and A*1120 (www.ebi.ac.uk/imgt/hla/allele.html). HLA-A*1101 is a common allele in East and South-East Asia, and is the most frequent allele in Thailand (91).

Control of HIV-1 infection by CTL

CTL play an important role in the suppression of viral replication both in animal model and in human viral infection, and is important mediators the protection of immunity against HIV infection. Multiple experimental approaches, including the adoptive transfer of virus-specific CTL in animal models to induce specific cellular immunity reactivity, have confirmed the importance of CTL in antiviral immunity (92, 93). In addition, the recent evidences support a role of CTL from CD8 depletion experiments. In monkeys depleted of CD8+ T cell, the viral replication during primary infection was shown to be poorly controlled. Similarly, the enhancement of plasma viremia were demonstrated following CD8 depletion which returns to baseline values when CTL loss (94, 95).

Evidence for a protective role of CTL in human viral infection showed in several studies. The adoptive transfer experiments have emerged from human studies involving therapeutic lymphocyte infusion following bone marrow transplantation. For example, transfer of enriched T-lymphocyte populations or virus-specific CTL prevented or improved post-bone marrow transplant Epstein-Barr virus (EBV)-associated lymphoproliferative disease (96-98). Similarly, protection from involving of human cytomegalovirus (CMV) reactivation was improved by the infusion of CMV-specific CTL into bone barrow recipients (3, 99).

In HIV infection, it is established that HIV-specific CTL appear early in the course of infection and associated with the control of viremia (4, 5). In addition, CTL are commonly found in high numbers during chronic infection (6) and decline with progression to AIDS (11-13, 100). For analysis of immune responses in individuals infected with HIV-1, the quantification of antigen-specific CD8+ T cell frequencies using specific tetrameric complexes in crossectional study demonstrated an inverse correlation with plasma viral load

(6, 100). Additional data from other studies using different methods of CTL quantification tend to be support these results (101). For protection of CTL against disease transmission, HIV-specific CTL have been documented in HIV-specific CTL have been study in HIV-1-exposed, persistently seronegative (HEPS) subjects (102-104), the uninfected heterosexual partners of HIV seropositive individuals (105, 106), and in repeatedly exposed but persistently seronegative female prostitutes in West Africa (14). These studies suggest that CTL may have the capacity to prevent transmission of HIV-1. In addition, the recent data suggested that CTL are also in at least a subset of persons with long-term non-progressing HIV-1 infection (8-13).

CTL escape in HIV-1 infection

The virus has developed mechanisms to avoid recognition of CD8+ T cells. These included both non-mutational and mutational mechanisms. The non-mutational mechanism involves CTL escape in HIV infection such as downregulation of HLA class I molecule, inhibition of antigen processing and up-regulation of FasL. For example, Nef has been shown to downregulate expression of HLA class I molecules (37, 107). However, this downregulation exhibits allele specificity and does not include HLA C and E. NK cell lysis is predominantly inhibited by HLA C and HLA E. Thus, this selective downregulation may allow HIV to escape from CTL while allowing infected cells to maintain a resistance to NK cell regulation (73, 108). In addition, Both SIV (109) and HIV (110) unregulated surface expression of FasL via a signaling mechanism involving Nef and the TCR. If HIV-infected cells express FasL, this may lead to the destruction of HIV-specific CTL via Fas/FasL apoptosis. Moreover, a recent report suggested that the HIV Tat protein inhibits the 20S proteasome and its 11S regulator (111). This may lead to defective presentation of some, but not all (112), HIV epitope, and thus aid escape from CTL.

For the mutation mechanism, it is well established that HIV-1 variants with impaired CTL recognition properties occur during the course of infection. However, it has been more difficult to determine whether this observed variation results from selection pressure exerted by CTL or represents merely a consequence of random sequence variation because of the

high frequency of mutations in HIV-1 and because of the complex dynamics caused by shifting immunodominance (113).

However, the evidences that sequence variation leads to CTL escape in HIV infection showed in several reports. The first evidence that mutations in HIV-1 could lead to escape from CTL recognition was obtained by Philip et al (114). In patients with HLA-B8-restricted CTL response against epitopes in the Gag protein, viral mutations were found to cluster in or near the epitope-encoding region of the *gag*. These mutant genes encoded variant peptides which were not recognised by CTL although the same patient's CTL still recognised the preceding sequence. This observation suggested that the mutant sequence survived because they avoid immune destruction. In addition, in a case of an immunodominant HLA-B44-restricted epitope in Env, escape can occur during primary infection. Variation appears to be specific for the CTL epitope (115). Price et al (116) demonstrated the selection of escape variants over time in a dominant CTL epitope of Nef during primary infection. This study further determined that coding mutations arose in the CTL epitope-encoding region and a standardized ratio of the rate of non-synonymous changes/the rate of synonymous changes (dN/dS) was higher in the region of the epitope showing that coding mutations predominated only in the epitope-encoding region; elsewhere, silent substitutions were common. They suggested that the amino acid mutations within epitope were driven by CTL-mediated selection. Additional data showing the selection of an escape mutant in a dominant HLA B27-restricted epitope in the late stages of HIV infection, which correlated with progression to AIDS, supports the argument for CTL-mediated selection (12).

The possible mechanism for CTL escape by sequence variation consists of several steps. (1) generation of peptide epitopes from proteins; (2) transport of peptides into the ER by TAP; (3) trimming of peptides at the N-termini; (4) assembly of peptides with HLA molecule; (5) recognition by the TCR of CTL (67, 113).

The amino acid sequence changes could lead to the destruction of CTL epitopes as a result of the formation of new sites of proteotytic cleavage within the epitope. In position where a single amino acid changes in the epitope sequence, it results in proteasome-mediated cleavage of the epitope, and a corresponding lack of CTL recognition (117). In addition, sequence variation within an epitope could also lead to a loss or reduction of its export from the cytosol by TAP. The length and sequence-specificity of TAP from a number of different

species has been studied, and there is clearly some peptide selection by TAP (118). Amino acid changes immediately adjacent to an epitope may also affect transport by TAP if the epitope is translocated as a larger precursor before trimming in ER (119). Moreover, the mutation in the flanking region of CTL epitopes can alter antigen processing at step of NH2-terminal trimming of N-extend antigenic peptides (67) for loading onto class I molecule. Sequence variation could lead to loss of presentation to CTL is loss of peptide binding to class I molecules. The alterations in the major anchor residues essential for binding of peptide to HLA molecule, leading to escape CTL recognition (120, 121). However, changes in residues other than the dominant anchor residues can also have significant effects on peptide binding to HLA molecules. Alternatively, sequence variation within epitopes may lead to loss of TCR recognition with minimal effect on peptide binding to HLA. Variation of CTL epitope at TCR contact residues may also generate peptides which are still able to interact with the TCR, but do not induce the normal stimulatory signal. These variation peptides, also called altered peptide ligands (APL), may antagonize TCR recognition of the normal epitope (122, 123).

Techniques for measurement of cytotoxic T lymphocyte

CTL plays critical roles in the regulation of immune responses and are responsible in many pathways of mechanisms of the immune system. For this reason, there has been need for assays to accurately measure the activity of CTL. This thesis reviews some of the established methods that are used for measurement of the CTL functions for the qualitative and quantitative analysis (http://www-ermm.cbcu.cam.ac.uk/).

1. Cytotoxicity assay

For many years, the standard assay for detection of CTL activity has been the cytotoxicity assay. In this assay, target cells have been labeled with sodium chromate ($Na_2^{51}CrO_4$). The labeled target cells are then incubated in co-culture with effectors cells for 4-6 hours. The target cells are lysed and release the amount of ^{51}Cr into supernatant. The amount of ^{51}Cr released into the supernatant is then measured and calculated for the cytotoxicity.

2. Limiting-dilution assay (LDA)

This assay provides method to examine the frequency of CTL presenting in PBMC population that are specific for a particular antigen and used to estimate the precursor frequency of a given cell type. Positive results for these results (proliferation or cytotoxicity) indicate the antigen-specific precursor from PBMC population in the early state which have become activated and have subsequently divided during the period of cell culture. The CTL functions can be assessed either for proliferation or cytotoxicity. LDA assay involves a range of dilutions of PBMC concentration. The other factor such as growth factor, antigen, and APC need to be added to the microtitre wells in excess.

3. Enzyme-linked immunospot (ELISpot) assay

The ELISpot assay is used to measure the local concentration of interferon-gamma (IFN-γ) which is secreted from antigen-specific CD8+T cells. PBMC have been activated with antigen in nitrocellulose-microtitre well plates which have been coated with the anti-interferon-gamma antibody. After effector cells activated with specific antigen, the local production of IFN-γ around producing cells can be detected by adding the secondary antibody and then label it with alkaline phosphatase and then adding substrate which can be changed by enzyme to insoluble coloured product. The spots can be enumerated under stereomicroscope or ELISpot reader. This assay has sensitivity more than the conventional LDA.

4. Intracellular cytokine staining (ICS)

Antigen-specific CD8+ T cell response can be activated with specific antigen before adding of brefeldin A which is used to block transport of cytokine across Golgi. After that, CD8+ T cell is fixed and permeabilised and stained intracellular cytokine with the conjugated anti-cytokine antibody.

5. Tetramer staining

HLA tetramerix complexes can be used to directly quantitate antigen-specific T cells by flow cytometry. HLA heavy chain is expressed in *Escherichia coli* (*E. coli*) with an

engineered COOH-terminal signal sequence containing a biotinylation site for the enzyme BirA. After refolding of heavy chain, β_2-microglobulin (β_2m), and peptide, the complex is biotinylated and tetramer formation induced by the addition of streptavidin. By means of fluorescently labeled streptavidin, the tetramer can be used to strain and sort antigen-specific cells. The staining is highly specific such that CTL clones and lined directed to different epitope peptides bound to the same HLA molecule do not stain (6).