Chapter 1

Introduction
1.1 Introduction to humoral response

Emil von Behring and Shibasaburo Kitasato (1890) demonstrated that serum from a rabbit immunized with tetanus protects mice from virulent strain of tetanus bacteria. They proposed that a mediator in serum reacts with foreign antigen and provides protection. Their idea prompted Paul Ehrlich to propose the side-chain theory in 1897. Ehrlich proposed that receptors (described as “side chains”) on the surface of cells bind to antigen in “lock and key” interaction and this binding is a trigger for production of antibodies (side chains that break off). In 1940, Linus Pauling confirmed the “lock and key” theory and showed that interaction between antigen and antibody depends more on their shape than their chemical composition. In 1954, Niles Jerne hypothesized that there is vast array of lymphocytes in the body prior to any infection. The entrance of an antigen into the body results in selection of one specific lymphocyte and production of corresponding antibody. Sir FM Burnett took this hypothesis further and proposed “clonal selection theory”. According to this theory, an individual lymphocyte expresses membrane receptors that are specific for a distinct antigen. This unique receptor specificity is determined before the lymphocyte is exposed to the antigen. Binding of antigen to its specific receptor activates the cell, causing it to proliferate into clone of cells that have the same immunologic specificity as the parent cell. The clonal selection theory is now accepted as underlying paradigm of immune response. Burnet also proposed that after infection there is selection of two types of lymphocytes. One type of lymphocyte acts immediately to combat infection while other remains long lasting and provides immunological memory.
In 1958, Gustav Nossal and Joshua Lederberg showed that one B cell produce only one antibody (Nossal and Lederberg, 1958) and provided evidence for “clonal selection theory”. In 1960s, Gerald Edelman and Rodney Porter described the structure of antibody (IgG) for which they were awarded with Nobel Prize in 1972. They showed that IgG is composed of disulfide-linked heavy and light chains and consists of Fab (fragment that binds to antigen) and Fc (crystallizable fragment) regions. After the early studies on IgG, other classes of immunoglobulin (IgM, IgA, IgD and IgE) were discovered. In 1976, Susumu Tonegawa and N Hozumi provided the evidence to the two-gene model for IgG (Hozumi and Tonegawa, 1976), which was proposed earlier by Dreyer and Bennett in 1965 (Dreyer and Bennett, 1965). Tonegawa et al showed that two separate genes encode the Fab and Fc region of immunoglobulin and the genes are rearranged in the course of B-cell differentiation. Subsequently it was shown that vast diversity among Fab region of antibodies was due to somatic recombination of immunoglobulin genes.

Antibodies play crucial role in clearing of various microbial infections by different mechanisms. In 1904, Almorth Wright suggested that antibodies bind to bacteria and label them for phagocytosis and killing, a process that he named as “opsonization”. Monocytes, macrophages and neutrophils, phagocytose the antibody coated foreign invaders and degrade them by proteolytic enzymes. Binding of IgG and IgM antibodies to virions, bacteria and infected cells activates complement cascade that lead to virolysis or cell lysis. Antibody-dependent cell-mediated cytotoxicity (ADCC) is another mechanism in which NK cells,
neutrophils and macrophages (cells which bear Fc receptor) recognize and destroy virally infected cells that have been labeled with binding of antibodies. Antibodies mediate protection against many viral infections or diseases due to their ability to neutralize the virus. The neutralizing antibodies bind to the virus and abrogate its infectivity. Vaccines against viruses such as measles, mumps, rubella, polio and human papillomavirus induces virus-specific antibodies that mediate virus neutralization and confer protection (Pantaleo and Koup, 2004; Robbins et al., 1995; Robinson, 2007). Licensed antibody products are also available for passive administration against rabies, hepatitis A, hepatitis B, varicella-zoster, respiratory syncytial and vaccinia virus (Mascola, 2003). Several new vaccines that are under development are also expected to induce neutralizing antibodies and provide protection. Such vaccines that are under development include Severe Acute Respiratory Syndrome corona virus (He and Jiang, 2005), Nipah and Hendra virus (Mungall et al., 2006), West Nile Virus (Martin et al., 2007), Dengue Virus (Chen et al., 2007), Hepatitis C virus (Law et al., 2008).

Antibodies mediate virus neutralization by various mechanisms. Common mechanisms of virus neutralization are:

**Interference to viral attachment:** Some virus specific antibodies bind to cell-free virion and inhibit attachment of virus to host cell. Such antibodies have been found against several viruses e.g. Vaccinia, Papillomavirus, Rotavirus, Influenza virus. Burton et al investigated relationship between surface area on different viruses and the number of antibody molecules required for neutralization (Burton et al., 2001). The number of antibody molecules required for neutralization...
increased along with size of the virus. This suggests that steric hindrance for interaction between virus and its receptor play important role in virus neutralization.

**Interference to post-attachment viral interactions:** Some viruses use ancillary receptors such as heparan sulphate proteoglycans for initial tethering to the target cell and use different molecules for internalization into the host cell (e.g. Herpes Simplex virus and Foot and Mouth Disease virus). Some viruses require additional co-receptor for entry into the host cell (e.g. adenovirus, HIV). All these interactions are potential targets for neutralizing antibodies.

**Inhibition of fusion:** Antibodies can interfere with fusogenic protein-protein interactions or conformational changes that are required for fusion of virus and cell membrane. Such antibodies have been identified against HIV-1.

**Formation of virus aggregates:** Cross-linking of virus particles by antibodies may lead to formation of high molecular weight aggregates, which show reduced infectivity and also render virions more susceptible for phagocytosis and destruction. Aggregation requires binding of bivalent/ multivalent antibody that links viral particles into lattice. Such cross-linking occurs at particular virus-antibody ratio and breaks due to excess antigen or antibody.

Interference with the uncoating of the virus and inhibition of transcription after viral internalization have been also described as potential mechanisms of virus neutralization (Klasse and Sattentau, 2002).

An individual infected with HIV builds a strong anti-HIV immune response (described in detail in section 1.2). Anti-HIV immune response is able to
control the virus multiplication leading to lowering of plasma HIV virus load. However, the virus cannot be eliminated from the body leading to complete cure. Correlates of protection against HIV are not defined. Hence it is critical to study different aspects of HIV-specific immune response for unraveling clues to protect from HIV. Although the role of innate immune response and mucosal immune response still remains to be fully understood, neutralizing antibody response, T helper response and Cytolytic T cell response are three important components. In this study, two aspects of humoral response are studied (Chapter 1, Section 1.3, page: 1-37).
1.2 Introduction to HIV-1

A clinical syndrome, which was later called Acquired Immunodeficiency Syndrome (AIDS), was identified in year 1981 at United States. This was followed by reports of around 1300 similar cases in next two years. These patients were either men having sex with men or injecting drug users (IDUs). They showed rare diseases like Kaposi’s sarcoma and Pneumocystis Jirovici (earlier known as Pneumocystis carinii) pneumonia and showed reversal of CD4+ : CD8+ T cell ratio in peripheral blood. These patients appeared to have lost their immune competence rendering them vulnerable to opportunistic infections as well as to lymphoid and other malignancies. Isolation of etiological agent from these patients was first reported in 1983 by Barre-Sinoussi et al at the Pasteur Institute (Barre-Sinoussi et al., 1983) as a reverse transcriptase containing virus (retrovirus) from lymph node of a man with persistent lymphadenopathy. They termed the virus as Lymphadenopathy Associated Virus (LAV). In 1984, Gallo et al from NIH, USA reported isolation of retrovirus from AIDS patient called ‘Human T cell Lymphotropic Virus III’ (HTLV-III) (Gallo et al., 1984). Few other investigators also reported isolation of AIDS associated Retroviruses (ARV). Subsequently the three prototype viruses (LAV, HTLV-III and ARV) were recognized as members of same group of retroviruses. In 1986, the International Committee on Taxonomy of Viruses (ICTV) recommended giving the AIDS virus a separate name, the Human Immunodeficiency Virus (HIV) (Coffin et al., 1986). Subsequently two types of HIV (type 1 and type 2) were identified.
Although AIDS was first detected in 1981, earlier there was phase of silent spread of HIV-1. Few cases of AIDS were identified retrospectively between year 1972 and 1976 in USA and Haiti (Korber et al., 2000). HIV-specific antibodies were seen in a serum sample from Kinshasa (Democratic Republic of Congo) that was stored in 1959 (Zhu et al., 1998). This is the earliest evidence of HIV infection. On the basis of HIV-1 Envelope gene from 1959 strain and 159 strains obtained at different time points, it has been estimated that HIV-1 was probably transmitted to humans in early thirties (Korber et al., 2000). The evidences such as similarities in genomic organization, phylogenetic relatedness, geographic coincidence, prevalence in the natural host and plausible routes of transmission suggest that HIV-1 was transmitted from chimpanzee (*Pan troglodytes*) to humans in central Africa (Stebbing et al., 2004). It is estimated that humans were first infected with HIV-2 about 60 years ago (Lemey et al., 2003) and has been speculated to have originated from SIVsm a lentivirus from the sooty mangabey (*Cercocebus atys*) of West Africa (Hahn et al., 2000). The HIV-2 show distinctly slower rate of disease progression compared to HIV-1 and has largely remained restricted to West Africa.

According to UNAIDS global estimates, 39.5 million (range: 34.1 to 47.1 million) individuals were living with HIV and 2.9 million (2.5–3.5 million) died due to HIV/AIDS in year 2006. Out of these deaths, 380,000 were children below 15 years of age. In some of the African countries (e.g. Kenya, Malawi, Zimbabwe, Swaziland) prevalence of HIV is >25%, which has significantly reduced the life expectancy. Since majority of individuals infected with HIV fall
in the age group of 15 to 50, which is prime time of working life, HIV/AIDS has devastated several millions of families.

**Structure of HIV-1 virion**

Mature, cell-free HIV-1 virion is around $110 \pm 8$ nm in diameter and is roughly spherical (Zhu et al., 2006) (Figure 1.1). The virus consists of two copies of non-complementary sense strands of RNA enclosed by capsid composed of 24 kD proteins (p24). This is surrounded by matrix, which is composed of 17 kD (p17) proteins. The viral capsid also encloses enzymes (reverse transcriptase, integrase and protease) and other viral proteins (Nef, Vif and Vpr) along with RNA. The entire virion is enclosed in a phospholipid bilayer (envelope) derived from host cell. The viral spikes (gp160 trimer glycoprotein composed of subunits gp120 and gp41) are embedded in the envelope. The gp120 is present on virus surface whereas its counterpart gp41 is present in transmembrane region. The gp120 and gp41 are held together by noncovalent interactions. It has been estimated that there are $14 \pm 7$ spikes (gp160 trimers) randomly distributed on HIV-1 surface (Zhu et al., 2003; Zhu et al., 2006). Several host cell proteins such as HLA class I, class II and Inter-Cellular Adhesion Molecules (ICAMs) are also present in virus envelope.
Figure 1.1: Schematic representation of HIV-1 structure
**Genome organization of HIV-1**

HIV-1 proviral genome consists of about 9200 nucleotides (Figure 1.2), which encode for three structural proteins (Gag, Env and Pol) & six accessory proteins (Tat, Rev, Nef, Vif, Vpr and Vpu). HIV-1 uses all three reading frames for transcription of mRNAs. The HIV-1 proteins are synthesized after differential splicing of primary mRNA transcripts (Figure 1.2).

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**Figure 1.2: Schematic representation of genome organization of HIV-1 provirus (A) and different RNA transcripts (B).** Unspliced RNA codes for Gag and Pol proteins. Incompletely spliced RNA code for Env, Vif, Vpr and Vpu proteins. The RNA transcript obtained after complete splicing code for Tat, Nef and Rev proteins.
**Genetic diversity of HIV-1**

Genetic diversity is a hallmark of HIV-1. The extreme heterogeneity of HIV-1 is a result of high mutation rate (0.2-2 mutations per genome per cycle) due to lack of proofreading ability of the reverse transcriptase, high replication rate and propensity for recombination. HIV-1 can be classified into three groups: M (Major), N (Non-M, Non-O / New) and O (Outlier). The M group is most prevalent and consists of nine subtypes (A-D, F-H, J and K). The virus strains belonging to the same subtype differ up to 20% in their envelope protein whereas differences between strains of different subtype may be up to 35% (Gaschen et al., 2002). Different HIV-1 subtypes predominate in different geographic regions; subtype A in Africa and Eastern Europe, subtype B in America, Europe and Australia; subtype C in India, China and South Africa. Apart from these subtypes there are HIV-1 strains that have mosaic genomes. They have been reported in geographic areas where more than one HIV-1 subtypes are circulating. These strains are product of recombination between two different HIV-1 subtypes that infect a single cell. The recombination occurs due to “template switching” during reverse transcription by reverse transcriptase enzyme. Recombination between different groups (Peeters et al., 2003), different subtypes (Lal et al., 2005), within subtype (Rousseau et al., 2007) as well as among recombinant strains of HIV-1 (Yang et al., 2003) has been reported. Approximately 8% of HIV-1 genome sequences available in Los Alamos HIV sequence database (http://www.hiv.lanl.gov) display mosaic genome structures (Kothe et al., 2006). When HIV-1 strains with similar mosaic structure are obtained from three or more
epidemiologically unlinked individuals, they are referred as Circulating Recombinant Forms (CRF). Recently 33rd CRF has been reported from Malaysia (Tee et al., 2006).

**HIV-1 transmission**

Majority of HIV-1 infections are acquired through sexual exposure with infected individual. HIV-1 infected women can transmit the virus to their infants during pregnancy, delivery or during breast-feeding. Mother-to-child transmission occurs in 15-30% cases in absence of any intervention. HIV-1 infection can also be acquired due to contact with contaminated blood, blood products and use of contaminated needles. Sharing of needles significantly contributes to HIV transmission among Intravenous Drug Users (IDUs).

Gray et al found that the probability of transmission of HIV-1 with each sexual encounter was approximately 0.001 and was related to viral dose (Gray et al., 2001). Direct relationship between viral load in peripheral blood and sexual transmission has been reported (Pope and Haase, 2003; Quinn et al., 2000). No evidence of HIV-1 transmission has been obtained when viral RNA level in blood is below 1,500 copies/ml. The risk of acquisition of HIV-1 during sexual contact increases due to breaches in the mucosal barrier.

**Mucosal acquisition of HIV-1 infection and dissemination**

HIV-1 crosses stratified squamous epithelium of vaginal mucosa and simple columnar epithelium of cervix either by transcytosis, capture of virus by
dendritic cells (DCs) or infection of intraepithelial lymphocytes (Pope and Haase, 2003). After crossing the mucosal barrier, HIV-1 predominantly infects CD4⁺ T cells (Zhang et al., 2004). Infection of these cells results in continuous virus production. Dendritic cells that express C-type lectin receptors (such as DC-SIGN/CD209/CD206) capture HIV-1 and transmit it to the lymph nodes. Infection of activated CD4⁺ T cells in lymphoid tissues results in explosive virus production.

**HIV-1 life cycle**

Life cycle of HIV-1 virus consists of following steps (Figure 1.3).

**Attachment to the host cell and entry:** HIV-1 binds to the host cell primarily through interaction between viral gp120 and CD4 receptor present on host cell. Interaction between viral gp120 and CD4 induces conformational change in gp120, which promotes its binding to the co-receptor. The chemokine receptors CCR5 and CXCR4 are commonly used as co-receptors by HIV-1. The interactions of viral gp120 with receptor and co-receptor promote conformational changes in gp41 that lead to fusion of viral and cell membrane. The viral core is then released into the cell interior.

HIV-1 uses CCR5 as preferential co-receptor for entry into the target cell at the time of transmission. Therefore individuals that carry defective homozygous CCR5 allele show resistance to HIV-1 infection. In some cases, HIV-1 isolated from patients during late stage of infection uses CXCR4 as coreceptor. Therefore based on phenotype, HIV-1 isolates are classified as CCR5
tropic (R5 phenotype) and CXCR4 tropic (X4 phenotype). HIV-1 isolates that use both i.e. CCR5 & CXCR4 coreceptors (R5/X4 phenotype) as well as coreceptors other than CCR5 and CXCR4 have been also reported.

**Viral uncoating and reverse transcription:** Host cell proteins including Mitogen Activated Protein (MAP) kinase, cyclophilin A and viral proteins (Nef and Vif) play important role in uncoating of viral matrix and capsid. After uncoating, there is formation of viral reverse transcription complex, which comprises diploid viral RNA genome, reverse transcriptase and primer for cDNA synthesis. The Vif protein acts as accessory factor for reverse transcription (Carr et al., 2007) as well as counteracts the anti-retroviral cellular factor APOBEC3G (Goncalves and Santa-Marta, 2004). APOBEC3G deaminates deoxyCytidine (dC) to deoxyUracil (dU) in cDNA and causes failure of viral replication. This failure is characteristic of Vif-defective virus.

After completion of c-DNA synthesis, there is formation of preintegration complex (PIC), which consists of viral double stranded DNA and virus proteins including integrase and host factor called High Mobility Group DNA binding protein. The PIC moves towards nucleus using microfilaments and microtubules.

**Integration into the host cell chromosome:** The PIC has a diameter of 28nm, which is larger than nuclear pore. PIC enters into the nucleus using nuclear import pathway. After translocation into the nucleus, the integrase cleaves the 3' termini of the viral dsDNA to generate two nucleotide 5' overhangs at each end. Subsequently the integrase triggers transesterification reaction in which the 3' hydroxyl group attacks phosphodiester bonds of chromosomal DNA and joins
viral DNA to host DNA. The viral DNA is randomly integrated at many chromosomal locations. The integrated form of the virus is called provirus. The nonintegrated linear DNA is circularized, which reduces signal for apoptosis by reducing number of linear DNA molecules.

**Transcription of viral RNA or viral latency:** The provirus remain silent (latent) or is actively transcribed depending on chromatin structure around the integration site and metabolic status of the host cell. The virus may remain latent due to integration into areas of repressed heterochromatin or due to absence of factors such as nuclear factor κB (NF-κB) and Nuclear Factor of Activated T cells (NFAT), which act as transcriptional enhancers. In an activated cell, NF-κB and NFAT bind to enhancer sequence of LTR and promote viral transcription. Host cell RNA Polymerase II binds to transcription initiation site and begins transcription but fails to elongate efficiently in absence of viral Tat protein. Tat protein binds to TAR (first 45 nucleotides of viral m-RNA, which is target sequence for viral transactivation) and prevent premature termination of transcription.

**Processing of viral RNA transcripts, nuclear export and expression of viral proteins:** Viral RNA transcripts are completely spliced, incompletely spliced or remain unspliced (Figure 1.3). The viral Rev protein plays an important role in controlling the splicing mechanism and transport of viral RNA transcripts to cytoplasm. In the cytoplasm these RNA molecules are translated into viral proteins. Multiply spliced m-RNA molecules encode Nef, Tat and Rev whereas incompletely spliced RNA transcripts encode Env, Vif, Vpr and Vpu proteins.
The unspliced RNA transcripts encode Gag (p55) and Gag-Pol (p160) precursor proteins. The Gag-Pol precursor is produced by ribosomal frame shifting near the 3' end of gag.

**Assembly of new virions and release:** The Env (gp160) protein is cleaved by cellular protease into gp120 and gp 41 proteins. These proteins are embedded in membrane of host cell. Approximately 2000 Gag proteins, 200 Gag-Pol proteins, two unspliced viral RNA and other proteins (Vif, Vpr and Nef) assemble below the cell membrane (Wilk et al., 2001). A human protein HP68 acts as molecular chaperone and facilitate conformational changes in Gag required for their assembly. These assembled viral components form immature virion which buds out of the host cell using cellular ESCRT (Endosomal Sorting Complex required for Transport) that mediates outward vesiculation (Marsh and Thali, 2003). In this process, host cell plasma membrane embedded with gp120 and gp41 proteins form HIV-1 envelope and encloses the nucleocapsid. After budding the viral protease enzyme cleaves Gag and Gag-Pol precursor proteins. The Gag precursor protein is cleaved into p24, p17 and other subunits whereas Gag-Pol precursor protein is cleaved into reverse transcriptase, protease and integrase. This results in formation of mature, infectious virion (Wilk et al., 2001).
Figure 1.3: Schematic representation of HIV-1 life cycle.
HIV-1 pathogenesis

The natural history of HIV-1 infection is characterized by an acute, primary phase that lasts for few weeks, followed by clinically latent phase that lasts for several years, and ultimately by immune deficiency syndrome (Figure 1.4).

**Acute phase:** Within 2-3 weeks after acquisition of HIV-1 infection, virus becomes well established in the lymphoid tissue. It has been estimated that, each infected cell generates about 20 infected progeny cells during its life-time (Little et al., 1999). Plasma viral RNA level increases exponentially with a doubling time of 10 to 20 hrs (Fiebig et al., 2003). The plasma viral RNA level up to 10^7 RNA copies/ ml has been reported (Lavreys et al., 2002). Plasma viral RNA level is highest at the start (primary stage) and end of infection (AIDS), whereas low and relatively steady level is maintained in between for a variable number of years (Figure 1.4). This steady state of viraemia (viral set point) is achieved within 6 to 12 months of infection and may depend on various host (immune response and genetic factors such as HLA) and viral factors as yet not fully understood. The viral set point may vary between < 50 to > 1 x 10^6 copies / ml. Patients with higher viral set point show rapid disease progression compared to patients with lower viral set point.

There is massive infection and loss of CD4^+ T cells predominantly in lymphoid tissues of gastro-intestinal track (Brenchley et al., 2004; Li et al., 2005b; Mattapallil et al., 2005; Mattapallil et al., 1998; Veazey et al., 1998). The immediate decline in CD4^+ T cell count in peripheral blood is also observed
during acute primary HIV-1 infection but their number rebounds after few weeks although not to pre-infection level (Figure 1.4).

About half of individuals in this phase of infection develop headache, fever, myalgia, anorexia, rash and diarrhea. These symptoms mimic many common febrile illnesses including influenza, malaria and rickettsial diseases. The symptoms subside in 1 to 2 month after their appearance.

**Clinically latent phase:** Acute primary HIV-1 infection is followed by a long period of clinical latency (usually 7 to 10 years). Although patients in this stage of infection do not show clinical disease and plasma viral RNA level is often low, virus multiplication continues in the lymphoid tissue. Ho et al showed that there is daily production and clearance of $0.05 \times 10^9$ to $2 \times 10^9$ HIV-1 virions and daily turnover of CD4$^+$ T lymphocytes ranges between $0.2 \times 10^9$ to $5.4 \times 10^9$ cells (Ho et al., 1995). This turnover in the CD4$^+$ cells leads to steady decline in peripheral CD4$^+$ T cell count leading to immune suppression. The rate of CD4$^+$ T cell decline may vary leading to slow, average or rapid disease progression. About 10% of the HIV-infected individuals progress to AIDS within 2 to 3 years after infection and are referred as *rapid progressors*. About 5-10% individuals do not show clinical disease even after 10 years of infection (Cao et al., 1995; Lefrere et al., 1997). These individuals show less than 5% decline in CD4$^+$ T cell count annually with plasma viral RNA level undetectable or very low without antiretroviral therapy. These individuals are called *Long-Term Non-Progressors (LTNP)*. However ultimately all individuals shows signs of disease progression.
(Lefrere et al., 1997). Majority of infected persons may lead to AIDS stage within 7 to 10 years.

**Acquired Immune Deficiency Syndrome (AIDS):** According to revised classification system for HIV infection (1993) by CDC (www.cdc.gov) the HIV-1 infected individual with $CD_4^+$ T cell count < 200 cells/mm$^3$ is reported as AIDS patient (CDC, 1992). The patients acquire various opportunistic infections and malignancies. Some of the conditions included in the 1993 AIDS surveillance case definition (CDC, 1992) are: Candidiasis of respiratory tract & lungs, Coccidioidomycosis (disseminated or extra pulmonary), Cryptococcosis (extra pulmonary), Cytomegalovirus disease (other than liver, spleen, or nodes), Herpes simplex infection (with chronic ulcer and greater than 1 month's duration), Histoplasmosis (disseminated or extra pulmonary), Isosporiasis (chronic intestinal with greater than 1 month's duration), Kaposi’s sarcoma, Burkitt's Lymphoma, Lymphoma of brain, Mycobacterium avium complex, Mycobacterium tuberculosis (pulmonary & extra pulmonary) and wasting syndrome. Patients in this stage show highest risk of death.
Markers of HIV-1 disease progression

The key characteristic of HIV-1 infection is gradual loss of CD4\(^+\) T lymphocytes (Figure 1.4). Profound loss of CD4\(^+\) T cells was reported in early description of AIDS (Lane et al., 1985). The peripheral blood CD4\(^+\) T cell count may predict future risk of progression to AIDS (Goedert et al., 1987; Phillips et al., 1989). CD4\(^+\) T cell count is one of the strongest predictors of clinical disease (Cozzi Lepri et al., 1998; Phillips, 2004). Risk of acquiring opportunistic infections remains relatively low until the CD4\(^+\) T cell count declines to 200 cells/mm\(^3\). There is exponential rise in risk for death due to infections and
malignancies when CD4$^+$ T cell count starts declining below 200 cells /mm$^3$ (Phillips, 2004).

The prognostic value of plasma viral RNA level was first described by Mellors et al. (Mellors et al., 1995). Results of Multicenter AIDS Cohort Study (MACS) showed association between risk of HIV-1 disease progression and plasma HIV RNA level (Mellors et al., 1997). Higher HIV RNA levels correlate with more rapid decline in CD4$^+$ T cell count and more rapid disease progression.

Plasma HIV-1 RNA level and peripheral blood CD4$^+$ T cell count are independent predictors of clinical outcome. These two markers are also used for assessment of response to Anti Retroviral Therapy (ART).

**Adaptive immune response to HIV-1 infection**

Adaptive immune response to HIV-1 infection consists of CD8$^+$ Cytotoxic T lymphocyte (CTL) response, CD4$^+$ helper T cell response and humoral antibody response.

**CD8$^+$ Cytotoxic T Lymphocyte (CTL) response:** During viral infection, intracelluarly synthesized viral proteins are digested by proteolytic enzymes and foreign peptides are presented on cell surface along with HLA class I proteins. CD8$^+$ T cells recognize foreign peptides that are 8 to 11 amino acids long (epitopes) and lyse virally infected cells. Therefore these cells are called as 'Cytotoxic T Lymphocytes (CTLs)'. During viral infection there is multiclonal expansion of CTLs bearing T cell Receptors (TCR) that can recognize different viral peptides.
HIV-1 specific CTLs were first detected in infected individuals in 1987 (Walker et al., 1987). Role of CTLs in controlling viraemia during primary HIV-1 infection was reported in 1994 (Borrow et al., 1994; Koup et al., 1994). The reduction in viraemia in acute infection was associated with the emergence of HIV-1-specific CTLs. The importance of CD8⁺ CTLs in control of viraemia was also evident in SIV-macaque model. In these studies, resolution of viraemia was not observed in absence of SIV-specific CTLs (Jin et al., 1999; Matano et al., 1998). A strong CTL response has been found to be associated with better virus control and slower disease progression in HIV-infected individuals (Cao et al., 2003; Harrer et al., 1996; Ogg et al., 1999). Therefore CTL response is considered to hold key for successful control of HIV disease (McMichael and Rowland-Jones, 2001; Ogg et al., 1998). CTL response against multiple epitopes is required for better control of HIV replication. HIV-positive individuals that are homozygous at any of the HLA class I alleles and therefore possess limited breadth of CTL response are more likely to show rapid disease progression (Carrington et al., 1999). CTLs also produce soluble anti-viral factors such as β-chemokines (RANTES, MIP-1α and MIP-1β), which can inhibit HIV-1 replication (Yang et al., 1997).

Although CTL response plays important role in controlling virus replication, there is emergence of escape mutants. As a result, plasma viral RNA level increases and leads to disease progression (Goulder and Watkins, 2004). Mutation in conserved region is likely to cause considerable fitness cost to the virus whereas escape in non-conserved region may cause little or no cost.
Therefore it has been speculated that CTLs that target highly conserved regions of HIV might show better virus control (Goulder and Watkins, 2004).

**CD4⁺ T cell response:** CD4⁺ T cells produce growth and differentiation factors for development of humoral and cell-mediated immune responses and therefore are referred as ‘helper T cells’. These cells recognize foreign peptides that are 10-18 amino acids long and are presented on cell surface along with HLA class II proteins. Lichterfeld et al showed that HIV-1 specific CD4⁺ T cell response is required for proliferation of HIV-1-specific CD8⁺ T cells (Lichterfeld et al., 2004). Vigorous HIV-1-specific CD4⁺ T cell proliferative response has been found to be associated with control of viraemia (Rosenberg et al., 2000; Rosenberg et al., 1997).

HIV-1 preferentially infects and eliminates the virus-specific CD4⁺ T cells (Douek et al., 2002). This may be the potential mechanism due to which there is loss of HIV-specific CD4⁺ T-cell responses and consequently the loss of immunological control of HIV replication. This is supported by the observation that progressive chronic HIV-1 infection is characterized by the absence of HIV-specific CD4⁺ T cell proliferative response and significantly lower number of IL-2 secreting CD4⁺ T cells compared to LTNPs (Harari et al., 2004; Harari et al., 2005).

HIV-1-specific cytotoxic CD4⁺ T cells (that express perforin, granzymes and display direct ex-vivo cytolytic activity) have been also reported in LTNPs (Zaunders et al., 2004).
**Humoral response:** About 3 to 6 weeks after infection (window period), HIV-specific antibodies become detectable in serum / plasma (referred as seroconversion). Antibodies against different proteins such as Gag, Env, Pol, Tat, Nef can be detected. This serves as the basis for serological diagnosis of HIV-1 infection. The antibodies directed against specific epitopes of Env protein (gp120 and gp41) show HIV-1 neutralizing activity. Characteristics of these antibodies and their role in HIV-1 immunopathogenesis are described in chapter 3.

Based on study of adaptive immune response in HIV-1 infected individuals and their role in immunopathogenesis attempts are being made to develop HIV-1 vaccines that can induce similar responses and provide protection against HIV-1 infection or disease progression.

**HIV-1 vaccine development**

Current thinking for HIV vaccine development indicates that there may be multiple immune mechanisms including neutralizing antibodies, T helper cell response and CTLs important for HIV control. DNA vaccines and vector-based vaccines are currently being explored in clinical trials. Several improvements such as codon optimization for expression of viral proteins in mammalian cells, alteration in regulatory elements, inclusion of cytokine expressing genes and novel formulations with different adjuvants are being pursued for induction of immune responses.

The two most widely used viral vectors are modified vaccinia Ankara (MVA) and serotype 5 adenovirus (Ad5). Live viral vectors have advantage of
achieving intracellular synthesis of immunogen. Since proteins are intracellularly synthesized, they can efficiently induce CD8+ T cell response. However limitations that can significantly reduce their efficiency are: pre-existing immunity to the vector and competition between vaccine and vector protein for antigen presentation to T cells. DNA vaccines have advantage of expressing only vaccine insert and absence of pre-existing immunity. However the cells poorly take up such molecules. Another concern for viral vectors and DNA vaccine is potential risk of oncogenesis due to chromosomal integration.

Priming with one construct and boosting with different vector is being explored to induce stronger and cross-reactive immune response by avoiding boosting of immune response against vector backbone (Amara et al., 2001). Till now, highest T-cell responses have been induced by prime / boost regimen and has been found to provide protection against disease progression in nonhuman primate model (Robinson, 2007). This concept is being evaluated in phase III human clinical trial at Thailand where more than 16,000 healthy volunteers have been enrolled. In this trial, ALVAC-HIV (canarypox vector carrying genes of gp120 of subtype E and gp41, gag and protease of subtype B virus) is administered for priming of immune response and AIDSVAX (gp120 envelope glycoprotein) is administered as a booster dose. The concern for this trial is that preliminary data from small number of breakthrough infections in phase I/II trials of ALVAC-AIDSVAX showed no evidence for suppressed viral load in immunized patients (Lee et al., 2004).
Despite extensive worldwide efforts for last two decades, HIV-1 vaccine development has remained elusive goal. Results of first phase III human clinical trial in which recombinant gp120 protein (AIDSVAX, VaxGen Inc, USA) was used for immunization were released in year 2005. HIV-1 was acquired by 6.7% of vaccinees and 7.0% of placebo recipients suggesting complete lack of vaccine efficacy (Flynn et al., 2005). Another disappointing result was obtained in the phase IIb clinical trial that was conducted by Merck & Co and HVTN (HIV Vaccine Trial Network). In this trial, adenovirus type 5 carrying three HIV-1 genes (gag, pol & nef) was used for immunization. The interim analysis of data from 1500 volunteers showed that vaccine did not prevent infection and also did not show reduced viraemia after infection. After this interim analysis vaccination and enrollment in the trial was discontinued in September 2007.

There are several challenges for HIV vaccine development. Some of them are:

1. **Genetic diversity of HIV-1 and emergence of escape mutants**: HIV-1 encountered by vaccinated individual may differ at least by 10% from the genome sequence of immunogen. HIV-1 vaccine needs to protect against diverse, circulating strains of HIV-1. Therefore development of HIV vaccine using polymeric protein sequences (Azizi et al., 2006) or centralized sequence is being pursued (Kothe et al., 2006). Polymeric vaccines use proteins from multiple viral isolates to increase the breadth of immune response. Centralized sequence is derived using consensus or ancestral method. Such sequence encodes most common amino acid at each position of viral protein. Due to minimization of
genetic distances between vaccine strains and circulating viruses they may be anticipated to elicit immune responses that recognize a broader spectrum of viral variants.

During HIV-1 replication, there is emergence of several viral mutants. The mutant virus that escapes action of neutralizing antibodies and CTL are rapidly selected in an infected individual (Goulder and Watkins, 2004; Mascola and Montefiori, 2003). Transmission of these mutants can contribute to evolution of HIV-1 strains that are more resistant to immune responses (Moore et al., 2002). It has been speculated that due to spread of such viruses, currently identified epitopes that are associated with successful containment of HIV may become extinct over time (McMichael and Klenerman, 2002). Therefore the vaccine constructs designed to induce immune responses against those epitopes may also become irrelevant in course of time.

Emergence of escape mutants and subsequent disease progression has been reported in vaccinated macaque as well as in human volunteer. Barouch et al vaccinated rhesus macaques and subsequently challenged with pathogenic strain of SHIV (Barouch et al., 2002). In this study, one animal initially controlled virus replication but subsequently there was single nucleotide mutation in Gag CTL epitope, which resulted in CTL escape of virus. This was followed by burst of viral replication, clinical disease progression and death from AIDS-related complications. Betts et al reported that anti-HIV-Gag CD4+ and CD8+ T cell responses were induced in HIV-seronegative individual after administration of
HIV-1 vaccine. However the individual acquired HIV-1 infection, showed emergence of escape mutant and rapid disease progression (Betts et al., 2005).

2. **Lack of understanding of correlates of protection:** Identification of the immune correlates of protection during natural infection is most crucial prerequisite for designing a vaccine. Like other viral infections, viral clearance by the host immune system is not observed in HIV-1 infection. Therefore the immune responses that vaccine needs to emulate are unknown. Although HIV-specific CTLs and neutralizing antibodies have gained attention, it is necessary to know what type, specificity and frequency of T-cell response correlate with protection. Some T-cell vaccines have provided protection in nonhuman primate models against development of AIDS. Therefore efforts are being made to identify subset of vaccine-elicited T cells that correlate with protection. The results suggest that frequency of central memory T cell has better correlation with protection than the frequency of effector cells (Vaccari et al., 2005).

Although neutralizing antibodies are considered important in protection against HIV-1, degree of cross-reactivity and titer of neutralizing antibodies required to be induced by HIV-1 vaccine is yet undefined (Burton et al., 2004).

3. **Lack of suitable animal model:** An animal model should permit the establishment of infection with the pathogen and exhibit pathology similar to human disease. Such animal models have contributed enormously in understanding pathogenesis of several infectious diseases as well as in the development of vaccines and novel therapeutics. However a suitable animal model is not available for HIV-1. The only non-human primate that HIV-1 can
infect is chimpanzee. However infection of chimpanzee with HIV-1 does not mimic the course of HIV-1 disease, which is seen in infected human being.

Since SIV (Simian Immunodeficiency Virus) is genetically closely related to HIV and shows pathogenesis in some species of macaques (e.g. sooty mangabeys, rhesus, pig-tailed macaques), infection of these macaques with SIV has been explored for testing of vaccine concepts. However inferences obtained from SIV-macaque model have limitations. For example, high dose of SIV that causes 100% infection and cause disease within few months is used in macaque model. However in case of HIV, the frequency of transmission is significantly low (1 in 1000 exposures) and causes disease after few years of infection. Therefore direct extrapolation of data from macaque to human is difficult. It is also observed that HIV-1 vaccines does not elicit T-cell response of same magnitude in human volunteers as they elicit in non-human primates (Robinson, 2007).

Despite several challenges and frustrating experiences for HIV vaccine development, it has been speculated that ‘delivery of correct combination of antigens in an effective formulation should enable induction of immunological protection in humans’ (Berkley and Koff, 2007). It has been suggests that an AIDS vaccine even with 50% efficacy, which covers only 30% of target population, can avert up to a third of HIV infections and thus save tens of millions of lives (Berkley and Koff, 2007).

Currently the only available strategy against HIV-1 is to improve quality of life of infected individuals by anti-retroviral therapy.
**Anti-Retroviral Treatment**

HIV-1 uses host cell machinery for replication. Therefore number of viral targets for drug designing is limited. The drugs that have been approved for clinical use are: reverse transcriptase inhibitors, protease inhibitors, virus-cell fusion inhibitors and integrase inhibitor.

**Reverse transcriptase inhibitors:** Nucleoside Reverse Transcriptase Inhibitors (NRTI) are nucleoside analogues, which lack 3'-hydroxyl group. When these molecules are incorporated in DNA during reverse transcription, they inhibit DNA polymerization. Examples of these drugs are: Zidovudin (AZT), Didanosine (ddl), Zalcitabine (ddC), Lamivudine (3TC), Stavudine (d4T) and Abacavir.

Non Nucleoside Reverse Transcriptase Inhibitors (NNRTI) binds to reverse transcriptase and inhibit enzymatic activity without interfering in DNA polymerization directly. Examples of these drugs are: Nevirapine, Delaviridine and Efavirenz.

**Protease Inhibitors:** Protease inhibitors bind to HIV-1 protease and inhibit processing of Gag & Gag-Pol polyprotein, which inhibit formation of many structural and enzymatic proteins. Examples of these drugs are: Saquinavir, Indinavir, Ritonavir, Nelfinavir and Amprenavir.

**Fusion Inhibitors:** Fusion inhibitor inhibits virus entry into the cell. Examples of these drugs are Enfuvirtide and Maraviroc. Enfuvirtide (commonly known as T-20) binds to gp41 and inhibit the conformational change, which is essential for virus-cell membrane fusion. Maraviroc binds to CCR5 co-receptor and inhibit entry of HIV-1 into host cell.
**Integrase inhibitor:** This category currently consists of only one drug, raltegravir. Raltegravir inhibits integrase enzyme and blocks virus integration into host cell chromosome.

Fusion inhibitors and integrase inhibitor are recently approved drugs by US-FDA. The first drug that was approved by US Food and Drug Administration (FDA), to treat HIV-1 infection in 1987 was Zidovudin (AZT). After approval of first protease inhibitor in year 1995, combination therapy was initiated for better virus control. HAART (Highly Active Anti Retroviral Therapy) that is now commonly administered to the patients consists of combination of three drugs (Two NRTIs and 1 protease inhibitor or NNRTI). The ART has significantly improved quality of life and prolonged life expectancy of treated individuals. Therefore to promote access of ART in developing countries, World Health Organization set “3 by 5” target in year 2003 with aimed to make ART available to 3 million people by the end of year 2005. Now the goal that has been set is “All by 2010” which describes universal access to ART by the year 2010.

Although ART has significantly improved quality of life; there are several limitations for its use. These include

**Toxicity:** Administration of all NRTIs is associated with mitochondrial toxicity resulting in increase in serum lactate concentration. Patients with moderate level of serum lactate concentration may show nausea, vomiting, weakness, muscle pains, diarrhea and loss of weight. If the serum lactate concentration increases
>10 mmol/l the patient may show respiratory disorder and NRTI treatment needs to be discontinued.

Administration of all protease inhibitors is associated with Lipodystrophy syndrome in which patient shows disorders of fatty acid distribution and metabolism. Increased serum levels of triglycerides, LDL-cholesterol, hyperglycemia and insulin resistance is observed.

**Drug resistance:** Drug resistant HIV-1 strains are consequence of incomplete suppression of HIV replication, which is commonly observed due to suboptimal adherence to ART. More than 300 mutations in reverse transcriptase as well as protease that lead to drug resistance have been reported. Spread of drug resistant HIV-1 strains represents major challenge for success of ART in future years.

**Life-long treatment:** The cells with latently infected virus are not eliminated by HIV-specific immune responses and act as a long-lived viral reservoir. The virus rebounds back from these reservoirs after discontinuation of ART. Siliciano et al showed that virus rebound back in treated individual despite undetectable viraemia for as long as 7 years (Siliciano et al., 2003). Mathematical model suggests that elimination of latently infected cells would require a minimum of 6 to 10 years if ART is started in the early stage of infection and several decades if initiated during the chronic phase of infection (Zhang et al., 1999). Administration of ART for life-long period is a challenging task.

Considering pandemic spread of HIV-1 and limitations of ART, prevention of transmission is a vital task. Prophylactic vaccine, vaginally applied microbicide and behavioral changes leading to reduction in risky behavior can
play role in prevention of HIV-1 transmission. It has been speculated that HIV epidemic is unlikely to be totally halted by behavioral changes. Therefore efforts are being made for development of prophylactic HIV-1 vaccine and microbicide using various novel strategies.

**Indian scenario**

The first case of HIV infection was detected in year 1986 at Chennai. In a survey done in next year 135 samples were found to be HIV positive. By the year 1990, cases of HIV infection were detected in every state of the country and rapid spread of HIV was observed among injecting drug users in Manipur, Mizoram and Nagaland.

Based on the data from 1,122 sentinel surveillance sites covering all districts in the country and a general population survey (National Family Health Survey III) conducted in year 2005-06 in which 230,000 people were tested, it has been estimated that 2 to 3.1 million people were living with HIV/AIDS in India in year 2006. Out of the estimated adults living with HIV, 38.4% were female. HIV prevalence among women attending antenatal clinics (ANC) ranges from 0 to 2% in different states of India. In 118 districts, >1% HIV prevalence among ANC attendees has been reported.

In India, ~85% of HIV-1 transmission is due to sexual intercourse (Marfatia YS, 2007). Majority of these are cases of heterosexual transmission but recently significant level of homosexual behaviour among males has been also reported (Gupta et al., 2006). Around 2% of transmission occurs due to
contaminated blood / blood products whereas another 2% is due to injecting drug usage. Around 4% cases of HIV-1 transmission are due to perinatal mode of transmission (Marfatia YS, 2007).

To characterize the HIV-1 strains that are spreading in India, several molecular characterization studies have been carried out. Studies carried out in different parts of India suggest that clade C is most predominant among HIV-1 subtypes circulating in India (Kandathil et al., 2005; Lal et al., 2005). Few cases of HIV-1 infection belonging to subtype B and A have been reported (Kandathil et al., 2005; Lal et al., 2005). The first genetically recombinant HIV-1 in India (mosaic of subtype A and C) was reported from Pune in 1999 (Lole et al., 1999). Another 3 A/C recombinant HIV-1 strains were reported in 2004 from Mumbai (Deshpande et al., 2004). B/C recombinant strains have been reported from Karnataka (Siddappa et al., 2005), Manipur (Bhanja et al., 2005; Tripathy et al., 2005) and Calcutta (Bhanja et al., 2007). Full-length genome sequencing of B/C recombinant strains from Manipur showed that these are novel recombinant strains of HIV-1 (Lakhashe et al., 2008a; Lakhashe et al., 2008b). However till now no CRF of HIV-1 has been reported from India.

To control spread of HIV, several awareness programs are being carried out. The Government of India started in year 2004 the program of providing free Anti Retroviral Therapy to all those who need it. Although more than 130,000 patients are now accessing the free ART, it is small proportion of all patients who need the treatment. The efforts are being made to make ART accessible to large number of patients.
1.3: Scope of the study

Among multiple immune mechanisms that may contribute to the control of HIV viremia and protection from HIV virus, CTL and humoral immune mechanisms are crucial. However, there is need to look at the other mechanisms that may help in the control of HIV infection. Studies on immune responses in subtype C HIV-1 infection in India are very limited. While our laboratory carries out studies in CTL responses as well as humoral immune mechanisms in HIV infected individuals, the scope of this study is limited to humoral immune mechanisms.

During budding out of the host cell, HIV-1 incorporates several host proteins including HLA in its envelope. Antibodies against HLA proteins may hence be able to bind HIV. Few investigators have reported anti-HLA antibody mediated HIV-1 neutralization. However the evidence available in literature for anti-HLA antibody mediated HIV-1 neutralization is inconclusive. Anti-HLA antibody mediated HIV-1 neutralization, if demonstrated conclusively, would offer anti-HIV strategy that would work independent of HIV-1 genetic variations. Hence potential of anti-HLA antibodies to neutralize HIV-1 was investigated.

Analysis of HIV-1 subtype C genome sequences from India show that these sequences are closely related to each other and form monophylactic lineage (Novitsky et al., 2002; Novitsky et al., 1999; Shankarappa et al., 2001). The low genetic diversity has been reported in gag (Gupta et al., 2005; Kurle et al., 2004; Mullick et al., 2006), nef (Jere et al., 2004; Kumar et al., 2006), tat (Mullick et al., 2006) as well as in the env (Agnihotri et al., 2004; Agnihotri et al., 2006; Khan et
al., 2007) gene. The low genetic diversity may result in cross-reactive immune responses. Cross-reactive immune responses suggest shared neutralization determinants among circulating viruses (Bures et al., 2002). This may have important implications for HIV-1 vaccine development strategies. However there is paucity of data about characteristics of immune responses in context of limited genetic diversity from India. Therefore cross-reactivity of neutralizing antibody response was investigated. The study involved investigation of cross-reactivity of neutralizing antibodies from HIV-1 infected individuals against a panel of HIV-1 isolates.

HIV-1 neutralizing antibodies are directed against Env protein of the virus. Therefore in Brief, antibodies directed against virally encoded Env proteins and host encoded HLA proteins were investigated for their HIV-1 neutralizing activity (Figure 1.5).
Figure 1.5: Targets of antibodies for virus neutralization. Antibodies against HLA proteins were studied for their potential to neutralize HIV-1 (chapter 2). HIV-1 neutralizing antibodies (which are directed against Env protein) were investigated for their intra-clade (subtype C) cross-neutralization (chapter 3).