MOLECULAR BASIS OF IMMUNOLOGICAL DYSFUNCTION IN PEOPLE LIVING WITH HIV AND AIDS IN ENUGU, NIGERIA

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TITLE PAGE

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A Thesis presented to the Department of Medical Laboratory Sciences, Faculty of Health Sciences and Technology, College of Medicine, University of Nigeria Nsukka in partial fulfilment of the requirement for the award of Degree of Doctor of Philosophy (Ph.D.)

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CERTIFICATION

Mr./Mrs./Miss OHOTU EDWIN OBIORA (PG/Ph.D./06/46140) a Postgraduate student of the Department of Medical Laboratory Sciences, Faculty of Health Sciences and Technology, College of Medicine, University of Nigeria, Enugu Campus, has completed the requirements for the research. The results embodied in the work have not been submitted in part or full to any diploma or Degree of this or any other University.

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DEDICATION

This work is dedicated to those who are infected with the acquired immunodeficiency virus (HIV), for the alleviation of pains and Godôs revelation of cure.

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ABSTRACT

Molecular basis of immunological dysfunction in people living with HIV and AIDS was studied among HIV-positive people attending clinics at the University of Nigeria Teaching Hospital Ituku-Ozalla, Annunciation Specialist Hospital Emene, Mother of Christ Specialist Hospital and Enugu State University Teaching Hospital, all in Enugu metropolis. A total of 90 subjects recruited for the study were divided into three groups: 30 diagnostically positive HIV subjects (A), 30 HIV-positive subjects on highly active antiretroviral therapy (HAART) (B) and 30 apparently healthy individuals with HIV seronegative status as control group (C). Blood samples (8ml from each subject) were collected for the estimation of IL-2 cytokines, malondialdehyde (MDA) and total antioxidant status (TAS) using ELISA method. $CD4^+$ and $CD8^+$ T-lymphocyte count, and expression of CD25 and CD38 were analyzed using Flow Cytometery. Also samples were tested for IL-2, Tim-3 and Fos gene expressions using standard gene extraction methods and real time polymerase chain reaction (RT-PCR) test. IL-2 and Fos genes were sequenced for single nucleotide polymorphisms (SNPs) using Sangerôs method with Big Dye Terminator chemistry. HIV viral load was estimated using RT-PCR method with TaqMan chemistry. The mean CD4 and CD8 T-cell count, percentage CD38 and CD4/CD8 ratio of group A subjects were statistically significantly lower ($p<0.05$) than those of group C. However, there was no statistically significant difference observed when the same variables in group A were compared with those of group B ($P>0.05$).The mean plasma IL-2 cytokines in group B was statistically significantly higher than that of group A ($p<0.05$) with corresponding statistically significant increase ($p<0.05$) in both IL-2 gene and Fos gene expressions. This may be as a result of statistically significant reduction in viral load ($p<0.05$), observed during antiretroviral therapy (B). Furthermore, the expression of IL-2, Tim-3 and Fos genes in group A were statistically significantly lower ($p<0.05$) than those of group B and C respectively. Also, the expression of Fos gene in group B was statistically significantly lower ($p<0.05$) than that of group C, while those of Tim-3 gene and IL-2 gene showed no statistically significant difference ($p>0.05$). On the other hand, the oxidative stress (MDA) in both A and B were statistically significantly higher ($p<0.05$) than that of group C while mean plasma concentration of TAS in group A was statistically significantly lower ($p<0.05$) than those of groups B and C. Finally, there was statistically significant difference in IL-2 gene SNPs among the three study groups ($p<0.05$) while that of Fos gene showed no statistically significant difference ($p>0.05$). The result of this study showed that HIV infection interferes with the activation of Fos gene which is a major regulatory factor for the

expression of IL-2 gene in T-helper cells, thereby down regulating the production of IL-2 cytokine during progressive HIV disease. The elevation of IL-2 cytokine secretion and reduction in viral burden during antiretroviral therapy does not show a corresponding increase in T-cell proliferation, indicating a qualitative defect in the IL-2 cytokines produced. The study also indicates that the expression of Tim-3 molecules on T-helper cells may be another mechanism for $CD4^+$ T-cell depletion in HIV infection through Tim-3-galactin-9 ligation-induced cell death. On the other hand, oxidative stress may be associated with viral load, $CD4^+$ T-cell depletion and total antioxidant capacity during HIV infection. The need for the initiation of drug intervention as soon as HIV diagnosis is established to enable drug and immune response act synergistically to prevent the observed qualitative and quantitative IL-2 cytokine defect after T-cell exhaustion is hereby suggested. Also, the use antioxidants should be included in the management of HIV and AIDS, while research should be designed to produce drugs which will be able to block Tim-3 signaling pathways to prevent Tim-3-galactin-9 ligation-induced T-cell death during HIV infection.

CHAPTER ONE

Introduction

Human Immunodeficiency Virus (HIV) is a member of the genus Lentivirus, in the family of Retroviruses. Lentiviruses infect many species of animals with characteristic illnesses of long duration and long incubation period (Levy, 1993). Lentiviruses are transmitted as single stranded RNA viruses. Upon entering into the target cell, the viral RNA genome is converted to a double-stranded DNA by a virally encoded enzyme, reverse transcriptase, present in the virus particle (Smith *et al.*, 2006). HIV is different in structure from other retroviruses. It is roughly spherical, around 60 times smaller than a red blood cell with a diameter of about 120nm. It is composed of two copies of single-stranded RNA that encodes for the virusôs nine genes enclosed in a capsid of 2,000 copies of the viral protein (McGovern *et al*., 2002).

Human Immunodeficiency Virus (HIV) is the causative agent of the fatal condition known as Acquired Immunodeficiency Syndrome (AIDS) (Weiss, 1993; Douck *et al.,* 2009), a condition in man in which the immune system begins to fail, leading to life-threatening opportunistic infections. HIV infection occurs by a transfer of blood, semen, vaginal fluid, pre-ejaculate, breast milk, or other body fluids, contaminated with the virus, to a new host. The four major routes of transmission are unprotected sex, contaminated needles, breast milk and prenatal transmission. Screening of blood products for HIV has largely eliminated transmission through blood transfusion in the developed world, though in Nigeria, it still accounts for the second largest source of HIV infection after heterosexual sex (FMH, 2009).

It was estimated that from the discovery of HIV infection in 1981, to the year 2006, that AIDS has killed more than 25 million people worldwide and infected about 0.6% of the world population. In 2005 alone, AIDS claimed an estimated 2.4 ï 3.3 million lives, of which more than 570,000 were children. One third of these deaths occurred in sub-Saharan Africa, retarding economic growth and increasing poverty (UNAIDS and WHO, 2010). In most infectious diseases, a number of factors contribute to the risk of acquisition of infection and to the occurrence of illness after exposure to the pathogen. These factors include the nature of the exposure (e.g. route and size of the inoculums), the ôvirulenceô of the microbes and the nature of the host susceptibility to infection. The nature of the exposure clearly determines the risk of infection. Parenteral exposure to blood infected with HIV carries a substantial risk

of infection. Among individuals transfused with blood from HIV-infected person has the risk of about 100% of being infected (Msellati *et al.*, 1990).

Trans-mucosal infection risks vary according to the site of exposure, with risk of transmission through rectal exposure exceeding the risk of transmission across oral mucosa. Mucosal inflammatory disease tends to enhance the risk of transmission particularly if associated with ulceration. Epidemiologic evidence of seroconversion after accidental needle stick injuries or sexual contact with infected persons, with different levels of plasma HIV RNA suggest that the magnitude of the inoculums also contributes to the risk of HIV infection (Cardo *et al*., 1997; Fideli *et al.*, 2001). Similarly, motherï toï children transmission of HIV is enhanced among women with high level of plasma HIV RNA, even after taking into account other known predictors of transmission (Garicia *et al.*, 1999). Insight gained from persons at high risk for infection yet who persistently remain seronegtive indicates that certain genetic loci can dramatically affect the risk for acquisition of HIV infection. Specifically, persons homozygous for 32-base-pair deletion (the so-called data-32 mutation) in the Cï C motif chemokine receptor 5 (CCR5) opens reading frame that results in failure of surface expression of this key viral co-receptor are protected from acquisition of HIV infection (Liu *et al.*, 1996).

The majority of HIV infections are transmitted through unprotected sexual relation. Sexual transmission can occur when infected sexual secretion of one partner comes into contact with the genital, oral or rectal mucous membranes of another partner. In high income countries, the risk of femaleï toï male transmission is 0.04 percent and maleï toï female transmission is 0.08 percent. For various reasons, these rates are 4 to 10% higher in lowï income countries (Boily *et al.*, 2009). Sexual assault greatly increase the risk of HIV transmission as condoms use is rarely employed and physical trauma to vagina or rectum occurs frequently, facilitating the transmission of HIV (Koenia *et al.*, 2004). Other sexual transmitted infections (STI) increase the risk of HIV transmission, because they cause the disruption of the normal epithelial barrier by genital ulceration and/or micro-ulceration and by accumulation of pools of HIVï susceptible or HIV-infected cells (lymphocytes and macrophages) in semen and vaginal secretions. Epidemiological studies from sub-Saharan Africa, Europe and North America suggest that genital ulcer, such as those caused by syphilis and/or chancroid, increase the risk of becoming infected with HIV by about fourfold (Laga *et al.*, 1991).

Transmission of HIV depends on the infectiveness of the index case and the susceptibility of the uninfected partner. Infectivity seems to vary during the course of illness and is not constant between individuals. An undetectable plasma viral load does not necessarily indicate a low viral load in the semen or vaginal section. However, each 10ï fold increase in the level of HIV in the blood is associated with about 81% increased rate of HIV transmission (Tovanabutra *et al.,* 2002). Women are more susceptible to HIVï 1 infection due to hormonal changes, vaginal microbial ecology and physiology, and a higher prevalence of sexually transmitted disease (Sagar *et al.*, 2004).

Transmission of HIV through blood products is particularly relevant to intravenous drug users, haemophiliacs and recipients of blood transfusion and other products. Needle sharing is the cause of about one third of all new cause of HIV infection in North America, China and Eastern Europe. The risk of being infected with HIV from a single prick with a needle that has been used on an HIV-infected person is thought to be about 1 in 150. Post exposure prophylaxis with anti-HIV drugs can further reduce this risk (Fan, 2005). Although HIV transmission through blood transfusion is highly reduced to negligible rate in developed countries the transfusion of unsafe blood still counts for the second largest source of HIV transmission in Nigeria (FRN, 2012). The transmission of HIV from mother to the child can occur in vivo during the last weeks of pregnancy and at childbirth. In the absence of treatment, the transmission rate between a mother and her child during pregnancy, labour and delivery is estimated to be 25%. However, when the mother takes antiretroviral therapy and gives birth by caesarean section, the rate of transmission is just 1% (Coovadia, 2004). This rate of infection is influenced by the viral load of the mother at birth, with the higher the viral load, the higher the risk. Breast feeding also increases the risk of transmission by about 4% (Coovadia and Bland, 2007).

HIV infects primarily vital cells in the human immune system such as helper T-cells (especially $CD4^+$ T-cells), macrophages, and dendritic cells (Cunningham *et al.*, 2010). HIV infection leads to low level of $CD4^+$ T-cells, through three main mechanisms which include; direct viral killing of infected cells, increased rate of apoptosis in infected cells and killing of infected $CD4^+$ T-cells by CD8 cytotoxic T-lymphocytes. When $CD4^+$ T-cell number decline below a critical level, cellï mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections. Most untreated people infected with HIVï 1 eventually develop AIDS (Migueles and Connors, 2010).

Most of HIV infected individuals die from opportunistic infection or malignancies associated with the protective failure of the immune system (Lawn, 2004). HIV progresses to AIDS at a variable rate, which is affected by the viral host and environmental factors. All those infected with HIV eventually progress to AIDS either within 10 years of infection or much sooner, or later (Buchbinder *et al.*, 1994). Treatment with highly active anti-retroviral therapy (HAART) increases the life expectancy of the infected people. Even after HIV has progressed to AIDS, the average survival time with antiretroviral therapy was estimated to be more than 5 years (Schneider *et al.*, 2005). The failure of the immune system to contain HIV is related to the functional impairment of HIVï specific $CD4^+$ and $CD8^+$ T-cells that accompanies progressive HIV infection, a phenomenon which is referred to as T-cell exhaustion (Day *et al*., 2006). It is known that the $CD4^+$ T-cells (Tï helper cells) play a major role in the overall immune responses by signalling other cells in the immune system to perform their respective functions (Deck *et al*., 2004; Ondoa *et al*., 2005). The mechanisms leading to T-cell exhaustion in HIV infection are clearly complex and cannot be attributed to a single pathway. In HIVï 1 infection, the deterioration of T cell responses involves the early loss of proliferative capacity, cytotoxic potential and the ability to produce interluikinï 2 (1L-2), followed by a progressive loss of the ability to produce interferon gamma (INF-y) (Kostense *et al*., 2002).

A cellular protoï oncogene called c-fos, belonging to the early gene family transcription factor is a protein encoded by the Fos gene. It dimerises with c-jun to form APï 1 transcription factor which up-regulates the transcription of genes involved in everything from proliferation and differentiation to defence against invarsion and cell damage (Van, 1983). It has been shown that cï fos is one of the major regulatory factors for the expression of ILï 2 gene in T cell activation (Ochi, 1992). On the other hand, a T-cell immunoglobulin mucinï 3 (Timï 3) has been identified as a T-helper-1 (Th1) specific marker and several in vivo studies have shown that Timï 3 regulates autoimmunity (Kobayashi *et al.*, 2007) and negatively regulates Th1-mediated inflammatory diseases. Timï 3 expressing T-cells have failed to produce cytokines or proliferate in responses to antigen and exhibit impaired Stat5, Erk1/2, and P38 signalling. It has been also reported that Tim-3 promotes immune tolerance induction and that it binds with its ligand, galectinï 9 to induce the death of T-cells expressing Timï 3 markers (Sabatos *et al*., 2003; Zhu *et al*., 2005; Jones *et al.*, 2008).

Though, a significant progress in the field of anti-HIV chemotherapy has been achieved with the development of highly active antiretroviral therapy (HAART)

(Sammodosis, 1993), the total eradication of HIV especially from the immune cells in the tissue sanctuary sites is still a mirage. In this project, therefore, we intend to analytically profile some of the immunological markers to assess the impact of HIV and HAART on gene expression, single nucleotide polymorphism, cytokine production, T-cell proliferation and lipid peroxidation, in order to expose the molecular picture of HIV and host interaction for new drug targets and better management strategy, for total eradication of HIV in an infected individual.

Objectives

1. To evaluate the levels of ILï 2 gene transcript number, synthesis of IL-2 cytokine, expression of IL-2 receptors on T-cells, and activation of CD4 and CD8 T-lymphocytes.

2. To determine the levels of dysfunctional T-helper cells (classified by the expression of Tim-3 molecules) in diagnostic HIV, those on HAART and HIV seronegative control subjects.

3. To determine the relationship between IL-2 gene transcript number, Fos gene transcript number and the level of IL-2 cytokine in the three study groups.

4. To determine the levels of single nucleotide polymorphisms (IL-2 gene and Fos gene), with respect to HIV infection and antiretroviral therapy (HAART).

5. To determine the relationship between oxidative stress, viral load, $CD4^{+}$ T-cell depletion and Highly Active Antiretroviral Therapy (HAART) in HIV-infected persons.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Cell

The cell is the basic structural and functional unit of all known living organisms. It is the smallest unit of life that is classified as a living thing (except the virus), and is often called the building block of life (Bruce-Alberts, 2002). Organisms can be classified as unicellular (including most bacteria) or multi-cellular (including plants and animals). Humans contain about 10 trillion (10^{13}) cells. Most plant and animal cells are between 1 and 100μm and therefore are visible only under the microscope (Campbell *et al.,* 2006). The cell was discovered in 1665 by Robert Hooke. The cell theory, first developed in 1839 by Mathias Jakoh Stchleiden and Theodor Schwann, states that all organisms are composed of one or more cells, that all cells come from pre-existing cells, that vital functions of an organism occur within the cell, and that all cells contain the hereditory information necessary for regulating cell functions and for transmitting information to the next generation of cells (Maton *et al.*, 1974). There are two types of cells: eukaryotic and prokaryotic. Prokaryotic cells are usually independent while eukaryotic cells are often found in multi-cellular organisms.

Cell Structure

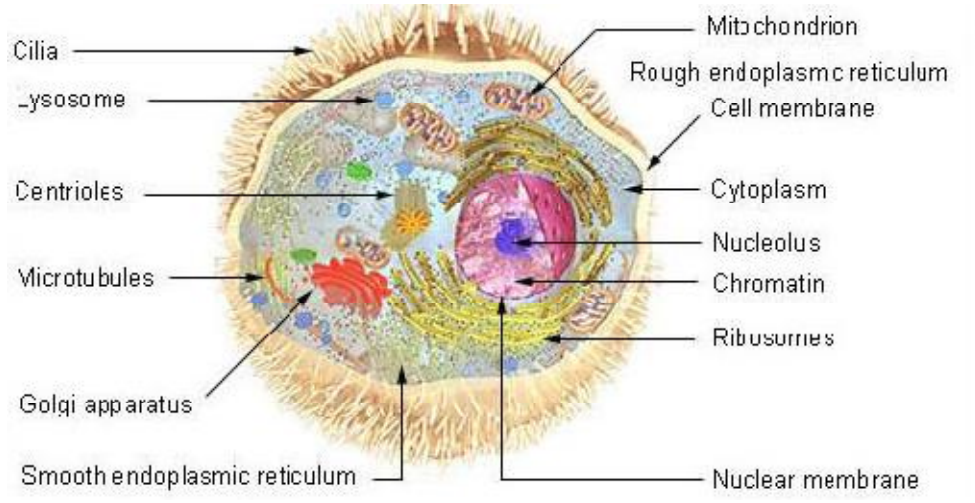


Fig. 2.1: Eukaryotic Cell

Plants, animals, fungi, slime moulds, protozoa, and algae are all eukaryotic. Their cells are about 15 times wider than a typical prokaryote and can be as much as 1000 times greater in volume. The major difference between prokaryotes and eukaryotes is that eukaryotic cells contain membraneï bound compartments in which specific metabolic activities take place. Most important among these is a cell nucleus, a membraneï delineated compartment that houses the eukaryotic cellôs DNA. This nucleus gives eukaryote its name which means ñtrue nucleusò.

The cytoplasm of a cell is surrounded by a cell membrane or plasma membrane. The plasma membrane serves to separate and protect a cell from its surrounding environment and is made mostly from a double layer of lipids (hydrophobic) and hydrophilic phosphorous molecules. Hence, the layer is called a phosphor lipid bilayer, or sometimes a fluid mosaic membrane. Embedded within this membrane is a variety of protein molecules that act as channels and pumps that move different molecules into and out of the cell. The membrane is said to be ñsemi-permeableò, in that it can either let a substance (molecule or ion) pass through freely, pass through to a limited extent or not pass through at all. Cell surface membrane also contains receptor proteins that allow cells to detect external signaling molecules such as hormones or cytokins (King *et al*., 2003).

The cytoskeleton acts to organize and maintain the cellôs shape: it anchors organelles in place: helps during endocytosis (the uptake of external materials by the cell) and cytokinesis (the separation of daughter cells after cell division) and moves parts of the cell in processes of growth and mobility. The eukaryotic cytoskeleton is composed of microfilaments, intermediate filaments and microtubules. There is great number of proteins associated with them, each controlling a cellôs structure by directing, bundling, and aligning filaments. The prokaryotic cytoskeleton is less well-studied but is involved in the maintenance of cell shape, polarity and cytokinesis (Michie and Lowe, 2006). The human body contains many different organs, such as the heart, lung, and kidney, with each organ performing a different function. Cells also have a set of ñlittle organs,ò called organelles that are adapted and/or specialized for carrying out one or more vital functions. Both eukaryotic and prokaryotic cells have organelles but organelles in eukaryotes are more complex. There are several types of organelles in a cell such as the nucleus, golgi apparatus, mitochondria, endoplasmic reticulum (ER), ribosome, vacuoles, centrosome, lysosomes and peroxisomes. The cytosol is the gelatinous fluid that fills the cell and surrounds the organelles.

The endoplasmic reticulum (ER) is the transport network for molecules targeted for certain modifications and destination. The ER has two forms: the rough ER, which has

ribosome on its surface and secretes protein into the cytoplasm, and the smooth ER, which lacks ribosome but play a role in calcium sequestration and release. The function of golgi apparatus is to process and package the macromolecules such as proteins and lipids that are synthesized by the cell. Ribosome is a large complex of RNA and protein molecules. They each consist of two subunits, and act as an assembly line where RNA from the nucleus is used to synthesize protein from amino acids. Ribosome can be found either floating freely or bound to a membrane of endoplasmic reticulum, in eukaryotes (Menetret *et al.*, 2007).

Mitochondria are self-replicating organelles that occur in various numbers, shapes, and sizes in the cytoplasm of all eukaryotic cells. Mitochondria play a critical role in generating energy in eukaryotic cell. It generates the cellôs energy by oxidative phosphorylation, using oxygen to release energy stored in cellular nutrients. Lysosomes contain digestive enzymes. They digest excess or worn-out organelles, food particles, and engulfed viruses or bacteria. Peroxisomes have enzymes that rid the cell of toxic peroxides. The centrosome produces the microtubes of a cell which is a key component of the cytoskeleton. It directs the transport through the ER and the golgi apparatus. Centrosomes are composed of two centrioles, which separate living cell division and help in the formation of the mitotic spindle (Heald *et al*., 1997). Cell nucleus is the most conspicuous organelle found in a eukaryotic cell. It houses the cellôs chromosomes, and is the place where almost all DNA replication and RNA synthesis (transcription) occur. The nucleus is spherical and separated from the cytoplasm by a double membrane called the nuclear envelope. Nuclear envelope isolates and protects DNA from various molecules that could accidentally damage its structure or interfere with its processing. During processing, DNA is transcribed or copied into a special RNA called messenger RNA (mRNA). This mRNA is then transported out of the nucleus, where it is translated into a specific protein molecule. The nucleolus is a specialized region within the nucleus where ribosome subunits are assembled. In prokaryotes, DNA processing takes place in the cytoplasm (Upangala, 2010).

Human Genome

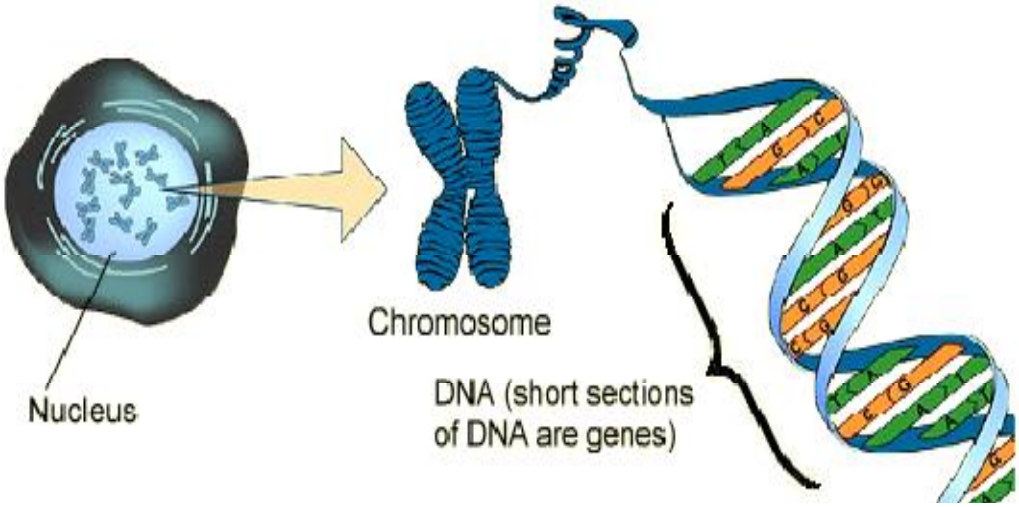


Fig. 2.2: Human Genome

The human genome is a complete set of human genetic information, stored as DNA sequences within the 23 chromosome pairs of the cell nucleus, and in small DNA molecules within the mitochondria. The haploid human genome (contained in egg and sperm cells) consist of three billion DNA base pairs, while the diploid genome (found in somatic cells) has twice the DNA content found in haploid genome. The haploid human genome contains approximately 20,000 protein-coding genes, significantly fewer than had been anticipated (IHGSC, 2004). Protein-coding sequences account for only a very small fraction of the genome (approximately 1.5%), and the rest is associated with non-coding RNA molecules, regulatory DNA sequences, introns, and sequences to which no function has yet been assigned (IGHSC, 2001). Although the human genome has been completely read by DNA sequencing, it is not yet fully understood. Many genes have been identified, yet much work still needs to be done to completely elucidate the biological functions of their protein and RNA products. Recent results indicate that most of vast quantities of non-coding DNAs within the genome have associated biochemical activities, including regulations of gene expression, organization of chromosome architecture, and signals controlling epigenetic inheritance (Elizabeth, 2012).

2.2: Coding DNA Sequence

The content of the human genome is commonly divided into coding and non-coding DNA sequences. Coding DNA is defined as those sequences that can be transcribed into mRNA and translated into protein during the human life cycle. Protein-coding sequences

represent the most widely studied and best understood component of the human genome. These sequences ultimately lead to the production of all human proteins, although several biological processes (e.g., DNA rearrangements and alternative pre-mRNA splitting) can lead to the production of many more unique proteins than the number of protein-coding genes. The complete modular protein-coding capacity of the genome is contained within the exome, and consists of DNA sequences encoded by exons that can be translated into protein. Because of its biological importance, and the fact that it constitutes less than 2% of the genome, sequencing of the exome was the first major milepost of the Human Genome Project. The categorized human protein by function is given as number of encoding-genes and the percentage of all the genes in human genome (Panther, 2011).

The number of protein-coding genes within the human genome remains a subject of active investigation. A 2012 analysis of the human genome based on in vitro gene expression in multiple cell lines identified 20687 protein-coding genes (Elizabeth, 2012). Historically, the estimate of the number of protein genes has varied widely, from as many as 2,000,000 in the late 1960s (Kauffman, 1969) to approximately 40,000 (Ohuo, 1972). Remarkably, the number of human protein-coding genes is significantly smaller than that of many less complex organisms, such as the roundworm and the fruit fly. This may be as a result of alternative pre-mRNA splicing, which provides the ability to build a very large number of modular protein through the selective incorporation of exons. Protein-coding genes are distributed unevenly across the chromosomes, with an especially higher gene density within chromosomes 19, 11 and 1. The significance of this uneven distribution of protein-coding genes is not well understood (Huang *et al*., 2009).

2.3: Non-Coding DNA Sequence

Non-coding DNA is defined as all of the DNA sequences within a genome that are not found within protein-coding exons, and so are never represented within the amino acid sequence of expressed proteins. By this definition, more than 98% of the human genome is comprised of non-coding DNA since protein-coding sequences constitute less than 1.5% of the human genome (IHGSC, 2001). Numerous classes of non-coding DNA have been identified, including genes for non-coding RNA (e.g., Transfer RNA: tRNA and ribosomal RNA: rRNA), pseudogenes, untranslated regions of mRNA, regulatory DNA sequences, repetitive DNA sequences, and sequences related to mobile gene elements. In addition, about 26% of the human genome is introns (Gregory and Ryan, 2005). Aside from genes (Exons and introns) and known regulatory sequences (8 ï 20%), the human genome contains regions of non-coding DNA. The exact amount of non-coding DNA that plays a role in cells

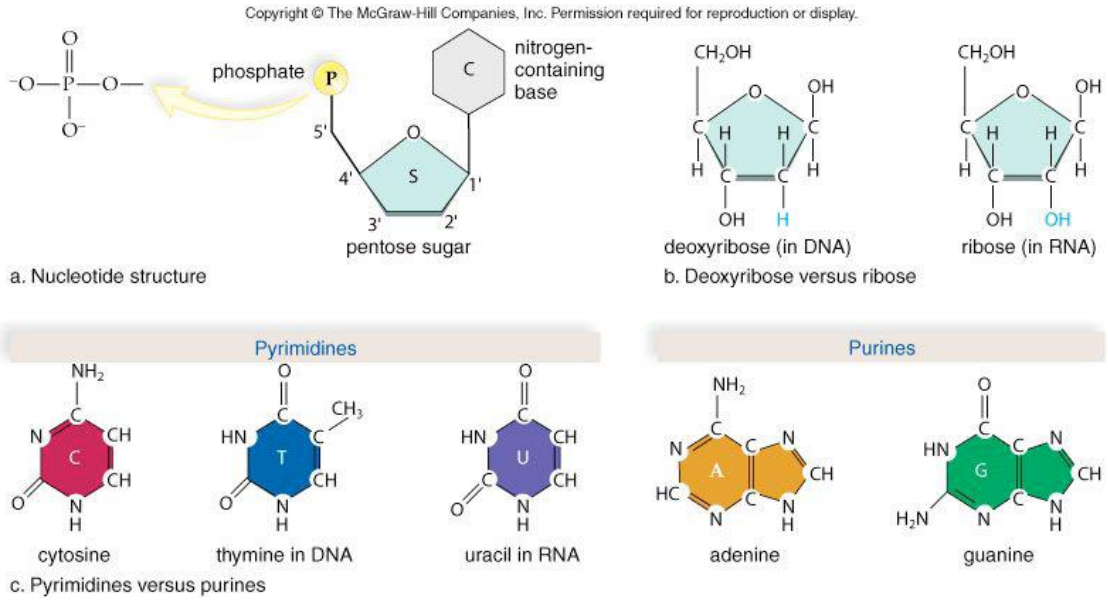
physiology has been hotly debated. Recent analysis by the ENCODE project indicates that 80% of the entire human genome is either transcribed, binds to regular proteins, or is associated to other biochemical activity (Elizabeth, 2012). It however remains controversial whether this biochemical activity contributes to cell physiology, or whether a substantial portion of this is the result of transcriptional and biochemical noise, which must be actively filtered out by the organism (Palazzo, 2012).

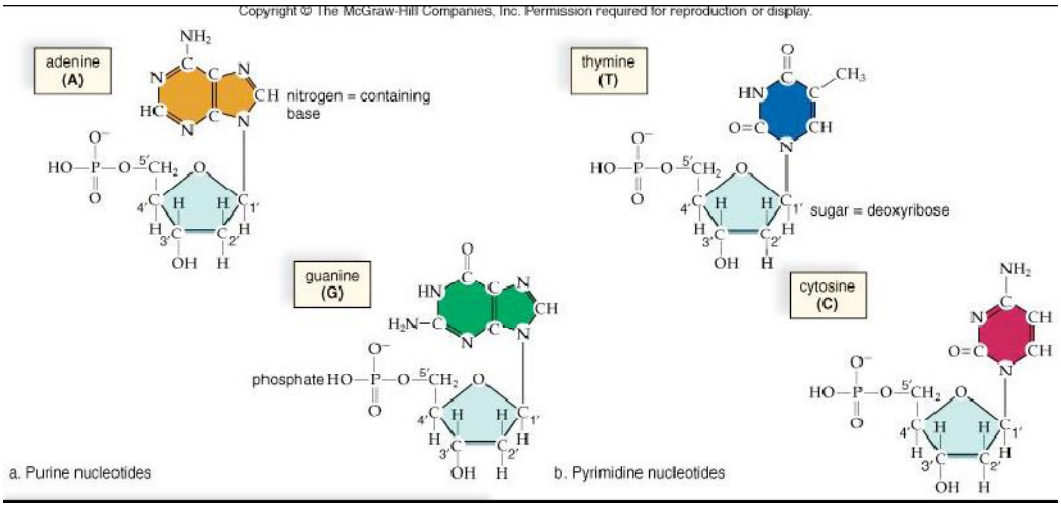
Excluding protein-coding sequences, introns, and regulatory regions, much of the non-coding DNA is composed of many DNA sequences that do not play a role in gene expression and have important biological functions. Comparative genomic studies indicate that about 5% of the genome contains sequences of non-coding DNA that are highly conserved, sometimes on time-scales representing hundreds of millions of years, implying that these non-coding regions are under strong evolutionary pressure and positive selection (Ludwig, 2002). Many of these sequences regulate the structure of chromosomes by limiting the region of heterochromatin formation and regulating the structural features of chromosomes, such as the telomeres and centromeres. Other non-coding regions serve as origins of DNA replication. Finally, several regions are transcribed into functional non-coding RNAs that regulate the expression of protein-coding genes for example: mRNA translation and stability, DNA methylation and DNA recombination (Martens *et al.*, 2004; Tsai *et al.*, 2010; Bartolomei *et al.,* 1991; Kobayashi 2005).

2.4: The Gene

Gene can be defined as a locatable region of genomic sequence, corresponding to a unit of inheritance, which is associated with regulatory region, transcribed regions, and/or other functional sequence of regions (Pearson, 2006; Elizabeth, 2007). Some viruses store their entire genomes in the form of RNA and contain no DNA at all. Because they use RNA to store genes, their cellular host may synthesize their proteins as soon as they are infected without any delay for transcription. On the other hand, RNA retroviruses, such as HIV, require the reverse transcription of their genome from RNA into DNA before their protein can be synthesized. While RNA is common as genetic storage material in viruses, in mammals RNA inheritance has been observed but very rarely (Rassoulzadegan, 2006).

Functional Structure of a Gene





The vast majority of living organisms encode their genes in long strands of deoxyribonucleic acid (DNA). DNA consists of a chain made from four types of nucleotide subunits, each composed of: a five-carbon sugar (2[1]ï deoxyribose), a phosphate group, and one of the four bases ï A, C, G and T (adenine, cytosine, guanine and thymine). The most common form of DNA in a cell is a double helix structure, in which two individual DNA strands twist around each in a right-handed spiral. In this structure, the base pairing rules

specify that guanine pairs with cytosine and adenine pairs with thymine. The base pairing between guanine and cytosine forms three hydrogen bonds, whereas the base pairing between adenine and thymine forms two hydrogen bonds. The two strands of DNA in a double helix must be complementary to each other. Due to chemical composition of the pentose residues of the bases, DNA strands have directionality. One end of a DNA polymer contains an exposed hydroxyl group on the deoxyribose sugar which is called 3^1 end of the molecule. The other end contains an exposed phosphate group, known as the 5^1 end. The directionality of DNA is vitally important to many cellular processes, such as DNA replication which occurs only in one direction. All nucleic acid synthesis in a cell occurs in the 5^1 ï 3^1 direction, because new monomers are added via a dehydration reaction that uses the exposed 3^1 hydroxyl group as a nucleophile (Gillham *et al*., 1997).

The expression of genes encoded in DNA begins by transcribing the gene into RNA, a second type of nucleic acid that is very similar to DNA, but whose monomers contain the sugar ribose rather than deoxyribose sugar. RNA also contains the base uracil in place of thymine. RNA molecules are also less stable than DNA and are typically single-stranded. Genes that encoded proteins are composed of series of three-nucleotide sequences called codons, which serve as the words in genetic language. The genetic code specifies the correspondence during protein translation between codons and amino acids. The genetic code is nearly the same for all known organisms (Berk and Cate, 2007). All genes have regulatory regions in addition to region that explicitly codes for a protein or RNA product. A regulatory region shared by almost all genes is known as promoter, which provides a position that is recognized by the transcription machinery when a gene is about to transcribe and expressed. A gene can have more than one promoter, resulting in RNAs that differ in how far they extend from the 5^1 end. Although promoter regions have consensus sequence some genes have ñstrongò promoters while others have ñweakò promoters. These weak promoters usually permit a lower rate of transcription than the strong promoters because the transcription machinery binds to them less frequently (Mortazavi *et al*., 2008). Other possible regulatory regions include enhancers, which can compensate for weak promoter. Most regulatory regions are ñup-streamò that is, before or toward the 5^1 end of the transcription initiation site. Eukaryotic promoter regions are much more complex and difficult to identify than prokaryotic promoters. Many prokaryotic genes are organized into groups (operons) of genes whose products have related functions and which are transcribed as a unit. By contrast, eukaryotic genes are transcribed only one at a time, but may include long stretch of DNA called introns which are not translated into protein (they are spliced out before translation).

Splicing can also occur in prokaryotic genes, but is less common than in eukaryotic (Woodson, 1998).

2.5: Chromosomes

The total complement of genes in a cell or organism is known as its genome. This may be stored on one or more chromosomes. The region of the chromosome at which a particular gene is located is called its locus. A chromosome consists of a single, very long DNA helix on which thousands of genes are encoded. Prokaryotes typically store their genomes on a single large, circular chromosome, sometimes supplemented by additional small circles of DNA called plasmids. Although some simple eukaryotes also possess plasmids, the majority of eukaryotic genes are stored on multiple linear chromosomes which are parked within the nucleus in complex with storage protein called histones. The manner in which DNA is stored on the histone, as well as chemical modification of the histone itself is one of the regulatory mechanism governing gene expressions (Scheen and Junien, 2012).

The ends of eukaryotic chromosomes are capped by long stretches of repetitive sequences called telomeres, which do not code for any gene product. They are present to prevent degradation of coding and regulatory regions during DNA replication. The length of the telomeres tends to decrease each time the genome is replicated in preparation for cell division. The loss of telomeres has been proposed as an explanation for cellular senescence (the loss of the ability to divide), and by extension, for the aging process in organisms (Braig and Schmitt, 2006). Whereas the chromosomes of prokaryotes are relatively gene-dense, those of eukaryotes often contain so-called ñjunk DNAò, or regions of DNA that serve no obvious function. Single-celled eukaryotes have relatively small amount of such DNA, whereas the genomes of complex multi-cellular organisms, including humans, contain an absolute majority of DNA without an identified function (IHGSC, 2004). However it now appears that although protein-coding DNA makes up barely 2% of the human genome, about 80% of the bases in the genome may be expressed, so the term ñjunk DNAò may be a misnomer (Elizabeth, 2007).

2.6: Gene Expression

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, but in non-protein coding genes such as ribosomal RNA (rRNA), transfer RNA (tRNA) or small nuclear RNA (snRNA) genes, the product is a functional RNA. The process of gene expression is used by all known life to generate the macromolecular machinery for life. Several steps in the gene

expression process may be modulated, including the transcription, RNA splicing, translation, and post-translational modification of protein. Gene regulation gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and adaptability of any organism.

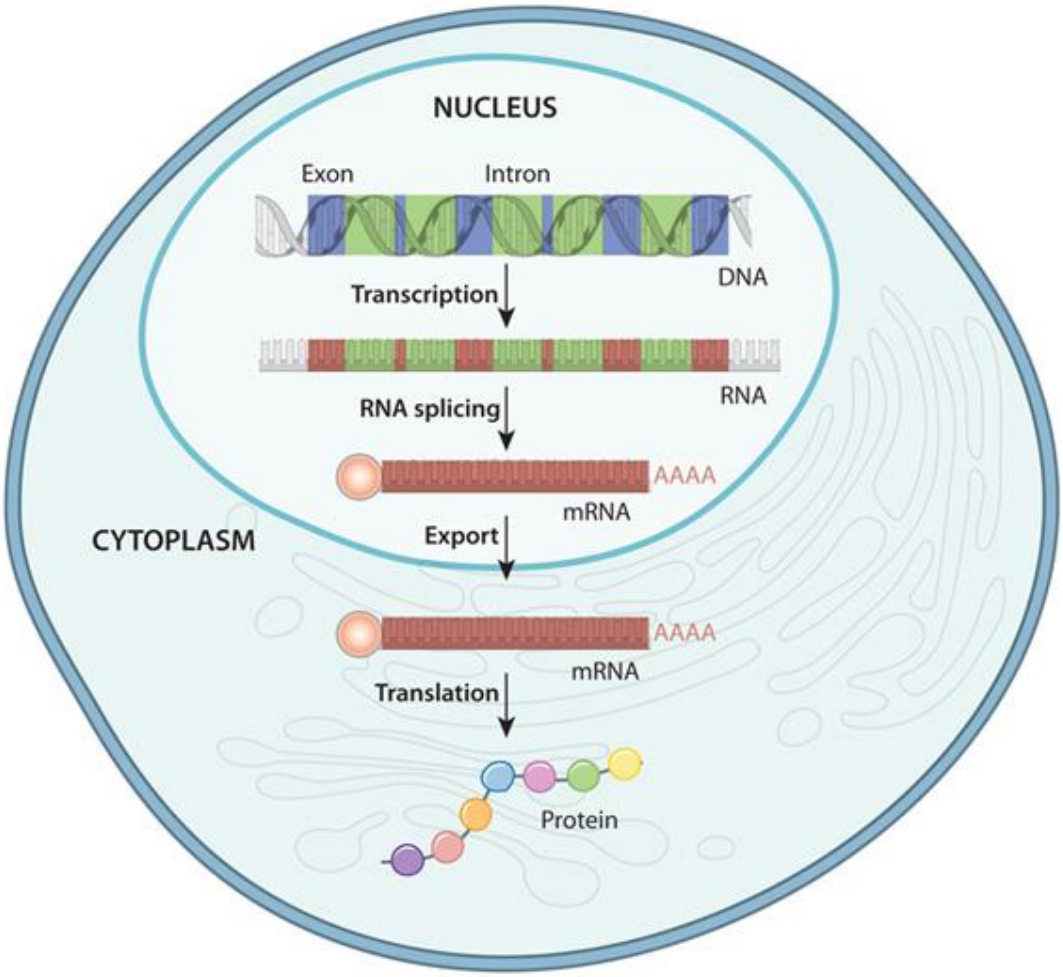


Fig.2.4: Gene Transcription

Genomic DNA consists of two antiparallel and reverse complementary strands, each having 5^1 and 3^1 ends. The production of RNA copies of the DNA is called transcription, and is performed by RNA polymerase, which adds one RNA nucleotide at a time to a growing RNA strand. This RNA is complementary to the template 3^1 ï 5^1 DNA strand which is itself complementary to the 5^1 ï 3^1 DNA strand (Brueckna *et al.*, 2009). Therefore, the resulting 5^1 ï 3^1 RNA strand is identical to the coding DNA strand with the exception that thymines (T) are replaced with uracils (U) in the RNA. A coding DNA strand reading ñATGò (Adenine,

Thymine and Guanine) is indirectly transcribed as ñAUGò in RNA. Transcription in prokaryotes is carried out by a single type of RNA polymerase, which needs a DNA sequence called a pribnow box as well as a sigma factor to start transcription. In eukaryotes, transcription is performed by three types of RNA polymerases, each of which needs a special DNA sequence called the promoter and a set of DNA binding protein known as transcription factors, to initiate the process. RNA polymerase **1** is responsible for transcription of rRNA genes. RNA polymerase **II** (Pol **II**) transcribes all protein coding genes but also some non-coding RNAs (e.g. snRNAs, snoRNAs). Pol **II** includes a C-terminal domain (CTD) that is rich in serine residues. When these residues are phosphorylated, the CTD binds to various protein factors that promote transcript maturation and modification. RNA polymerase **III** transcribes 5s rRNA, tRNA genes and some small non-coding RNAs (e.g. 7sK). Transcription ends when the polymerase encounters a sequence called the terminator (Connelly and Manley, 1988).

While transcription of prokaryotic protein-coding genes creates messenger RNA that is ready for translation into protein, transcription of eukaryotic genes leaves a primary transcript of RNA (pre-mRNA), which first has to undergo a series of modifications to become a mature mRNA. These modifications include 5^1 capping, which is set of enzymatic reactions that add 7-methylguanosine to 5^1 end of pre-mRNA and thus protect the RNA from degradation by exonucleases. The 7-methylguanosine cap is then bound by cap binding complex heterodimer (CBC20/CBC80) which aids in mRNA export to cytoplasm and also protects the RNA from decapping. Another modification is 3^1 cleavage and polyadenylation. They occur if polyadenylation signal sequence (5^1-AAUAAA-3^1) is present in pre-mRNA, which is usually between protein-coding sequence and terminator. The pre-mRNA is first cleaved and then a series of about 200 adenines (A) are added to form poly-(A) tail which protects the RNA from degradation. Poly-(A) tail is bound by multiple poly-(A)-binding proteins (PABP) necessary for mRNA export and translation re-initiation (Vinciguerra and Stutz, 2004). A very important modification of eukaryotic pre-mRNA is RNA splicing. The majority of eukaryotic pre-mRNAs consist of alternative sequences called exons and introns. During the process of splicing, an RNA-protein catalytic complex known as spliceosome catalyzes two trans-esterification reactions, which remove an intron and release it in form of lariat structure, and then splice neighboring exons together. In certain cases, some introns or exons can be either removed or retained in mature mRNA. This so-called alternative splicing creates series of different transcripts originating from a single gene. Because these transcripts

can be potentially translated into different proteins, splicing extends the complexity of eukaryotic gene expression (Moniatis and Tasi, 2002).

In most organisms, non-coding genes are transcribed as precursors which undergo father processing. In the case of ribosomal RNA (rRNA), they are often transcribed as a pre-rRNA which contains one or more rRNAs. The pre-rRNA is cleaved and modified (2^1-O-Methylation and pseudouridine formation) at specific sites by approximately 150 different small nucleolus-restricted RNA species, called snoRNAs. SnoRNAs associate with proteins, forming snoRNPs. While snoRNA part basepair with the target RNA and thus position the modification at a precise site, the protein part performs the catalytical reaction. In eukaryotes, in particular a snoRNP called RNase, cleaves the 45s pre-rRNA into the 28s, 5.8s and 18s rRNAs. The rRNA and RNA processing factors form large aggregates called the nucleolus (Sirri *et al.*, 2008). In the case of transfer RNA (tRNA), for example, the 5^1 sequence is removed by RNase P, whereas the 3^1 end is removed by the tRNase Z enzyme and the non-templated 3^1 CCA tail is added by a nucleotidyl transferase. In the case of micro RNA (miRNA), miRNAs are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, 70-nucleotides stem-loop structures known as pre-miRNA in the cell nucleus by the enzymes Drosha and Pasha. After being exported, it is then processed to mature miRNAs in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Frank and Pace, 1998; Weiner, 2004; Ceballos and Vioque, 2007). While some RNAs function in the nucleus, many RNAs are transported through the nuclear pores into the cytosol. Notably this includes all RNA types involved in protein synthesis (Kohler and Hurt, 2007). In some cases RNAs are additionally transported to specific parts of the cytoplasm, such as synapse. They are then towed by motor proteins that bind through linker proteins to specific sequences (called Zipcodes) on the RNA (Jambhekar and Derisi, 2007).

2.7: Translation

For some RNA (non-coding RNA) the mature RNA is the final gene product. In the case of mRNA the RNA is an information carrier coding for the synthesis of one or more proteins. Messenger RNA carrying a single protein sequence (common in eukaryotes) is monocistronic whilst mRNA carrying multiple protein sequences (common in prokaryotes is known as polycistronic (Amaral *et al.*, 2008). Every mRNA consists of three parts - 5^1 untranslated-region (5^1UTR), protein-coding region or open reading frame (ORF) and 3^1 untranslated regions (3^1UTR). The coding region carries information for protein synthesis encoded by the genetic code to form triplets. Each triplet of nucleotides coding region is

called a codon and corresponds to a biding site complementary to an anticodon triplet in transfer RNA. Transfer RNAs with the same anticodon sequence always carry an identical type of amino acid. Amino acids are then chained together by the ribosome according to the order of triplets in the coding region. The ribosome helps transfer RNA to bind to messenger RNA and takes the amino acid from each transfer RNA and makes a structure less protein out of it (Hansen *et al*., 2003; Berk and Cate, 2007). Each mRNA is translated into many protein molecules on average of 900 in mammals (Schwanhausser *et al*., 2011).

In prokaryotes translation generally occurs at the point of transcription, often using a messenger RNA that is still in the process of transcription. In eukaryotes, translation can occur in a variety of regions of the cell depending on where the protein is supposed to be. Major locations are the cytoplasm for soluble cytoplasmic proteins and the membrane of the endoplasmic reticulum for proteins that are for export from the cell or insertion into a cell membrane. Proteins which supposed to be expressed at the endoplasmic reticulum are recognized port-way through the translation process. This is governed by the signal recognition particle, a protein which binds to the ribosome and direct it to the endoplasmic reticulum when it finds a signal sequence on the growing amino acid chain (Hegde and Kang, 2008).

2.8: Folding and Protein Transport

The polypeptide folds into its characteristic and functional three dimensional structures from a random coil (Alberts *et al*., 2002). Each protein exists as an unfolded polypeptide or random coil when translated from a sequence of mRNA in a linear chain of amino acids. This polypeptide lacks any developed three dimensional structures. Amino acids interact with each other to produce a well-defined three dimensional structure, the folded protein known as the native state. The resulting three-dimensional structure is determined by the amino acid sequence (Anfinsen, 1972). The correct three-dimensional structure is essential to function, although some parts of functional proteins may remain unfolded (Jeremy, 2002). Failure to fold into the intended shape usually produces inactive proteins with different properties including toxic prisons. Several neurodegenerative and other diseases are believed to result from the accumulation of misfolded proteins (Dennis, 2003). Many allergies are caused by the folding of the proteins, for the immune system does not produce antibodies for certain protein structures (Alberts *et al*., 2010). Enzymes called chaperones assist the newly formed protein to fold into its three-dimensional structure. Similarly, RNA chaperones help RNAs attain their functional shape. Assisting protein folding is one of the main roles of the endoplasmic reticulum in eukaryotes (Hebert and

Molinari, 2007; Russel *et al.*, 2008). Many proteins are destined for other parts of the cell than the cytosol and a wide range of signaling sequences are used to direct them to where they are supposed to be. In prokaryotes this is normally a simple process due to limited compartmentalization of the cell. However, in eukaryotes there is a great variety of different targeting processes to ensure the protein arrives at the correct organelle. Not all proteins remain within the cell, hence many are exported; for example digestive enzymes, hormones and cytokines. In eukaryotes the export pathway is well developed and the main mechanism for the export of these proteins is translocation to the endoplasmic reticulum, followed by transport via the Golgi apparatus (Moreau *et al*., 2007; Prudovsky *et al*., 2008).

2.9: Regulation of Gene Expression

Regulation of gene expression refers to the control of the amount and timing of appearance of the functional product of a gene. Control of expression is vital to allow a cell to produce the gene products it needs only when it needs them. Any step of gene expression may be modulated, starting from DNAï RNA transcription step to post-translational modification of a protein. The stability of the final product, whether it is RNA or protein, contributes to the expression level of the gene. In general, gene expression is regulated through changes in number and type of interaction between molecules that collectively influence transcription of DNA and translation of RNA (Zaidi *et al*., 2004; Mattick *et al.*, 2009; Martinez and Walhout, 2009; Tomilin, 2008).

2.10: Transcriptional Regulation

Regulation of transcription can be divided into three main routes; genetic (direct interaction of a control factor with the gene), modulation (interaction of a control factor with the transcription machinery) and epigenetic (non-sequence changes in DNA structure which influence transcription). Direct interaction with DNA is the simplest and the most direct method by which a protein can change transcription levels. Genes often have several protein binding sites around the coding region with the specific function of regulating transcription. There are many classes of regulatory DNA binding sites known as enhancers, insulators and silencers. The mechanism for regulating transcription are varied, from blocking the key binding sites on the DNA for, RNA polymerase to acting as an activator, and promoting transcription by assisting RNA polymerase binding.

The activity of transcription factors is further modulated by intracellular signals causing protein post-translational modification including phosphorylated, acetylated, or glycosylated protein. These changes influence a transcription factorôs ability to bind, directly or indirectly, to promoter DNA, to recruit RNA polymerase, or to favor elongation of a newly

synthesized RNA molecule. The nuclear membrane in eukaryotes allows further regulation of transcription factors by the duration of their presence in the nucleus which is regulated by reversible changes in their structure and by binding of other proteins. Environmental stimuli or endocrine signals may cause modification of regulatory proteins eliciting cascades of intracellular signals, which result in regulation of gene expression (Veitia 2008; Nguyen *et al*., 2009; Paul, 2008; Los *et al*., 2009). More recently it has become apparent that there is a huge influence of non- DNA-sequence specific effects on translation. These effects are referred to as epigenetic and involve the higher order structure of DNA, non-sequence specific DNA binding proteins and chemical modification of DNA. Epigenetic are a variety of features of the human genome that transcend its primary DNA sequence, such as chromatin packing, histone modifications and DNA methylation, which are important in regulating gene expression, genome replication and other cellular processes. Epigenetic markers strengthen and weaken transcription of certain genes but do not affect the actual sequence of DNA nucleotides. DNA methylation is a major form of epigenetic control over gene expression and one of the most highly studied topics in epigenetics. During development, the human DNA methylation profile experiences dramatic changes. In early germ line cells, the genome has a very low methylation levels. These low levels generally describe active gene. As development progresses, parental imprinting tags lead to increase methylation activity (Mistch, 2007; Beinstein *et al*., 2007).

2.11: Mutation

DNA replication is for the most part extremely accurate, with an error rate of 10^{-6} to 10^{-10} per site, in eukaryotes (Watson *et al*., 2004), prokaryotes and viruses, the rate is much higher. Rare spontaneous alterations in the base sequence of a particular gene arise from a number of sources, such as error in DNA replication and the aftermath of DNA damage. These errors are called mutation. The cell contains DNA repair mechanisms for preventing mutations and maintaining the integrity of the genome. In some cases, such as breaks in both DNA strands of a chromosome, repairing the physical damage to the molecule is a higher priority than producing an exact copy of the DNA. Some mutations in protein-coding genes are silent, or produce no change in the amino sequence of the protein for which they code. For example, the codons UCU and UUC both code for serine, so the U ï C mutation has no effect on the protein. Mutations that do have phenotypic effects are most often neutral or deleterious to the organism but sometimes they confer benefits to the organismsô fitness (Michael and Daniel, 2007). Mutations propagated to the next generation lead to variations within a speciesô population. Variants of a single gene are known as alleles and differences in

alleles may give rise to differences in traits. Although it is rare for the variants in a single gene to have clearly distinguishable phenotypic effects, certain well-defined traits are in fact controlled by single genetic loci. A geneôs most common allele is called wide-type allele and rare alleles are called mutants. However, it does not imply that the wild-type allele is the ancestor from which the mutants are descended (Semon and Wolfe, 2007).

2.12: New Concept of Gene

The concept of the gene has changed considerably from the original definition of gene as a unit of inheritance to where gene is taken as a DNA-based unit that can exert its effects on the organism through RNA or protein products. It was also previously believed that one gene makes one protein but the discovery of alternative splicing and trans-splicing has changed the previous concept (Gerstein *et al*., 2007). The first case of RNA-base inheritance has been discovered in mammal by Rassoulzadegan *et al*. (2006). Evidence is also accumulating that the control regions of a gene do not necessarily have to be close to the coding sequence on the linear molecule or even the same chromosome. Promoter region of the interferon-gamma gene on chromosome 10 and the regulatory regions of the Th2 Cytokine Locus on chromosome II was discovered to come into close proximity in the nucleus possibly to be jointly regulated (Spilianakis *et al*., 2005). The concept that genes are clearly delimited is also being eroded. There is evidence for fused proteins stemming from two adjacent genes that can produce two separate protein products. While it is not clear whether these fusion proteins are functional, the phenomenon is more frequent than previously thought (Parra *et al*., 2006). Even more striking than the discovery of fused gene is the observation that some proteins can be composed of exons from far away regions and even different chromosomes (Kapranov *et al*., 2005). The new data have led to an update, and probably tentative definition of a gene as ña union of genomic sequences encoding a coherent set of potentially overlapping functional productsò. This new definition categorizes genes by functional products, whether they are protein or RNA, rather than specific DNA loci. All regulatory elements of DNA are therefore classified as gene-associated region (Gerstain *et al*., 2007)

DNA replication and cell cycle

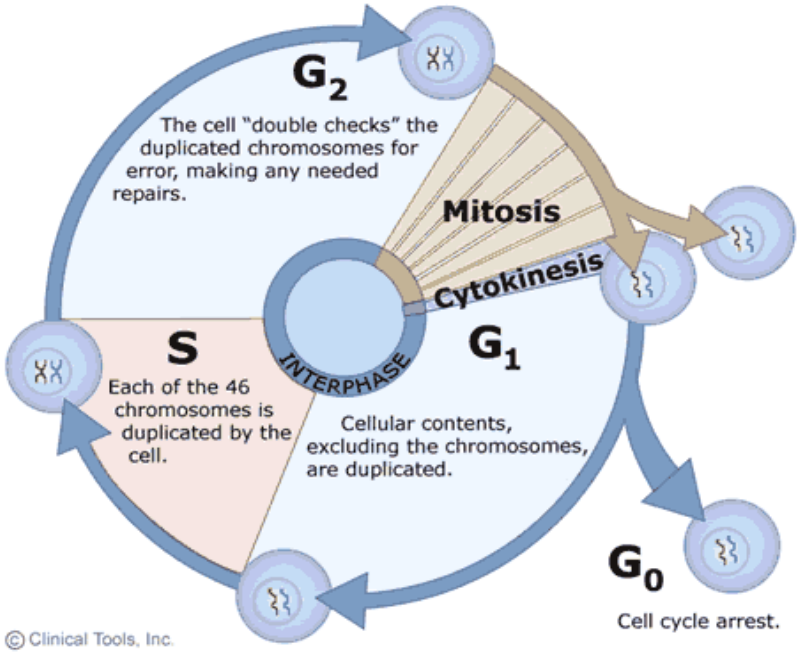


Fig.2.5: **Cell Cycle**

The growth, development, and reproduction of organisms rely on cell division. This requires first making a duplicate copy of every gene in the genome in a process called DNA replication. The copies are made by specialized enzymes known as DNA polymerases, which ñreadò one strand of the double-helical DNA, known as the template strand, and synthesize a new complementary strand. Because the DNA double helix is held together by base pairing, the sequence of one strand completely specifies the sequence of its complement; hence only one strand needs to be read by the enzyme to produce a faithful copy. The process of DNA replication is semi-conservative; this is, the copy of the genome inherited by each daughter cell contains one original and one newly synthesized strand of DNA (Watson *et al*., 2004).

After DNA replication is complete, the cell must physically separate the two copies of the genome and divide into two distinct membrane-bound cells. In prokaryotes, this usually occurs via a relatively simple process called binary fission. In this process, each circular genome attaches to the cell membrane and is separated into the daughter cells as the membrane invaginates to split the cytoplasm into two membrane-bound portions. Binary fission is extremely fast compared to the rates of cell division in eukaryotes. Eukaryotic cell division is a more complex process known as the cell cycle; DNA replication occurs during

the phase of this cycle known as S phase, whereas the process of segregating chromosomes and splitting the cytoplasm occurs in M phase (Albert *et al*., 2002).

2.13: Structure of HIV

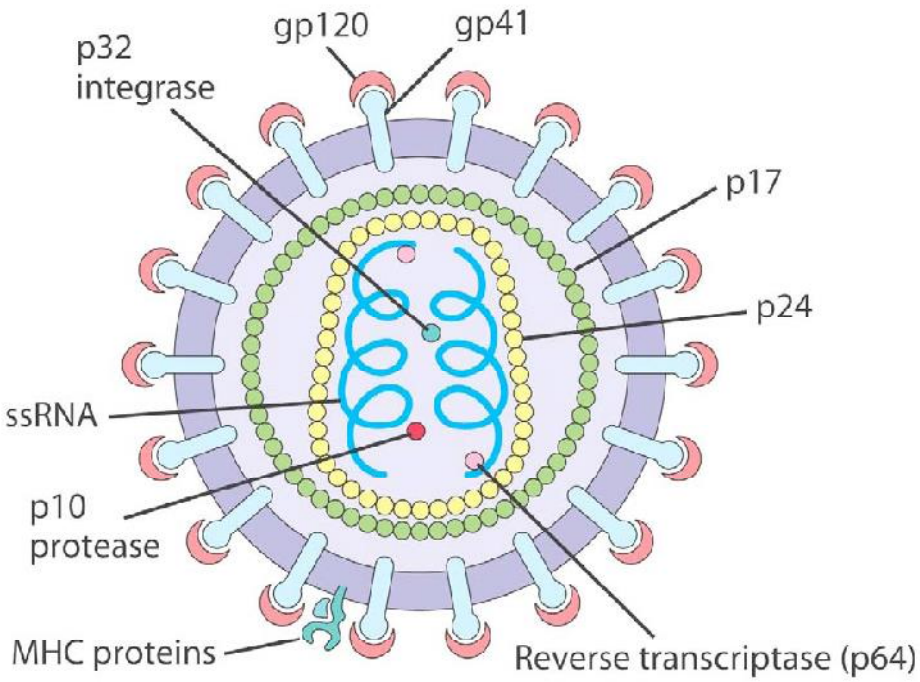


Fig.2.6: Human immunodeficiency virus

Human Immunodeficiency Virus (HIV) isolates are currently grouped into two types, HIV type-I (HIV-1) and HIV type-II (HIV-II). The main agent of AIDS is HIV-1, while HIV-II is restricted to some regions of Western and Central Africa. The retrovirus genome is composed of two identical copies of single-stranded RNA (ssRNA) molecule and it is characterized by the presence of structural genes; *gag*, *pol* and *env* (Luciw, 1996). HIV-1 and HIV-II viruses differ in organization of their genome, although, the basic structure (*gag*, *pol* and *env* genes) is the same as in all retroviruses. In addition to having these three genes, the HIV-1 and HIV-II genomes present a complex combination of other regulatory/accessory genes. The structure of HIV particle is similar for both HIV-1 and HIV-II. Similar to other retroviruses, HIV *gag* gene encodes the structural proteins of the core (p24, p7 and p6), and Matric (p17). The *env* gene encodes the viral envelope glycoproteins gp120 and gp41, which

recognize cell surface receptors. The *pol* gene encodes for enzymes crucial for viral replication, which are the reverse transcriptase that converts viral RNA into DNA, the integrase that incorporates the viral DNA into host chromosomal DNA (provirus) and the protease that cleaves large Gag and Pol protein precursors into their components.

HIV vital particles have a diameter of 100nm and are surrounded by a lipoprotein-rich membrane. Each viral particle membrane includes glycoprotein heterodimer complexes composed of trimers of external surface gp120 and the trans-membrane spanning gp41 glycoproteins bound together. The biding between gp120 and gp41 is not covalent and therefore the gp120 may be shed spontaneously within the local environment and detected in the serum and within the lymphatic tissues of HIV infected patients. During the process of budding from the infected cell, the virus may also incorporate into its membrane different proteins from the host cell membrane, such as human leucocytes antigen (HLA) class I and II proteins, or adhesion proteins such as ICAM-1 that may facilitate adhesion to other target cells. A matrix protein (p17) is anchored to the inside of the viral lipoprotein membranes. Virus membrane and the matrix protein include the capsid composed of polymers of the core antigen (p24). The capsid contains two copies of HIV RNA combined with a nucleoprotein and enzymes reverse transcriptase, integrase and protease (Gelderblom *et al*., 1989).

HIV viruses are characterized by other accessory/regulatory genes that play key roles in modulating virus replication (Emerman and Malim, 1998). Among these, are the *tat* gene encodes for a protein (Tat) that is expressed very early after infection and promotes the expression of HIV genes. The Rev protein, coded by the *rev* gene, ensures the export of the correctly processed mRNA and gRNA from nucleus to cytoplasm. The function of the other accessory HIV proteins is less understood. It is believed that the Vpr protein is involved in the arrest of the cell cycle. This protein also enables the reverse transcribed DNA to gain access to the nucleus in non-dividing cells such as macrophages, a function that is performed in HIV-II by Vpx protein. Vpu is a protein necessary for the correct release of virus particle, whereas the *vif* gene codes for a small protein (Vif) that enhances the infectiveness of progeny virus particles. Finally, the Nef protein has multiple functions including cellular signal transduction and the down regulation of the CD4 receptor on the cell surface to allow virus budding in the late stages of the virus replication cycle.

2.14: Mechanism of HIV Infection and Replication Cycle

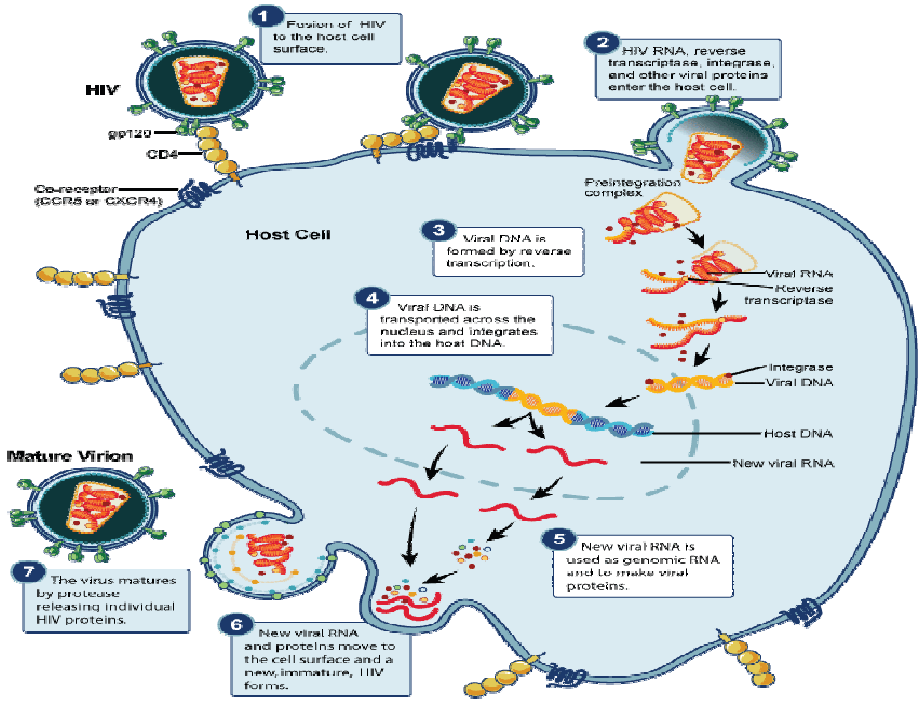


Fig.2.7: HIV Replication Cycle

The entry pathway of HIV-1 and HIV-II can be divided into three major events: virus binding to the cell, activation and fusion. The viral envelope trimeric complex, composed of the heterodimer proteins gp120 and gp41, is essential for virus recognition and entry into target cells. The gp41 subunit contains a fusogenic hydrophobic peptide at its amino terminus, which is essential for fusion of the viral and cellular membranes (Weiss, 1993). HIV gp120 binds a 58 kDa monomeric glycoprotein, designed as CD4, which is expressed on the cell surface of about 60% of circulating T-lymphocytes, T-cell precursors, monocytes/macrophages, eosinophils, dendritic cells and microglia cells of the central nervous system. The CD4 molecule normally functions as a co-receptor of the major histocompatibility complex class II molecule during T-cell recognition of a foreign antigen (Miceli *et al*., 1993). Upon gp120 binding with the CD4 protein the virus envelope complex undergoes a structural change, exposing a specific domain in the gp120 to be able to bind chemokine receptors on the cell membrane. These molecules are recognized by chemotactic cytokines that mediate the homing and recruitment of immune cells in the course of inflammation. These receptors are classified on the basis of the position of disulfide-like cysteine residues, as well as their angioneic effects. At least 17 members of these chemokine ligands, working as HIV receptor, have been identified. Amongst these, the most common

co-receptors used by HIV are CXCR4 and CCR5, but other potential co-receptors have been described (Alkhatib and Berger, 2007). The Ŭ-chemokine stromal cell-derived factor (SDF-1) is the natural ligand of CXC4, whereas CCR5 is a receptor for the ɓ-chemokine family (RANTES, macrophage inflammatory proteins; MIP-1-Ŭand MIP-1-ɓ). CXCR4 is expressed on many cells, including T-lymphocytes, whereas CCR5 is present on monocyte/macrophages, dendritic cells and activated T-lymphocytes. The differential expression of chemokine receptors on cell targets has been shown to be a major determinant of the HIV-1 tropism (Broder and Berger, 1995). There are strains of HIV-1 preferentially binding of ɓ-chemokine receptor CCR5 present mainly in macrophages and CD_4^+ T-cells expressing CCR5. These strains are also known as macrophage tropic (M-tropic) or R5 viruses. CCR5 is used by almost all primary HIV-1 isolates regardless of viral genetic subtype. Conversely, other isolate used preferentially CXCR4 for entry and replicate in primary CD_4^+ T-cells that express CXCR4. These strains are known as T-lymphocyte-tropic (T-tropic) or X4 viruses. Finally, there are HIV isolates that are able to bind to both CCR5 and CXCR4 receptors. These strains are termed dual tropic or X4R5 viruses (Coakley *et al*., 2005).

The double binding of gp120 to both the CD4 and one chemokine receptor allows a more stable two-pronged attachment of the virus, which in turns allows the N-terminal fusion peptide gp41 to penetrate the cell membrane. The HRI and HR2 repeat sequence in gp41 interact, causing the collapse of the extracellular portion of gp41 into a hairpin. This loop structure brings the virus and cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid. Following the entry of viral capsid, the virus core uncoats into the cytoplasm of the target cells freeing the virus RNA. The conversion of viral RNA into proviral DNA takes place because of the action of the reverse transcriptase and the integrase. The viral RNA is reverse-transcribed into complementary DNA (cDNA) by the virus reverse-transcriptase through the use of a cellular lysine tRNA molecule as a primer (Cen *et al*., 2001). Through the viral ribonuclease H active site the reverse-transcriptase begins the reverse transcription of viral RNA in the cytoplasm. This occurs as a minus-strand polymerization, starting at the primer binding site, until viral RNA is transcribed into a RNA/DNA hybrid double helix. The ribonuclease H site brakes down the RNA strand and the polymerase active site of the reverse transcriptase completes a complementary strand to form a double helix DNA molecule.

The reverse transcriptase incorporates an incorrect nucleotide every 1500 to 4000 bases, which explains the rapid occurrence of mutations. Some of the resulting mutations

provide a survival advantage, leading to drug resistance strains (Tantillo *et al.*, 1994). The newly synthesized HIV-1 cDNA is transported to the nucleus. Two HIV proteins, Vpr and Vif, participate in this nuclear transport. Vpr is thought to enhance the HIV-1 pre-integration complex transport to the nucleus. Vif associates with cytoskeletal elements and increase the infectious potential (Miller and Sarver, 1997; Zhang *et al*., 2001). Vpr and the matrix protein provide a nuclear localization signal to enter the nucleus. The viral cDNA integrates randomly into the host cell genome in a reaction catalyzed by the viral enzyme integrase which cleaves the nucleotides of each 3^1 end of the double helix DNA creating two sticky ends. The integration of proviral DNA and the expression of the provirus require that target cell is in activated state. Monocyte/macrophage, microglial cells, and latently infected quiescent $CD4^+$ T-Cells contained integrated provirus and important long-living cellular reservoirs of HIV (Chun *et al*., 1997). After integration, cellular transcription factors are able to activate viral gene transcription, producing low levels of short, multiply spliced mRNA transcripts. They encode the regulatory proteins Tat, Rev, and Nef. Tat transactivates transcription by binding the 5^1 end of the viral DNA sequence, increasing the viral transcription rate 1000-fold (Parada and Roeder, 1996). Rev protein binds an RNA structure in the *env* gene region and mediates the nuclear export of incompletely spliced transcripts. Rev favours the export of partially spliced mRNA transcripts encoding structural proteins and full-length mRNA transcripts that constitute the viral RNA genome (Emerman and Malim, 1998).

The viral mRNA coding for long fragments migrate into the cytoplasm, where structural proteins of new virus are synthesized. The proteins coded by *pol* and *gag* genes form the nucleus of the maturing HIV particle while the gene product coded by the *env* gene form the glycoprotein spikes of the viral envelope. The components of the viral core are initially translated into pr55, a pre-protein resulting from a long singly spliced *gag* mRNA and then cleaved during maturation. Gag-Pol is another pre-protein that is cleaved to produce the viral enzymes: protease, integrase, and reverse transcriptase. HIV protease mediates the specific cleavage of these pre-proteins and its inhibition results in marked suppression of viral replication (Weller *et al*., 2001). Large gp160 precursor molecules are cleaved by HIV protease into gp120 and gp41. The *gag* and *pol* proteins are also derived from a large 160kD precursor molecule, from which the HIV protease cleaves the p24, p17, p9 and p7 Gag final products and Pol proteins. Pr55 and Gag-Pol are the result of 2 reading frames that also control the relative amount of these proteins to produce more structural components than viral enzymes. The first reading frame encodes pr55 and is more efficient than the second,

which is responsible for Gag-Pol synthesis (Jacks *et al.*, 1988). An intermediate-length mRNA produces gp120, which migrates to the cell membrane and is cleaved during the virion formation to originate the regulatory proteins Vpr, Vpui and Vif, and the envelope proteins gp41 and gp120.

The formation of new viral particle is a stepwise process: two viral RNA strands associates together with replication enzymes, while core proteins assemble over them forming the virus capsid. These proteins and host membrane protein are incorporated on the plasma membrane to form the viral envelope around the capsid, which already contain the viral RNA genome. The assembly process is energy-dependent and likely involved unidentified cellular factors (Tritel and Resh, 2001). During the process of budding, the virus lipid membrane may incorporate various host cell proteins and become enriched with phospholipids and cholesterol as it buds through the host cell membrane. Unlike T-lymphocyte where budding occurs at the cell surface and virions are released into the extracellular space, the budding process in monocytes and macrophages results in the accumulation of virions within intracellular vocules which are then released into the extracellular space.

2.15: Immune Response to HIV Infection

HIV does not survive outside the blood stream or lymphatic tissues. Virus is easily inactivated by the exposure to common detergents and disinfectants. Thus, virus transmission requires the direct exposition to infected blood or secretions in the presence of skin damage (by needle or sharp objects, or abrasion in mucosal tissues during sexual intercourse (Suliqoi *et al*., 2010). Transmission of HIV is highly dependent on biological properties of the virus isolate, its concentration in the infected individual and the host susceptibility. HIV is mainly integrated or replicated into the infected cells, which are the main vehicle of virus transmission (Martin and Sattentaus, 2009). HIV infected cells can transfer the virus to cells of the local immune system (e.g., T-Cells, macrophages, dendritic cells), as well as cells lining vaginal or anorectal mucosa. Infected cells can undergo lyses or allow the establishment of latent infection, particularly in macrophages and resting $CD4^+$ T-cells, which are permanent viral reservoirs. This represent a great obstacle in the complete eradication of the infection since it allows viral persistence even in the presence of effective regimens of highly active antiretroviral treatment (Alexaki *et al*., 2008).

2.16: Innate Immune System

A wide range of innate immune mechanisms operate as a first line of defense upon exposure to infection. The physical barriers to infection include skin, mucous secretions,

ciliary action, lachrymal secretions, gastric acid, and the antagonistic effect of normal microbial flora. If penetration occurs, bacteria are destroyed by soluble factors, such as lysosomal enzymes and through ingestion by phagocytic cells. Once engulfed by phagocytes, these organisms are destroyed by a number of microbicidal mechanisms, including reactive oxygen intermediates or the synthesis of nitric oxide, which is released within cytoplasmic granules. Following activation of the complement system, with the accompanying activation and attraction of phagocytic cells and neutrophils, an inflammatory response occurs. There is also an elaboration of other acute phase proteins, such as C-reactive and mannose-binding proteins, which argument the inflammation response. Extracellular killing of virally infected cells can be carried out by natural killer (NK) cells. NK cells, an important component of innate immune system, participant in early responses against infected or transformed cells, either through the production of cytokines or by direct cytotoxicity. These responses are mediated through interactions involving killer cell immunoglobulin-like receptors (KIRs) which are found on the cellôs surface. Various KIR isotypes promote stimulation of NK cell activity, whereas others cause inhibition of activity. KIR isotypes function in close association with human leukocyte antigen (HLA) molecules. The KIR allele KIR3DSI, in the presence of HLA-B BW4-80ILE, has been shown to delay HIV disease progression (Martin *et al*., 2002). Lyses by NK cells can also take place against cells recognized by host antibodies through binding of immunoglobulin to fragment constant (Fc) receptors on the NK cells.

Toll-like receptors (TLRs) play a key role in the response to microbial infections. TLRs recognize structurally conserved molecules derived from microbes and activate an immune response once these molecules have breached physical barriers, such as skin or intestinal tract mucosa. TLRs are believed to be crucial component of the innate immune system and appear to be one of the most ancient, conserved components of the immune system (Medzhitov, 2001). TLRs, which are a type of pattern-recognition receptor, recognize molecules that are broadly shared by pathogens but that are distinguishable from host molecules, collectively referred to as pathogen-associated molecule patterns (PAMPs). During acute primary HIV infection, there is an acute depletion of CD4 cells in the intestinal mucosa, leading to compromise in the integrity of mucosal epithelium. A consequence of this is the translocation of microbial products from the lumen. These products include lipopolysaccharide (KLPS), peptidoglycan, and bacterial 16sDNA, which all interact with TLRs and have the capacity to cause intense immune activation of a broad range of T-cells. Upon engagement with TLR, the T-cells enter cells cycle but fails to complete division and

instead die by apoptosis (Funderberg *et al*., 2007). Further evidence of the role of TLRsô response to microbial products suggests that there is a direct correlation between LPS in plasma and the individualôs state of immune activation. The highest level of LPS was found in progressive disease, whereas the lowest level was found in long-term non-progressor. Levels of bacterial products were also correlated with the state of activation of T-cells and CD4 T-lymphocyte levels (Brenchley *et al*., 2006).

The recently identified protein called APOBECZA has both antiviral and an antiretroviral effect (Bieniaesz, 2004). This protein is from the APOBEC family of cytidine deaminase, which hypermutate retroviral genomes and renders them unstable. These proteins are expressed in the natural target cells for HIV infection, namely CD4 T-lymphocytes and macrophages. When the virus infects these cells, APOBEC3G is packaged into the viral particle. When the virus subsequently infects a new cell, the APOBEC3G within the virus particle hyper-mutates the nascent viral complementary DNA (cDNA), which is then degraded. The *vif* gene product of HIV attempts to overcome this innate antiviral response by binding to the protein and overcoming its effect (Sheehy *et al*., 2002). TRIM5 alpha is another cellular factor shown to have activity against HIV-1. This cellular protein interacts with the viral capsid and interferes with the uncoating process through which viral RNA is released into the cytoplasm of an acutely infected cell (Stremlau *et al*., 2004).

2.17: Cellular Immune Response

Bone marrow-derived lymphocytes actively divide in the thymic cortex and then migrate to the medulla, where they are induced to differentiate into cells that will eventually participate in cellular immune responses. The thymus gland is very active in infants and responsible for the selection of T-cells in the body that will provide protection throughout life. During a personôs lifetime, the thymus gland atrophies and becomes smaller. Until recently, it was thought that the gland becomes inactive after the third decade of life. Recent evidence suggests however, that the gland retains activity throughout a personôs life, but the thymic reserve declines with age (Douck and Koup, 2000). These emigrant naïve T-Cells migrate to the secondary lymphoid tissues, such as the inner cortex of the lymph nodes, the periaticular sheaths in the lymph nodes, and the payerôs patches in the gut, tonsils, and appendix. Once at these sites the thymic-dependent T-lymphocytes become immunologically competent. T-cells only recognize antigen when it is on the surface of a body cell. Accordingly, the T-cell surface receptors recognize antigen when it is presented in conjunction with another surface marker belonging to a group of molecules known as the major histocompatibility complex (MHC). When this occurs, a naïve T-cell is introduced to

an antigen and becomes ñprimedò and activated as an effector cell. There is a constant movement of naïve, effector, and memory cells from the blood circulation to the lymphatic circulation and lymph nodes. Antigen-primed T-cells migrate to tissues where HIV replication occurs. In these tissues, clonal expansion and recruitment of other effector cells take place, leading to hyperplasia of the tissue, which manifests as lymphadenopathy, a condition often seen in early phase of HIV infection (Kuby, 1997).

Cyotoxic T-lymphocytes (TLs) bear the CD8 molecule and are a major immunological mechanism in the control of viremia. They inhibit viral replication in at least two distinct ways (Goulder *et al.,* 1999). When a virus invades a cell, a proteolytic degradation of viral proteins takes place. These proteins are then transported into the endoplasmic reticulum, where they form a complex with a developing MHC class-I molecule and are transported to the cell surface. The presence of the viral protein (usually 8ï 10 amino acids in length) within the protein-binding cleft of the classï 1 molecule acts as an immunological signal to CTLs. This event activates the CTL to kill the infected cell, through a direct recognition by the T-cell receptors (TCR) on the CTL. This cell killing is carried out through the production of granzymes and

Perforins (Keefe *et al*., 2005). Although most cytolytic activities are mediated through this route, CD8 cells expressing Fas ligand can also bind to fas (CD95) on the surface of target cells, thereby inducing apoptic cell death (Lowin *et al*., 1994). At the same time, activation of CTL leads to the release of soluble antiviral factors, which inhibit progeny viruses from entering target cells. These antiviral factors include the beta chemokines RANTES (regulated on activation normal T-cell expressed and secreted), MIPI Ŭ (macrophage inflammatory I alpha, and MIPI ß) (Cocchi *et al*., 1995). These chemokines prevent HIV from entering cells by competing for binding to certain co-receptors and promoting internalization of the HIV co-receptor. RANTES, MIPI Ŭ and MIPI ß are active against CXC chemokine receptor 4 (CXCR4, or R4) viruses.

Recent studies have reported additional antiviral factors that are produced by CD8 cells but are clearly not chemokines. A group of defensins produced by neutrophils and CD8 cells have a similar action. This compound is probably one constituent of soluble antiviral factors loosely termed CD8 antiviral factor (CAF) (Zhang *et al*., 2002). These factors act by inhibiting viral transcription and appear to have an action that is distinguishable from chemokine or defensin-mediated viral suppression (Chang *et al*., 2003). CD8-mediated response may be related to progression of HIV disease and disease outcome (Barker *et al.*, 1998). In addition to CTLs, the cellular immune response also relies on virus T-helper cells.

T-helper cell bears the CD4 molecule and recognize viral proteins that have been taken up in the lysosomes of antigen-presenting cells (APC). These proteins are processed at the cell surface in conjunction with MHC class II molecules. The Tï helper cells become activated and drive immunological signal to other categories of immune cells by direct cell-to-cell interactions and by the release of soluble factors. A hallmark of progressive HIV infection is the lack of strong HIVï specific T-helper cell responses, which are lost early in the infection. There are interesting clinical correlation between patients who have strong T-helper responses and viral suppression. This is also seen in so-called elite controllers, where T-helper responses are optimal (Rosenberg *et al*., 1997).

2.18: Humoral Immune Response

The differentiation of B-lymphocytes probably takes place in the bone marrow itself. The B-lymphocytes carry surface markers that serve both as antigen receptors and as identification markers. Upon stimulation, these cells undergo blast transformation, proliferation, and differentiation, eventually becoming plasma cells that produce antibody. Majority of antibodies in HIV infection are non-neutralizing and are often directed against virion debris rather than against conformational epitopes on intact virions. Neutralizing antibodies are directed against a number of different epitopes, including antibodies that prevent CD4 binding (CD4ï binding site antibodies) and entry of virus into cells (directed against the V_3 loop). Some antibodies are directed against linear portions of the viral envelope, whereas others are directed against conformational structure. Some potent neutralizing antibodies do not prevent binding of gp120 to CD4; instead, they interact with the viral-receptor complex, thus preventing the necessary conformational changes that allow viral entry to be mediated by gp41 (Park *et al*., 2000).

Neutralizing antibodies as a means of immune control have been problematic in that they tend to be weak and lack broad cross-reactivity. The viral envelop displays considerable adoptability, with the capacity to revise its glycosylation sites, resulting in a changed three-dimensional configuration that allows it to escape antibody-mediated neutralization (Wei *et al*., 2003). Lack of the capacity to neutralize new strains arising within an individual results in an expansion of the viral ñquasi speciesò. The relationship of autologous neutralizing antibody responses to disease progression has been studied. Studies comparing the strength and breadth of neutralizing antibodies in long-term non-progressors and rapid prorgressors showed that responses in ñcontrollersò were significantly greater than responses in progressors (Barker *et al*., 1998). Like cellular immune responses, the humonal responses are often deregulated and are characterized by paradoxical hyper-activation and hypo-

responsiveness. Hyper-activation is reflected by polyclonal hyper-globulinemia, only a portion of which is directed against HIV antigens. Other features of the hyper-activation include bone marrow plasmacytosis, heightened expression of activation molecules on B-lymphocytes, and the presence of auto-reactive antibodies in plasma (Moir *et al.*, 2001; Chretien *et al.*, 2003). The humoral hypo-responsiveness is manifested by a decrease in protective antibody responses to antigens and after immunization with protein or polysaccharide vaccines (Steinhoff *et al.*, 1991).

2.19: Other Components of the Immune System

2.20: Macrophages:

Infection of monocytes/macrophages occurs via the CD4 receptor which is expressed on the cell surface; however, this infection is in smaller numbers as compared with CD4 lymphocytes (Orenstein *et al*., 1997). Because macrophages do not undergo lyses, these cells become significant reservoirs of HIV. Monocyte migration from the blood to tissue takes place continuously, facilitating the transport of the virus to various tissues and anatomical compartments. Both monocytes and macrophages are antigen-presenting cells that stimulate Tï and Bï lymphocyte responses. They are also primary effector cells. They both have an extensive array of antimicrobial, antifungal, chemotactic, and secretary functions, including the production of proinflammatory cytokines. Although many of these functions are preserved in HIV infection, many others are not (Biggs *et al*., 1995).

2.21: Dendritic Cells

Dendritic cells (DCs) are an important group of antigen-presenting cells that are derived in the bone marrow from precursor CD34 stem cells and act as a primer of the immune response. Dendritic cells, which are found in lymphoid and non-lymphoid tissues (e.g., lungs, skin, and brain), trap antigens and migrate to lymphoid tissue, where the antigens are presented to immune-competent cells. There is also a concentration of DCs within lymph nodes; these DCs form part of the follicular dendritic network. Over the course of HIV disease, there is a loss of the follicular dendritic network, presumably due to a loss of interactive signals with immune-competent cells and a disturbance in the tissueôs cytokine milieu (Macatonia *et al*., 1990). Dendritic cells are thought to play an important role in the early events to HIV infection. The major pathway of viral transmission is by cell-associated virus, which is taken up by interdigitating dendritic cells (Langerhans cells) situated in the cervical, vaginal, and rectal mucosa. These cells are characterized by the expression of CD1a and the presence of birbeck granules. Virus has been shown to be carried on dendritic cells by

way of DC-SIGN (dendritic cell-specific ICAM-glabbing non-integrin) to the regional lymph nodes, where a bridgehead of infection is established. Two populations of DCs can be identified in blood: myeloid dendritic cells (characterized by CD11c expression) and plasmacytoid dendritic cells (CD123). Circulating numbers of these cells tend to be diminished in HIV infection, and this may occur early in primary infection (Pacanowski *et al*., 2001).

2.22: Cytokines

CD4 cells communicate with other cellular components of the immune system by either cell contact or the elaboration of soluble factors known as cytokines. There is a number of different cytokines, each having different and distinct actions. A bipolar Th1/Th2 concept was originally described in the mouse model, in which there was a division of CD4 cells into Th1 and Th2 cells based on the cytokine production from the respective cells (Gray *et al*., 2005). Due to the enormous complexities of the human immune response, this model is not directly applicable in humans. However, relevant features can provide some insights into immunopathogenesis during the course of HIV infection. Th1 cells secrete cytokines that will derive cell-mediated immunity, affected predominantly by CD8 cells. Examples of these cytokines are IL-1, IL-2, IL-6, IL-12, IL-15, tumor necrosis factor alpha (TNF-Ŭ), and interferon-ɔ. A th2 response derives a humoral immune response and will stimulate B-cells. Th2 cytokines include IL-4, IL-5, and IL-10. Th1 and Th2 cells are derived from processor Th0 cell that is thought to be a naïve cell capable of secreting a broad range of cytokines. It is postulated that differentiation into Th1 or Th2 response, is a function of the type of antigen initially encountered and the immunological environment predominating in the tissue at that time. During HIV disease progression, CD4 cell loss leads to a progressive decline in the Th1 responses and therefore to a progression decline in cell-mediated immunity, with a switch to Th2-type immunity in late-stage disease (Than *et al.,* 1997). There is also a group of CD4 cells, classified as T-regulatory (Treg) cells, that serves a regulatory function. These cells, which bear the CD25 marker, are able to inhibit both Th1 and Th2 responses (Maloy and Powrie, 2001). The Treg cells express cytokine IL-10 and transforming growth factor beta (TGFɓ). Some contrasting effects have been attributed to Treg cells, including impairment of HIV-specific responses on one hand and suppression of immune activation on the other hand (Sempere *et al*., 2007).

Recent research has demonstrated the possible importance of Th17 cells which elaborate a cytokine IL-17. Interleukin 17 is important in host defense against extracellular pathogens, such as bacteria and fungi. Th17 cells are profoundly depleted in the gut mucosa

of HIV-infect individuals and in pathogenic models of simian immunodeficiency virus infection (Cervasi *et al*., 2008). With the loss of Th17 cells, bacteria commensals within the gut lumen may gain access to deeper tissues and contribute to the events involving the gut-associated lumen tissue (GALT) in primary infection, leading to immune activation and CD4 cell loss. The proinflammatory cytokines particulary TNF-Ŭ, can up-regulate HIV replication and derive high viral loads, as was seen in a cohort of HIV-infected African patients during and after treatment for tuberculosis (Lawn *et al*., 1999). Abnormal levels of immune activation have been shown to be a feature in HIV-negative subjects in Africa when compared with their European counterparts, suggesting that environmental factors may be responsible for this observed difference (Rizzardini *et al.*, 1998). Some investigators speculate that hyper-immune activation demonstrated in Africans may be a contributing factor in the pathogenesis of AIDS in Africa (Bentwich *et al*., 2000). This immune activation has the potential to create a situation where HIV can be more readily acquired and, if acquired, can lead to a rapid progression of disease.

2.23: Host Genetic Influence on Immune Response

Differences in the host genetic composition may also affect the immune response and, consequently, the rate of disease progression. Polymorphisms in the chemokine co-receptors necessary for viral entry have been associated with differing disease progression. These polymorphisms are seen in people who are heterozygous for the delta-32 base pair deletion in the CCR5 open reading frame. These individuals are characterized by decreased expression of cell surface CCR5, lower viral load, and slower disease progression (Huang *et al.*, 1996). Viral peptides are presented to the immune system for recognition in association with HLA alleles, and certain HLA alleles may vary in their efficiency of antigen presentation (Carrington and OôBrien, 2003). It has also been shown that even a single substitution in an HLA molecule can determine peptide binding and presentation (Gao *et al.*, 2001). Certain HLA class-1 alleles have been associated with a greater risk of disease progression, such that patients with a greater heterozygosity in HLA Class-1 alleles have demonstrated better outcomes (MacDonald, 2000). In essence, this would imply that patients with broader immune response enjoy a better prognosis.

Certain HLA backgrounds have been associated with protection against HIV infection; this background has been described in seronegative partners of HIV-infected individuals and in certain groups of sex workers who have been resistant to infection. For instance, a study of seronegative sex workers in South Africa found a clustering of HLA-A24

that was not found in HIV-infected women, suggesting that this allele may confer protection (Puren *et al.*, 2000). Certain HLA molecules (e.g., HLA-B27 and HLA-B14) are associated with an immunodominant state, which results in a slower progression to AIDS. In these cases, the slow disease progression results from the HLA recognition of immunodominant epitopes, leading to a restriction in the viral diversity within the viral quasi species and thus in a greater immune control. This in turn, leads to a lower viral set point. In contrast, HLA-A29 and HLA-B22 are significantly associated with rapid progression to AIDS, possibly a result of limited antigen presentation. A persuasive example of the influence of the HLA system on the immune control of HIV infection is seen among long-term non-progressors (LTNPs), or elite controllers, among whom there is a significant association with HLA-B*57 (Migueles *et al.*, 2000).

2.24: Immunological Dysfunction in HIV Infection

HIV infection induces a profound immune dysfunction, with abnormalities in every arm of the immune system. The study of long-term non-progressors has revealed that several immune mechanisms are significant in controlling HIV infection. Such patients might have low but detectable viremia, which seems to be important in maintaining the host-specific immune response. These mechanisms include the following; increased production of Th1-type cytokines (such as IL-2 and IFN-y), HIV-specific $CD4^+$ T-cell proliferative responses and cytotoxic $CD8^+$ T-cell activity, increased synthesis of $CD8^+$ T-cell suppressive activity, and increased synthesis of $CD8^+$ T-cell suppressive factors and ß-chemokines. HIV has several inherent strategies by which to escape this vigorous immune response and continue replicating. The most studied of these strategies are antigenic variation, down regulation of the surface expression of MHC molecule, and reduction of specific $CD8^+$ T-cells (Goulder *et al.*, 1997; Pantaleo *et al.*, 1997). HIV does not replicate in B-cells but produces severe B-cell dysfunction, mediated by viral protein toxicity and cytokine dysregulation (Patke and Shearer, 2000). HIV infected patients present with B-cell hyperplasia, circulating immune complexes, elevated auto antibodies, and polyclonal hyper-gammaglobulinemia, with approximately 20% specific anti-HIV antibodies (Shirai *et al.,* 1992). There is impairment in the production of specific antibodies to the new and recalled antigens. A subpopulation of B-cells with low CD21 expression has been described in high viremia patients. These cells are enhanced in immunoglobuline secretors and poor antibody responders, and might be partly responsible for the humoral defects in HIV infection (Gibb *et al.*, 1995; Moir *et al.*, 2001).

HIV gp120 protein modulates B-cell function, apparently by binding the VH3 domain of the membrane immunoglobuline, similar to superantigens (Patke and Shearer, 2000).

Experiments in primates have shown that passive HIV-specific antibody transfer might be useful in protection against HIV infection, and IgG3 appears to have more neutralizing potency than IgG1, according to results of in vitro assays to block viral fusion. However, passive antibodies might induce a selective pressure on viral replication, resulting in viral escape mutants (Schwarf *et al.*, 2001; Parren *et al.*, 1999). In acute infection, partial viral clearance occurs before the specific antibody response is generated. There is a general lack of correlation between the magnitude of the humoral response and the decrease of viral load. These facts argue against a significant role of neutralizing antibodies in controlling HIV infection (Koup *et al.*, 1994; Poignard *et al.*, 1999). HIV activates complement through alternative and classic pathways. Although complement C3 is deposited on the viral surface, there is poor function of the complement C5ï C9 membrane attack complex. HIV might infect cells using complement receptors. Soluble CD16 has been shown to inhibit C3 receptor-mediated HIV-1 infection in monocytes (Reisinger *et al*., 1990: Stoiber *et al.*, 2001). Available report has shown that untreated HIV disease is characterized by a gradual deterioration of immune function. Most prominently, the $CD4^+$ T-cells are disrupted and destroyed during typical course of HIV infection. It is known that the $CD4^+$ T-cells play a major role in the immune response, signaling other cells in the immune system to perform their specific roles (Ondoa *et al*., 2005). It has been shown that a healthy, HIV-negative individual usually has 800 to 1,000 $CD4^+$ T-cells per mm^3 of blood. During untreated HIV infection, the number of these cells in a personôs blood progressively decline. When the $CD4^+$ T-cell count falls below $200mm^3$, a person becomes particularly vulnerable to the opportunistic infections that are commonly associated with AIDS. Most scientists believe that HIV causes AIDS by inducing the death of $CD4^+$ T-cells or interfering with their normal function and by triggering other processes that weaken the immune system. For example, the network of signaling molecules that normally regulates the immune response is interrupted during HIV disease, impairing the ability to fight other infections. The HIV-mediated destruction of the lymph nodes and related immunologic organs also plays a major role in causing the immunosuppression seen in people living with HIV and AIDS (Dandekar, 2007; Vajpayee *et al.*, 2009).

CD4 is a molecule found on the surface of helper T-lymphocytes and other $CD4^+$ cells like monocytes, macrophages and dendritic cells. The designation CD stands for ñCluster of Differentiationò and refers to a nomenclature applied by immunologists who generate

monoclonal antibodies against surface proteins of blood cells as means of identifying the surface proteins for further studies. When a ñClusterò of monoclonal antibodies (antibodies produced in vitro by single clones of B-lymphocytes) is found to react with the same protein, it represents a group of reagents defining a specific marker and that marker is given a CD number (Appay and Sauce, 2008). The $CD4^+$ T-cells perform a central and coordinating role in immune response (Vajpayee *et al.*, 2009). These cells, also known as T4 or helper/inducer T-lymphocytes, recognize antigens presented by cells bearing MHC class II molecules such as monocytes and macrophages. The $CD4^+$ molecules help to stabilize the binding of these T-lymphocytes to the MHC class II molecules on the antigen presenting cells (APC) (Wildson *et al.*, 2004). Once an antigen is recognized, $CD4^+$ T-lymphocytes orchestrate the bodyôs antigen-specific immune response by coordinating B-lymphocyte production of antibodies to these antigens, producing cytokine and inducing cytotoxic lymphocyte response. These functions make $CD4^+$ T-lymphocytes critical element of the immune system and their dysfunction/destruction in HIV-1 infection seriously impairs the ability to respond to diverse pathogens and opportunistic infections (Vajpayee *et al.*, 2005).

In the absence of suppressive antiviral therapy, HIV infection advances and progressively infects more cells in the follicular dendritic cell network, resulting in the destruction of lymph node architecture. This promotes the release of more viral particles into the circulation (SAHIVCs, 2001). The release of more HIV particles into circulation causes more $CD4^+$ T-cells to be infected and destroyed at a faster rate than can be replaced, thereby shifting the dynamic equilibrium in favour of the virus (Leng *et al*., 2001). Studies have shown that an important course of declining $CD4^+$ T-cell count is the failure of the regenerative capacity of the immune system to produce immune cells. This is a result of HIV infection of precursor stem cells and also due to failure of ñprogrammingò of $CD4^+$ T-cells in the HIV infected thymus gland (Hezenburg *et al.*, 2000). The initial process in the life cycle of HIV infection is the binding of HIV gp120 that is present on the surface of the virus to CD4 molecule. Once the virus gains entrance into the cell, it begins the process of viral replication. A direct cytopathic effect of HIV on $CD4^+$ T-cells may occur via the destruction of the cell membrane that is subsequent to massive viral budding, presence of large amounts of non-integrated viral DNA, heterodisperse RNAs and viral core proteins in the cytoplasm of the infected cell (Eggena *et al.*, 2005; Appay and Sauce, 2008).

The T-cell immunoglobulin mucin-3 (Tim-3) recognizes apoptotic cells through the Fg loop in the Igv domain, and is crucial for clearance of apoptotic cells by phagocytes (Masafumi *et al*., 2011). Tim-3 has been identified as a T-helper 1 (Th1) specific marker, and

several in vivo studies have shown that Tim-3 regulates autoimmunity (Kobayashi *et al*., 2007), and negatively regulates Th1-mediated inflammatory diseases such as experimental autoimmune encephalomyelitis (EAE), type-1 diabetes, and acute graft versus- host disease. Moreover, it has been reported that Tim-3 promotes tolerance induction (Sabatos *et al*., 2003). Recently, Zhu *et al.* (2005) identified galectin-9 as Tim-3 ligand, and have demonstrated that galectin-9 binds to the carbohydrate chains on Tim-3, and induces cell death of Th1 cells in vitro. This may explain the mechanism by which Tim-3 suppresses Th1 immune responses. Tim-3 appears to have multiple roles for immune regulation in vivo. However, it remains unknown whether these multiple functions of Tim-3 are mediated solely through galectin-9 ligand. In progressive HIV-1 infection, Tim-3 expression was up-regulated on HIV-1-specific $CD8^+$ T-cells. Tim-3 expressing T-cells failed to produce cytokine or proliferate in response to antigen and exhibited impaired Stat5, Erk1/2, and p38 signaling. Blocking the Tim-3 signaling pathway restored proliferation and enhances cytokine production in HIV-1-specific T-cells. Thus, Tim-3 represents a novel target for the therapeutic reversal of HIV-1-associated T-cell dysfunction in HIV and AIDS (Jones *et al.,* 2008)

2.25: Possible causes of $CD4^+$ T-cells depletion

Several mechanisms have been proposed to explain HIV-mediated depletion of $CD4^+$ T-cells. The total body $CD4^+$ T-cells may be depleted in absolute number because they are destroyed or because their productions are impaired. In addition, the fraction of circulating cells may decrease if viral infection results in their redistribution out of the intracellular space and into the confines of lymphoid organs (Appay and Sauce, 2008).The balance of destruction and production is one important factor that can be explained by multiple mechanisms. It is possible, for example, that $CD4^+$ T-cells depletion is related directly to the virally-mediated destruction of infected cells. On the other hand, physiological responses to HIV infection might initiate events that result in the destruction of uninfected cells. In either case, loss of mature cells should be compensated for by increased production of new cells and mature $CD4^+$ T-cells depletion should occur only if cells lost in the periphery cannot be replaced. The devastating features of HIV infection is that the virus can have direct and indirect pathogenic effect on both mature $CD4^+$ T-cells and on the progenitor cells from which they arise (Brenchley *et al*., 2004; Choudhry *et al*., 2007).

The provision of potent antiretroviral medications to patients with advanced HIV disease caused the viral load to drop and the $CD4^+$ T-cells count to rise. By making reasonable and largely accepted assumptions about T-cell destruction and by assuming that

antiretroviral therapy does not alter the production rate of T-cells, which means that, before therapy, continuous rounds of $CD4^+$ T-cells infection sustained the viral load and that 2×10^9 infected $CD4^+$ T-cells are destroyed per day. In HIV infected human subject, quantitative image analysis revealed decreased number of $CD4^+$ T-cells and increased level of cellular proliferation and apoptosis in lymphoid tissues (Wilson *et al*., 2004). It is also reported that during the cause of HIV infection, about one billion HIV particles are produced per day, resulting in increasing number of infected $CD4^+$ T-cells. The infection spreads in the memory cells, in the naïve $CD4^+$ T-cells and in the thymus. The source is therefore progressively exhausted, surpassing the capacity to produce new $CD4^+$ T-cells (Autran, 2000; Appay and Sauce, 2008).

It is agreed that $CD4^+$ T-cells death may occur in uninfected cells as a by-product of HIV infection of other cells. According to Grossman and Herberman (1997), HIV disease is typified by a state of chronic activation driven in part by the antigenic stimulus of HIV and in part by an antigen-independent mechanism. For instance cytokines are released by apoptotic cells and activated T-cells. Multiple bursts of activated cells spread throughout the body and would be characterized by apoptotic cell-mediated activation of resting lymphocytes, cytokine driven expansion of responding cells and contraction of the responding population by activated induced cell death. It is believed that if apoptotic cells are infected by, or otherwise carry HIV, antigen-specific cell activation could support virus dissemination to responding $CD4^+$ T-cells, irrespective of their TCR specificity (Hazenberg *et al.,* 2003; Odoa *et al.,* 2005). During the asymptomatic phase of infection when the fraction of infected cells is much lower than the fraction of activated cells, these activated cell burst would continue in a local, recurrent and asynchronous fashion, and $CD4^+$ T-cells depletion might be driven by several mechanisms (Bentwich *et al*., 1999; Ondoa *et al*., 2005). It has been suggested that the activation of naïve cells into the activated/memory pool may not be fully compensated for by replacement of new naïve cells from the thymus or by the generation of viable memory cells. Alternatively, chronic stimulation of resting T-cells might have a negative effect on the homeostatic regeneration of these cells. The relevance of this process to $CD4^+$ T-cells depletion can be explained by the observation that disease progression is associated with immune activation and vice versa (Wilson, 1990; Bentwich *et al.*, 1999; Hazenberg *et al*., 2003; Vajpayee *et al*., 2005).

Immune activation hypothesis proposed that HIV adopted strategies to increase the availability of target cells by activating $CD4^+$ T-cells. It could be argued that increasing target cell may be likened to fueling a fire, consequently causing more viral replication and $CD4^+$

T-cells depletion. In a recent investigation, the correlation between the runaway $CD4^+$ T-cells depletion process and the slow scale of memory $CD4^+$ T-cells depletion in person with HIV infection was conducted to explain the depletion of $CD4^+$ T-cells by activation hypothesis. It was observed that immune activated $CD4^+$ T-cells have a very short life-span which shows that immune activated $CD4^+$ T-cells are lost by activated induced cell death. This demonstrated that the immune activation model provides an explanation for the depletion of $CD4^+$ T-cells and that the rate of destruction is dependent on the rate of immune activation (Grossman *et al*., 2002; Yates *et al*., 2007).

Laboratory findings showed that when late stage patients initially presented with opportunistic infections, they were not just lymphopenic, but anemic, neutropanic and thrombocytopenic as well. These findings led to multiple diagnostic bone-marrow biopsies, the results of which were frequently abnormal. Microscopic examination revealed hyper cellularity or hypo cellularity, plasmacytosis, myeloid or erythroid dysplasia and variety of other pathological changes. Also phenotypic and functional analysis of bone-marrow progenitor cells showed a decrease in the number of lineage-restricted colony-forming units and in some, but not all instances, infection and/or apoptotic death of $CD4^+$ T-cells progenitors (McCune and Kaneshima, 1995; Bialecki *et al*., 2009). Examination of paediatric and adult specimens has revealed thymocyte depletion, loss of corticumedullary demarcation and development of thymic medullary B-cell follicles. These changes are associated with immuno-histochemical visualization of structural proteins within thymocytes and are evidence of viral infection. It has proven difficult to study the thymus in HIV-infected humans, the frequency of circulating $CD4^+$ and $CD8^+$ naïve T-cells have been found to decrease as disease progress (Roederer, 1995; Dandekar, 2007). It has also been noticed that peripheral lymphoid organs undergo marked alterations after HIV infection. These changes include the accumulation of virus on and eventual destruction of the follicular dendritic cell network, decompartmentalisation and depletion of both the $CD4^+$ and $CD8^+$ T-cells population. Thus, HIV infection leads to profound disruption of the bone-morrow, thymus and peripheral lymphoid organs, with measurable quantitative and qualitative defects in $CD4^+$ T-cells progenitor cells (Brenchley *et al.*, 2004; Vajpayee *et al*., 2005).

Research reports have shown that there is a relationship between the extent of apoptosis and disease progression in HIV-infected persons. It is believed that one main pathway of T-cell apoptosis is mediated via the tumor necrosis factor family of receptors. It was also demonstrated that peripheral blood lymphocytes from HIV-positive persons express higher Fas and that the proportion of Fas-expressing T-cell increases with disease

progression. The viral protein Tat, has been found to induce receptor-mediated apoptosis in $CD4^+$ T-cells by up-regulation of Fas liagand (FasL) expression and also increase the sensitivity of Fas-mediated apoptosis by up-regulation of caspase 8 (Catton *et al.*, 1997; Selliah and Finkel, 2001). Nef has been shown to increase surface expression of both fas and FasL, and has the capacity to interact with cellular kinases for the increased expression and apoptosis (Appay and Sance, 2008).

A cohort study of HIV-positive persons conducted at different stages of HIV disease showed that the degree of apoptosis was significantly higher in $CD4^+$, $CD8^+$ and B-cells compared to HIV-negative persons and correlated with HIV disease progression. The study also reported low level of apoptosis in long-term progressors and a high level of apoptosis in fast progressors. Increase apoptosis has been reported in the lymph nodes of HIV-positive individual. Apoptosis is known to occur in the absence of viral replication when infected and uninfected cells were cultured together indicating that viral proteins interact with uninfected cells and induce an apoptotic signal. The binding of HIV-1 Env to $CD4^+$ and CXCR4 or CCR5 has been shown to induce apoptosis in primary T-lympocytes (Pantaleo *et al*., 1993; Cicala *et al*., 2000; Dandeker, 2007).

2.26: Mechanism of HIV persistence in the monocyte-macrophage lineage

Several advances have been made in curing HIV disease since the introduction of the highly active antiretroviral therapy (HAART) in 1996. This therapy can reduce plasma virus level bellow detection limits (Ò 50 copies/mL). It induces a biphasic decline of HIV-1 RNA with a rapid decline of infected $CD4^+$ T-cells followed by a decline originating from infected tissue macrophages (Geeraert *et al*., 2008). However, a residual viremia is still detected in patients on HAART. Moreover, HIV RNA returns to a measurable plasma level in less than two weeks when HAART is interrupted. These observations suggest that even long term suppression of HIV replication by HAART cannot totally eliminate HIV, the virus persist in cellular reservoirs because of viral latency, cryptic ongoing replication or poor drug penetration (Zhang *et al.*, 1999; Zhang *et al.*, 2000).

There are essentially two theories of persistent infection: latency and ongoing replication. Latency is best described as a lack of proviral gene expression. On the other hand, ongoing replication requires continuous viral expression without cytopathic effect. Resting memory $CD4^+$ T-cells are the major cellular and the best characterized reservoir in the natural host. The presence of latent proviral HIV-I DNA in this cell population has been undoubtedly proven (Finzi *et al*., 1997; Chun *et al*., 1997; Wong *et al*., 1997). Genetic studies

have shown that during rebound viremia (when HAART was interrupted) the virus could be detected from another reservoir than the $CD4^+$ T-cells (Dybul *et al.*, 2003).

Cells of myeloid lineage including monocytes, macrophages and dendritic cells play an important role in the initial infection and therefore contribute to its pathogenesis throughout the course of infection. This is mainly because these cells are critical immune cells responsible for a wide range of both innate and adaptive immune functions. All cells from the monocyte/macrophage lineage appear to derive from the same progenitor multipotent cell, the haematopoietic stem cell (HSC). The HSC, located in the bone marrow, may differentiate into myeloid or lymphoid precursor. The myeloid precursor is then able to migrate into the blood stream and to differentiate into a monocyte. Monocytes migration to specific tissues and their differentiation occur upon a different cytokines, interleukins and/or other factors cocktail. Depending on the location, the monocytes become either interstitial dendritic cells, macrophages or microglia cells. Lymphoid precursor runs parallel with the myeloid precursor, but can directly differentiate into another type of dendritics cells, the plasmacytoid dendritic cell.Infected monocytes have been recovered from the blood of HIV-1 infected patients, even from those on HAART and with a viral load below detectable limits. Early studies have shown that monocytes harbor latent HIV-1 proviral DNA (McElrath *et al*., 1991; Zhu *et al*., 2002). Interestingly, a minor monocyte subset, the $CD16^+$ is more permissive to the infection than the more abundant $CD14^+$ $CD16^-$ monocyte subset. Although HIV-1 proviral DNA is only in less than 1% of circulating monocytes, these cells are important viral reservoirs as they are responsible for the dissemination of HIV-1 into sanctuary places such as the brain (Ellery *et al.*, 2007; Perno *et al*., 2006).

Macrophages harboring the CD4 receptor and CCR5 co-receptor are now recognized as early cellular targets for HIV-1. These cells are able to produce and harbor the virus for a longer period. This is partly due to the higher resistance of these cells to cytopathic effects (Permo *et al*., 2006). In patients on HAART, very few lymph node macrophages are infected (about 0.005%). However, the finding of in vivo reactivation of those infected macrophages in response to opportunistic infections is in vafour of macrophages as HIV-1 reservoirs (Orenstein *et al*., 1997; Caselli *et al.*, 2005). Resident macrophages in central nervous system (CNS) are involved in the pathogenesis of HIV-1-associated dementia (Williams and Hickey, 2002). Four types of macrophages were described in the CNS, the meningeal macrophages, the macrophages of the chranoid-plexus, the perivascular macrophages and microglial cells. Among these four types, the perivascular macrophages and microglial cells are the main target for HIV-1 in CNS. Perivascular macrophages have a turnover of 2 ï 3 months while

that of microglial cells is several years. These features make these cells potential reservoirs for HIV-1 in infected individuals (Lassmann *et al*., 1993; Williams *et al*., 2001; Garden, 2002).

Haematopoietic cells (HPC) have been proposed to serve as a viral reservoir, since a subpopulation of $CD34^+$ HPCs express CD4 and CCR5 and/or CXCR4 they are susceptible to HIV-infection. Interestingly, the $CD34^+$ $CD4^+$ HPC subset has an impaired development and growth when HIV-1 is present. This HPC will then generate a sub-population of monocytes permissive to HIV-1 infection with a low level of CD14 receptor and an increase of CD16 receptor ($Cd14^+$ $CD16^{++}$). This population of monocyte may differentiate in dendritic cells in tissues such as lymph nodes and help in the spread of HIV-1 to the sanctuaries (Slobod *et al.*, 1996; Roundolph *et al.*, 1999; Ancuta *et al*., 2000).

2.27: Viral Induced Cytokine Production

Immediately following a viral infection, a strong host response is initiated. For a range of viruses it has now been clarified that the mere interaction of viral surface proteins with cellular surface proteins starts a cellular reaction that in many cases leads to the first wave of cytokine production after infection. In addition many viral proteins not present in the infection particles but produced during the course of the viral life cycle are able to affect cellular signaling in a manner leading to cytokine production. Moreover, accumulation of viral RNA and overload of the cellular protein synthesis machinery induces signals that are able to trigger an early host response to infection. The importance of such alert signals in the clearance of viral infections is illustrated by the fact that many viruses have adopted mechanisms to interfere with these processes (Burysek *et al*., 1999; Melville *et al.*, 1999). Interferon (IFN) regulatory factor 3 (IRF-3) and IRF-7 are recently discovered virus-activated transcription factors that have been ascribed an important role in IFN-Ŭƃ expression (Marei *et al*., 1998). These transcription factors become activated by serine/threonine phosphorylation. The mitogen-activated protein (MAP) Kinase P^{38} and Jun N-terminal Kinase (JNK) are also activated in response to many viruses. Following activation, the serine/threonine kinase phosporylate their downstream targets, notably activating transcription factor 2 (ATF-2) and Jun, thus promoting their trans-activating potential. Jun can form homodimers as well as heterodimers with ATF-2 and Fos. The ATF-2/Jun dimer binds to the cyclic AMP response element (CRE), whereas the Jun homodimer and the Jun/Fos heterodimer recognize the TPA-responsive element (Whitmarsh and Davis, 1996).

Another transcription factor activated in response to virus infection is nuclear factor of activated T-cells (NF-AT). NF-AT is constitutively present in the cytoplasm in a latent phosphorylated form. Increasing levels of cytoplasmic calcium activate the calmodulin-dependent phosphatase calcineurin, which activates NF-AT by dephosphorylation (Crabtree, 1999). Activation of NF-kB is a hallmark of most infections including viral infections. This transcription factor is normally found in the cytoplasm complex with an inhibitory protein, IkB, of which various isoforms exist (Hatada *et al.*, 2000). Upon infection, signaling events are initiated leading to activation of MAP kinase kinase kinase (MAP3K), which promotes the activation of a large kinase complex able to phosphorylate IkB at two specific amino-terminal serine residues. The kinases responsible for IkB phosphorylation are IkB kinase Ŭ (IKKŬ) and IKKɓ. Phosphorylated IkB is subsequently targeted for degradation through the ubiquitin-dependent 265 proteasome pathway. Degradation of IkB unmasks the nuclear localization signal of NF-kB, which then migrates to the nucleus and activates transcription (Siebenlist *et al.*, 1994).

The acute host response to a primary HIV infection is characterized by a Th0 cytokine profile including the proinflammatory cytokines IL-1, IL-2, IL-6, TNE Ŭ, IFN-Ŭɓ, and IFN-y, as well as the anti-inflammatory cytokines IL-4, IL-10, and IL-13 (Graziosi *et al.*, 1996; Patella *et al.*, 2000). At later stages of infection, as full-blown AIDS progresses, the pattern of cytokine production shift towards strongly biased Th2-like response (Clerici and Sheorer, 1993). The HIV glycoprotein gp120, which interacts with CD4 and the chemokine receptors CXCR4 and CCR5, is able to induce the secretion of many proinflammatory cytokines including IL-1Ŭ, IL-1ɓ, IL-6, IL-8, TNF-Ŭ, IFN-Ŭɓ and IFN-y. It is also able to induce the secretion of IL-4 and IL-13 in basophils, and IL-10 in mononuclear cells, indicating that the acute Th0-like host response to HIV is explained largely by the interaction of gp120 with host cell receptors (Schols and Clereq, 1996; Capobianchi *et al.*, 1997; Patella *et al.*, 2000). While most gp120 induced functions are explained by its interaction with CD4, the mechanism through which IL-4 and IL-13 are induced appears somewhat different. Recombinant gp120 from various divergent HIV-1 isolates was found to induce secretion of IL-4 and IL-13 in basophils, and this occurred by the action of gp120 as superantigen, where gp120 binds to the V_H3 region of immunoglobuine E (Patella *et al.*, 2000). Another HIV protein, Tat, is known to stimulate the production of many cytokines. Tat is produced by HIV-infected cells and has pleiotropic effects on viral replication and cell growth. Tat is predominantly cytoplasmic but can be released from infected cells and enter adjacent cells. The protein contains an arginine-rich domain that permits Tat to efficiently cross membranes

(Schwarze *et al*., 1999). The cytokines and chemokines induced by Tat include IL-1ϐ, IL-2, IL-6, IL-8, TGF-ϐ1, TNF-Ŭand MCP-1 (Westendorp *et al*., 1994; Hofman *et al.*, 1999; Lim and Garzino-Demo, 2000).

A third HIV protein able to induce cytokine production is Nef. As with Tat, Nef is predominantly cytoplasmic, but soluble Nef as well as anti-Nef antibody can be detected in sera from HIV patient (Fuji *et al.*, 1996). Although cytoplasmic Nef is immunomodulatory, it is not as potent an inducer cytokine production as is extracellular Nef, which induces the production of IL-1 ϐ, IL-6, IL-10, IL-15, TNF-Ŭand IFN-y in various leukocyte populations (De *et al.*, 1998; Quaranta *et al.,* 1999). Finally there is evidence that viral protein Vpr induces the expression of IL-6, IL-8, IL-10, TNF-Ŭand IFN-2 in a variety of cell types (Roux *et al*., 2000).

2.28: Signal Transduction and Immunological Dysfunction

HIV infection affects cellular signaling activity in a very profound manner. At present, signaling properties have been reported for HIV proteins. Glycoprotein gp120 induces signaling mainly through interaction with CD4, but there is also evidence that the chemokine receptors CXCR4 and CCR5 signal following interaction with gp120. The CD4-dependent signaling has been elucidated. The binding of gp120 to CD4 results in activation of the tyrosine kinase Lck and the serine/threonine kinase Raf-1 (Popik and Pitha, 1996). This receptor engagement also induces activation of the MAP kinases; ERK1, ERK2, and JNK, as well as the transcription factors NK-kB, AP-1 and C/EBPϐ (NF-IL6) (Lannuzel *et al*., 1997; Popik *et al*., 1998). Two studies have shown that gp120 triggers signaling from the coreceptors CXCR4 and CCR5, as evidenced by phosphorylation of the protein tyrosine kinase Pyk2. However, whereas the natural CXCR4 ligand stromal cell-derived factor 1Ŭalso activate MAP kinase pathways, this was not seen in response to gp120, indicating that gp120-induced chemokine receptor singnaling does not fully mimic the response to chemokines (Devis *et al*., 1997; Misse *et al*., 1999).

The Tat protein activates cellular signaling cascades and transcription factors associated with a proinflammatory host response. It is a potent inducer of many proinflammatory cytokines. However, giving that the HIV LTR is regulated by transcription factors of the NF-kB, NF-AT, Sp1, and C/EBP families, this property of Tat is beneficial for viral replication (Kinoshita *et al.*, 1997; Ruocco *et al*., 1996). Activation of NF-kB by Tat proceeds via Ikk, which is constitutively active in HIV-infected cells. Moreover, the ability of Tat to activate NF-kB also seems to require PkR. The involvement of this in NF-kB activation is not restricted to Tat since PkR-deficient cells also display an impaired ability to

activate NF-kB in response to TNF-Ŭ and dsRNA. In addition to the IKK pathway, some earlier studies showed that activation of PKC by Tat leads to nuclear translocation of NF-kB (Deluca *et al*., 1999; Demarchi *et al.*, 1999; Conant *et al.*, 1996; Zamanian *et al.*, 2000). It has been shown that Tat primarily forgets 1kBŬ for degradation. Tat expression leads to constitutive activation of NF-kB, which is normally associated with degradation of 1kBƃ rather than 1kBŬ. IkBŬ degradation and synthesis are subjected to auto regulation due to the presence of NF-kB-responsive sites in the 1kBŬ promoter. Hence, 1kBŬ-dependent activation of NF-kB is self-limiting (Demarchi *et al.*, 1996; Chiao *et al.*, 1994). One study has shown that nuclear 1kBƃ is at least partly responsible for the observed constitutive NF-kB activity in HIV infected cells. Nuclear hypophosphorylated 1kBƃ is known to maintain NF-kB DNA binding by rendering the protein-DNA complex insensitive to 1kBŬ-mediated association from DNA. This mechanism contributes to the sustained NF-kB activity in HIV-infected cells (Deluca *et al*., 1999; Attar *et al*., 1998).

JNK which is responsible for phosphorylation of c-Jun and, to a lesser extent ATF-2, is also regulated by Tat. It was demonstrated that Tat activates JNK and AP-1 in the human histocytic lymphoma cell line U937, whereas the effect on ATF-2/c-Jun activity was not examined. Similar findings have been achieved in another study, where it was further shown that Tat activates the ERK kinases (Kumar *et al.*, 1998; Mischiati *et al*., 1999). Another transcription factor regulated by Tat is SpI, which is essential for optimal activation of HIV LTR (Harrich *et al.*, 1989). Sp1 is a ubiquitous transcription factor involved in basal and inducible expression of many genes. Tat enhances SpI DNA binding and arguments SpI phosphorylation, which was shown to be associated with enhanced promoter activity (Lim and Garzino, 2000). Of other transcription factors affected by Tat, notably NF-AT and C/EBPƃ have received attention due to their ability to regulate HIV LTR activity, NF-AT activation by Tat is cyclosporine A sensitive, implying a role of calcineurin in the process. In addition, Tat is able to associate with NF-AT, thus enhancing NF-AT-driven transcription. Similarly, Tat-dependent DNA binding of C/EBPƃ is at least partly attributed to complex formation between the two proteins, which enhances the DNA affinity of C/EBPƃ (Ambrosino *et al.*, 1997; Macian and Rao, 1999; Kinoshita *at al.*, 1997; Ruocco *et al*., 1996; Westendorp *et al*., 1994).

The effects of Vpr on cellular signaling and gene transcription have also been studied, and the data suggest that Vpr modulates the function of a number of DNA-binding proteins through direct protein-protein interaction. It was shown that the ability of Vpr to stimulate HIV LTR transcription was mediated mainly through physical interaction between Vpr and

SpI bound to the HIV LTR (Wang *et al*., 1995). Subsequent studies have shown that Vpr interacts with p53 and that this association antagonizes Vpr/SpI-driven transcription, also that Vpr enhances the trans-activating function of NF-kB and C/EBPϧ (Sawaya *et al*., 1998; Roux *et al.,* 2000). The extensive knowledge about cytokine induction and signal transduction by HIV gp120 is not accompanied by a similar in-depth understanding of which signaling pathways are responsible for gp120-induced cytokine synthesis. For the mechanism of Tat-induced cytokine production, a substantial amount of knowledge has been gathered. There is evidence that NF-kB is pivotal for expression of IL-2, IL-6, IL-8, TNF-Ŭ and MCP-1. Other studies have shown that Tat-supported IL-2 expression relies on the ability of Tat to activate the NF-kB and NF-AT, both of which are required to activate the IL-2 promoter (Vacca, 1994; Lim and Garzino, 2000).

IL-2 Production and Signal Transduction in T-Cell Proliferation

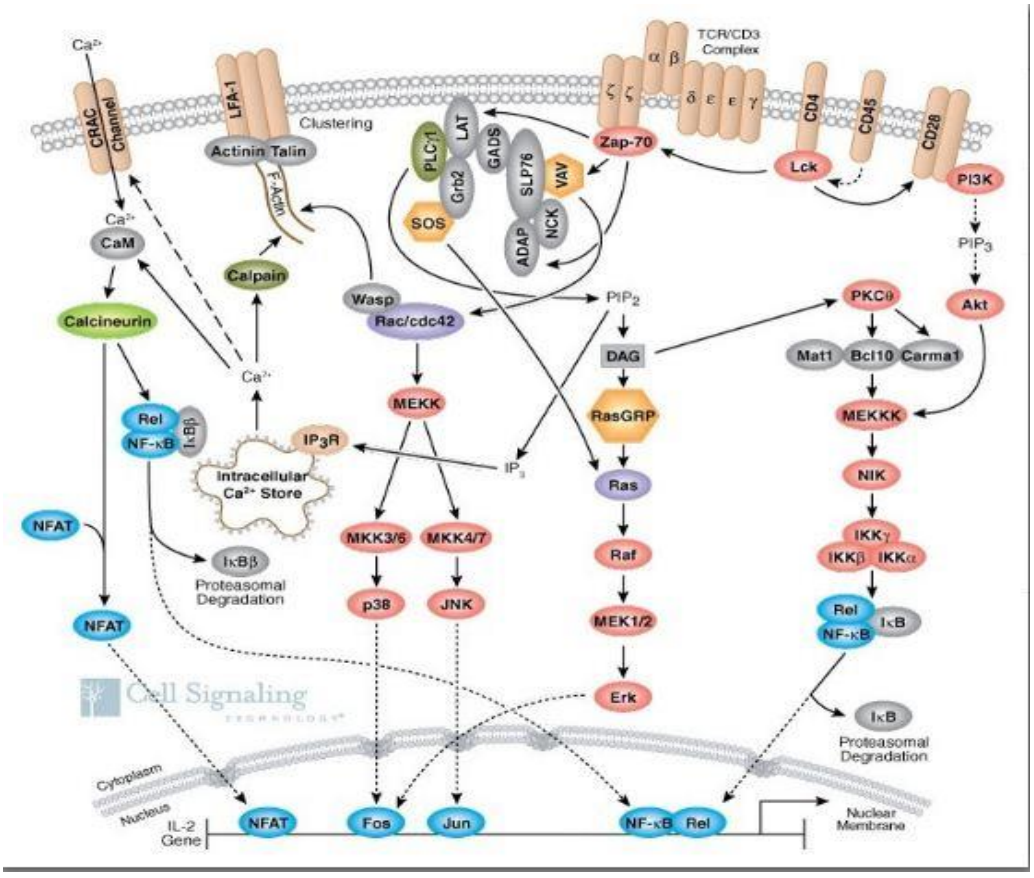


Fig.2.8: IL-2 Signalling Pathways

IL-2 (Interleukin-2) is a T-cell-derived cytokine important in the regulation of growth and differentiation of T-cells, B-cells, natural killer cells, glioma cells and cells of the monocyte lineage after specifically interacting with its receptors. Human IL-2 is a 133-amino acid polypeptide with a molecular mass of 15ï 18 KDa. IL-2 signaling is mediated by multichain receptor complex consisting of an alpha (CD25); beta (CD122), and Gamma (CD123) chain. The IL-2R (IL-2 receptor) alpha subunit primarily increased the affinity of ligand binding and is known to contain a signaling domain, whereas the beta and gamma subunits participate in both ligand binding and signal transduction (Smith, 1988). The IL-2R signaling system proceeds through at least three different pathways, which mediates the flow of mitogenic and survival-promoting signals. One of the pathways proceeds through protein tyrosine kinase activity, Ras and the MAPK (Mitogen-Activated Protein Kinase) cascade, leading to expression of proto-oncogenes; c-Fos, c-Jun, and Elk-1. The Syk, that is responsible for c- Myc gene induction, initiates the second pathway. The final pathway results in BCL-2 (B-Cell Leukemia-2) expression, and progression through a Rho, P13k (Phosphoinositide-3 kinase) and Akt/PKB (Protein Kinase-B) mediated signaling pathway which is also involved in IL-2-prompted regulation of actin cytoskeleton organization (Gomez *et al.*, 1997).

IL-2R signaling activates P13K which catalyses phosphorylation of inositol phosphates. These act as second messengers and recruit molecules such as Akt kinase to the cell membrane. Akt kinase is further activated by phosphorylation and subsequently positively or negatively regulates the activity of downstream targets like PKB. Proapoptotic proteins which can be phosphorylated and inhibited by PKB include BAD (BCL 2 Antagonist of cell Death), human caspase 9, and the forkhead family of transcription factors. PKB can also cause stimulation of NF-kB (Nuclear factor Kappa B) activity by upregulating 1-Kappa B degradation via phosphorylation of 1kks (1-Kappa B kinases) and by affecting NF-kB itself, thereby allowing the transcription of genes involved in promoting survival, such as the BCL2 homologue bfl-1. In addition to the fork head and NF-kB families, E2F-mediated transcription can also be activated by the hyperphosphorylation and subsequent inactivation of Rb (Retinoblastoma Protein) in response to signals from PI3K and its downstream effectors, PKB and p70S6K. The transcription factors activated by PI3K and PKB are of great interest in the IL-2 response, as they regulate the genes responsible for determining whether activated T-cells survive, proliferate or die (Lindemann *et al.*, 2003).

IL-2 also activates Lck (Lymphocyte-Specific Protein Tyrosine Kinase) which is involved in T-cell receptor signaling. Signaling from the T-cell receptor does activate PLC-Gamma, but this requires ligand binding. The protein tyrosine kinases JAK1 and JAK3 (Janus Kinase-1 and -3), which are associated with the IL-2R beta and gamma subunits, respectively, are also activated after binding of IL-2 to its receptor. Phosphonylation of the cytoplasmic domains of the beta än- and gamma än- subunits of the IL-2R provides docking sites for the JAK 1/3, which after phosphorylation, in turn provide docking sites for and phosphorylates STAT3 (Signal Transducer and Activator of Transcription-3) and STAT5. Phosphorylation induces dimerisation and nuclear translocation of STAT3 and STAT5 complexes, where they promote specific target gene transcription. IL-2 also stimulates ERKs (Extracellular Signal Regulated Protein Kinases) and/or p38 in Mitogen-activated T-lymphocytes. Several transcription factors include NF-AT. AP-I and NF-kB have been identified as major regulators of IL-2. NF-AT is activated mainly by a $Ca2^{+}$ (Calcium)-dependent protein phosphate; calcinuerin. The increase of intracellular calcium activates this phosphate and induces nuclear translocation of NF-AT. AP-I, which consists of two transcription factors, Fos and Jun is another key component for activation of IL-2 promoter. AP-I binding sites are found in juxtaposition to NF-AT and NF-kB binding sites and it has been shown that NF-AT and AP-1 function in a cooperative manner. Binding sites for NF-kB, a group of transcription factors involved in the regulation of many genes are found in the IL-2 promoter region and members of the NF-kB family, RelA and c-Rel, are activated by T-cell receptor (TCR) stimulation (Iwashima *et al.,* 2002).

2.29: Reactive Oxygen Species and Immunological Dysfunction

Aerobic organisms, which derive their energy from the reduction of oxygen, are susceptible to the damaging actions of the small amounts of $.O_2^-$, .OH and H_2O_2 (superoxide, hydroxyl radical and hydrogen peroxide respectively) that inevitably form during the metabolism of oxygen, especially in the reduction of oxygen by the electron transfer system of mitochondria. These three species together with unstable intermediates in the peroxidation of lipids are referred to as Reactive Oxygen Species (ROS). Many diseases are linked to damage from ROS as a result of an imbalance between radical generating and radical scavenging systems ï a condition called oxidative stress (Brain *et al*., 1997). Cells generate energy aerobically by reducing molecular oxygen (O_2) to water. The cytochrom c oxidase-catalyzed reaction involves transfer of four electrons (e) to oxygen in principle, without intermediates, but in fact, partially reduced oxygen species are produced. Other enzymes,

especially flavin enzymes, also generate partially reduced oxygen species. One or two percent of total oxygen consumption may, in fact, be converted to superoxide anion radical ($.O_2^-$). Other sources of ROS include radiation (e.g., UV light), toxic chemicals and drugs. Formation of superoxide anion radical leads to a cascade of other reactive oxygen species (Grisham, 1992; Moslem, 1994).

Mammalian cells possess elaborate defense mechanisms to detoxify radicals. The key metabolic steps are superoxide Dismutase (SOD) catalysis of the dismutation of superoxide to hydrogen peroxide and oxygen, and the conversion of H_2O_2 to $2H_2O$ (water) by glutathione peroxidase (GPX), or to O_2 and H_2O by catalase. Since the reaction catalyzed by glutathione peroxidase requires glutathione (GSH) as substrate and depends in part on the ratio of GSSG: GSH, the concentration of these reactants and their ratio, which is a reflection of the redox state of the cell, are important to ROS detoxification. Similarly, the concentration of redox-active metals, such as iron, catalyzes formation of some ROS. This is minimized by keeping the concentration of these metal ions very low due to binding to storage and transport proteins (e.g., ferritin, transferrin, lactoferrin), thereby minimizing hydrogen peroxide formation. Finally, the radical scavenging antioxidants (e.g., Vitamin E), interrupts the chain reactions by capturing the radical and thus terminate the chain reaction of the radical damage (Brain *et al*., 1997).

Neutrophils have a variety of functions including chemotaxis, adhesion to the endothelium and foreign agents, phagocytosis, and microbicidal activity. Neutrophils can penetrate and migrate into infected tissues, where they internalize and destroy invading micro organisms by producing toxic agents such as ROS, proteases, and proteins that interfere with bacterial development (Lactoferrin). ROS are generated during the complex process of respiratory burst, during which superoxide anion is formed immediately after the reduction of molecular oxygen by single electrons through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. Superoxide anion is then rapidly transformed through enzyme activity (super oxide dismutase, catalase, myeloperoxidase) into other ROS, which include hydrogen peroxide, hydroxyl radicals, and hypochlorous (HOCL) acid (Suzuki *et al*., 1996). The importance of superoxide and hydrogen peroxide production for neutrophil bactericidal activity is illustrated by patients with chronic granulomatous disease who do not produce enough $\_O_2^-$ and H_2O_2, thus becoming more susceptible to bacterial infection (Lakshman *et al.*, 2005). Despite the intrinsic function of neutrophils in the innate immune response, potent cytotoxic compounds are released into the extracellular space, which damage host tissues (Suzuki *et al*., 1996). Hermanôs free radical theory, which has been

accepted as a plausible explanation of the primary chemical reactions involved in ageing process, proposes that oxygen-derived free radicals are responsible for the age-associated damage at the cellular and tissue levels through the oxidative modification of biological molecules (lipids, proteins, and nucleic acids), which leads to functional impairments (Harman, 1956).

From a physiological perspective, endogenous ROS produced by the NADPH oxidase system regulate tyrosine phosphorylation dependent pathways that in turn modulate host defense responses such as phagocytosis. Also, via NF-kB, they modulate the expression of cytokines and chemokines that further modulate the inflammatory response (Sela *at al*., 2005). Under pathological circumstances such as inflammatory tissue injury, excess production of ROS may destroy vicinal cells such as endothelium or epithelium. In addition, since ROS are membrane permeable, they may influence intracellular signaling pathways in the cell as well as the adjacent cells in the inflammatory milieu. During a systemic inflammatory response, it is feasible that ROS act as signaling molecules leading to modulation of crucial events including phagocytosis, secretion, gene expression, and apoptosis, resulting in dysregulation of inflammation (Van *et al*., 2003; Falkow *et al.*, 2007).

2.30: Antiretroviral Drugs and Mechanism of Action

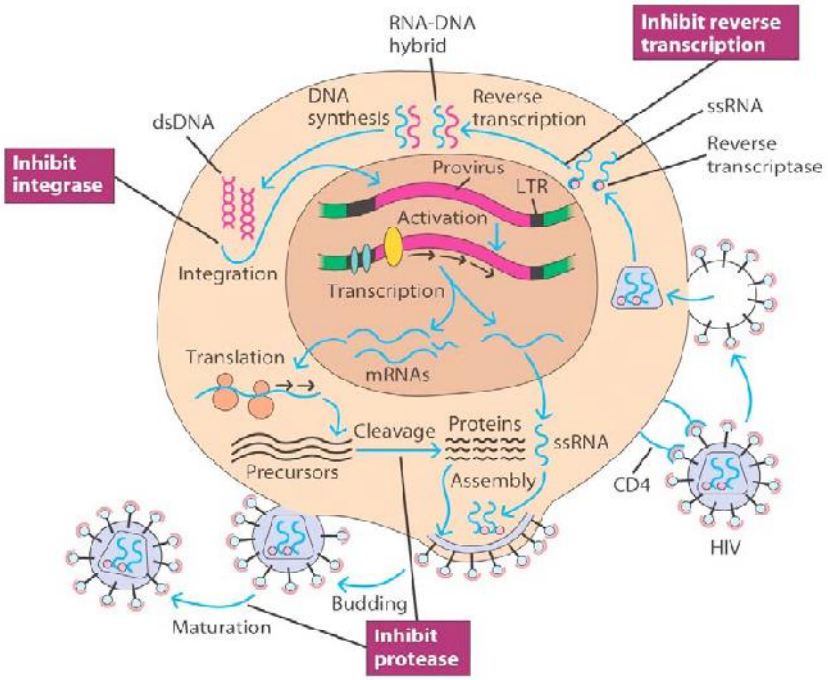


Fig.2.9: Antiretroviral drugs and mechanism of action

The clinical management of HIV-I largely consisted of prophylaxis against common opportunistic pathogens and managing AIDS-related illnesses. The treatment of HIV-1 infection was revolutionalized in the midï 1990s by the development of inhibitors of the reverse transcriptase and protease, two of the three essential enzymes of HIV-1, and the introduction of drug regimens that combines these agents to enhance the overall efficacy and durability of therapy. To date, an arsenal of 24 FDA (Food and Drug Administration)-approved drugs are available for treatment of HIV-1 infections. These drugs are distributed into six distinct classes based on their molecular mechanism and resistance profiles: (1) nucleoside reverse transcriptase inhibitors (NRTIs), (2) non-nucleoside reverse transcriptase inhibitors (NNRTIs) (3) Integrase inhibitors (4) Protease inhibitors (PIs), (5) Fusion inhibitors and (6) Co-receptor antagonists (Arts and Hazunda, 2012).

The first step in the HIV-1 replication cycle, viral entry is the target for several classes of antiretroviral agents: attachment inhibitors, chemokine receptor antagonists, and fusion inhibitors. The HIV-1 envelope gp120/gp41 has affinity for the CD4 receptor and directs HIV-1 to $CD4^+$ immune cells. Interaction of the gp120 subunit of the HIV-1 envelope with CD4 is followed by binding to an additional co-receptor, either CCR5 or CXCR4 chemokine receptor (Dalgleish *et al.*, 1984; Alkhatib *et al*., 1996; Doranz *et al.*, 1996). The disposition of these co-receptors on the surface of lymphocytes and monocyte/macrophages, and co-receptor recognition by the viral envelope, are major determinants of tropism for different cell types. These sequential receptor-binding events trigger conformational changes in the HIV-1 envelops, exposing a hydrophobic domain of gp41 that mediate fusion with the cellular membrane. Gp120 and CD4 are targets for small-molecules and antibody-based attachment inhibitors BMS-378806 and TNX-355, each of which have shown some clinical promise, although neither is approved for use in HIV-1 patient (Lin *et al.*, 2003; Kuritzkes *et al*., 2004).

Viral entry and fusion of HIV-1 envelope with the host cell membrane allow for uncoating of the viral core and initiates a slow dissolution process that maintains protection of the viral RNA genome while permitting access to deoxyribonucleosid triphosphate (dNTPs) necessary for reverse transcription and proviral synthesis. Reverse transcription (RT) was the first HIV-1 enzyme to be exploited for antiretroviral drug discovery. RT is a multifunctional enzyme with RNA-dependent DNA polymerase, RNase-H, and DNA-dependent DNA polymerase activities, all of which are required to convert the single-stranded HIV-1 viral RNA into double-stranded DNA (Hu and Hughes, 2011). Reverse transcriptase is the target for two distinct classes of antiretroviral agents: the nucleoside

reverse transcriptase inhibitors (NRTIs), which are analogs of native nucleoside substrates, and the non-nucleoside reverse transcriptase inhibitors (NNRTIs), which bind to a non-catalytic allosteric pocket on the enzyme. Although the NRTIs and NNRTIs differ with respect to their site of interaction on the enzyme and molecular mechanism, both affect the DNA polymerization activity of the enzyme and block the generation of full-length viral DNA.

The pre-integration complex generated after reverse transcription comprised of viral as well as cellular components, is transported to the nucleus where the second essential HIV-1 enzyme, integrase, catalyzes the integration of the viral DNA with the host DNA. Integrase orchestrates three sequence-specific events required for integration; assembly with the viral DNA, endonucleolytic processing of the 3^1 ends of the viral DNA, and strand transfer or joining of the viral and cellular DNA. The newest class of approved integrase inhibitor (INIs or InSTIs), specifically inhibit strand transfer and block integration of the HIV-1 DNA into the cellular DNA (Craigie and Bushman, 2011). Following integration, the cellular machinery can initiate transcription; however transcript elongation requires binding of the HIV-1 regulatory protein Tat to the HIV-1 RNA element (TAR) (Karn and Stoltzfus, 2011). This mechanism is unique to HIV-1 and is thus considered a highly desirable therapeutic target. A variety of candidate small-molecules inhibitor of either HIV transcription, or more especially the Tat-Tar interaction, has been identified (Hwang *et al*., 2003). Unfortunately, none of these compounds were sufficiently potent and/or selective to progress beyond phase I clinical trials. Recently, reports describe a new cyclic Tat peptidomimetic that binds to TAR with high affinity and show broad and potent HIV-1 inhibition. Surprisingly, this drug inhibits both HIV-1 reverse transcription and Tat-mediated mRNA transcription (Davidson *et al.*, 2009; Lalonde, 2011).

The final class of approved antiretroviruses (ARVs) in the context of HIV-1 life cycle is the HIV-1 protease inhibitors (PIs). Protease inhibitors block proteolysis of the viral polyprotein, a step required for the production of infectious viral particle (Sundquist and Krausslich, 2011). Protease inhibitors are among the most potent agents developed to date, but are large peptide-like compounds that generally require the co-administration of a ñboostingò agent to inhibit their metabolism and enhance drug level. Therefore, P1-containing regimens contain a fourth drug, albeit one, that does not directly contribute to overall antiviral actively. Ritonavir (RTV) is the only boosting agent or pharmacokinetic enhancer (PKE) available for use, although other compounds are in early stages of clinical development (Hsu *et al.*, 1998).

2.31: Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs)

NRTIs are administered as pro-drugs, which require host cell entry and phosphorylation by cellular kinases before enacting an antiviral effect (Hart *el al*., 1992). Lack of a 3^1-hydroxyl group at the sugar (2^1-deoxyribosyl) moiety of the NRTIs prevents the formation of a 3^1-5^1-phosphodiester bond between the NRTIs and in coming 5^1ï nucleoside triphosphates, resulting in termination of the growing viral DNA chain. Chain termination can occur during RNA-dependent DNA or DNA-dependent DNA synthesis, inhibiting production of either the (-) or (+) strands of the HIV-1 pro-viral DNA (Richman, 2001). Currently, there are eight FDA-approved NRTIs: abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, zidovudine and Tenofovir disoprovil fumarate. Resistance to NRTIs is mediated by two mechanisms: ATP-dependent pyrophosphorolysis, which is the removal of NRTIs from the 3^1 end of the nascent chain, and reversal of chain termination and increased discrimination between the native deoxyribonucleotid substrate and the inhibitor (Boyer *et al.*, 2001).

2.32: Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

NNRTIs inhibit HIV-1 by binding and inducing the formation of a hydrophobic pocket proximal to, but not overlapping the active site. The binding of NNRTIs changes the spatial conformation of the substrate-binding site and reduces polymerase activity. Unlike NRTIs, these non/uncompetitive inhibitors do not inhibit the RT of other lentiviruses such as HIV-2 and simian immunodeficiency virus (SIV) (Tantillo *et al*., 1994; Spence *et al*., 1995; Witvarouw *et al*., 1999). Currently, there are four approved NNRTIs: etravirine, delavirdine, efavirenz, and nevirapine. NNRTI resistance generally results from amino acid substitutions in the NNRTI-binding pocket of RT. The most common NNRTI mutations are K103N and Y181C (Tantillo *et al*., 1994; Demeter *et al.*, 2000; Dykes *et al*., 2001). Interestingly, the majority of NNRTI-resistance mutation selected under NNRTI treatment are commonly found as wild-type sequence in HIV-1 group O and HIV-2 group O can actually be subdivided into lineages based on a C181 or Y181 amino acid in reverse transcriptase (Tebit *et al*., 2010).

2.33: Integrase Inhibitors

Integrase was the most recent HIV-1 enzyme to be successfully targeted for drug development. Raltegravir (RAL) was FDA approved in 2007, and other integrase inhibitors including Elvitegravir, are progressing through clinical development. All integrase inhibitors in development target the strand transfer reaction and are thus referred to as either INIs or more specifically, integrase strand transfer inhibitors (InSTIs) (Hazuda *et al.*, 2004a; Shimura

et al., 2008; McColl and Chen, 2010). The selective effect on strand transfer is a result of a now well-defined mechanism of action in which inhibitors (1) bind only to the specific complex between integrase and the viral DNA and (2) interact with the two essential magnesium metal ion cofactors in the integrase active site and also the DNA. Therefore, all InSTIs are comprised of two essential components: a metal-binding pharmacophore, which sequesters the active site magnesium, and a hydrophobic group, which interacts with the viral DNA as well as the enzyme in the complex (Grobler *et al*., 2002). Mutations that engender resistance to InSTLs almost always within the inegrase active site near the amino acid residues that coordinate the essential magnesium co-factor (Hare *et al*., 2010).

2.34: Protease Inhibitors (PIs)

The HIV-1 protease is the enzyme responsible for the cleavage of the viral gag and gag-pol polyprotein precursors during virion maturation (Miller, 2001). Ten protease inhibitors are currently approved: amprenavir, atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, sequinavir, and tipranavir. All Protease inhibitors share relatively similar chemical structures and cross resistance is commonly observed. For most Protease inhibitors, primary resistance mutations cluster near the active site of the enzyme, at positions located at the substrate/inhibitor binding site. These amino acid changes usually have a deleterious effect on the replicative fitness (Quinones-Mateu *et al.*, 2008). In addition to mutations in the protease gene, changes located with eight major protease cleavage sites (gag and pole genes), have been associated with resistance to protease inhibitors (Miller, 2001). With protease inhibitors resistance, HIV-1 appears to follow a ñstepwiseò pathway to overcome drug selection: (1) acquisition of primary resistance mutation in the protease gene, (2) selection of secondary/compensatory protease mutations to repair the enzymatic function and rescue viral fitness, and (3) selection of mutation in the major cleavage sites of the gag and gag-pol polyprotein precursors that restore protein processing and increase production of the HIV-1 protease itself (Berkhout, 1999; Nijhuis *et al*., 2001).

2.35: Entry Inhibitors

HIV-1 entry exploits several host proteins for a set of intricate events leading to membrane fusion and virus core release into the cytoplasm. HIV-1 entry inhibitors can be subdivided into distinct classes based on disruption/inhibition of distinct targets/steps in the process.

2.36: Fusion Inhibitors

The crystal structure of the gp41 ectodomain and of the ectodomain partnered with an inhibitory peptide (C34) revealed that the fusion-active conformation of gp41 was a six-helix bundle in which three N helices form an interior, trimeric coiled coil onto which three antiparelleled C helices pack (Doms and Wilen, 2011). Peptide fusion inhibitors were designed based on the discovery that two homologous domains in the viral gp41 protein must interact with each other to promote fusion. So the mimicry of one of these domains by a heterologous protein can bind and disrupt the intramolecular interactions of the virus protein. Alpha-helical peptides homologous to the leucine zipper domain of gp41 had significant antiviral activity against HIV-1. Rational design of helical inhibitors ultimately produced a molecule (T-20, enfuvirtide) with potent antiviral activity in vivo (Wild *et al*., 1993; Lalezari *et al*., 2003).

Resistance to early alpha-helical inhibitors was shown to be mediated by mutation in the amino-terminal helptad repeat region of gp41 which provide further evidence for binding these peptides to the virus (Rimsky *et al.*, 1998). Mutations that confer resistance to enfavirtide result in reduced replication capacity/replicative fitness presumably because mutations that reduce enfuviride binding also reduce the efficiency of six-helix bundle formation and overall fusion rate. These mutations do not confer cross resistance to other entry inhibitors (attachment or co-receptor inhibitors) but can sensitize viruses to neutralization by monoclonal antibodies that target the gp41 domain by prolonging the exposure of fusion intermediates that are specifically sensitive to these antibodies (Reeves *et al*., 2005; Ray *et al*., 2005).

2.37: Small-Molecule CCR5 Antagonists

Small-molecule CCR5 antagonists bind to hydrophobic pockets within the transmembrane helices of CCR5 (Tsamis *et al*., 2003). This site does not overlap the binding sites of either agonists or HIV-1 envelope. Instead, drug binding induces and stabilizes a receptor conformation that is not recognized by either. Thus, these molecules are considered allosteric inhibitors. Ideally, a small-molecule inhibitor of CCR5 would block binding by HIV-1 envelope but continue to bind native chemokines and allow signal transduction. Most small-molecule inhibitors however are pure antagonists of the receptor. Oral administration of small-molecule antagonists has been shown to inhibit viral replication in macaque models and prevents vaginal transmission of the virus. Thus for, three antagonists (VCV, MVC and Aplaviroc) have been shown to inhibit viral replication in human (Veazey *et al.*, 2003; Veazey *et al*., 2005; Dorr *et al*., 2005). Since MVC binds to a host cell protein, resistance to

MVC is unlike that of other antiretroviruses. Potential resistance mechanism include: (1) tropism switching (utilization of CXCR4 instead of CCR5) for entry, (2) increased affinity for the receptor (3) utilization of inhibitor-bound receptor for entry, and (4) faster rate of entry. Evaluation of co-receptor tropism of viruses from patients who failed MVC therapy during trials have suggested that tropism change occurred only when X4 tropic viruses were preexisting in the patient quasispecies before initiating treatment with MVC (Westby *et al*., 2006).

2.38: Conclusion of literature review

Although neutralizing antibodies are present in HIV infected individuals, it is clear that they fail to effectively inhibit virus replication. This failure was observed to be due to the rapid appearance of HIV variants during the course of HIV infection (Trembley *et al*., 1990; Pathak *et al*., 1990; Oxenius *et al*., 2004). The extensive production of HIV is closely related to the destruction and replacement of $CD4^+$ T-cells (Ondoa *et al*., 2005; Kaushik *et al*., 2006). It is reported that during the course of HIV infection, about one billion HIV particles are produced per day, resulting in increasing number of infected $CD4^+$ T-cells. The infection spreads in the memory cells, in the naïve $CD4^+$ T-cells and in the thymus; the source is therefore progressively exhausted (Autran, 2000; Appay and Sauce, 2008).

Though, several researches have been carried out on $CD4^+$ T-cell replication/depletion during different stages of HIV infection, the true definition of $CD4^+$ T-cell loss in HIV disease is still unclear (Deeks *el al*., 2004; Brenchley *el al*., 2004; Dandekar, 2007). On the other hand, despite the significant progress in the field of anti-retroviral chemotherapy with the development of highly active antiretroviral therapy (HAART) (Sammodosis *et al*., 1993), the total eradication of HIV, especially from the immune cells in the tissue sanctuary sites of an infected individual is still a mirage. Also, the withdrawal or interruption of the therapy returns a measurable plasma level of HIV in less than two weeks (Greeraert *et al*., 2008). Therefore, it is urgently needed that more studies are designed to find out the true picture of $CD4^+$ T-cell depletion in HIV disease and possibly identify a better drug target for total eradication of HIV in an infected individual.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Subjects and Study Design

This project was designed to have a total of three study groups:

(1) Diagnostic HIV Subjects; people infected with human immunodeficiency virus but have not started taking anti-retroviral drugs.

(2) HIV-positive subjects on highly active anti-retroviral therapy (HAART).

(3) HIV-negative subjects that were used as control subjects.

Members of the three groups were recruited to participate in the project after clinical and laboratory assessment as stated in the criteria for inclusion and exclusion.

A total of ninety subjects participated in this project under the three study groups. Each of these groups had a total of thirty (30) subjects as a study population. The test subjects were recruited from HIV/AIDS patients attending clinics at the University of Nigeria Teaching Hospital Ituku ï Ozalla, Enugu State University Teaching Hospital (Park Lane), Annunciation Specialist Hospital Emene and Mother of Christ Specialist Hospital, all in Enugu, Nigeria. Control subjects were recruited from member of the public, students and staff of the hospitals stated above. Sample collection was completed within three months.

Criteria for Inclusion and Exclusion

Qualification for recruitment into this study was based on the outcome of oral-interview/questionnaire, clinical and laboratory assessments. Each person recruited after the assessment, was included as a participant after an informed consent has been duly signed by him/her. Such participant was then grouped according to his/her HIV status and whether he/she has started anti-retroviral drug or not. The research considered only those on first line HAART drug combination and basically those on Zudovudine, lamivudine and nevirapine drug combination. This is nucleoside/nucleotide and non-nucleoside reverse transcriptase inhibitor drug combination. With the approval of the ethics committee, the research was carried out at the Immunology Research Unit of the Department of Haematology and Immunology, University of Nigeria Teaching Hospital, Itukuï Ozalla, Enugu State.

Sample Collection

A total of 8mls of blood was collected from each subject using dipotassium ethylendiamine tetra-acetic acid (EDTA) bottle. The samples were maintained at 2 to 8^0C temperature range, and transferred immediately to the laboratory for pre analytical sample

processing. After pre-analytical procedure, the sample aliquots were stored according to the analytical procedure for each variable. 500µl of the whole blood was delivered into 2ml cryovial and stored in liquid nitrogen for cluster of differentiation (CD) marker studies. The remaining whole blood was separated into plasma and packed cells. The plasma samples were frozen at -85^{0}C in two aliquots while the sedimented cell samples were immediately used to harvest white blood cell pellets. The plasma samples were used for biochemical analysis and HIV viral load estimation.

METHODOLOGY

3.4: Pre–Extraction Processing of Whole Blood Sample

Whole blood samples were processed to white blood cell pellets using ammonium chloride red cell lysis buffer (RCLB) method. The white blood cell pellets were subsequently lysed in guanidine Isothiocyanate (GITC) buffer to obtain GITC lysate.

Materials and Reagents

(1) 5 ï 10ml whole blood
(2) 15ml plain tube or falcon tubes
(3) 20ml Universal container
(4) Cold Red Cell Lysis Buffer (RCLB, pH 7.4).
(5) Gene Vortex Machine
(6) Centrifuge (Microï centrifuge).
(7) Blunt end needle (18G) and 2ml syringes.
(8) Laminar flow hood (Biological class 2)
(9) Phosphate buffered saline (PBS, pH 7.2)
(10) GITC buffer and 2ï B Mercapto ethanol (BME) (10µl of BME per 1ml of GITC buffer).
(11) Waste bucket containing virkon
(12) Bucket of Ice

Fig.3.1a Gene vortex machine (UK)

Fig.3.1b Spectrafuge 16M (USA)

Procedure

(1) Whole blood sample was transferred into a 15ml tube or falcon tube and labeled accordingly.

(2) 10ml of cold RCLB was added to the blood sample and mixed by inversion.

(3) Tube was placed on ice for 10 minutes.

(4) Wiped and centrifuged at 1870s for 7 mins.

(5) Supernatant was carefully decanted into the waste bucket with virkon.

(6) 10ml of cold RCLB was added to the pellet, mixed by vortexing and steps 3 to 5 repeated

(7) 10ml of cold RCLB was again added to the cell pellet when traces of rbc were still seen, mixed by vortexing and repeated as in steps 3 ï 5

(8) 10ml of PBS was added to the cell pellets, mixed by vortexing and centrifuge at 1870g for 7 min.

(9) Supernatant decanted into the waste bucket; 5ml of PBS added, mixed by vortexing and centrifuged at 1870g for 5 min.

(10) The supernatant was decanted into the waste bucket, and the tube drained on a clean towel.

(11) 500µl of PBS was added to the pellets, mixed by vortexing and 200µl transferred to cryovial and stored at minus 20^0C.

(12) 1ml of GITC buffer containing 10µl of BME was added to the remaining white cell pellets in the tube.

(13) Using sterile Pasture pipette, the contents of the tube was transferred to a 20ml universal container.

(14) Using blunt end needle (18G) and 2ml syringe, the lysate was homogenized by aspirating and discharging for about 20 times.

(15) Using sterile pasture pipette, the GITC lysate was transferred into 2ml cryovial, labeled accordingly for and stored at minus 20^0C otherwise the lysate was used immediately for nucleic acid extraction.

(16) For quality control purposes, 1ml of the GITC buffer containing 10µl BME was also transferred into a cryovial, labeled accordingly and treated as a sample during nucleic acid extraction.

3.5: WBC Estimation and Viability Testing Using Countess Invitrogen Automated Cell Counter (10064-080, Korea.)

Principle

Blood samples are frequently analyzed by Laboratories and required standardized cell concentrations for most experiments. The Countess Automated Cell Counter introduces a great advance in cell counting. It uses the standard trypan blue technique for viability determination; digital image capture and sophisticated image analysis program to determine the cell count as well as the percentage viability of the population. Countess Automated Cell Counter is capable of counting Red and White blood cells from a wide range of collection media and processing technique but it gives best results with fresh, homogeneous samples diluted to approximately 10^6 cells/ml in a clear, isotonic, lowï protein buffer like Phosphate Buffered Saline (PBS).

Materials and Reagents

(1) Pre-extraction processed white cell pellet in 5ml PBS.

(2) 0.4% trypan blue (cat no. T10282).

(3) Countess Invitrogen Chamber Slides (cat no. C10228).

Fig.3.2: Countess Invitrogen automated cell counter (10064-080, Korea)

Procedure

(1) White cell pellet in 5ml PBS was homogenized using vortex.

(2) 10µl of homogenized white cell was added to a clean 5ml test tube.

(3) 10µl of trypan blue was added to the test tube and mixed well with the cells.

(4) 10µl of the mixture was transferred into one side of the countess chamber slide.

(5) The slide was inserted into the countess invitrogen automated cell counter

(6) The zoom function knob was used to adjust and focus the image to bring the objects into proper position.

(7) ñCount-cellò button was pressed to begin the process of acquiring and analyzing the image. The data readout was given in total cells/ml, life cells/ml, dead cells/ml, and percentage viability.

(8) ósaveô button was pressed to archive the result while a copy of the result was also made manually.

3.6: Total RNA Extraction using RNeasy Spin Column Extraction Kit (Qiagen-UK).

Materials and Reagents

(1) GITC and Reagents

(2) GITC lysate

(3) RNeasy Mini Kit Columns

(4) RW1 Buffer

(5) RPE Buffer

(6) Eppendorf tubes

(7) Sterile 1.5ml tubes and 2ml collection tubes.

(8) RNAase free water (ultra pure water).

(9) Centrifuge

(10) Biophotometer and cuvattes

(11) Bucket of ice cubes

(12) DNAse digesting Reagents

Procedure:

(1) 350µl of tissue lysate was transferred into eppendorf tube, 350µl of 70% ethanol added and mixed by pipetting.

(2) Sample mixture was transferred to RNeasy column in 2ml collection tube, closed and centrifuged for 15s at 8000g.

(3) Flow through in the collection tube was discarded and tube drained on a clean towel.

(4) 650µl buffer RW1 was added to RNeasy column, and centrifuge for 15s at 8000g, and flowï through discarded as in (3)

(5) 10µl of DNAse1 and 70µl of DNA buffer were mixed and transferred to the RNeasy column. Lid closed and incubated at room temperature for 15 min. 2µl of EDTA was added into the RNeasy column and allowed to stand for 2 min at toom temperature.

(6) 500µl Buffer RPE was added to RNeasy column. Lid closed and centrifuged for 15s at 8000g. Flowï through was discarded with collection tube and RNeasy column placed in a new collection tube.

(7) 500µl Buffer RPE was added to RNeasy column, lid closed and centrifuged for 2 min at 8000g. Flowï through discarded and collection tube changed.

(8) Column was centrifuged at 8000g for 1 min.

(9) RNeasy column was placed in a new 1.5ml sterile tube and 50µl RNase free water added. Lid was closed and centrifuged for 1min at 8000g, and the tube containing RNA elute was placed on ice, ready to be used for cDNA synthesis.

(10) 5µl of RNA elute was added to 95µl of RNAseï freeï water and read in a Biophotometer at A260/A280. The expected ratio of the reading was 1.70 ï 2.10, for good RNA extraction.

3.7: Estimation of DNA/RNA suitability (yield and purity) using Eppendorf Biophotometer Plus (AG22331 Hamburg, Germany) for the optimization and validation of DNA and RNA Extraction.

Principle

The ultra violet (UV) absorbance spectrum of DNA exhibits an Amax at 260nm based on the aromatic ring structures of the DNA bases. This is the most convenient way to estimate DNA concentration and calculate yield, as long as the DNA preparation is relatively free of contaminants that absorb in the UV. Proteins and residual phenol left from the isolation procedure are typical contaminants that may lead to an overestimated DNA concentration. The ratio of absorbance at 260nm and 280nm is used to assess the purity of DNA and RNA. A ratio of approximately 1.8 is generally accepted as ñpureò for DNA while a ratio of approximately 2.0 is generally accepted as ñpureò for RNA. If ratio is appreciably lower in

either case, it may indicate the protein, phenol or other contaminants that absorb strongly at or near 280nm.

Fig.3.3: Biophotometer (Eppendorf AG22331 Hamurg, Germany)

Procedure

(1) 100 µl of ultrapure water was transferred to Biophotometer cuvate and used for standardization of equipment.

(2) 95 µl of ultrapure water was transferred into a new cuvate and 5 µl of RNA or DNA eluate added.

(3) The cuvate was inserted into the Biophotometer and set at A260/A280.

(4) The reading expected for a good quality DNA or RNA to be used for gene analysis was a ratio of 1.70 to 2.10.

3.8: Complementary DNA Synthesis Using Total RNA Eluate

Principle:

Total RNA is converted to complementary DNA by random hexamer priming using murine moloney leukemia virus reverse transcriptase (Mï MuLVï Revers Transcriptase). Complementary DNA is more stables than RNA which is considered labile and easily degradable by ubiquitous RNases.

Materials and Reagents

(1) Total RNA elute on ice

(2) cDNA mix (210µl for 10 samples)

(3) RNasin (RNAse inhibitor ï 40 U/µl)

(4) M ï MuLV ï Reverse Transcriptase (200 U/µl).

(5) 2 dry blocks or water baths (one set at 65^0C and the other at 37^0C).

Procedure

(1) The RNA sample was placed in water bath or dry block at 65^0C for 10 min.

(2) cDNA cocktail was prepared by adding 12µl of RNasin and 24µl of Mï MuLVï Reverse Transcriptase to the 210µl of cDNA mix in a 2ml tube. Cocktail was mixed by brief vortex.

(3) 21µl of the cDNA cocktail was added into the RNA elute of sample or control (placed on ice during pipetting) and incubated at 37^0C for 2 hours in the dry thermal block.

(4) After 2 hours, the tube was transferred to water bath or dry block set at 65^0C for 10 min

(5) After 10min, pulseï centrifuged, labeled and stored accordingly for molecular analysis.

3.9: Extraction of Genomic DNA using QIAamp DNA Mini Kit (Qiagen, UK)

Materials and Reagents

(1) Tissue lysate

(2) AL buffer, TE buffer

(3) 100% Ethanol

(4) QIAamp DNA Mini Kit (Qiagen, UK).

(5) Pipettes and pipette tips with aerosol barrier.

(6) Micro-centrifuge (with rotor for 2ml tubes).

(7) Vortex Machine

Procedure:

(1) 200µl of tissue lysate was transferred to 2ml tube and 200µl of AL buffer added and mixed by pulseï vortex for 15 sec (White precipitate may appear).

(2) 200µl of 100% Ethanol was added and mixed by pulseï vortex for 15 sec (precipitates should disappear).

(3) Sample was incubated at room temperature for 5 min.

(4) Sample was applied to the QIAamp DNA micro column attached to a 2ml collection tube and centrifuged at 8,000rpm for 1 min to bind the DNA. Flowï through was discarded.

(5) 500µl AWI buffer was applied and column centrifuged at 8000rpm for 1 min. Flowï through was discarded and column returned to the collection tube after draining with a clear towel.

(6) 500µl AW2 buffer was applied and centrifuged at 8000 rpm for 1 min. Flowï through was discarded.

(7) The column was transferred to a new collection tube and centrifuged at 14,000rpm for 3 min.

(8) Column was transferred to a new 1.5ml collection tube.

(9) 25µl of TE buffer was applied and incubated at room temperature for 5 min to elute DNA.

(10) The 1.5ml tube was centrifuged at 14,000 rpm for 1 min to collect DNA solution.

(11) 5µl of DNA eluate was added to 95µl of ultra pure water for A260/A280 reading in a Biophotometer. Expected ratio is 1.60 ï 1.90. Can be stored at -20^{0}C or proceed to PCR quality control test.

3.10: HIV RNA Extraction using Thermo Scientific Viral RNA Purification Kit from Thermo Fisher Scientific Inc.

Principle

The viral DNA/RNA purification kit uses wellï established nucleic acid isolation and purification technique comprising of the following:

(1) The sample was lysed by incubation with lysis solution and proteinase K under denaturating conditions at elevated temperature (56^{0}C). The lysis solution and proteinase K inactivate both RNases and DNases, ensuring protection of viral nucleic acids against degradation.

(2) The lysed sample was transferred to a spin column where released viral nucleic acid immediately binds to the silica-based filter in the presence of chaotropic salts. The remaining lysate was removed by centrifugation.

(3) The remaining contaminants were removed during three wash steps using Wash Buffers 1 and 2, whereas pure nucleic acids remain bound to the membrane.

(4) Pure viral nucleic acids were released from the spin column filter using eluent. The resulting purified nucleic acids were ready for subsequent use in downstream nucleic acid applications.

Materials and Reagents

1. Plasma Sample
2. Column Preparation Liquid (CPL)

3. Lysis Solution (LS)
4. Wash Buffer 1 (WB1)
5. Wash Buffer 2 (WB2)
6. Eluent (EL)
7. Proteinase K (PK)
8. Carrier RNA (CR)
9. Spin Columns preassembled with Wash Tubes (SC)
10. Wash Tubes ï 2ml (WT) and Elution Tubes ï 1.5ml (ET).

Procedure

1. Spin Column Preparation:

50µl of column preparation liquid is added to the center of spin column membrane, so that the membrane will be entirely moistened.

2. Sample Lysis:

a. 200µl of plasma sample was loaded to an empty 1.5ml lysis tube.
b. 200µl of Lysis solution (supplemented with carrier RNA), and 50µl of proteinase K were added and mixed thoroughly using vortex.
c. The sample was incubated for 15 min at 56^0C in a thermomixer.
d. Centrifuged for 5s at full speed to collect any sample solution from the inside of the lid.

3. Adjusting Binding Condition

a. 300µl of ethanol (100%) was added and mixed using vortex.
b. Sample incubated at room temperature for 3 min.
c. Centrifuged for 5 sec at 8000g, to collect drops from the inside of the lid.

4. Binding nucleic acids to the spin column:

a. The lysate was transferred to the prepared spin column pre assembled within the wash tube.
b. Column was centrifuged for 1 min at 6,000g.
c. The wash tube containing flowï through was discarded.
d. Spin column was placed into a new 2ml wash tube.

5. Washing with Wash Buffer 1:

a. 700µl of Wash Buffer1 supplemented with ethanol was added to the spin column.
b. Column was centrifuged for 1min at 6,000g.
c. Wash tube containing the flowï through was discarded.

d. Column was placed into a new 2ml wash tube.

6. Washing with Wash buffer2:

a. 500µl of wash Buffer2 supplemented with ethanol was added to the spin column.

b. The column was centrifuged for 1 min at 6,000g.

c. The wash Tube containing the flowï through was discarded.

d. Spin column was placed into a new 2ml wash tube.

7. Washing with Wash Buffer2 is repeated:

8. Spin dry:

a. Spin column was centrifuged for 3min at 16,000g.

b. The wash tube containing the flowï through was discarded.

9. Elution of Pure Nucleic Acids:

a. Spin column was placed into a new 1.5ml elution tube.

b. 50µl of eluent preheated to 56^0C was added to the center of spin column membrane.

c. Incubated for 2min at room temperature

d. Colum centrifuged for 1 min at 13,000g.

e. Spin column was discarded.

10. Storage and Use in Downstream Application

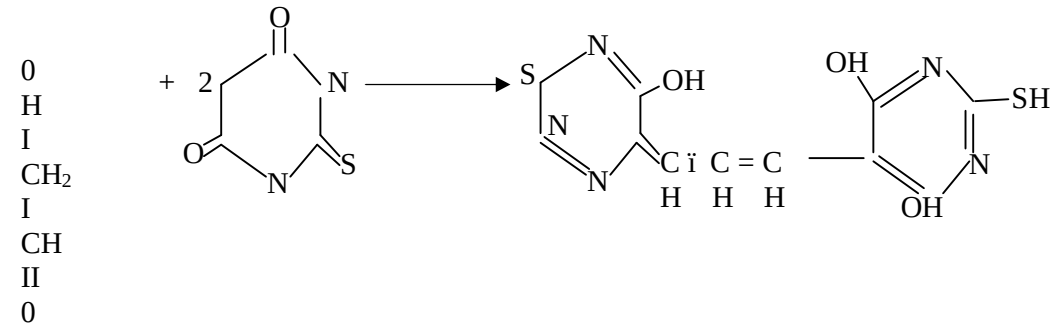
a. 1.5ml elution tube containing pure viral nucleic acids was labeled and used immediately or stored atï 20^0C for further use in downstream qPCR application

b. The eluted viral RNA was converted to cDNA by reverse transcription

3.11: Malondialdehyde (MDA) estimation using Northwest Life Science Specialties (NWLSS^{TM}) MDA Assay Kit, from AMs Biotechnology (Europe) LTD.

Principle:

The NWKï MDA01 assay is based on the reaction of MDA with thiobarbituric acid (TBA); forming an MDAï TBA_2 adduct which absorbs strongly at 530mm



Reaction between MDA and TBA

Butylate hydroxytoluen (BHT) and EDTA were added to the sample and reaction mixture to minimize oxidation of lipids that contribute artifactually during sample processing and the TBA reaction. The temperature of the reaction mixture has also been reduced to minimize the decomposition of lipid hydroperoxides. Because much of the MDA is protein bound, mostly as a Schiff base, the pH of the reaction has been optimized to facilitate hydrolysis of the MDA. Additionally, the reaction mixture is subjected to derivative spectrophotometric analysis that resolves the problem of the variable and nonlinear baseline observed when attempting to measure the A532 absorbance in various biological samples.

Materials and Reagents

2 ï Thiobarbituric acid, dry powder (TBA Reagent) Butylated hydroxytoluene in ethanol (BHT Reagent), 1M phosphoric acid (Acid Reagent). Phosphate buffer, pH 7.0 with EDTA (Assay Buffer) Tetramethoxypropropane in a stabilizing buffer at 4,3,2,1 and 0 µM MDA equivalents (Calibrators), Deionized water, magnetic stir bar.

Reagent Preparation

Into the bottle of TBA reagent, 10.5ml of deionized water was added and magnetic stir-bar inserted, and mixed until the TBA powder dissolved.

Assay Protocol

1. 10µl of BGT reagent was added to microcentrifuge vial.
2. 250µl of Acid Reagent was added
3. 250µl of TBA Reagent was added and the vial vigorously vortexed.
4. The microcentrifuge vial was incubated for 1hr at 60^0C and centrifuged at 10,000g for 3 minutes.
5. Reaction mixture was transferred to ELISA micro-well plate and read in 96 ELISA micro-well plate Reader at 400 ï 700nm spectra.
6. 3^{rd} derivative analysis was performed using calibrator data of 3^{rd} delivation peaks at 514nm.

3.12: Total Antioxidant Capacity estimation using Abcam Plc Assay Kit, 330 Cambridge Science Park, Cambridge, CB4 OFL, UK.

Principle:

Measurement of the combined non enzymatic antioxidant activity of biological fluids and other samples provides an indication of the overall capacity to counteract reactive oxygen

species (ROS), resist oxidative damage and combat oxidative stressï related diseases. In some cases, the antioxidant contribution of protein is desired whereas in other cases only the contribution of the small molecule antioxidants is needed. The Total Antioxidant Capacity Assay Kit can measure either the combination of both small molecule antioxidants and proteins or small molecules alone in the presence of the protein mask. Cu^{++} ion is converted to Cu^{+} by both small molecule and protein. The protein mask prevents Cu^{++} reduction by protein, enabling the analysis of only the small molecule antioxidants. The reduced Cu^{+} ion is chelated with a colorimetric probe giving a broad absorbance peak around 570nm, proportional to the total antioxidant capacity.

Reagents and Reagent's Reconstitution

Reagents included Cu^{++} Reagents, Assay Diluent Protein Mask and Trolox Standard.

1. Cu^{++} Reagent, Assay Diluent and Protein Mask were ready to use as supplied in the kit.
2. Trolox standard (lyophilized) is dissolved in 20µl of DMSO by vortexing. 980µl of distilled water is added and mixed to give a final concentration of 1mM solution.
3. Just before use, one part of Cu^{++} Reagent is diluted with 49 parts of Assay diluents to obtain a working solution of the Cu^{++} Reagent.

Procedure

(1) Trolox standard curve was prepared by adding 0, 4, 8, 12, 16, 20µl of Trolox standard to individual wells.

(2) Volume of each well was adjusted to 100µl with distilled water to give final concentration of 0, 4, 8, 12, 16 and 20nmol of Trolox Standard respectively.

(3) 100µl of serum samples were added to separate wells.

(4) 100µl of Cu^{++} working solution was added to all standard and sample wells.

(5) Plate was covered and incubated at room temperature for 1½ hrs.

(6) Absorbance was read at 570nm using micro plate reader.

Calculation

Absorbance of Trolox standard at 570nm was ploted in a standard curve as a function of Trolox concentration. Sample antioxidant Trolox equivalent concentrations were determined from the standard curve using the following equation:

Sample antioxidant capacity = (Sample absorbance ï blank absorbance) x (µl of sample)/slope of standard curve). nmol/µl or mM trolox equivalent.

3.13: Estimation of IL-2 concentration using Abcam Human IL–2 ELISA Kit from Abcam PLs, 330 Cambridge Science Park, Cambridge, CB4 OFL, UK.

Principle:

The 1Lï 2 Kit is a solid phase sandwich enzyme linkedï immuno-sorbent assay (ELISA). A polyclonal antibody specific for 1Lï 2 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known 1Lï 2 concentrations, control specimens and unknown samples are pipetted into the wells. During the first incubation, the 1Lï 2 antigen and a biotinylated monoclonal antibody specific for 1Lï 2 are simultaneously incubated. After washing, the enzyme (stieptavidinï peroxidase) is added. After incubation and washing to remove all unbound enzyme, a substrate solution which is acting on the bound enzyme is added to induce a colored reaction product. The intensity of this colored product is directly proportional to the concentration of 1Lï 2 present in the sample.

Materials and Reagents

96 well microtiter plates, plastic cover, standard (1000pg/ml), control, Standard Diluent Buffer, Standard Diluent (human serum), Biotinylated antiï 1Lï 2, Biotinylated Antibody Diluent, Streptavidinï HRP Washing Buffer, Chromogen TMB, Stop Reagent (H_2SO_4).

Reagent Preparation

(1) **Standard**

Because biological fluids might contain proteases or cytokineï binding proteins that could modify the recognition of the cytokine to be measured, the standard vials were reconstituted with human serum as recommended by the manufacturer. This reconstitution produces a stock solution of 1000pg/mL 1L2. The standard was allowed to stand for 5 minutes with gentle swirling prior to making dilutions. Serial dilution of standard was made before the assay. Standard buffer diluents 10X concentrate was diluted 10 times with distilled water before use to obtain 1X.

(2) Control

Freezeï dried control vials were reconstituted with human serum. Reconstitution of freezeï dried material with the recommended volume gave a solution for which the 1Lï 2 concentration is stated on the vial. It was allowed to stand for 5 minutes with gentle swirling prior to distribution in the control wells.

Dilution of biotinylated anti–1L–2

Biotinylated anti-IL-2 was diluted with the biotnylated antibody diluents in a clean glass vial according to the number of wells to be used. For 96 wells, 6360µl of biotinylated antibody diluent was used to dilute 240µl biotinylated antibody. This was done immediately before use.

Dilution of Streptavidin–HRP

0.5ml of HRP diluents was used to dilute 5µl vial of streptavidinï HRP. Furth dilution of streptavidinï HRP was made with HRP diluent in a clean glass vial according to the number of wells to be used. 10ml of streptavidinï HRP diluent was used to dilute 150µl of streptavidineï HRP for 96 well assay.

Washing Buffer 200X Concentrate:

This was diluted 200 times to obtain 1X wash buffer.

Procedure:

1. Reagents were thoroughly mixed before use without allowing it to foam.
2. The number of samples to be run, including standards and controls were asertianed to determine the number of wells and microwell strips to be used for each batch.
3. 100µl of standard diluents was added to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2. 200µl of standard was transferred from A1 and A2 to B1 and B2 respectively. The contents of B1 and B2 were mixed by pipetting and 100µl transferred to wells C1 and C2 respectively. The above procedure is repeated from wells C1 and C2 to D1 and D2 and so on until the last wells F1 and F2 from where 100µl were discarded. This created two parallel rows of 1Lï 2 standard dilutions ranging from 1000 to 31.25 pg/ml.
4. 100µl of standard diluent was added to each of the blank wells (G1 and G2).
5. 100µl of the reconstituted control was added to each of the control wells (H1 and H2).
6. 100µl of the sample was added to the respective sample well.
7. 50µl of the diluted biotinylated antiï 1L2 was added to each well.
8. The plate was covered with the plastic plate cover and incubated for 1hr at room temperature.
9. Plastic plate cover was removed and the micro well titer plate washed three times by dispensing 0.3ml of wash solution to each well and aspirating carefully without dislodging the bound antibodies.
10. 100µl of HRP solution was dispensed into each well, including the blank wells.

11. The micro titer well plate was covered and incubated at room temperature for 3 minutes.
12. Micro well Plate cover was removed and plate washed with wash solution as in step 9.
13. Immediately after the last wash, 100µl of readyï toï use TMB substrate solution was pipetted into each well including the blank wells and incubated in the dark for 15 minutes. The covered plate was wrapped in aluminium foil to prevent direct exposure to light.
14. 100µl of H_2SO_4: stop reagent was quickly pipetted into each well immediately after incubation to completely and uniformly inactivate the enzyme.
15. Plate was read in 96 well micro-titer plate reader using 450nm as the primary wavelength and 620nm as the reference wavelength.
16. 1Lï 2 concentration in the samples was determined using the standard calibration curve.

3.14: HIV Screening Test Using Genscreen ULTRA HIV Screening Kit for the Detection of HIV P24 Antigen and Antibodies to HIV–1 and HIV–2 in Human Serum/Plasma by Enzyme Immunoassay (BIO–RAD).

Principle:

The Genscreen™ ULTRA HIV Agï Ab is an enzyme immunoassay based on the principle of the sandwich technique for the detection of HIV antigen and of the various antibodies associated with HIVï 1 and / or HIVï 2 virus in human serum or plasma. The solid phase is coated with:

(1) Monoclonal antibodies against P24 HIVï 1 antigen.

(2) Purified antigen: gp160 recombinant protein, synthetic peptide mimicking a totally artificial HIVï 1 group Oï specific epitope and a peptide mimicking the immunodominant epitope of the HIVï 2 envelope protein.

The conjugates are based upon the use of:

(1) Biotinylated polyclonal antibodies to HIV Ag (conjugate 1).

(2) Streptavidin and HIV antigensï peroxidase conjugate (gp41 and gp36 peptides mimicking the immunodominant epitopes of the HIVï 1 and HIVï 2 envelope glycoproteins, and the same synthetic peptide mimicking a totally artificial HIVï 1 group Oï specific epitope used for the solid phase) (conjugate 2).

Conjugate 1 is added into the micro-plate wells. Serum samples to be assayed and controls are pipetted into their respective wells. If present, HIV antigens bind with the monocloral antibody bound to the solid phase and the conjugate 1. HIVï 1 and/or HIVï 2 antibodies, if any, bind to the antigens immobilized on the solid phase. Deposition of conjugate 1 and sample is validated through a colour change, from yellowï green to blue. After incubation and washing, and addition of conjugate 2, streptavidin reacts with biotinylated Abï Agï Ab complexes. Peroxidase labeled, purified HIVï 1 and HIVï 2 antigens bind in turn to the 1gG, 1gM or 1gA antibodies captured on the solid phase. After incubation and washing, and incubation in the presence of substrate, the presence of the complexed conjugate is shown by colour change which can be read spectophotometrically at 450/620 ï 700nm to determine the presence or absence of HIV Ag or HIVï and/or HIVï 2 antibodies.

Materials and Reagents

(1) Microplate Wells, coated with monoclonal antibodies to p24 HIVï 1 (Mouse) and purified HIVï 1 and HIVï 2

(2) Washing solution (Tris NaCl buffer ï pH 7.4).

(3) Heat inactivated human plasma, negative for HBs antigen, HIV antigen, antiï HIVï 1, antiï HIVï 2 and antiï HCV antibodies as negative control.

(4) Heat inactivated human plasma positive for antiï HIV antibodies, negative for HIV and HBs antigens and antiï HCV antibodies, in synthetic diluents as HIV antibody positive control.

(5) Purified HIVï 1 antigen inactivated with a chaotropic agent, in synthetic diluents, as HIV antigen positive control.

(6) Biotinylated polyclonal antibodies to p24 HIVï 1 (sheep) as conjugate 1ï yellowï green.

(7) Lyophilised peroxidase labeled streptavidin and purified HIVï 1 and HIVï 2 antigens as conjugate 2.

(8) Kimmed red milk solution as conjugate ï 2 diluent.

(9) Sodium citrate and sodium acetate solution pH - 4.0, with H_2O_2 (0.015%) and DMSO (4%) as peroxdase substrate buffer.

(10) Solution containing tetramethyl benzidine (TMB) as chromogen.

(11) 1N sulphuric acid solution as stopping solution.

(12) Distilled water.

Assay Procedure

(1) 25µl of conjugate 1 was pipette into each well, 75µl of HIV Ag positive control into well A1, 75µl of HIV Ab positive control into well B1.

(2) 75µl of negative control was pipetted into wells C1, D1 and E1.

(3) 75µl of specimen 1, 2, 3 etc were pipetted into the following wells respectively.

(4) After pipetting step, the microplate was covered with adhesive film and the mixture homogenized by shaking the micro-plate.

(5) It was incubated at 37^0C for 1hr.

(6) After incubation, the plate was washed with automatic washer, allowing a soak time of 30 seconds. The residual wash solution was removed by turning the plate upside down on absorbent paper.

(7) 100µl of conjugate-2 solution was quickly dispensed into all wells.

(8) Micro-plate was covered with adhesive film and incubated at room temperature for 30 minutes.

(9) After incubation the plate was washed as in step 5.

(10) 80µl of freshly prepared substrate solution was quickly dispensed into each well and allowed to stand in the dark without cover, for 30 minutes at room temperature.

(11) 100µl of stopping solution was added to each well using the same sequence as for substrate distribution.

(12) Optical density of coloured solution was read within 30 minutes of stopping the reaction, at 450/620 ï 700nm using micro-plate reader.

(13) Calculation and interpretation of results. The presence or absence of detectable HIV antigen or antibodies to HIVï 1 and/or HIVï 2 was determined by comparing the absorbance measured for each sample to the calculated cut ï off value. Mean absorbance of the negative control (ODR3):

$$ODR3 = \frac{OD(C1) + OD(D1) + OD(E1)}{3}$$

Cut ï off (CO) value is given as:

$$CO = ODR3 + 0.200.$$

Sample with absorbance values less than the cutï off value (CO) was considered negative by the Genscreen ULTRA HIV Agï Ab test.

3.15: HIV–1/2 Confirmatory Test Using Quali Code HIV–1/2 Kit, from Immunetics; Inc. 27 Drydock Avenue, Boston USA.

Principle

The Qualicode HIVï 1/2 kit is a Qualitative Immunoblot Assay on the Western Blotting Principle. The assay is performed on an immunoblot membrane containing HIVï 1 viral lysate protein (HTLVï III B Strain) and a recombinant HIVï 2 protein. To produce the membrane, HIVï 1 viral lysate proteins are fractionated according to molecular weight by electrophoresis on a polyacrylamide slab gel (PAGE) in the presence of sodium dodecyle sulfate (SDS). The separated HIVï 1 polypeptides are then transferred via electrophoretic blotting from the gel to a nitrocellulose membrane. Two bands are directly striped on the membrane: (1) a control Band comprising staphylococcal protein-A; (2) a recombinant HIVï 2 specific envelop antigen. The membrane is then cut into strips for individual sample testing. During the procedure, the strips containing HIVï 1/2 proteins are reacted with serum specimens and washed to remove unbound antibodies. Visualization of human immunoglobulin specifically bound to HIVï 1 or HIVï 2 proteins is performed by sequential reaction with goat antiï human immunoglobulin ï alkaline phosphatase conjugate and BCIP/NBT substrate. Band positions are compared to those on the reference, and developed using the HIVï 1/2 positive control serum. Intensity of the bands is monitored by comparison with the HIVï 1/2 weakly reactive control.

Materials and Reagents

(1) HIVï 1/2 membrane strips
(2) HIVï 1/2 positive control
(3) HIVï 1/2 weakly reactive control
(4) Human 1gG Negative control
(5) Buffer
(6) Milk based dilution buffer as sample diluents
(7) Alkaline phosphatase conjugated antiï human 1gG (Fc fragment specific)
(8) Alkaline phosphatase substrate solution.
(9) Incubation trays, record sheet, reference card.

Assay Procedure

(1) 1ml of 1X wash buffer was added to each active channel of the incubation tray.

(2) Individual strips were placed into wells (using forceps) with the strips number facing up, and incubated at room temperature on rocking platform until strips were homogeneously wet (1 minute).

(3) Wash buffer was aspirated and 1ml of sample diluents added to each active channel.

(4) 10µl of test sample, HIVï 1/2 controls, human 1gG negative control were added to the appropriate active channels and mixed by pipetting.

(5) Incubated for 2 hrs on rocking platform at room temperature, and solution aspirated.

(6) Active channels were rinsed 3 times by rocking with wash buffer for 3 minutes and aspirated.

(7) 1ml of antiï human 1gG conjugate was added to each active channel and incubated for 15 minutes on the rocking platform.

(8) It was aspirated and rinsed once by rocking with 1ml of 1X wash buffer for 3 minutes and aspirated.

(9) Active channel was rinsed with 1ml of distilled water once by rocking for 3 minutes.

(10) After aspiration, 1ml of substrate solution was added to each channel using multiï channel pipette to ensure a uniform reaction in all the channels.

(11) Active channels were incubated for 8 minutes on the rocking platform to initiate the colour reaction.

(12) Active channels were aspirated and rinsed two times with brief changes of distilled water to stop colour development.

(13) Strips were transferred face-up (using forceps) to a paper towel and allowed to air-dry.

(14) The dried strips provide a permanent record of the test result which can be interpreted at any time by aligning the strips and matching the bands with the band on the reference card provided in the kit.

3.16: Visualization of standard PCR product using Agarose gel Electrophoresis and UV–light To Determine the Quality of Primers and Gene Extraction.

Principle

Agorose is a threeï dimensional matrix, formed of helical agarose molecules in supercoiled bundles that aggregated into a threeï dimensional structure with channels and pores through which biomolecules can pass. The 3ï D structure is held together with

hydrogen bonds and can therefore be disrupted by heating back to liquid state. Depending on the source, it has a gelling temperature of 35 ï 42^{O}C and a melting temperature of 85 - 95 ^{O}C. Agarose gel has large pore size and good gel strength that makes it particularly suitable as an anti-convection medium for the separation of DNA and large protein molecules under electric current.

Materials and Reagents

(1) Agarose powder, 1X TAE buffer used to desolve agarose powder and as medium in the electrophoretic tank.

(2) Bromophenol blue as a loading dye and ethidium bromide as staining substance for UV visualization.

(3) Standard Ultraviolet (UV) trans-illuminator with wave length of 302/312 -nm and camera.

(4) Eletrophonetic tank and power-park.

(5) Microwave machine for heating the agarose in TAE buffer.

(6) Electrophoretic cast and comb for casting the gel after melting.

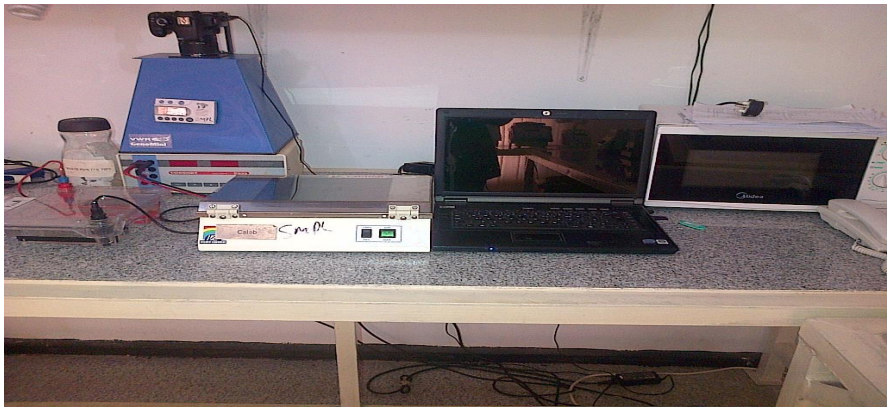


Fig.3.4: Agarose gel electrophoresis system.

Procedure

(1) Agarose powder (2g) was placed into a conical flask containing 100ml of 1X TAE buffer.

(2) The content is mixed by whirling the flask to allow the agarose to homogenize.

(3) The flask is loosely covered and placed in the microwave for 2 minutes to dissolve.

(4) It is allowed to cool after mixing with 10µl of ethidium bromide.

(5) The molten gel was poured into the cast with comb put in place.

(6) Agarose gel was removed from the cast after removing the comb, and placed on the electrophoretic tank.

(7) The gel was submerged wit 1X TAE buffer.

(8) 10µl of samples (Standard PCR Products), controls and ladder were mixed with loading dye and placed into separate wells in the gel.

(9) Electrophoretic tank was covered and connected to power-park, set at 100V and 5 AMP. System was allowed to run for about 25 minutes while observing the extent of the movement of loading dye.

(10) At the end of run, the gel was removed from the tank and placed on the UV trans-illuminator for visualization.

(11) Photograph of the bands were taken and saved in the computer.

(12) Bands of the tests and controls were compared with the standard bands of the ladder to determine if there was amplification, the number of base pair (bp) of the gene amplified and the intensity which somehow determine the concentration.

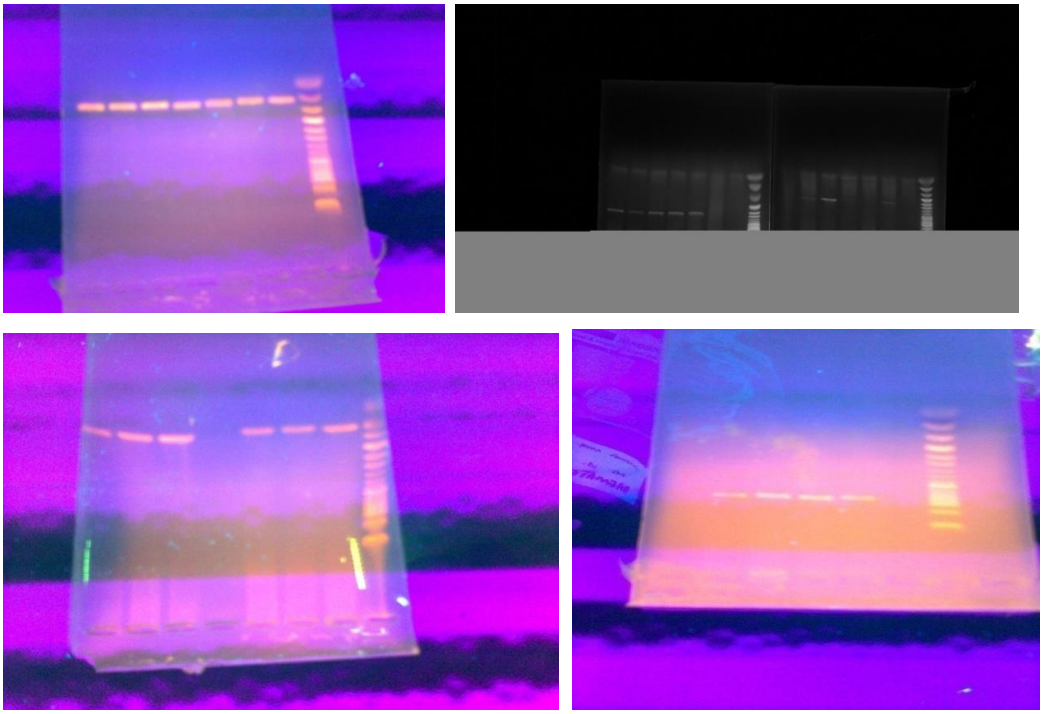


Fig.3.5: PCR product of cDNA and DNA in agarose gel electrophoresis

3.17: Primer Designing for standard PCR using Primer Express 3 online software from Applied Bio systems.

Principle

Polymerase Chain Reaction (PCR) is widely held as one of the most important inventions of the 20th century in molecular biology. Small amount of the genetic material can now be amplified to be able to identify and manipulate DNA, detect infectious organisms, including the viruses that cause AIDs, hepatitis and tuberculosis, detect genetic variations, including mutations, and numerous other tasks. PCR involves three steps namely: Denaturation, Annealing and Extension. The genetic material is denatured converting the double stranded DNA molecules to single strands. The primers are then annealed to the complementary regions of the single stranded molecules. Thirdly they are extended by the action of DNA polymerase. Good primer design is essential for successful reaction with specific amplification and high yield.

Procedure/Important Considerations

(1) **Primer Length:** The generally accepted optimal lent of PCR primer: 18 ï 22bp is considered. The length was made long enough for adequate specificity and short enough for easy binding at the annealing temperature.

(2) **Primer Melting Temperature (Tm):** The G-C content of the primer sequence was adjusted to give the melting temperature within the range of 52 ï 58^{O}C which produces the best result.

(3) **Primer Annealing Temperature:** The primer annealing temperature (Ta) was considered using the formula

$$Ta = 0.3 \text{ X } Tm \text{ (primer)} + 0.7 \text{ Tm (product)} ï 14.9$$

Where

Tm (Primer) = Melting temperature of the primers

Tm (Produt) = Melting temperature of the products

(4) **GC Clamp:** The presence of G or C bases within the last five bases from the 3^{1} end of the primers was made not more than three.

(5) **Primer Secondary Structure:** Presence of primer secondary structure was considered to avoid hairpins, self dimer and cross dimer.

(6) **Repeats:** Occurance of di-nucleotide more than four times was avoided during designing, to prevent misprime.

(7) **Runs:** consistent appearance of single base more than four in a primer was also avoided to prevent misprime.

(8) **Template Secondary Structure:** The primers were designed in the regions of the template that do not form stable secondary structure during PCR reaction.

(9) **Cross Homology:** Primers were BLASTed to test for specificity. Also, template was BLASTed to avoid regions of cross homologies during primer designing.

Fos (110)	-	Forward	CTGCTGAAGGAGAAGGAAAAACTAG
Fos (110)	-	Reverse	TCAAGGGAAGCCACAG ACATC
	-		
ILï 2(102)	-	Forward	TGGAACTAAAGGGATCTGAAACA
ILï 2(102)		Reverse	TGCTTTGACAAAAGGTAATCCA
Tim3(103)		Forward	CCAAGGTCACCCCTGCAC
Tim3(103)	-	Reverse	CCCAGTGTCTGTGTCTCTGC

3.18: Optimization and validation of gDNA and cDNA Extractions by Amplifying and visualizing House Keeping Genes (KLK_2 and ABL) using Thermo-Cycler (standard PCR) and Agarose Gel Electrophoreses.

Principle of PCR

Polymerase Chain reaction (PCR) is an invitro technique based on the principle of DNA polymerization reaction. It relies on thermal cycling consisting of repeated cycle of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using thermostable DNA polymerase, primer sequence (complementary to target region) and dNTPs. It thus, can amplify a specific sequence of DNA by as many as one billion times. Most PCR methods can amplify DNA fragments of 10 to 40 kilo base (kb) in size.

Materials and Reagents

(1) 2X Master Mix (Taq polymerase enzyme, dNTPs, $MgCl_2$ and PCR buffer) already optimized.

(2) 0.2ml PCR tubes, 1.5ml tubes, pipettes and pipette tips.

(3) Ultrapure water (DNAse-free and RNAse-free water)

(4) KLK_2 and ABL gene primers for gDNA and cDNA respectively.

(5) Standard PCR machine (thermocycler).

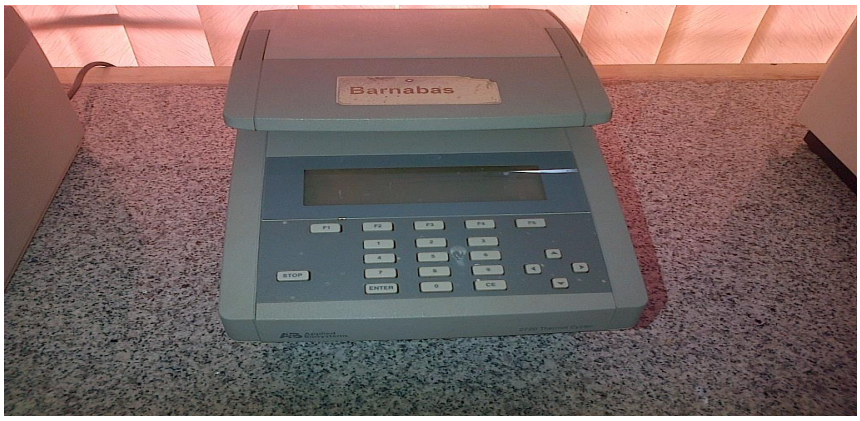


Fig.3.6: Thermocycler (2720) Applied Biosystems, UK.

Protocol/Procedure

(1) Working concentration of primer was calculated against PCR total volume of 20µl per PCR tube.

(2) Primer mix was prepared as follows:

	For one sample	For 5 samples; x 5
Forward primer	0.5µl	2.5µl
Reverse primer	0.5µl	2.5µl
Ultrapure water	7.0µl	3.50µl
Total	**8.0µl**	**40.0µl**

PCR Analysis

(3) Master mix 10µl
Primer mix 8µl
Sample elute (DNA) 2µl
Total volume 20µl

(4) PCR tubes were set up according to the number of samples and controls.

(5) 10µl of master mix was added to all the PCR tubes.

(6) 8µl of primer mix was added to the tubes.

(7) 2µl of control samples (ultrapure water, No amplicon control, No template control) were added to their respective tubes.

(8) 2µl of sample elute (DNA) were added to the sample tubes.

(9) PCR tubes were covered with their lids and puls-centrifuged to allow the contents to mix.

(10) PCR tubes with the materials were placed in the thermocycler and closed.

(11) Thermal profile of the PCR was set as follows:

Steps	**Temperature (^{O}C)**	**Duration**	**Description**	**No**
1	95	5 min	Initial Deneturation	X1
2	95	30 sec	Denaturation	
3	55	30 sec	Annealing	X35
4	72	2 min	Extension	X1
5	72	5 min	Final Extension	X1
6	4	Hold		X1

(12) After the selection of the contract programme from the thermocycler, the PCR was set to run for 40 cycles.

(13) After running the PCR products were subjected to Agarose gel electrophoresis.

(14) Presence of bands at the appropriate level of the ladder match, determined the amplification of the target region of the House keeping gene which validated the DNA extraction.

3.19: Optimization and Validation of Primers (Fos gene, IL–2 gene and Tim–3 gene) by amplification and visualization of the genes using cDNA samples.

Materials and Reagents

(1) 2X Master Mix (Taq Polimerase enzyme, dNTPs, MgCL2, and PCR buffer).

(2) Primers:

Fos (110)	-	Forward	CTGCTGAAGGAGAAGGAAAAACTAG
Fos (110)	-	Reverse	TCAAGGGAAGCCACAG ACATC
ILï 2(102)	- -	Forward	TGGAACTAAAGGGATCTGAAACA
ILï 2(102)		Reverse	TGCTTTGACAAAAGGTAATCCA
Tim3(103)	-	Forward	CCAAGGTCACCCCTGCAC
Tim3(103)	-	Reverse	CCCAGTGTCTGTGTCTCTGC

(3) PCR tubes were set up in the rack according to the number of samples and controls.

(4) 10µl of Master Mix was added to the PCR tubes

(5) 8µl of Primer mix was added to the tubes.

(6) 2µl of control samples were added to the control tubes.

(7) 2µl of sample elute (cDNA) was added to the PCR sample tubes

(8) PCR tubes were covered with their lids and pulsï centrifuged to mix the contents.

(9) PCR tubes were transferred from rack to the thermocycler and closed.

(10) Thermal profile programmed, complification cycle and total volume were selected and adjusted.

(11) The PCR machine was set to run.

(12) PCR products were subjected to Agarose gel electrophoresis and visualized using UV trans-illuminator.

3.20: Real–Time PCR quantification of Fos Gene, IL-2 gene, Tim-3 gene and ABL gene transcript number using Taqman Chemistry.

Principle

The Polymerase Chain Reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of DNA strand across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Almost all PCR applications employ a heatï stable DNA polymerase which enzymatically assembles a new DNA strand from DNA building block, the nucleotides, by using a simpleï stranded DNA as a template, and DNA oligonucleotides as a DNA primer. The vast majority of PCR methods use thermal cycling. In the first step the two strands of the DNA helix are physically separated at a high temperature in the process called melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA using primers. In Real Time PCR (RTï PCR), reaction exploits the 5ô nuclease activity of the ampliTaq Gold DNA polymerase to cleave a TaqMan probe during PCR. The Taq Man probe contains a reporter dye at the 5ô end of the probe and a quencher at the 3ô and of the probe. During extention, the 5ô nuclease activity cleaves the probe to separate the reporter and quench resulting in the emission of fluorescence light. This process occurs only if the target sequence is complementary to the probe and is amplified during PCR reaction. Accumulation of PCR products is detected directly by monitoring the

increase of fluorescence by reporter dyer. Transcript number is inversely proportional to the log of the cycle threshold (Ct) value.

Fig.3.7: ABI-Step one plus RT-PCR (USA)

Materials and Reagents

(1) 10X master mix (Taq polymerase enzyme, dNTPs, $MgCL_2$ and PCR buffer).

(2) Working concentration of primer and prob was calculated using PCR total volume of 20µl and sample volume of 5µl per tube.

		One Test	10 Samples
PCR Mix	-	10µl	100µl
Primer/Probe	-	2µl	20µl
Ultrapure water	-	3µl	30µl
cDNA	-	5µl	50µl
Total volume	-	20µl	200µl

(1) RTï PCR plate was set up in the rack.

(2) 10µl of PCR mix was transferred to all the wells

(3) 2µl of primer with probe was added to the wells

(4) 3µl of ultrapure water was added to the wells

(5) 5µl of cDNA of sample was added to the wells.

(6) 2µl of the primer with probe of the standard (ABL) was placed into the 5 wells for standard calibration.

(7) 3µl of ultrapure water was added into the five wells.

(8) 5µl of the five standards were added to the respective wells.

(9) 2µl of water was added to the negative control well.

(10) Plate was covered with cellophane paper and placed into the pre-set RTï PCR machine.

(11) Thermal profile was set up as follows:

Steps	**Incubation Hold**	**Enzyme Activation Hold**	**PCR**	
			40 Cycles	
Time	2 min	10 min	15 sec	1 min
Temp.	$42^{O}C$	$95^{O}C$	$95^{O}C$	$60^{O}C$

(12) RTï PCR was set to run for 40 cycles while the computer system plot the graph of fluorescence emission.

(13) At the end of 40 cycles the cycle threshold was set and the exponential fluorescence emission of the standard was plotted to determine the Ct value of the tests.

(14) Using the formula $2^{-ææCT}$ the relative transcript numbers of the samples were calculated after normalization.

3.21: Estimation of HIV Viral Load using Real Time–PCR method and TaqMan Chemistry.

Principle

This is based on extraction of viral RNA from both test and standard HIV samples, reverse transcription of RNA to complementary DNA (cDNA), amplification of target cDNA using HIVï 1 specific complementary primers with target-specific probe (TaqMan probe) and computerized detector system that monitors and records fluorescence emission. Plotting a graph between the Log of the starting amount of template in the standard and the corresponding increase in the fluorescence of the reporter signal during exponential phase of the PCR reaction, transcript number of the tests can be calculated using electronic device attached.

Materials

(1) PCR master mix (hot start)

(2) Primers

HIV 1-F	ACC CAT GTT TAC AGC ATT ATC AGA AG
HIV 1-R	GCT TGA TGT CCC CCT ACT GTA TTT
HIV 1-Probe	AGC CAC CCC ACA AGA TTT AAA CAC CAT GT

(3) cDNA of standard HIV and test samples

(4) ultra pure water

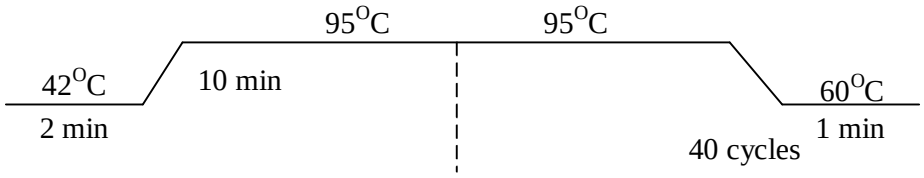
(5) TaqMan Probe with FAM and TAMRA dyes as reporter and quencher dyes respectively.

Procedure

(1) PCR Reaction

	One well	**10 wells**
PCR Master Mix	10µl	100µl
Primer/Probe	2µl	20µl
Ultra Pure Water	3µl	30µl
cDNA	5µl	50µl
Total volume	**20µl**	**200µl**

Thermal Profile



(1) PCR plate was set on a rack.

(2) 10µl of PCR master mix was transferred to all wells

(3) 2µl of Primer/Probe was added to the wells.

(4) 3µl of ultra pure water was added to the wells including standard.

(5) 5µl of standard was added to the five wells set out for standard.

(6) 5µl of tests cDNA were added to the wells designated tests.

(7) 5µl of ultra pure water was added to the negative control well.

(8) Plate was covered with transparent cellophane sticker and plateôs content mixed by gentle shaking.

(9) Plate was inserted into the pre-set RTï PCR machine and lid closed.

(10) Machine was set to run for 40 cycles while the electronic monitor system was set to record the signals and plot the amplification curve.

3.22: Estimation of CD markers (CD4, CD8, CD25 and CD38) using Accuri cytometer (C6 flow cytometer system).

Principle

A beam of light (usually laser light) of a single wavelength is directed onto a hydrodynamically focused stream of liquid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (forward scatter ï FSC) and several perpendicular to it (side scatter ï SSC) and one or more fluorescence detectors. Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analyzing fluctuations in brightness at each detector (one for each fluorescent emitting peak), it is then possible to derive various types of structures of each individual particle. FSC depends on the inner complexity of the particle (ie, shape of the nucleus, the amount and types of cytoplasmic granules or the membrane roughness).

Fig.3.8: C6 Accuri flow cytometer system

Procedure

(1) HPLC reagent grade filtered, deionized H_2O (0.22 fitter) with Bacteriostatic concentrate solution (Part # KRï 220) was added to fill the sheath bottle (sheath fluid).

(2) Diluted decontamination concentrate (Part # KRï 2000) and cleaning concentrate (Part # KRï 225) solutions were added to fill decontamination and cleaner bottles respectively.

(3) The bottles were placed in the fluidics bottle tray. The fluidics harness was connected to the cytometer and to the appropriate bottles in the correct orientation.

(4) For absolute CD count, 20µl of whole blood sample was added to the same tube.

(5) The content of the tube was mixed and incubated for 15 minutes in the dark at room temperature. 800µl of no lyse buffer was added and read in the flow cytometer.

(6) For percentage CD count 10µl of antibody A (antibody to the CD marker) was added into a test tube.

(7) 10µl of antibody B (antibody to CD45) was added to the tube.

(8) 20µl of whole blood sample was added to the tube, mixed and incubated in the dark at room temperature for 15 minutes.

(9) 400µl of no lyse buffer A was added after incubation.

(10) 400µl of no lyse buffer B was added and the solution read immediately in the flow cytometer.

(11) Using the computer software, the result (absolute CD count or percentage CD count) was determined and recorded.

3.23: Sequencing of IL-2 and Fos genes for single nucleotide polymorphisms (SNPs) using Sanger's method and Big Dye X Terminator chemistry

Principle

DNA sequencing is based on the ability of dideoxynucleotide triphosphates (ddNTPs) to prevent elongation of the newly synthesized strand if they are incorporated at the 3ô end to the daughter strand of DNA instead of the relevant deoxynucleotide triphosphate (dNTP). The product from four separate reactions, each containing all four dNTPs but a different ddNTP, are size separated on a gel in separate tracks. As the fragments migrate proportional to their length, a ladder pattern is generated in each lane in which each band represents a specific termination event, and the sequence can be read from the shortest fragment at intervals of one nucleotide across all four lanes. The process can be automatically detected and read electronically.

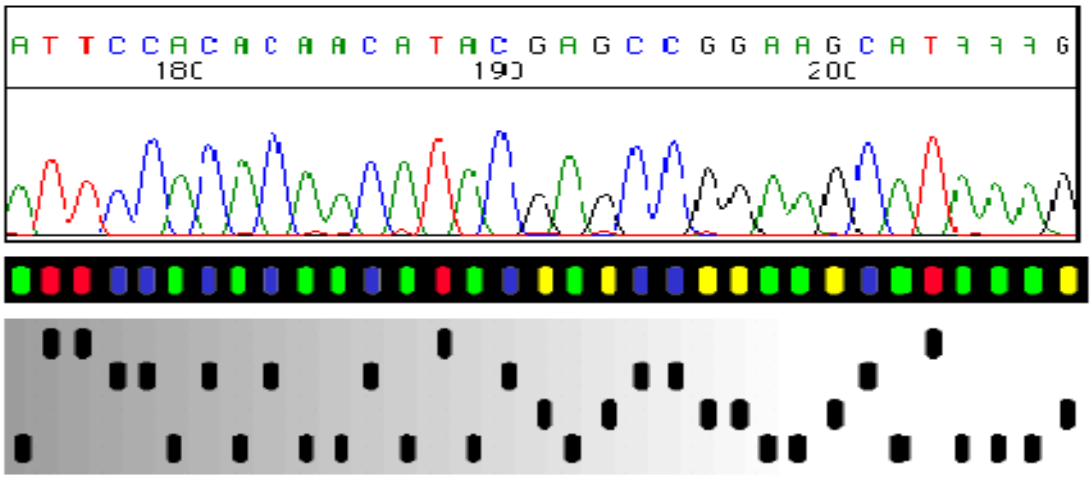


Fig.3.9: Gene sequence product

Procedure

1 in 10 dilutions of the PCR products were made. A sequencing reaction was set up in a 96 well plate as follows

DNA template	1µl
Primer (F or R)	1µl
Big Dye v 1.1	0.5µl
Sequencing buffer	2µl
Water	5.5µl
Final volume	10µl

The Big Dye and Sequencing buffer were purchased from Applied Biosystems, UK. A larger reaction mix could be set up by scaling all other reagents appropriately except the DNA template. The plate was sealed with microfilm and pulse-centrifuged. Covered with a compression pad (to prevent evaporation), the PCR sequencing reaction was performed for 25 cycles using the thermal profile:

96^{o} C for 10s

50^{o} C for 5s

60^{o} C for 4 min

The Sequencing PCR product was then purified using the Big Dye X terminator purification kit: To each well of PCR product, 45µl of SAM solution (Applied Biosystems, UK) and 10µl of Big Dye X terminator solution (Applied biosystems, UK) were added, the plate firmly sealed and mixed by vortex at 2000 rpm for 15 min. The plate was centrifuged at 1000g for 2 min and run on the Genetic Analyzer 3130 (Applied Biosystems, UK) according to the manufacturerôs instructions using the protocol BDX_x36cm_POP7_SetE.

CHAPTER FOUR

4.0 RESULTS

Plasma levels of IL-2 cytokine in diagnostic HIV positive subjects, HIV subjects on highly active antiretroviral therapy (HAART) and HIV seronegative control groups were presented in Table 4.1 the mean value of IL-2 cytokine secreted in the plasma of diagnostic HIV positive subjects (52.57 ± 23.53) differs significantly from that of HIV positive subjects on antiretroviral (ARV) drugs (99.32 ± 43.83) when compared statistically ($p<0.05$). There is also statistically significant difference between the mean values of IL-2 plasma levels of HIV positive subjects on ARV drugs (99.32 ± 43.83) and HIV seronegative control subjects (66.50 ± 29.90) ($p<0.05$). There was no significant difference observed when the mean values of IL-2 plasma level of diagnostic HIV positive subjects (52.57 ± 23.53) was statistically compared with that of the control subjects (66.50 ± 29.90) at 5% level of significance.

Table 4.1

Plasma IL–2 levels in diagnostic HIV positive subjects, HIV positive subjects on ARV drugs (HAART) and HIV Sero-negative control subjects.

Parameters (pg/ml)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	47.26	107.7	65.82
Mean	52.57	99.32	66.50
Standard Deviation	23.53	43.83	29.90
Minimum (pg/ml)	34.26	17.06	17.79
Maximum (pg/ml)	143.10	180.80	130.20
Normality	No	Yes	Yes
No of Samples	30	30	30

In Table 4.2, the malondialdehyde (MDA) levels in the plasma of diagnostic HV positive subjects, HIV positive subjects on ARV drugs and HIV seronegative control subjects were presented. The mean plasma MDA level of diagnostic HIV positive subjects (1.02±0.21) showed no statistically significant change when compared with that of HIV positive subjects on ARV drugs (0.85±0.23), ($p>0.05$). There was an observed significant increase when the mean plasma MDA level of diagnositic HIV positive subjects (1.02±0.21) and that of HIV positive subjects on ARV drugs (0.85±0.23) were statistically compared with the mean MDA level of HIV seronegative control subjects (0.44±0.18), ($p<0.05$).

Table 4.2

Plasma MDA levels in diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV Sero-negative control subjects.

Parameters (µM)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	0.99	0.85	0.40
Mean	1.02	0.85	0.44
Standard Deviation	0.21	0.23	0.18
Minimum	0.66	0.35	0.11
Maximum	1.56	1.21	0.85
Normality	Yes	Yes	No
No of Samples	30	30	30

The mean plasma level of total antioxidant status (TAS) in diagnostic HIV positive subjects (13.51±6.76) shown in Table 4.3 was statistically significantly reduced at 5% level of significance when compared with that of HIV seronegative control group (19.70±7.96). There was no statistically significance difference (P>0.05) observed when compared with the mean antioxidant capacity of HIV positive subjects on antiretroviral drugs (16.54 ± 8.00) with those of diagnostic HIV positive subjects (13.512 ± 6.76) and HIV seronegative control subjects (19.70 ± 7.96) respectively (p>0.05).

Tables 4.3

Plasma levels of TAS in diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV Sero-negative control subjects.

Parameters (mMol/L)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	11.06	13.88	19.42
Mean	13.51	16.54	19.70
Standard Deviation	6.76	8.00	7.96
Minimum	5.26	6.45	6.45
Maximum	32.75	43.57	40.86
Normality	No	No	Yes
No of Samples	30	30	30

Absolute $CD4^+$ T-lymphocyte count of diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV seronegative control subject were presented in Table 4.4. The mean value of $CD4^+$ T-lymphocyte count in diagnostic HIV positive subjects (246.60±186.10) was statistically significantly reduced ($P<0.05$) in comparison with that of the HIV seronegative control subjects (996.40 ± 207.10). Also, the mean $CD4^+$ T-lymphocyte count of HIV positive subjects on antiretroviral drugs (255.40±168.70) showed statistically significant, ($P<0.05$) when compared with that of the control group (996.40±207.10). At 5% level of significance, there was no statistically significant difference observed when the mean $CD4^+$ T-lymphocyte count of diagnostic HIV positive subjects (246.60±186.10) was compared with that of the HIV positive subjects on antiretroviral drugs (255.40±168.70), ($p>0.05$).

Table 4.4

$CD4^+$ T-lymphocyte levels in diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV Sero-negative control subjects.

Parameters (cells/µl)	Diagnostic HIV	ARV Drugs	HIV Seronegative
Median	177.00	202.50	976.50
Mean	246.60	255.40	996.40
Standard Deviation	186.10	168.70	207.10
Minimum	85.00	97.00	681.00
Maximum	920.00	819.00	1366.00
Normality	Yes	Yes	Yes
No of Samples	30	30	30

In Table 4.5, the mean absolute $CD8^+$ T-lymphocyte count of diagnostic HIV positive subjects (449.20±273.50) in comparison with that of the HIV positive subjects on ARV drugs (463.80±208.10) showed no significant difference, statistically ($p>0.05$). Furthermore, there was no statistically significant difference ($P>0.05$) when the mean $CD8^+$ T-lymphocyte level in HIV positive subjects on ARV drugs (463.80±208.10) was compared with that of the control group. On the other hand, there was significant difference ($p<0.05$) between the mean $CD8^+$ T-lymphocyte counts of diagnotstic HIV positive subjects (449.20±273.50) and HIV seronegative controls (613.20 ± 258.20).

Table 4.5

$CD8^+$ T-lymphocyte levels in diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV Sero-negative control subjects.

Parameters (cells/µl)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	405.50	407.50	562.00
Mean	449.20	463.80	613.20
Standard Deviation	273.50	208.10	258.20
Minimum	89.00	196.00	289.00
Maximum	1355.00	1048.00	1086.00
Normality	No	Yes	No
No of Samples	30	30	30

In Table 4.6, the percentage CD38 expression in diagnostic HIV positive subjects, HIV subjects on ARV drugs and HIV seronegative controls were presented. The mean percentage CD38 expression in diagnostic HIV positive subjects (25.59±7.31) was statistically none significant when compared with that (25.05±11.95) of the HIV positive subjects on ARV drugs, ($p>0.05$). When compared the mean percentage CD38 expression in diagnostic HIV positive subjects (25.59±7.31) with that of HIV seronegative controls (36.08±13.47), there was statistically significant difference ($p<0.05$). Also, there was statistically significant difference ($P<0.05$) between the mean percentage CD38 expressions of HIV seronegative control subjects (36.08±13.47) and HIV positive subjects on ARV drugs (25.05±11.95).

Table 4.6

Percentage CD38 expression in diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV Sero-negative control subjects.

Parameters (%)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	24.40	21.80	37.95
Mean	25.59	25.05	36.08
Standard Deviation	7.31	11.95	13.47
Minimum (%)	15.40	5.30	13.00
Maximum (%)	47.50	51.00	62.70
Normality	No	Yes	Yes
No of Samples	24	24	24

The mean percentage CD 25 expression of diagnostic HIV positive subjects (1.77±1.38) presented in Table 4.7, showed no statistically significant difference ($p>0.05$) when compared with those of HIV positive subjects on ARV drugs (2.26±1.18) and HIV seronegative control subjects (1.97±1.46). Also, HIV positive subjects on ARV drugs and HIV seronegative control subjects showed no statistically significant difference in their mean percentage CD25 expression (2.26±1.18 and 1.97±1.46 respectively) when compared at 5% level of significance, ($p>0.05$).

Table 4.7

Percentage CD25 expression in Diagnostic HIV positive subjects, HIV patients on ARV Drugs and HIV Sero-negative control subjects.

Parameters (%)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	1.45	2.30	1.55
Mean	1.77	2.26	1.97
Standard Deviation	1.38	1.18	1.46
Minimum (%)	0.00	0.00	0.30
Maximum (%)	5.10	4.50	5.50
Normality	Yes	Yes	Yes
No of Samples	24	24	24

Table 4.8 presents the CD4/CD8 T-lymphocyte ratio of diagnositic HIV positive subjects, HIV positive subjects on ARV drugs and HIV seronegative control groups. Mean CD4/CD8 T-lymphocyte ratio of diagnostic HIV subjects (0.767±0.661) and HIV seronegative control subjects (1.867±0.723) showed statistically significant difference ($P<0.05$) when compared at 5% level of significance. Also, between HIV positive subjects on ARV drugs and HIV seronegative control groups, the mean CD4/CD8 ratios (0.647±0.471 and 1.867±0.723 respectively) showed statistically significant difference ($P<0.05$) when compared at 5% level of significance. On the other hand, the comparison between the mean CD4/CD8 T-lymphocyte ratios of diagnostic HIV positive subjects (0.767±0.660) and that (0.647±0.471) of HIV positive subjects on ARVdrugs showed no statistically significant difference ($p>0.05$).

Table 4.8

CD4/CD8 T-Lymphocyte Ratio in Diagnostic HIV positive subjects, HIV positive subjects on ARV Drugs and HIV Sero-negative control subjects.

Parameters	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	0.500	0.500	1.650
Mean	0.767	0.647	1.867
Standard Deviation	0.661	0.471	0.723
Minimum	0.100	0.200	0.900
Maximum	2.700	1.900	3.400
Normality Test	No	No	Yes
No of Samples	30	30	30

Quantitative HIV viral load in diagnostic HIV positive subjects and HIV positive subjects on antiretroviral drugs were presented in Table 4.9. The mean viral load of HIV positive subjects on ARV drugs (145591.00±259499.00) was significantly reduced when statistically compared with that (24407.00±106479.00) of diagnostic HIV subjects ($p<0.05$).

Table 4.9

Quantitative HIV in Diagnostic HIV Positive Subjects and HIV positive subjects on Antiretroviral Drugs (HAART).

Parameters (copies/ml)	Diagnostic HIV	ARV Drugs
Median	20646.00	0.00
Mean	145591.00	24407.00
Standard Deviation	259499.00	106479.00
Minimum	1905.00	0.00
Maximum	926246.00	581442.00
Normality	No	No
No of Samples	30	30

Gene expression and transcript number of ILï 2 gene in diagnostic HIV positive subjects, HIV positive subject on ARV drugs and HIV seronegative control subjects were presented in Table 4.10. There was significant difference between the mean IL-2 gene transcript number of diagnostic HIV positive subjects (0.0618±0.1617) and HIV positive subjects on ARV drugs (0.1990 ± 0.2631), when compared statistically, at 5% level of significance ($p<0.05$). The mean IL-2 gene transcript number of diagnostic HIV positive subjects (0.0618±0.01617) showed statistically significant difference in comparison with that of the control subjects (1.2370±2.4770), ($p<0.05$). There was no significant difference observed between the mean ILï 2 transcript numbers of HIV seronegative control group (1.2370±2.4770) and HIV positive subjects on ARV drugs (0.1990±0.2631) when compared statistically ($p>0.05$).

Table 4.10

Transcript number of IL–2 gene in Diagnostic HIV positive subjects, HIV positive subjects on ARV Drugs and HIV Sero-negative control subjects.

Parameters (copies/5µl cDNA)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	0.0145	0.1035	0.3235
Mean	0.0618	0.1990	1.2370
Standard Deviation	0.1617	0.2631	2.4770
Minimum	0.0000	0.0010	0.0200
Maximum	0.8770	1.3070	11.3900
Normality	No	No	No
No of Samples	30	30	30

Transcript number of Tim-3 gene expression in diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV seronegetive control group were presented in Table 4.11. The mean transcript number of Tim-3 gene expression in diagnostic HIV positive subjects (7.506±16.220) was significantly reduced ($p<0.05$) when compared statistically with Tim-3 gene expression in HIV positive subjects on ARV drugs (194.400±657.900). There was also significant increase when the mean Tim-3 gene transcript number of the HIV seronegative control group (23.100±38.400) was statistically compared with that of diagnostic HIV positive subjects (7.506±16.220), ($p<0.05$). No significant difference was observed when the mean Tim-3 gene transcript number of HIV positive subjects on ARV drugs (194.40±657.90) was compared statistically with that (23.10±38.40) of HIV seronegative controls ($P>0.05$).

Table 4.11

Transcript number of Tim-3 gene in Diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV Sero-negative control subjects.

Parameters (copies/5µl cDNA)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	0.9460	14.3500	6.6140
Mean	7.506	194.400	23.100
Standard Deviation	16.220	657.900	38.400
Minimum	0.004	0.093	0.036
Maximum	69.000	3365.000	159.800
Normality	No	No	No
No of Samples	30	30	30

In Table 4.12, the Fos gene RNA transcript number in the three study groups were presented. There was statistically significant difference when the mean Fos gene transcript number of diagnostic HIV positive group (2.936 ± 10.910) was compared with that (32.30±52.59) of HIV positive subjects on antiretroviral drugs ($P<0.05$). Also there was significant difference when the mean transcript number of Fos gene in diagnostic HIV positive subjects (2.936 ± 10.910) was statistically compared with that of HIV seronegative group (453.70 ± 568.70), ($P<0.05$). The difference between the mean transcript number of Fos gene in HIV positive subjects on ARV drugs (32.30 ± 52.59) and HIV seronegative control group (453.70 ± 568.70) was statistically significant ($P<0.05$).

Table 4.12

Transcript number of Fos gene in Diagnostic HIV positive subjects, HIV positive subjects on ARV Drugs and HIV Sero-negative control subjects.

Parameters (copies/5µl cDNA)	Diagnostic HIV	ARV Drugs	HIV Sero-negative
Median	0.0565	6.8640	261.3000
Mean	2.9360	32.3000	453.7000
Standard Deviation	10.9100	52.5900	568.7000
Minimum	0.000	0.0070	22.2500
Maximum	59.2100	208.9000	2424.0000
Normality	No	No	No
No of Samples	30	30	30

Single nucleotide polymorphism (SNP) of ILï 2 gene in diagnostic HIV positive subjects, HIV positive subjects on ARV drugs and HIV seronegative control group were presented in Table 4.13. There was significant difference when the total SNPs in different study groups were compared statistically.

Table 4.13

IL-2 gene Single Nucleotide Polymorphisms (SNPs) in diagnostic HIV positive subjects, HIV positive subjects on ARV and HIV seronegative subjects.

SNP ID	Nucleotide change	SNP type (Variance)	No of SNPs (Diagnostic)	No of SNPs (ARV)	No of SNPs (control)
rs 77806995	A/G	Missense	3		5
rs 370039955	A/C	Missense	1		4
rs 3087209	A/G	Missense			4
rs 72558016	A/G/C	Missense	1		4
rs 202027273	A/G	Missense		1	1
rs 369900603	C/T	Synonymous	1		
rs 1479921	C/T	Intron		1	1
rs 374465594	A/G	Missense	1	1	
rs 374889117	C/T	Intron	1		
rs 206964	A/T	Intron	1		
rs 2069772	A/T	Missense	1		
Total			10	3	19

In Table 4.14, the single nucleotide polymorphisms (SNPs) of Fos gene in the three study groups were presented. There was no significant difference observed when the sum of the SNPs in each study group was compared statistically among the three study groups using chi-square.

Table 4.14

Fos gene Single Nucleotide Polymorphisms (SNPs) in Diagnostic HIV positive subjects, HIV positive subjects of ARV and control subjects.

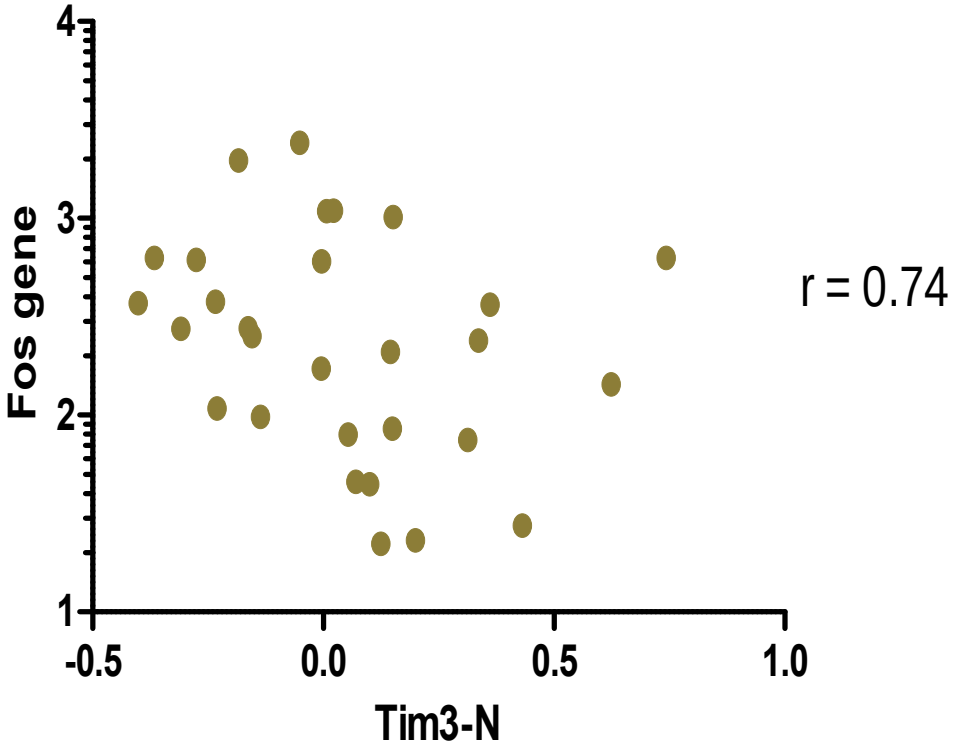
SNP ID	Nucleotide change	SNP type (Variance)	No SNPs (Diagnostic)	No SNPs (ARV)	No SNPs (Control)
rs 370915106	C/G	Missense	3		
rs 138334429	C/G	Missense		1	2
rs 150284892	C/G	Missense	1		
rs 75898085	C/T	Synonymous		1	1
rs 373065227	G/T	Missense			2
rs 202129176	C/T	Missense		2	3
Total			4	4	8

Table 4.15 presents the correlation matrix of all the markers that appeared strongly correlated in this study. In this table óNô represents HIV seronegetive group C. The variables that have strong correlation were presented with their correlation coefficient rank values. There were eleven strong positive correlations, five strong negative correlations, four weak positive correlations and two weak negative correlations among the studied variables. Some of the strongly correlated variables are presented bellow, graphically.

Table 4.15

Correlation coefficient of all the variables studied

Parameters			Correlation coefficient rank (r)
Tim 3 gene (A)	vs	IL-2 gene (A)	0.603
Tim 3 gene (N)	vs	IL-2 gene óBô	0.624
Fos gene óBô	vs	ILï 2 gene óBô	0.614
Fos gene óNô	vs	ILï 2 gene óNô	0.710
CD 38 óAô	vs	ILï 2 gene óAô	-0.521
CD 38 óBô	vs	ILï 2 gene óBô	0.503
Fos gene óBô	vs	Tim-3 gene óBô	0.525
Fos gene óNô	vs	Timï 3 gene óNô	0.743
MDA ï óNô	vs	TAS ï óNô	0.506
CD4/CD8-R óNô	vs	TAS ï óNô	-0.426
CD 38 ï óNô	vs	TAS ï óNô	0.443
CD 38 ï óNô	vs	MDA ï óNô	0.465
CD4/CD8-R óAô	vs	CD4 ï óAô	0.712
CD4/CD8 ï R óBô	vs	CD4 ï óBô	0.691
CD4 ï óN	óvs	CD8 ï óNô	0.474
CD4/CD8 ï R óAò	vs	CD8 ï óAô	-0.720
CD4/CD8 ï R óBô	vs	CD8 ï óBô	-0.661
CD4/CD8-R óNô	vs	CD8 ï óNô	-0.869
CD38 ï óNô	vs	CD8 ï óNô	-0.532
CD38 ï óNô	vs	CD4/CD8-RôNô	0.507
Fos gene óBô	vs	CD38 ï óBô	0.403
TAS ï óBô	vs	CD 38 ï óBô	-0.490



Correlation between Tim3 and fos gene transcripts

Fig. 4.1

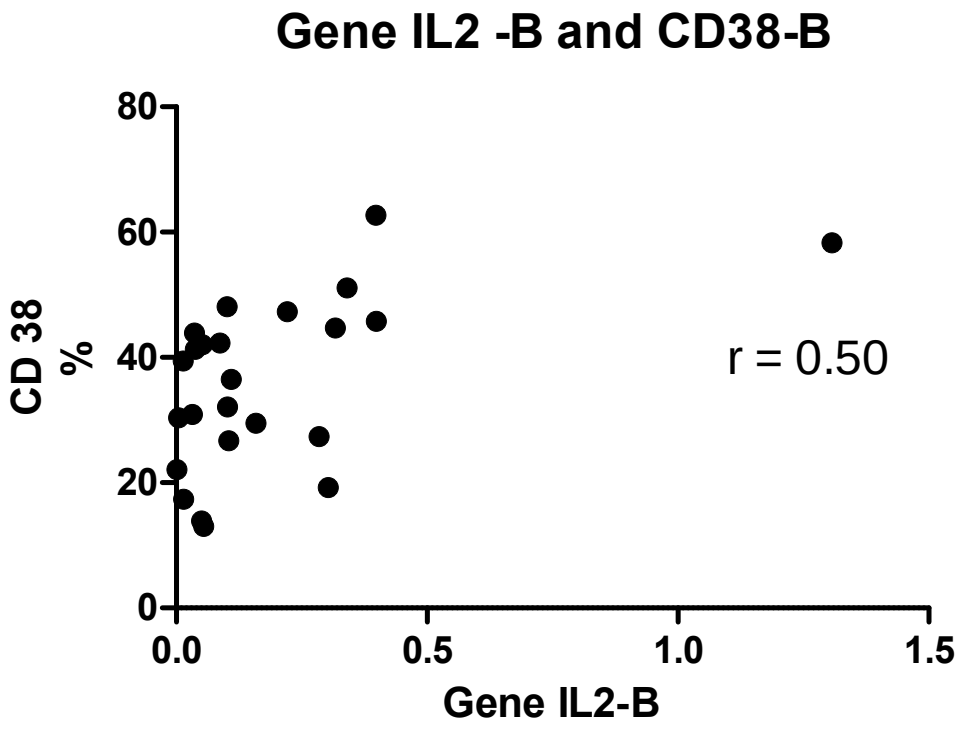
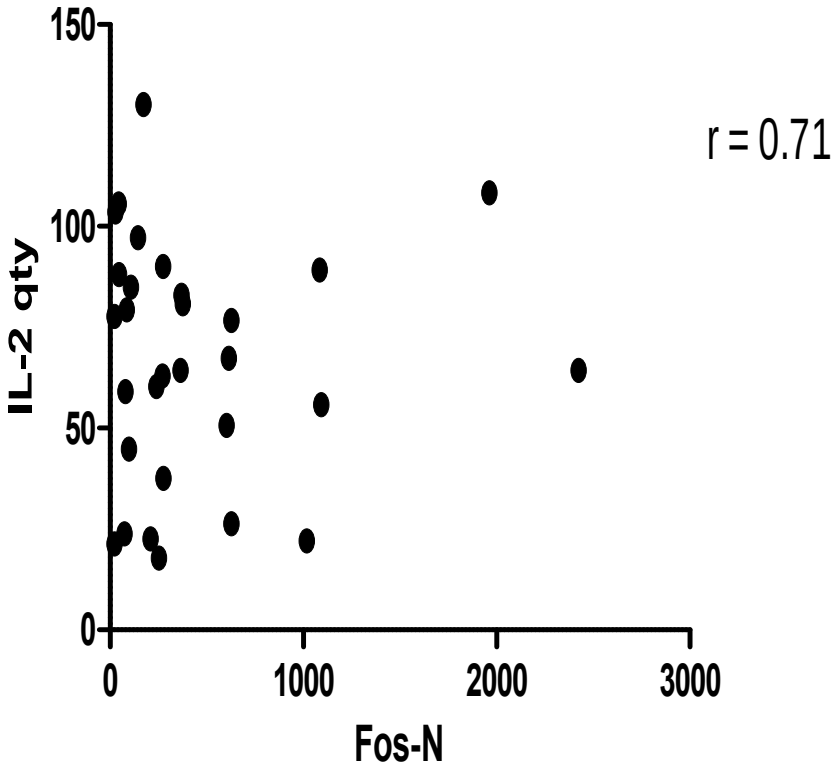


Fig. 4.2: **Correlation between % CD38 'B' and IL-2 gene 'B'**



Correlation between IL-2 and Fos gene transcript numbers

Fig. 4.3

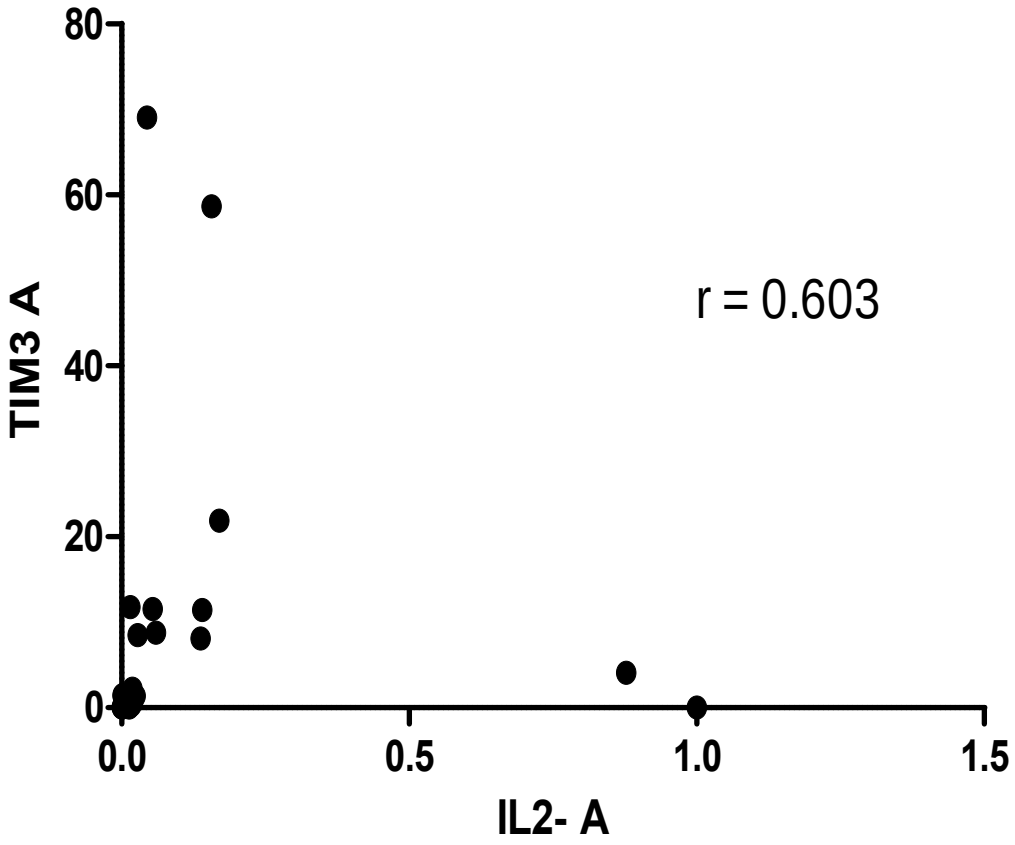


Fig.4.4: **Correlation between Tim-3 and IL-2 genes in diagnostic HIV positive**

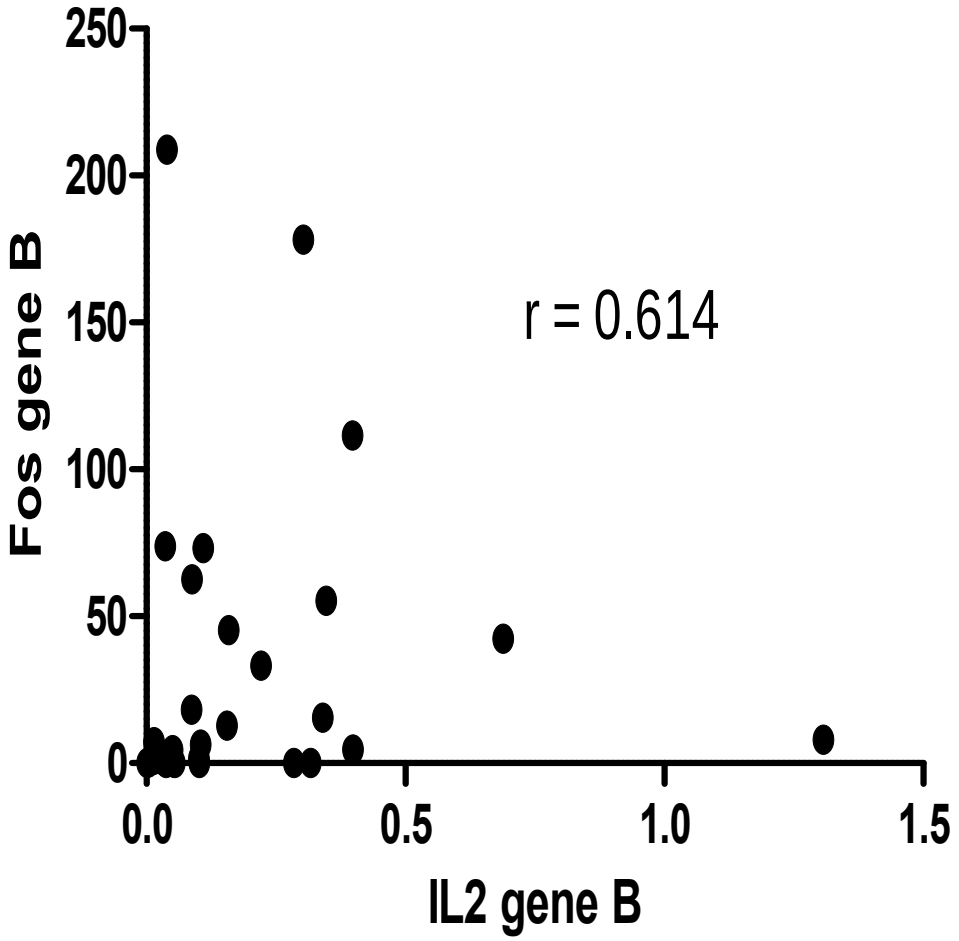


Fig.4.5: **Correlation between Fos gene and IL2 gene transcript numbers in HIV positive subjects on ARV drugs.**

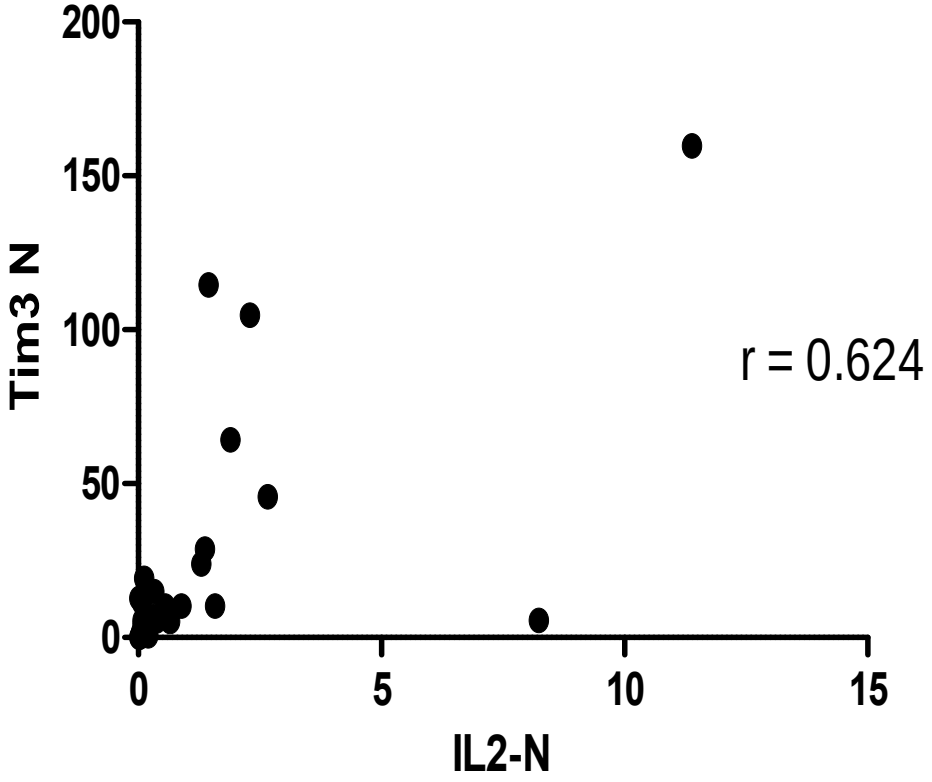


Fig.4.6: Correlation between Tim-3 and IL-2 genes transcription in control subjects.

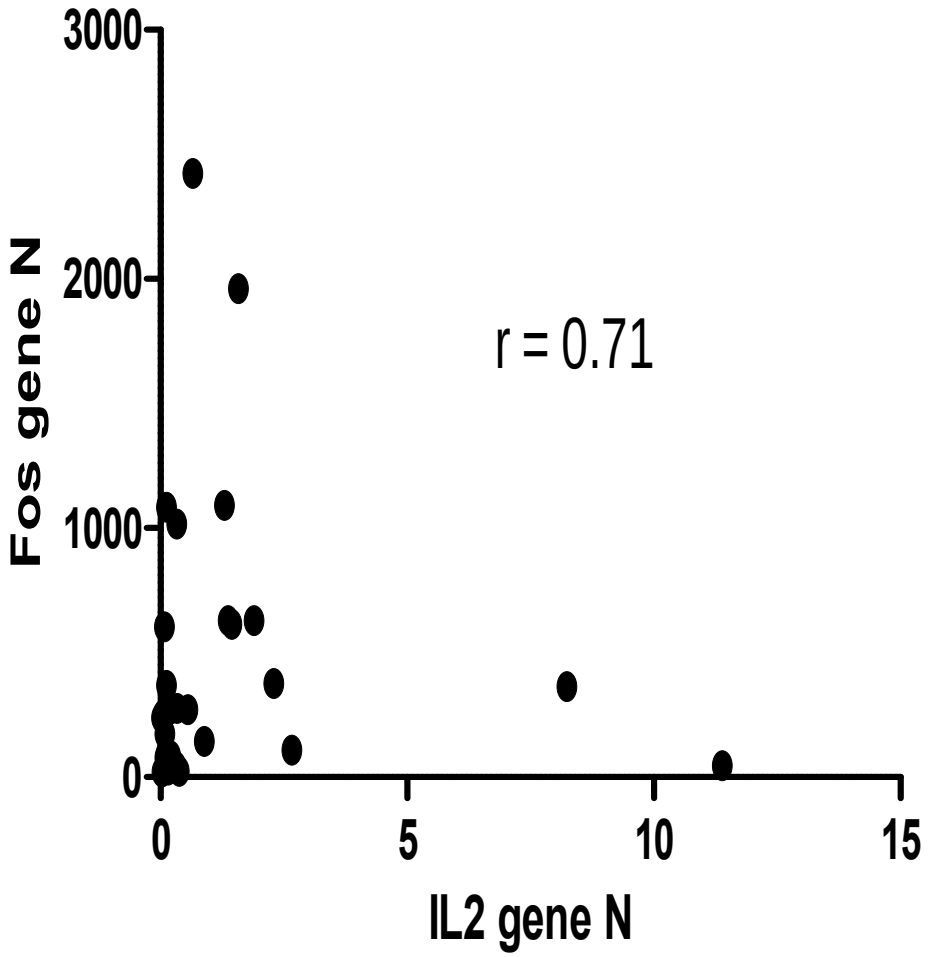


Fig.4.7: Correlation between Fos gene and IL-2 gene transcription in conrol subjects.

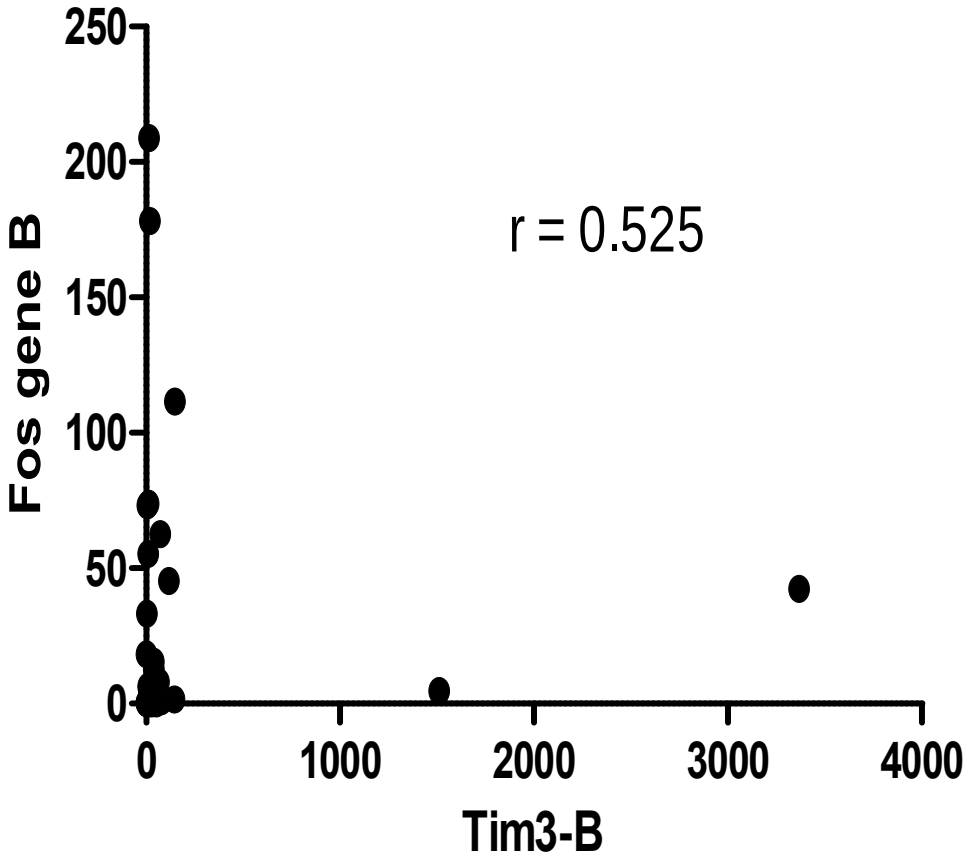


Fig.4.8: Correlation between Fos gene and Tim-3 gene transcription in HIV positive subjects on ARV drugs.

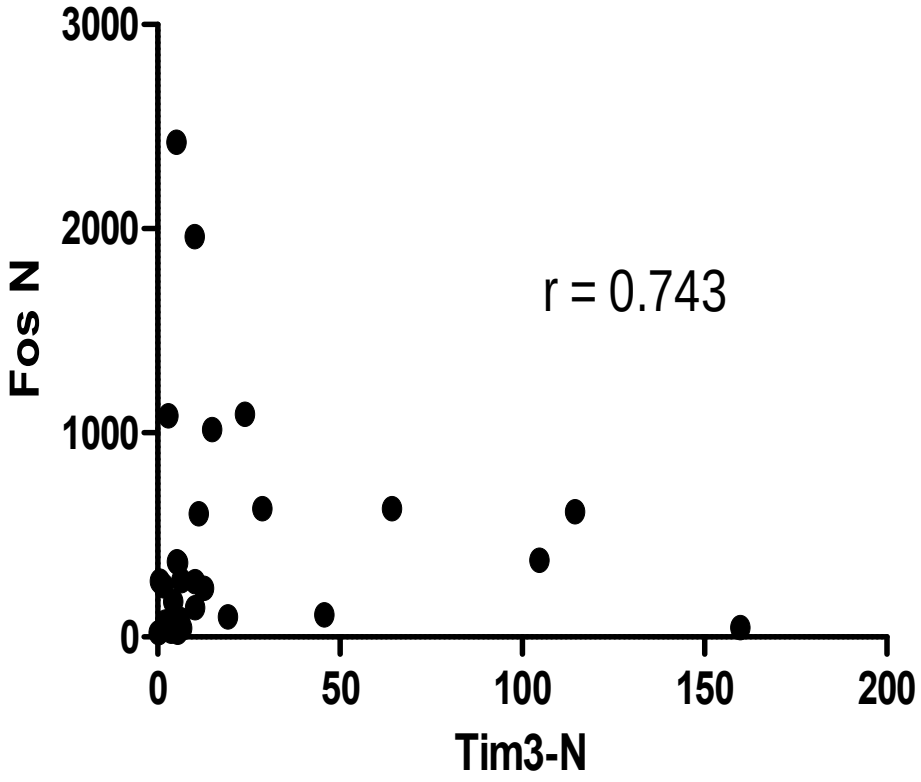


Fig.4.9: Correlation between Fos gene and Tim-3 gene transcript number in control subjects

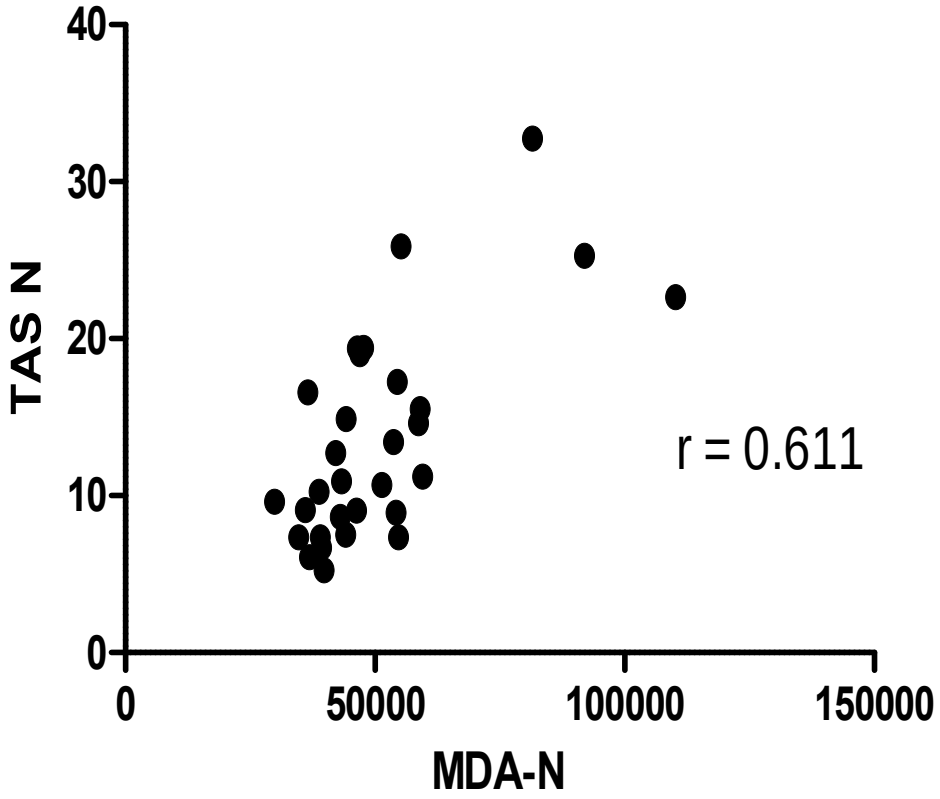


Fig.4.10: Correlation between TAS and MDA in control subjects.

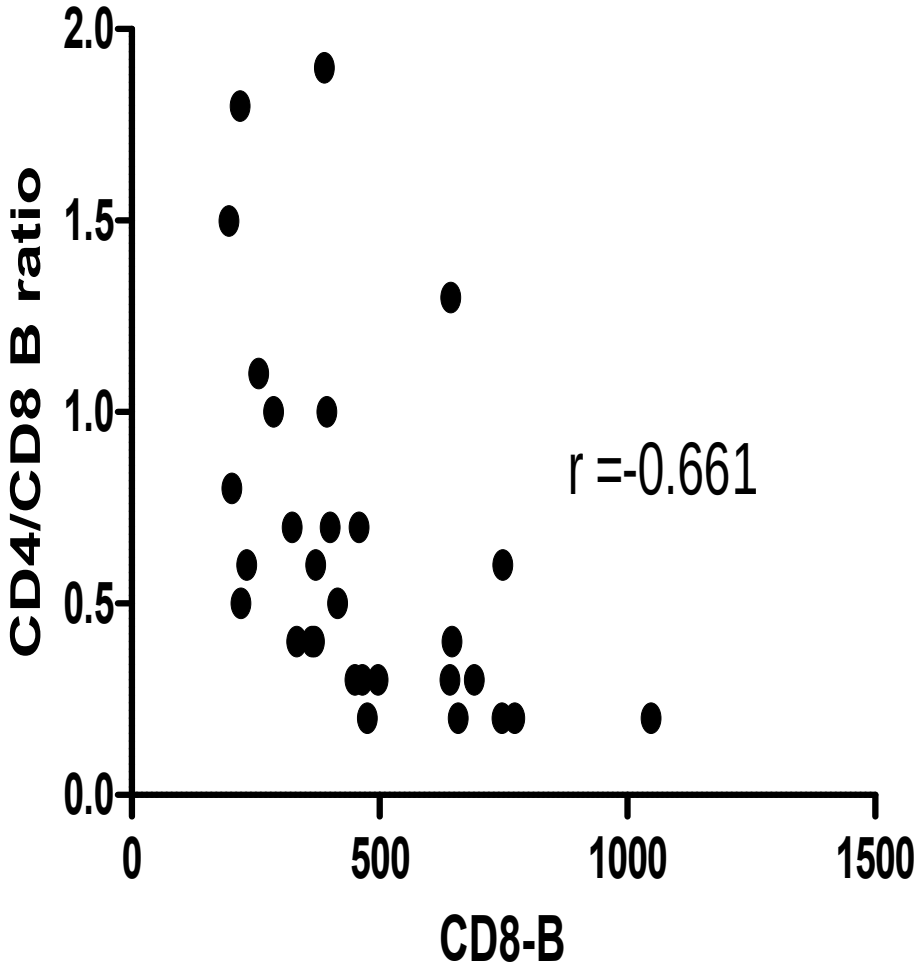


Fig.4.11: Correlation coefficient of CD4/CD8 ratio and CD8 in HIV positive subjects on ARV drugs

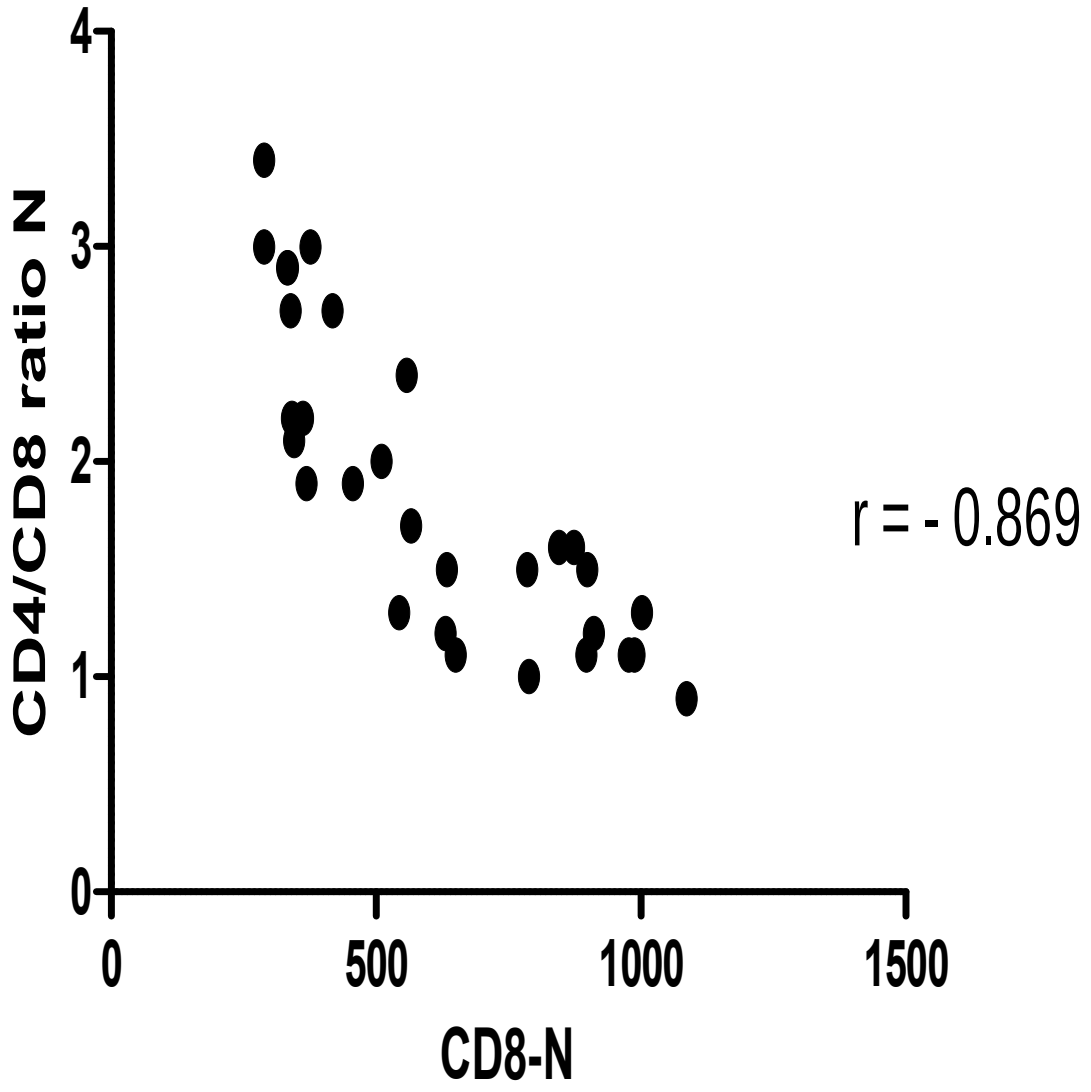


Fig.4.12: Correlation coefficient of CD4/CD8 ratio and CD8 in HIV seronegative subjects

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

The role of immunology in both clinical and pathological manifestation of human immunodeficiency virus infection has been extensively studied by different scholars since the discovery of HIV and AIDS. These studies have elucidated so many aspects of the disease pathogenesis which lead to the discovery of highly active antiretroviral drug combination (HAART). The use of such drug has reduced the viral particles to the barest minimum from the blood of an infected individual. Though the efficiency of HAART has brought life back to the people living with HIV and AIDS, the fact remains that cure is still not achieved, since the withdrawal or interruption of HAART therapy brings back the full blown disease within a space of time.

In the present study, we concentrated on immunological dysfunction by analytically profiling some of the immunological markers from genomic level through protein synthesis to assess the impact of HIV and HAART on gene expression, cytokine secretion and T-cell depletion in people living with HIV and AIDS. Failure of the immune system to contain HIV is related to the functional impairment of HIV-specific $CD4^+$ and $CD8^+$ T-cells that accompany the progression of HIV infection (Day *et al.*, 2006). It is the role of $CD4^+$ T-cells to signal other cells of the immune system to perform their respective functions (Deck *et al*., 2004; Ondoa *et al*., 2005). The deterioration of T-cell responses or T-cell exhaustion in HIV infection involves the early loss of proliferative capacity, cytotoxic potential and the ability to produce interleukin-2 (Kostense *et al*., 2002).

Low level of plasma IL-2 cytokine observed in the diagnostic HIV-positive subjects in spite of high viral load and constant immune challenges, by the viral particles, may be attributed to $CD4^+$ T-cell depletion and other suppressive activities of HIV on the $CD4^+$ T-lymphocytes (Kostense *et al*., 2002; Ondoa *et al.,* 2005). The significant increase in the plasma IL-2 level of HIV-positive subjects on ARV drugs in comparison with both diagnostic HIV-positive and HIV-seronegative control groups may also be attributed to the impact of the highly active antiretroviral drugs on the viral particles. Clearance or reduction in the plasma viral load can lead to increase in absolute $CD4^+$ T-cell count because HIV has direct and indirect cytolytic effect on both mature $CD4^+$ T-cells and on the progenitor cells which is the reason for progressive exhaustion that surpasses the capacity to produce new cells (Antran, 2000; Brenchley *et al*., 2004; Choadhry *et al*., 2007; Appay and Sauce, 2008). Increase in

$CD4^+$ T-cells count may lead to increase in IL-2 syntheses which may up-regulate the activation and proliferation of $CD4^+$ and $CD8^+$ T-lymphocytes. $CD8^+$ T-cell activation may increase the immune activity against the virus, augmenting the action of HAART therapy and restoring the physiological condition of the patient.

CD4 molecules found on the surface of helper T-lymphocytes, and other $CD4^+$ - cells like monocytes, macrophages and dendritic cells help to stabilize the binding of T-lymphocytes to the major histocompatibility complex (MHC) class II on the antigen presenting cells (APC). Activation of T-helper cells through such binding orchestrates the bodyôs antigen-specific immune response by co-ordinating B-lymphocyte production of antibodies, secretion of cytokines by various immune cells and induction of cytotoxic lymphocyte response to the antigens. These functions make $CD4^+$ T-lymphocytes critical elements of the immune system (Wilson *et al*., 2004; Vajpayer *et al*., 2005; Vajpayer *et al*., 2009). The significant reduction of $CD4^+$ T-lymphocytes observed among diagnostic HIV-positive subjects in comparison with the HIV-seronegative control group may be attributed to the devastating cytolytic effect of the virus on both mature and progenitor $CD4^+$ T-lymphocytes that leads to the total collapse of immune responsiveness during chronic and progressive HIV disease.

Specific cytotoxic $CD8^+$ T-cells kill HIV-infected cells with the help of $CD4^+$ T-lymphocytes that are needed to prime $CD8^+$ T-cell responses and maintain both immunologic memory and cytolytic response. During acute HIV infection, the $CD8^+$ T-cell count increases up to 20-folds with a vigorous specific anti-HIV response (Koup *et al*., 1994). The observed significant decrease of $CD8^+$ T-cell count in diagnostic HIV-positive subjects with respect to that of HIV-seronegative control group may be associated with the decrease in the $CD4^+$ T-lymphocyte counts of the diagnostic HIV-positive subjects. Depletion of $CD4^+$ T-cell count and its dysfunctional activity leads to decrease in the ability to recognize and respond to the HIV peptides on the surface of antigen presenting cells (APC), which may lead to low $CD4^+$ T-cell activation and IL-2 production. Absence or reduced IL-2 production may adversely affect $CD8^+$ T-cell activation and proliferesion, and specific cytotoxic $CD8^+$ T-cell killing of HIV-infected cells. This dysfunctional activity may give HIV allowance to proliferate, increase the viral load, infect more cells, deplete more $CD4^+$ T-cells and possibly cause permanent injury to the immune system.

Administration of antiretroviral drug (HAART) may cause reduction in the viral load, elevation of $CD4^+$ T-cells, increased IL-2 production and possibly activation of $CD8^+$ T-cells for proliferation and cytolytic activities (Kostense *et al.*,2002). This may be the reason for the

observed non-significant difference between the $CD8^+$ T-cell count in HIV-positive subjects on antiretroviral drugs and that of the HIV-seronegative control group. Such finding may imply that highly active antiretroviral therapy has tried to restore the immune response by up-regulating the $CD4^+$ T-cell, IL-2 cytokines and cytotoxic T-lymphocytes quantitatively and qualitatively. This may probably be emphasized by the observed significant reduction in the plasma viral load of the HIV-positive subjects on antiretroviral drugs when compared with those of the diagnostic HIV-positive subjects.

The number of circulating $CD4^+$ T-cells is widely used to monitor the degree of immune suppression in HIV infection and provides a predictor of the immediate risk for opportunistic illness (Masur *et al*., 1989; Chiappini *et al*., 2006). In early HIV infection, $CD8^+$ T-cell number tends to increase, reflecting expansion of memory $CD8^+$ T-cells, particularly HIV-specific $CD8^+$ T-cells. In contrast to this, the proportion of naive $CD8^+$ T-cells tend to fall in early infection but absolute number of $CD8^+$ T-cell do not fall until HIV disease progresses (Margolick *et al.,* 1995; Chinen *et al*., 2001; Mttapallis *et al*., 2004). In the present study, the CD4/CD8 ratio significantly reduced in both diagnostic HIV-positive subjects and HIV positive subjects on antiretroviral therapy compared with the CD4/CD8 ratio of HIV-seronegative control subjects. This finding may be as a result of persistent destruction of $CD4^+$ T-cells throughout the stages of HIV disease (Day *et al*., 2006). Absolute fall in $CD4^+$ T-cells count in relation to $CD8^+$ T-cells count may result to the fall in CD4/CD8 ratio observed in this study.

There is a strong positive correlation between the CD4/CD8 ratio and $CD4^+$ T-lymphocyte count ($r = 0.712$) of diagnostic HIV-positive subjects, and that of HIV-positive subjects on antivetroviral drugs ($r=0.691$). On the other hand, strong negative correlation exists between the CD4/CD8 ratio and $CD8^+$ T-lymphocyte count in diagnostic HIV-positive subjects, HIV-positive subjects on antiretroviral drugs and HIV-seronegative control group. Correlation coefficientôs rank (r) include -0.720, - 0.661 and -0.869 respectively.

The present study observed a significant reduction in the mean percentage T-cells expressing CD38 molecules in both diagnostic HIV-positive subjects and HIV-positive subjects on antiretroviral drugs when compared with that of the HIV-seronegative control group. This observation may be as a result of HIV-induced destruction of T-cells expressing CD38 molecules by Fas-mediated cell death or by suppressing the expression of CD38 molecules geared to reduce the capacity of T-cells to produce IL-2 and INF gamma. CD38 molecule is a type II trans-membrane glycoprotein expressed by haematopoietic and non-haematopoietic cells. Its surface expression is under complex control and varies during

lymphocyte development, activation, and differentiation. T-cells expressing high level of CD38 displayed reduced proliferative activity, but exhibited an improved potential to produce IL-2 and INF gamma, suggesting an important role of this molecule during T-cell activation and differentiation (Sandovol-Montes and Santos-Argumedo, 2005). It was observed that a substantial proportion of $CD8^+$ T-cells expressing CD38 in HIV-infected individuals with active viral replication was susceptible to spontaneous and Fas-mediated cell death (Chien *et al*., 2004). Non-significant increase in the percentage T-cells expressing CD38 molecule in HIV-positive subjects on antiretroviral therapy when compared with diagnostic HIV-positive subjects may indicate low T-cell activation due to down regulation of viral replication by antiretroviral therapy.

High-affinity interleukinï 2 receptor (IL-2R) is a heterotrimeric complex composed of the *a*-chain (CD25), ɓ-chain (CD122), and the common *y*-chain (CD132). Engagement of the IL-2R complex on activated T-cell initiates a complex signalling that can induce proliferation, increase survival as well as prime for activation-induced cell death (Refachi *et al*., 1998). IL-2R (CD25) is expressed on activated T-cells and regulatory T-cells and is capable of binding with low affinity IL-2 by itself. Percentage CD25 expressing T-cells in both diagnostic HIV-positive subjects and HIV-positive subjects on antiretroviral drugs presented in this work showed no significant changes when compared with the expression of CD25 molecules in HIV-seronegative control subjects. This result does not agree with that of Weiss *et al*. (2004), who reported that a subset of $CD4^+$ T-cells expressing CD25 increased in the peripheral blood of HIV-infected individuals. It is possible that he worked specifically on the regulatory T-cells (Treg) that express $CD4^+$ $CD25^+$ molecules while the present study is on both activated and regulatory T-cells. Therefore, non-expansion of T-cells expressing CD25 molecules in HIV-infected individuals observed in this study may be as a result of elimination of activated T-cells that express $CD4^+$ $CD25^+$ molecules by activated induced cell death modulated by HIV tat protein (Yang *et al*., 2002).

Dioxygen is a highly important, yet toxic molecule that reacts in vivo to produce reactive oxygen species (ROS) such as superoxides, peroxides, hydroxyl radicals, and other related species. These radicals play important roles in healthy organisms as immunological arsenal against infectious organisms. They are also implicated in ageing and a wide range of disease processes. Measurement of a combined nonenzymatic antioxidant capacity of biological fluids provides an indication of the overall capability to counteract reactive oxygen species, resist oxidative damage and combat oxidative stress-related diseases. In this study, oxidative stress measured by malondialdehyde (MDA) concentrations, was significantly

increased in diagnostic HIV-positive subjects when compared with the HIV-seronegative controls. There was also significant increase when the lipid peroxidation of HIV-positive subjects on ARV drugs was compared with that of the HIV-seronegative control group. On the other hand, there was no significant difference observed when the oxidative stress of diagnostic HIV-positive subjects was compared with that of the HIV-positive subjects on antiretroviral therapy (HAART). The combination of both small molecules and protein antioxidant capacity measured in the three study groups of this work showed that diagnostic HIV-positive subjects have significantly lower antioxidants in comparison with HIV-seronegative control subjects. There was no significant difference in total antioxidant capacity of HIV-positive subjects on ARV drugs, compared with those of diagnostic HIV-positive subjects and the control group.

The finding of increased lipid peroxidation in both diagnostic HIV-positive subjects and HIV-positive subjects on ARV drugs in comparison with that of the control group is in agreement with the findings of other scholars who reported oxidative stress in HIV-positive patients by increased plasma lipid peroxide and malondiadehyde concentration (Halhwell and Cross, 1991; Revillord and Vincent, 1992; Postaire *et al*., 1994). Though an excessive production of ROS which may be explained by polymorphonuclear leukocyte activation during infectious conditions or by a pro oxidant effect of tumor necroses factor alpha produced by activated macrophages (Das *et al*., 1990), a decrease in antioxidant defence mechanism may contribute to the observed oxidative stress in HIV-infected individuals. Non-existence of significant reduction of oxidative stress in HIV-positive subjects on ARV drug even when there was a significant reduction in viral load when compared with the diagnostic HIV-positive subjects could be explained by the possibility of ARV drug itself inducing oxidative stress.

Antioxidant deficiency in people living with HIV and AIDs is probably due to increased in the consumption of antioxidant micronutrients because of increased oxidative stress rather than inadequate dietary intake or malabsorption (Dworkin *et al*., 1990; Kapembwa *et al.,* 1990). Increased oxidative stress may be associated with low IL-2 cytokine production, reduced $CD4^+$ T-cell proliferation and decreased $CD8^+$ T-cell activation. This process may result in immunological collapse and progressive viral replication. An in vitro experiment (Schreck *et al*., 1991) has shown that ROS such as hydrogen peroxide can specifically activate the nuclear factor kB to induce the expression and replication of HIV-1 in a human T-cell line, and an addition of antioxidant vitamins blocked activation of nuclear factor kB and inhibited HIV-replication (Harakeh *et al*., 1990; Harakeh and Jariwalls, 1991).

In the present study, there is a strong positive correlation (r = 0.506) between peroxidative activity and total antioxidant capacity in HIV-seronegative control individuals. Total antioxidant capacity (TAS) also had a positive correlation (r=0.443) with T-cell activation (CD38) in apparently healthy individuals used as control group. On the other hand, there was negative correlation (r = -0.426) between the total antioxidant capacity of diagnostic HIV-positive subjects and CD4/CD8 ratio of the same group. CD4/CD8 ratio also correlates positively (r=0.416) with oxidative stress (MDA) in diagnostic HIV-positive subjects, while negative correlation (r = -0.490) exists between the total antioxidant capacity and T-cell activation of HIV-positive subjects on highly active antiretroviral therapy. Such findings most likely emphasize the good relationship that exists between oxidative stress, immune cell activation, absolute CD4 and CD8 count, total antioxidant status and highly active antiretroviral therapy in people living with HIV and AIDS.

The c-Fos protein, a component of AP-1 transcription factor complex is a member of a multi-gene family which includes Fos-related and jun-related genes. The AP-1 binding site is recognized by dimeric protein complex, composed of Jun homodimer or Fos/Jun heterodimers. The Fos/Jun heterodimer complex shows greater affinity than Jun/Jun homodimer for the HP-1 binding site (Franza *et al*., 1989). Activation of c-Fos contributes to cell cycle progression of haematopietic cells (Tay *et al*., 1996). It has been shown that c-fos is one of the major regulatory factors for the expression of IL-2 gene in T-cell activation (Ochi, 1992).

A significant decrease in c-fos gene expression observed in the present work, in both diagnostic HIV-positive subjects and HIV-positive subjects on ARV drugs, may possibly indicate that HIV infection may have suppressive ability on expression of c-fos gene during chronic stage of HIV infection. It was reported that structural integrity of the activation domain of HIV Tat protein was required for the promotion of c-Fos promoter in both Jurkat and U937 cells. Mutation in cys^{22} results in the complete loss of the transactivation of Tat on HIV-1 long terminal respect (Gibellini *et al*., 1995). Gibellini *et al.* (1997) suggested that the c-Fos up-regulation mediated by Tat, might play a significant role in the control of viral gene transactivation. Since c-fos is a major contributor to IL-2 gene expression in T-cell activation, low expression of c-fos gene may probably contribute to low expression of IL-2 gene.

Consistent activation of T-cells and aggressive immune response against HIV replication may probably induce mutation in the cys^{22} of Tat protein, thereby suppressing the activity of Tat on upregulation of c-fos expression. HIV on the other hand may transform the Tat activity on up-regulating c-fos gene expression in order to down-regulate the expression

of IL-2 gene so as to decrease the production of IL-2 cytokines, and proliferation of $CD4^+$ and $CD8^+$ T-cells. This will enable the virus to upregulate the viral gene transactivation, increase the viral burden, induce more immunological dysfunction and achieve a faster rate of disease progression.

This study observed a significant decrease in IL-2 gene expression in diagnostic HIV-positive subjects when compared with HIV-positive subjects on ARV drugs and HIV-seronegative control group. Though the expression of IL-2 gene in HIV-positive subjects on ARV was lower than that of the control group, there was no significant difference observed. This may imply that the significant reduction in HIV load due to HAART administration may have contributed to the upregulation of IL-2 gene expression at a significant level. The significant decrease in IL-2 gene expression in diagnostic HIV-positive group probably emphasizes the activity of c-fos in the expression of IL-2 gene in T-cell activation.

There is strong correlation between IL-2 gene expression and c-fos gene expression in both HIV-positive subjects on ARV drugs (r=0.614) and HIV-seronegative control group (r=0.710). Also a positive correlation exists between T-cell activation (CD38) and c-fos gene expression in HIV-positive subjects on ARV drugs (r=0.403). A strong negative correlation was observed between expression of IL-2 gene and the activation of T-cells in diagnostic HIV-positive subjects (r=-0.521). On the other hand, a strong positive correlation exists between T-cell activation and IL-2 gene expression in HIV-positive subjects on ARV drugs. In spite of consistent T-cell activation, the expression of IL-2 gene remained reduced in diagnostic HIV-positive subjects. This may be explained by low c-fos gene expression observed in diagnostic HIV subjects. It may also possibly explain the reason why IL-2 cytokine was significantly reduced in diagnostic HIV-positive subjects. The quantitative and qualitative status of c-fos gene, IL-2 gene IL-2 protein and total antioxidant capacity observed in diagnostic HIV-positive subjects are significantly improved in HIV-positive subjects on ARV drug, possibly due to dramatic down regulation of viral gene transactivation and proliferation.

Intracellular contents of apoptotic and secondary necrotic cells could be autoantigene and capable of inducing autoimmune responses when released in the body system. Phagocytes such as macrophages and dendritic cells must engulf these dying cells rapidly and efficiently to prevent detrimental inflammatory reactions and autoimmune diseases (Luckashnek *et al*., 2008). T-cell immunoglobulin mucin-3 (Tim-3) recognizes apoptotic cells through the FG loop in the immunoglobulin variable (Igv) domain, and is crucial for the clearance of apoptotic cells by phagocytes (Nakayama *et al*., 2009). In the present study, a

significant decrease in Timï 3 gene expression was observed in diagnostic HIV-positive subjects when compared with those of HIV-positive subjects on ARV drugs and the control group. There was no significant difference when the Tim-3 gene expression in HIV-positive subjects on ARV drugs was compared with that of HIV-seronegative control group. Initially, Tim-3 was identified as a molecule exclusively expressed on the surface of T-helper type I (Th1) cells and conferred sensitivity to cell death through a mixture of apoptotic and necrotic pathways upon ligation with its ligand, galactin-9 (Monney *et al*., 2002; Zhn *et al*., 2005). Recently, it has been detected on the surface of Th17 cells, Natural Killer (NK) T-cells, dendritic cells, T-cytotoxic ($CD8^+$) cells and macrophages (Anderson *et al*., 2007; Hasting *et al*., 2009; Ju *et al*., 2010). The observed low Tim-3 gene expression in this work may be associated with viral infection of T-cells that bear Tim-3 molecules. Such infection may upregulate galactin-9 ligation with Tim-3 expressing T-cells, causing T-cell functional arrest and Tim-3-galactin-9 induced cell death. However, HIV by itself do not directly induce Tim-3 expression on the T-cells but it is demonstrated that Tim-3 expressing Th1 and T-cytotoxic type I T-cells are the population of exhausted T-cells during chronic HIV infection (Jones *et al*., 2008; Mujib *et al*., 2012). Such findings may imply that Tim-3 expression on T-cells may contribute to $CD4^+$ T-cell depletion in chronic and progressive HIV disease by initiating the elimination of Tim-3 expressing T-cells through galactin-9-Tim-3 ligation. During antiretroviral therapy (HAART) the viral load is dramatically reduced, thereby reducing the activation of apoptotic and necrotic pathways, and Tim-3-galactin-9 induced cell death. Such reductions may explain the reasons behind the significant increase in Tim-3 expression observed in HIV positive subjects on antiretroviral drugs as obsereved in the present study.

Accumulating evidence suggests that immune response needs to balance the expression of Tim-3 to tone down the initial immune activation for appropriate immune outcome. Due to the chronic nature of HIV infection, a constant immune activation may increase the Tim-3-Galactin 9 interaction and render $CD4^+$ and $CD8^+$ T-cells exhausted. Exhaustion of these cells involve the loss of proliferative capacity, cytotoxic potential and the ability to produce IL-2, followed by progressive ability to produce Tumor necrotic factor alpha and interferon gamma (Kostense *et al*., 2002; Wherry, 2011; Odorizzi and Wherry, 2012). There is strong positive correlation between Tim-3 gene expression and IL-2 gene expression in diagnostic HIV-positive subjects (r=0.603) and HIV-seronegative control group (r=0.624). Also strong positive correlation exists between Tim-3 gene expression and Fos gene expression in HIV-positive subjects on ARV drugs (r=0.525) and HIV-seronegative control subjects (r=0.743). Such findings may imply that HIV infection up-regulates the

expression of Tim-3, IL-2 and Fos genes, but due to exhaustion during chronic and progressive HIV infection, the expressions of the three genes become significantly reduced.

Gene expression is the fundamental level at which the genomic information is transcribed into phenotypic expression. Such phenotypes are often expressed by synthesis of proteins that control the organismôs shape, or that act as enzymes catalysing specific pathways or as cytokines activating T-lymphocytes for appropriate immune responses. DNA sequencing exposes DNA fragments skeletal structure as individual nucleotides in a single-stranded form. When such strand is aligned with a reference sequence, differences in the nucleotide arrangement can be observed as variance, mutation or single nucleotide polymorphism (SNP). Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, drugs, vaccines and other agents (Carlson, 2008).

In this study, a significant increase in single nucleotide polymorphism was observed in the IL-2 gene of HIV-seronegative control subjects in comparison with those of diagnostic HIV-positive subjects and HIV-positive subjects on antiretroviral drugs. Out of eleven different types of IL-2 gene SNPs observed in this work, seven types were missense variance while three others occurred in the intergenic regions of the IL-2 genes. The remaining one was a synonymous SNP. Though, the observation of SNPs alone cannot be sufficient to confirm resistance to HIV infection or HIV non-progressive nor susceptibility to HIV infection, but it may possibly be associated with HIV infection since such mutations can cause changes in RNA expression and protein synthesis. Therefore such findings may likely imply that those SNPs may probably have protective ability against HIV viral integration to human genome or replication to express viral mRNA.

The significant difference observed in IL-2 gene single nucleotide polymorphisms (SNPs) among the study groups was not based on individual single nucleotide polymorphism but on the total SNPs observed in each group. According to Singn *et al*. (2010), for complex diseases, SNPs do not usually function individually rather, they work in coordination with other SNPs to manifest a disease condition. Though, the observation of significant difference in IL-2 gene SNPs may not be enough to conclude on genetic disease manifestation, it may imply that IL-2 gene single nucleotide polymorphism has an association with HIV infection. This may possibly be associated with quantitative and qualitative defect in IL-2 cytokine production in chronic and progressive HIV disease. The non-significant difference observed in Fos gene SNPs among the three study groups in contrast to that of IL-2 gene probably

indicates that IL-2 gene may have more direct and significant roles to play than the Fos gene in HIV-host interaction.

5.2 Conclusion

1. Fos gene expression which is a major regulatory factor for IL-2 gene activation in T helper-cells may be interfered with during HIV infection.
2. HIV infection down-regulates the production of IL-2 cytokine, possibly by down-regulating the expression of IL-2 gene through interfering with Fos gene expression.
3. Though there was elevation of IL-2 cytokine secretion during highly active antiretroviral therapy (HAART), with down regulation of viral burden, there was no corresponding increase in T-cell proliferation, indicating that there might be a qualitative defect in the IL-2 cytokines produced during antiretroviral drug intervention.
4. Expression of T-cell immunoglobulin mucin-3 (Tim-3) molecules on T helper-cells may be another mechanism for $CD4^+$ T-cell depletion in HIV infection through Tim-3-galactin-9 ligation-induced cell death.
5. Oxidative stress may be associated with viral load, $CD4^+$ T-cell depletion and total antioxidant capacity during progressive HIV disease.
6. Though the significant single nucleotide polymorphisms (SNPs) observed from IL-2 gene sequencing may not be enough to conclude a genetic disease manifestation, it may probably be associated with HIV infection or disease progression.

5.3 Recommendation

1. There is need for drug intervention to be initiated as soon as HIV diagnosis is established to enable drug and immune response act synergistically to prevent the qualitative and quantitative IL-2 cytokine defect that is observed after T-cell exhaustion.
2. The use of antioxidants should be included in the management of HIV and AIDS.
3. There is need for new drugs which can block Tim-3 signaling pathways in order to prevent Tim-3-galactin-9 ligation-induced T-cell death of T-helper cells (CD4 T lymphocytes depletion) in HIV disease.

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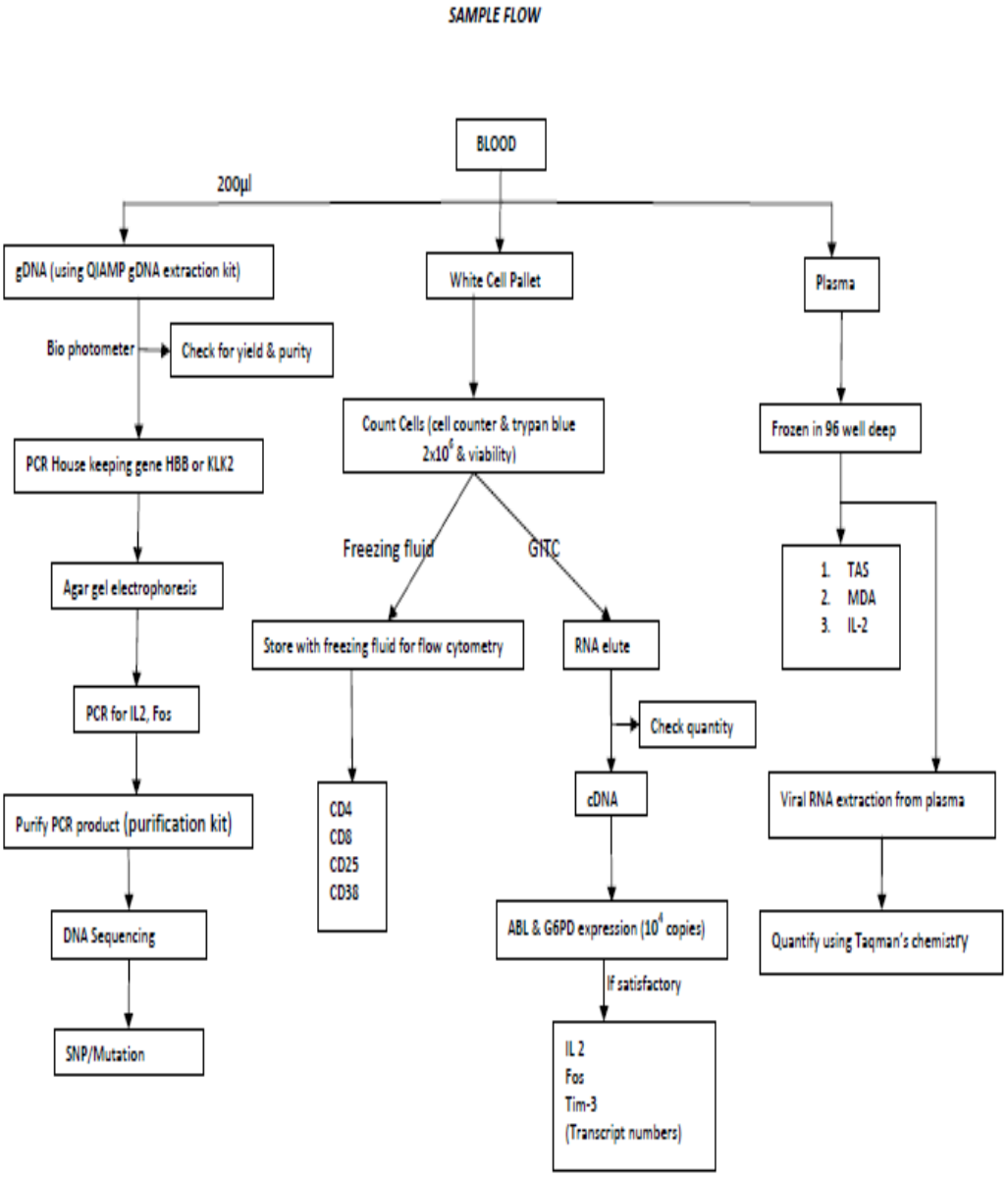
APPENDIX 1


SAMPLE FLOW
BLOOD
200µl
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White Cell Pallet
Plasma
Bio photometer
Check for yield & purity
Count Cells (cell counter & trypan blue 2x10^6 & viability)
Frozen in 96 well deep
PCR House keeping gene HBB or KLK2
Freezing fluid
GNC
1. TAS
2. MDA
3. IL-2
Agar gel electrophoresis
Store with freezing fluid for flow cytometry
RNA elute
PCR for IL2, Fos
Check quantity
Purify PCR product (purification kit)
CD4
CD8
CD25
CD38
cDNA
Viral RNA extraction from plasma
DNA Sequencing
ABL & G6PD expression (10^4 copies)
Quantify using Taqman's chemistry
If satisfactory
SNP/Mutation
IL 2
Fos
Tim-3
(Transcript numbers)

Appendix 2

Sample Size Calculation

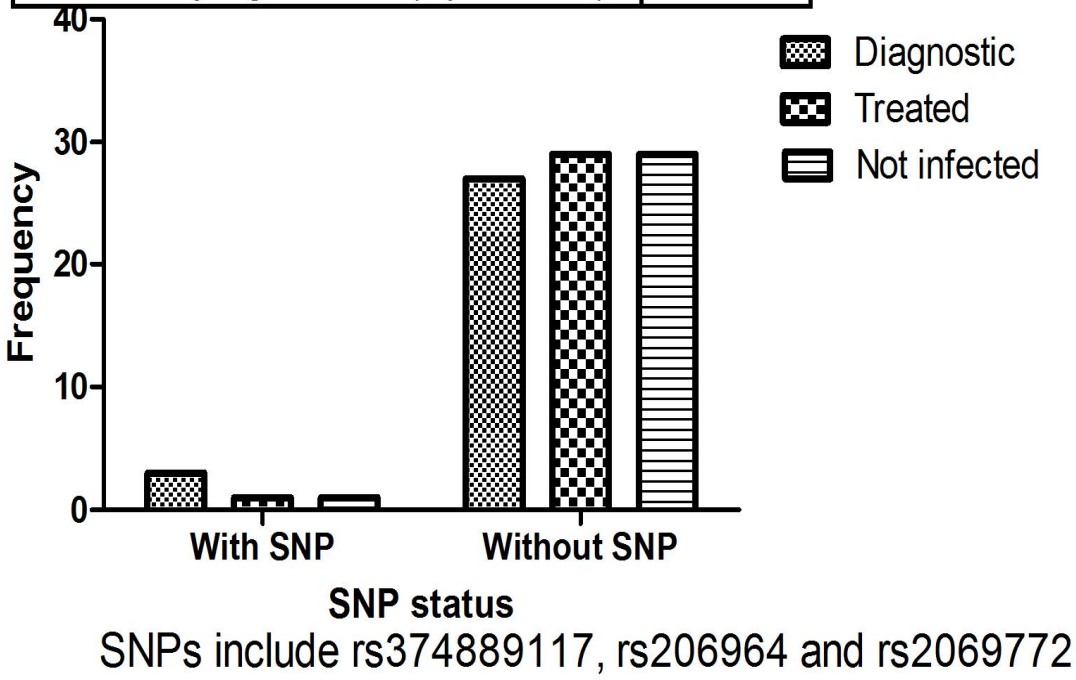
Sample size was calculated using GraphPad StatMat software, version 2.0.
A sample size of thirty (30) in each group has 80% power to detect a difference between means of 0.37 with a significance level (alpha) of 0.05.

Statistical Analyses

Statistical analyses of the results were carried out using GraphPad Prism software, version 5.03. Kruskal-Wallis statistic and Dunnôs Multiple Comperison Tests were used to test for levels of significance in IL-2 Cytokine, MDA, TAS, CD4 and CD8 concentrations. The same instrument was also used for percentage CD25 and CD38, and the expression of Fos gene, Tim-3 and IL-2 genes. The level of significance was set at $p < 0.05$. Chi-square was used to test SNPs while Mann-Whitney test was used to test viral load.

Appendix3

Chi-square	
Chi-square, df	1.694, 2
P value	0.4287
P value summary	ns
One- or two-sided	NA
Statistically significant? (alpha<0.05)	No

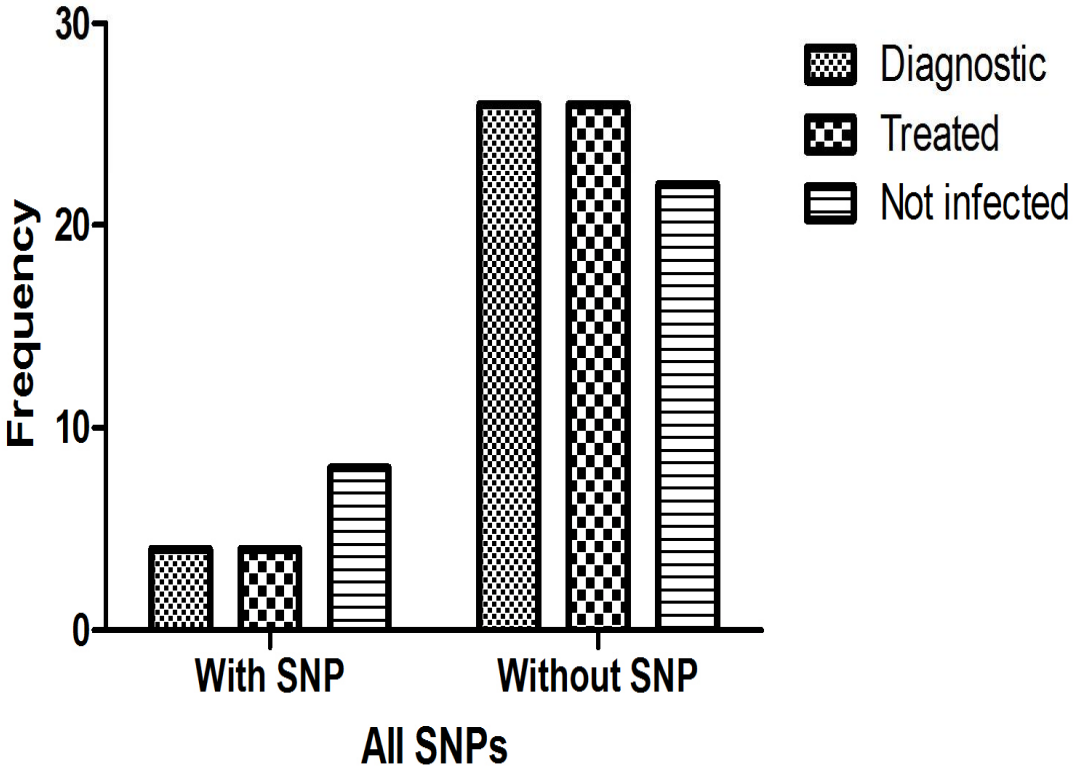


SNPs include rs374889117, rs206964 and rs2069772

Fos gene single nucleotide polymorphisms

Appendix 4

Chi-square	
Chi-square, df	2.432, 2
P value	0.2963
P value summary	ns
One- or two-sided	NA
Statistically significant? (alpha<0.05)	No



All single nucleotide polymorphisms of Fos genes