Evaluation of Immunological Factors in HIV Pathogenesis

BY

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Evaluation of Immunological Factors in HIV Pathogenesis

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A thesis submitted in partial fulfillment of the requirements for the degree of

MS Healthcare Biotechnology

Supervised by: **Dr. Aneela Javed**

Co-supervisor: **Dr. Sobia Manzoor**

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Rida e Zainab

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Dedicated to

Mama, Baba, Aizaz, and my sons

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All praises for Allah Almighty, the utmost Merciful and the Gracious, this success is not only mine but also of many other people. I am extremely thankful to my parents who have supported me to this level where I am able to finish my MS. The success belongs to my mother, more than me who instigated me to finish my degree even though there were a million reasons not to. She was my guiding star.

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Rida e Zainab

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Abstract

Since its advent in the 1980s, HIV has created an undisputed havoc for mankind by its intelligence and ability to replicated quickly, develop resistance to host immune factors and utilizing the host machinery for its own use. The virus has infected more than 39.9M people worldwide and although much research has been carried out for its treatment, no cure is available till now. As the virus completely relies on the host machinery, the virus has multiple accessory proteins to counteract the action of host defense mechanisms and evolutionary proteins. Purinergic receptors, P2X have a role in aiding the entry of the HIV to the host cells. As HIV infects the body, extracellular ATP is increased. This ATP increase serves as a means for inducing conformational changes in p2x receptor present on the surface of plasma membrane and allows the virus to enter the host cell. Inhibitors designed for P2X receptors are involved in preventing the virus from entry to host cells. APOBEC3G is a host factor that induces hypermutations into the HIV ssDNA and prevents the virus from successfully replicating, ultimately destroying its structure. However, vif, inhibits the action of apobec3g by recruiting the protein to proteosomal degradation. Tetherin is also a host protein that tethers the virion to the surface of the host plasma membrane. The protein is of unique structure having unique topology and anchors nascent virions to the cell surface using GPI anchors for attachment of the virions. The protein is responsible for inhibiting the virus to escape from the cell without interacting with the viral proteins. Vpu interacts with tetherin and destroys its activity, however, when vpu is absent, tetherin can still act to inhibit the virus. TRIM 5α is pattern recognition protein which recognizes the virus by its capsid protein, prevents the uncoating of the virus and even before reverse transcription, the protein acts to inhibit the virus by its E3 ubiquitinase activity and triggering MAPK pathway.

Chapter no 1

Introduction

HIV & AIDS

Human Immunodeficiency Virus (HIV) is a retrovirus discovered in the 1980s, in homosexuals, also transferring to heterosexuals in a short time, belongs to lentiviruses that leads to the infamous acquired immunodeficiency syndrome (AIDS). AIDS is in fact a late stage of progressed HIV infection and happens when the immune system is badly damaged virus. There are basically two types of HIV that are held responsible for infections in humans, i.e. HIV-1 and HIV2. They primarily differ in their virulence and global prevalence.

Most of the reported AIDS cases worldwide are attributed to HIV- type 1. Transfer of HIV occurs by numerous modes i.e., transfer of, breast milk infected blood, vaginal fluid and other fluids within the body. HIV exist as either free viral particles or as a virus within infected cells within body fluids. Major causes of transmission of HIV are unprotected sexual intercourse, transmission contaminated needles and vertical transmission from infected mother to baby during or after birth (through breast milk).

The virus was discovered in chimpanzees but due to absence of receptors in them, it doesn't cause disease in the monkeys. HIV-1 has four distinctive lineages by which it is transmitted to humans from the other primates. These distinct HIV-1 lineages are grouped into M, N, O and P. Of these, the most significant and persisting lineage throughout the world has been the M group of HIV-1, mostly responsible for the human infections. The M group is further

classified into 9 subtypes which include A, E, F, J and K subtypes along with other sub-subtypes such as A1, A2, F1 and F2, unique recombinant forms (URFs) and circulating recombinant forms CRFs; others such as O group has O subtype, N group has N subtype.

These HIV-1 subtypes are critical in respect of determining virus' etiology, epidemiology, laboratory diagnosis, clinical symptoms, drug screening and vaccine development techniques.

The primary target of HIV is the human immune system and its cells i.e., helper T cells

(Mainly CD4+). A body, infected with HIV, contains low levels of CD4+ T cells in comparison to healthy controls. After the drop-down of these cells under a critical threshold, cell-mediated immunity is lost, and body is immune to opportunistic infections until body loses its potential to fight off pathogens and develops AIDS (acquired immunodeficiency syndrome). The most common infections developed at this stage include cytomegalovirus-induced eye infection, chronic cryptosporida diarrhea pneumocystis pneumonia, toxoplasmosis, and tuberculosis as well as infections with members of the *Mycobacterium avium* complex. Additionally, the many different types of cancer are also developed, such as invasive lymphoma, cervical cancer and Kaposi sarcoma is frequently observed. The disease is still incurable.

HIV Prevalence in World

Introduction

According to the sources of UNAIDS, altogether an average of 38.0 million people were living with HIV at the end of 2019. A typical of 0.7% of adults with ages between 15 to 49 years worldwide are currently living with HIV, though the prevalence of this epidemic fluctuates

suggestively between countries and regions. The most strictly affected is the WHO African region, in which 1 in every 25 adults (that makes up 3.7% of adults) are currently living with HIV and about two-third of the people are living with HIV around the globe (UNAIDS).

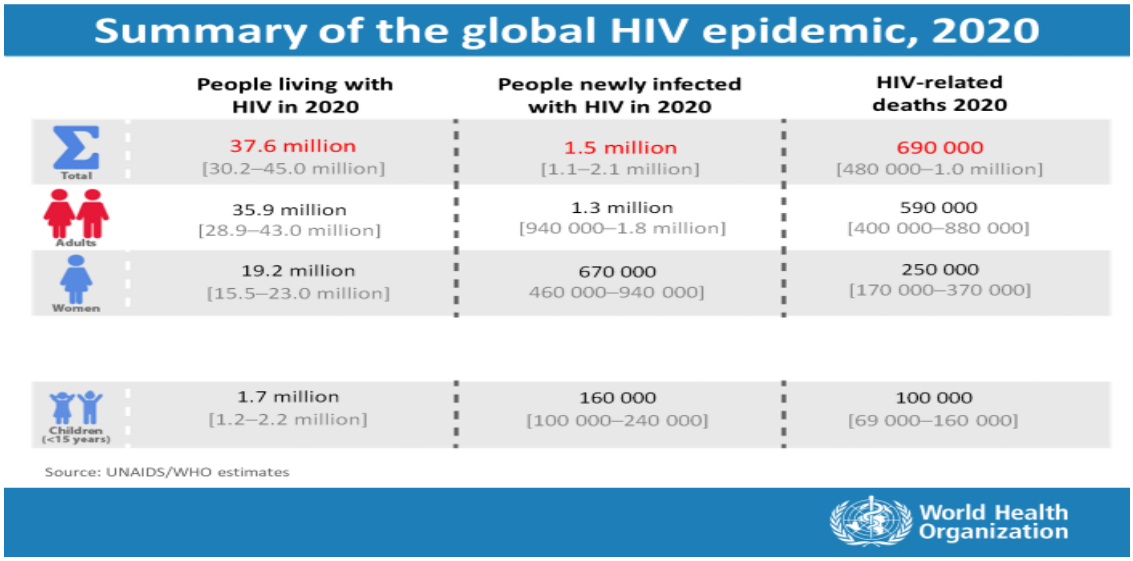

Fig no 1.4

The Incidence of HIV in 2020, report published by WHO (https://www.who.int/data/gho/data/themes/hiv-aids)

Chapter no 2

Literature Review

HIV

HIV came into highlights as a lethal, silent and mysterious killer in the earlier 1980s in gay individuals. The virus was first recognized in chimpanzees but due to the absence of host entry protein, it doesn't cause disease in them. After about 40 years, due to rapid advances in molecular immunology and virology, AIDS has now been transferred from a lethal infection to a manageable disease although it still remains to be a global threat because 40 M people in the world with deaths related to HIV increasing with each passing year. (Ruiz et al., 2020)

Life Cycle of HIV

The main target of HIV is CD4 T lymphocytes of immune system. CD4 are our immune cells i.e., the white blood cells that play their role in protection of the body from infection. HIV utilizes the cellular mechanism of the host cells for its multiplication and spread in the body. It enters the CD4 cells and takes control over the host machinery in 7 steps known as the HIV life-cycle. (Ruiz et al., 2020)

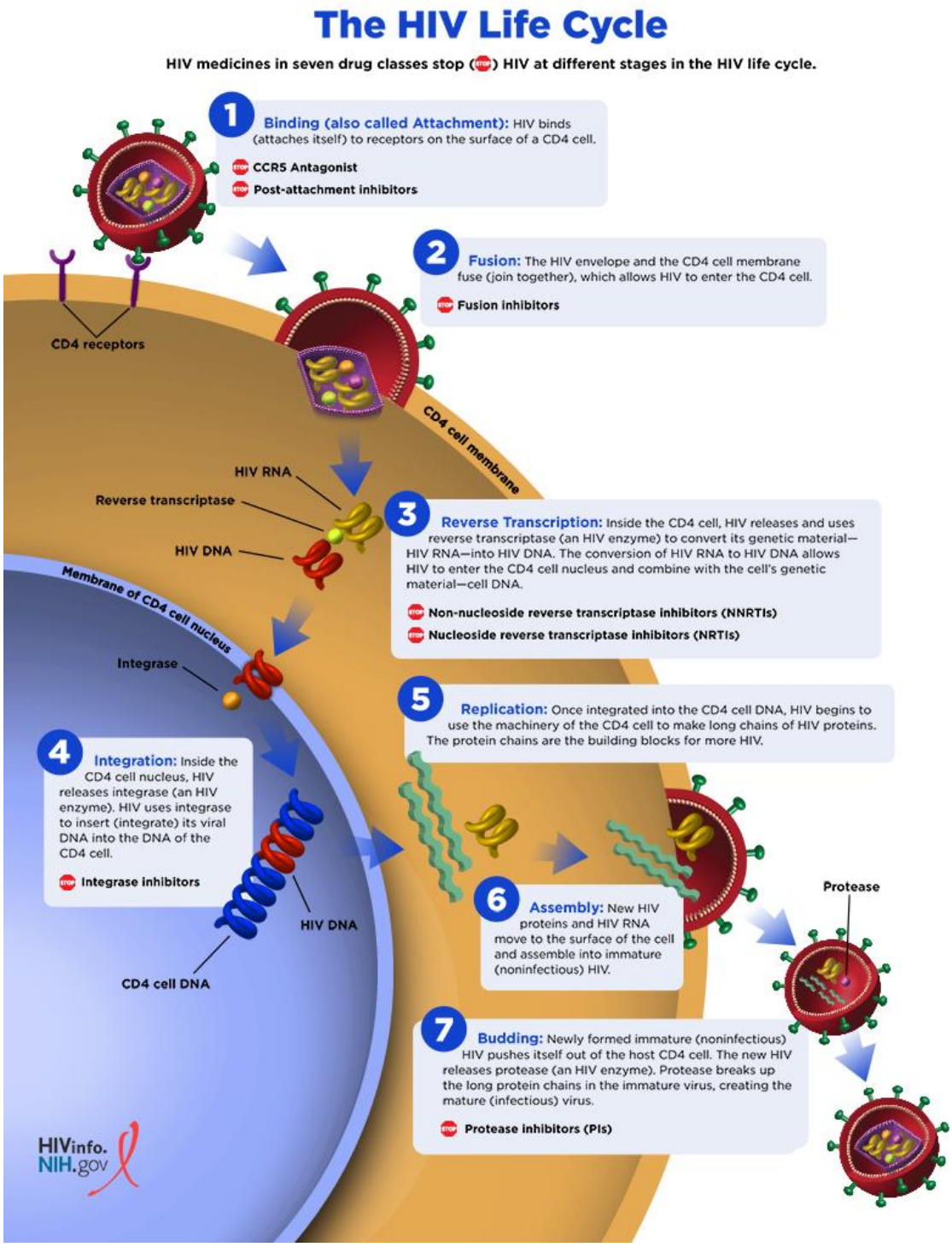

Fig no 2.1 ***The seven steps of HIV life-cycle in CD4 Lymphocytes. The virus enters the host cells by the interaction of its envelop protein to the host p2x1. The genome is released and***

integrates to the host genome. New copies of the virus are formed and released into the host cytoplasm. **(Ruiz et al. 2020)**

Host Factors Involved in HIV Replication

Human Immunodeficiency Virus (HIV) is totally dependent on host molecular machinery for its replication and spread. Many systematic attempts to identify these host factors biochemically or genetically have led to the generation of hundreds of candidates, but only few of them have been validated in primary cells. Better-quality understanding of these molecular mechanisms underlying pathogenesis HIV replication and persistence is serious for the growth of new curative and therapeutic strategies. (Hiatt et al., 2022)

Additionally, CD4+ T lymphocytes, differentiated macrophages and myeloid cells are therapeutic targets to HIV-1 infection through communication of gp 120 Env with or CCR5, CD4 CXCR4. Both macrophages and T cells play their role in viral replication, though with minor differences. As compared to activated CD4+ T lymphocytes, HIV-1 replication inside macrophages occurs in nondividing cells, also it is established by the absolute deletion of cytopathic pathway both in vivo and in vitro. (Pagani et al., 2022)

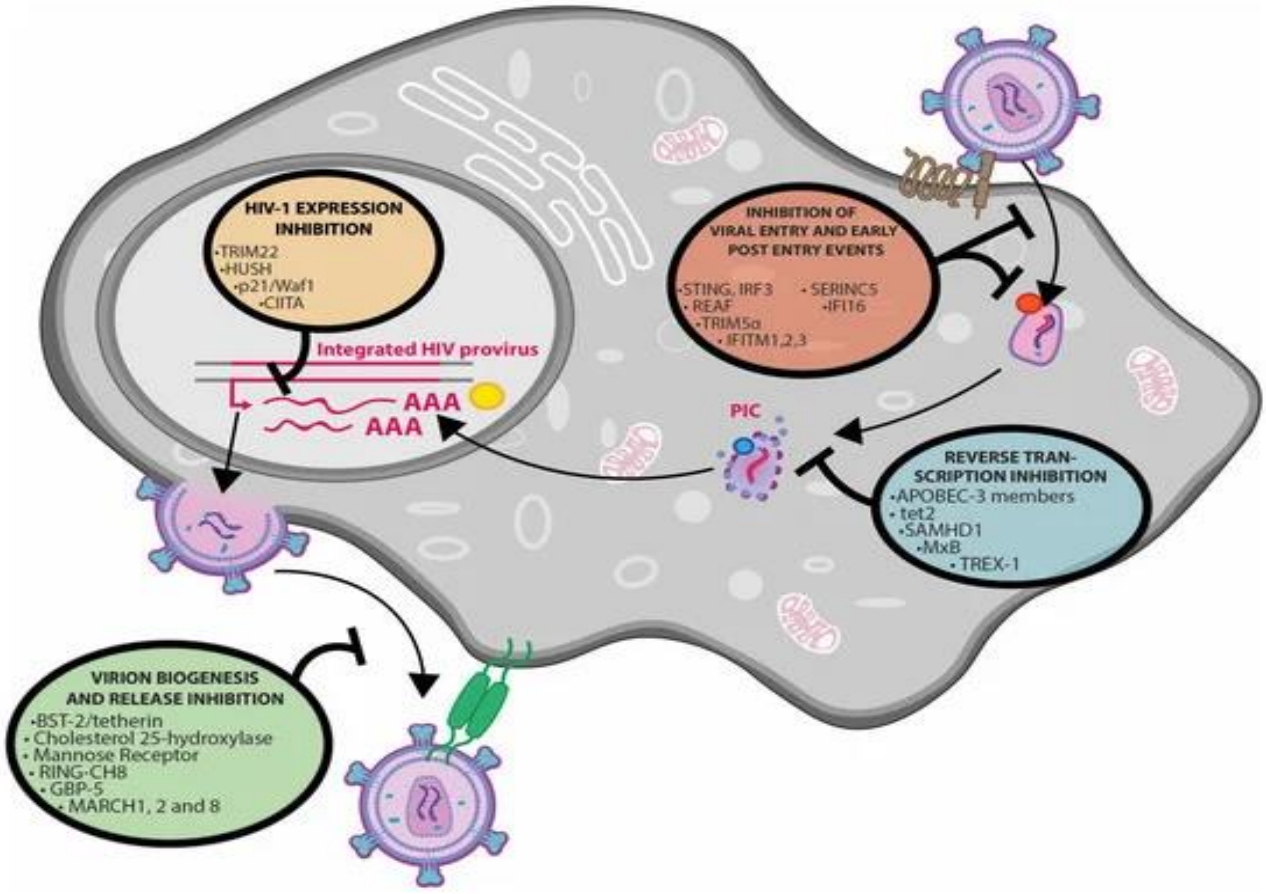

Fig no 2.2 ***Restriction factors interfering with HIV-1 infection of macrophages. PIC: Pre-Integration Complex. HIV enters the cell in 7 main steps and each of these steps a target of potent antivirals and host resistance factors. (Pagani et al., 2022)***

APOBEC 3

Apobec3 is human apolipoprotein 3 mRNA editing enzymatic catalytic subunit 3 belonging to a polynucleotide cellular deaminase which comprises of about 7 followers (A, B, C, D, F, G and H). They are responsible for catalyzing the conversion, on ssDNA, of cytosine to uracil. A3G subunit exhibit the most potential antiviral activity against retroviruses and retrotransposons which includes HIV 1. Though the HIV-1, (Vif) acts as an antagonist to A3G mediated defense system

and promotes HIV-1 propagation in human cells. Vif is responsible for the recruitment of A3G to Ubiquitin ligase complex i.e., E3 which include ElonginB/C, Cullin5 and also CBFβ, are the factors responsible for promoting the degradation of A3G through ubiquitin proteosome pathway.

In absence of Vif, A3G binds to domain of either Gag or to any viral or non-viral RNAs. It is released and incorporated into HIV-1 virions and exhibit its antiviral activity. Within cells infected with HIV, A3G hinders viral replication specifically through deamination of C in the minus-stranded DNA, thus leading to large-scale G-to-A hypermutation of the newly formed viral DNA genome during the process of reverse transcription. It is keenly observed as "all or nothing" phenomenon i.e., the A3G induced hypermutation. Mostly, A3G directly stops reverse transcriptase elongation and inhibits the incorporation of pro-viral DNA to the host chromosome. These associated molecular mechanisms are probably significant in enhancing the anti-HIV-1 activity. (Okada et al., 2016)

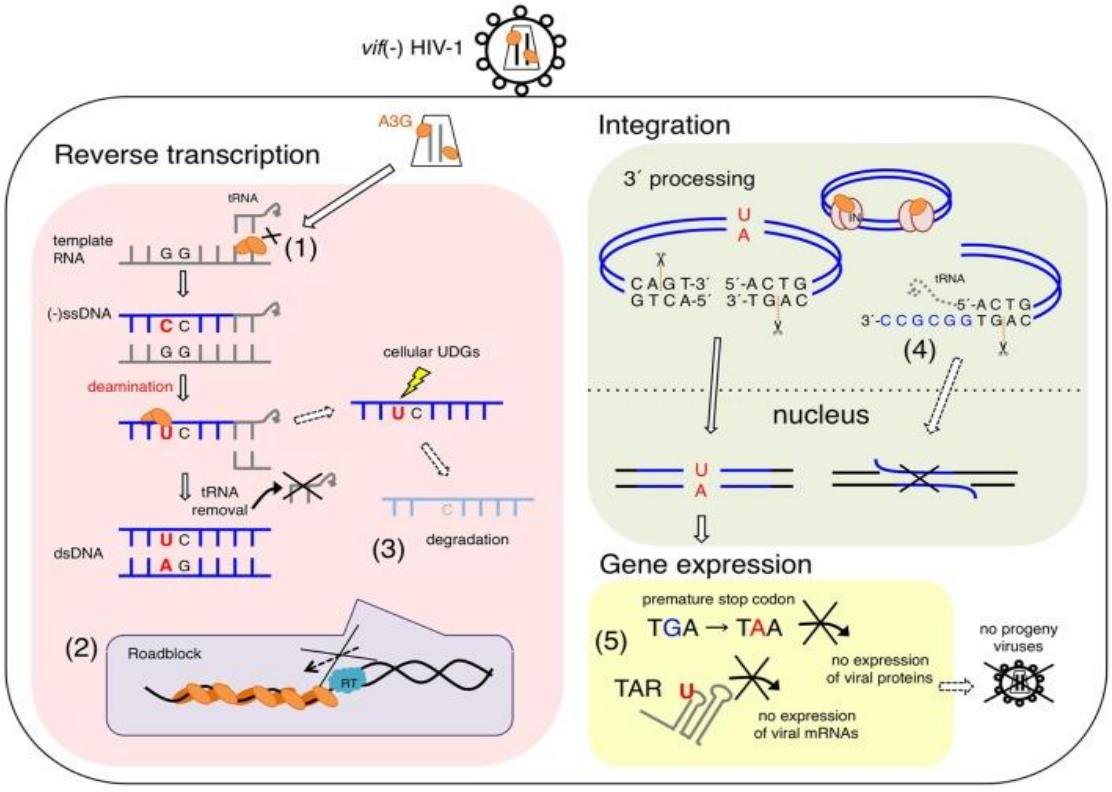

Fig no 2.3.

Fig no. 2.3 Antiviral mechanism of APOBEC3G (1) APOBEC3G interferes with the annealing of tRNA primer for reverse transcription (2) it binds to RNA and physically block reverse transcription (3) it induces hypermutations in ssDNA (4) it decreases efficieny of primer tRNA (5) mutations from dC to dU leads to viral replication failure *(Okada et al., 2016)*

Tetherin\ Bone Marrow stromal cell antigen 2 (bst2)

Tetherin (also known as bone marrow stromal antigen-2 (BST-2), (CD317), or HM1.24) cluster of differentiation 317 is an IFN-inducible, type II transmembrane (TM) glycoprotein that is responsible for affecting the late stage of the viral cycle by securing virions to the cell surface

It was originally recognized as indicator for bone marrow stromal cells; it is stated constitutively in terminally differentiated monocytes, dendritic cells, B and T cells and is up-regulated in cancer cells. Tetherin comprises 180 amino acids and is localized in lipid rafts at the cell surface and on intracellular membranes. Tetherin blocks viral release of HIV, without interacting with the viral proteins, by blocking and tethering the nascent viral particles to the surface of the cell. The viral Vpu counteracts the effects of tetherin and blocks it. However, in the absence of Vpu, tetherin still works in blocking viral release. Their exceptional topology allows them to be in cell through their N-terminus using GPI anchor for attachment to budding virions. HIV-1 has managed to overcome this restriction through vpu. Vpu counteracts with tetherin by interrelating with this protein at its transmembrane domain, employing β-TrCP2, which sources ubiquitination and degradation of tetherin. (Waheed et al., 2020)

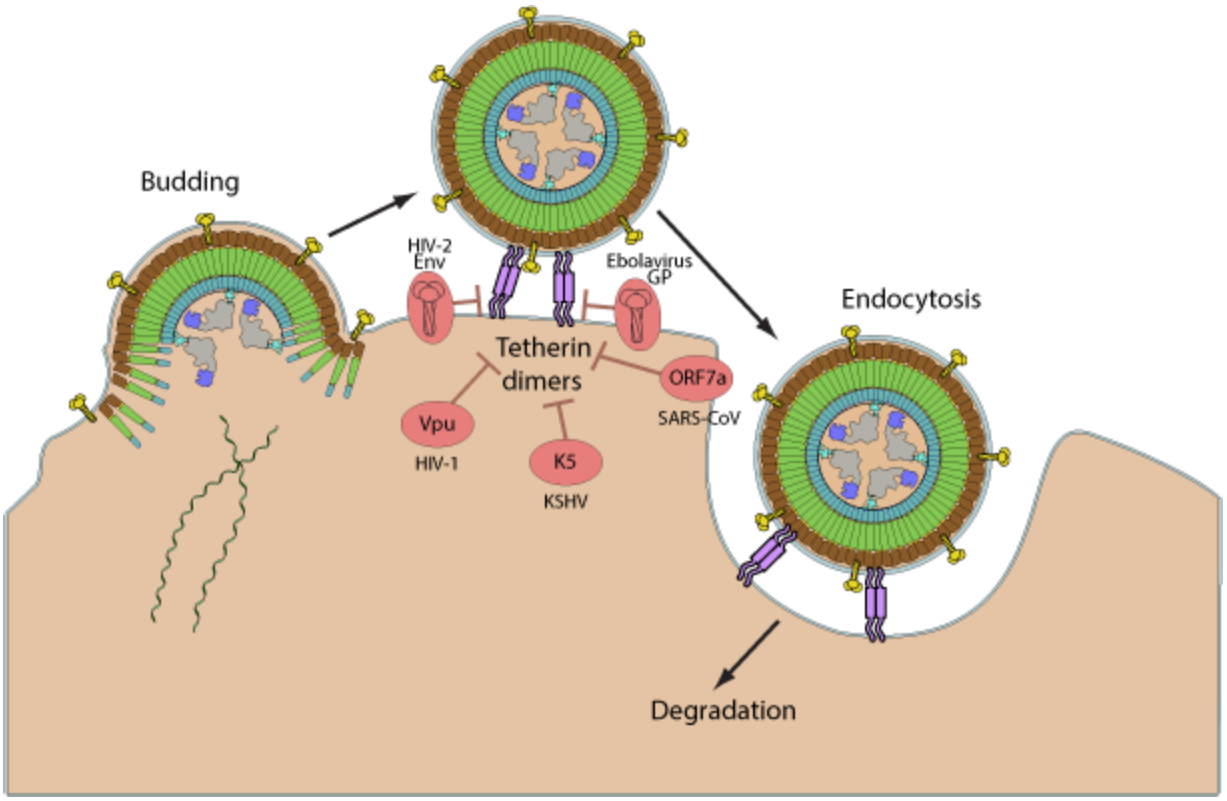

Fig no 2.4. ***Inhibition of host Tetherin by virus. Each of the RNA virus has evolved certain factors for inhibition of tetherin. HIV possess vpu for inhibition of tetherin activity. (Courtesy of Viral Zone*****)***

<u>Tripartite motif containing 5 (Trim 5 alpha)</u>

TRIM 5 is capsid-specific restriction factor that blocks infection from the non-host-adapted retroviruses. Also functions in blocking the viral replication earlier in the life cycle, after viral entry before reverse transcription. Additionally acting as a pattern recognition receptor, it also acts as a capsid-specific restriction factor that enhances innate immune signaling against the retroviral capsid. When it binds to the viral capsid, it activates its E3 ubiquitin ligase activity, and in performance with ubiquitin conjugating enzyme complex UBE2V1- which generates 'Lys-63'-linked polyubiquitin chains, and in turn are catalyzers in autophosphorylation of MAP3K7/TAK1 complex (includes TAK1, TAB2, and TAB3). Activation of the complex, MAP3K7/TAK1 complex by autophosphorylation, results in the initiation and expression of MAPK-responsive and

NF-kappa-B inflammatory genes, which leads to innate immune response in infected cell. Restricts infection by N-tropic murine leukemia virus (N-MLV), simian immunodeficiency virus of macaques (SIVmac), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), and bovine immunodeficiency virus.

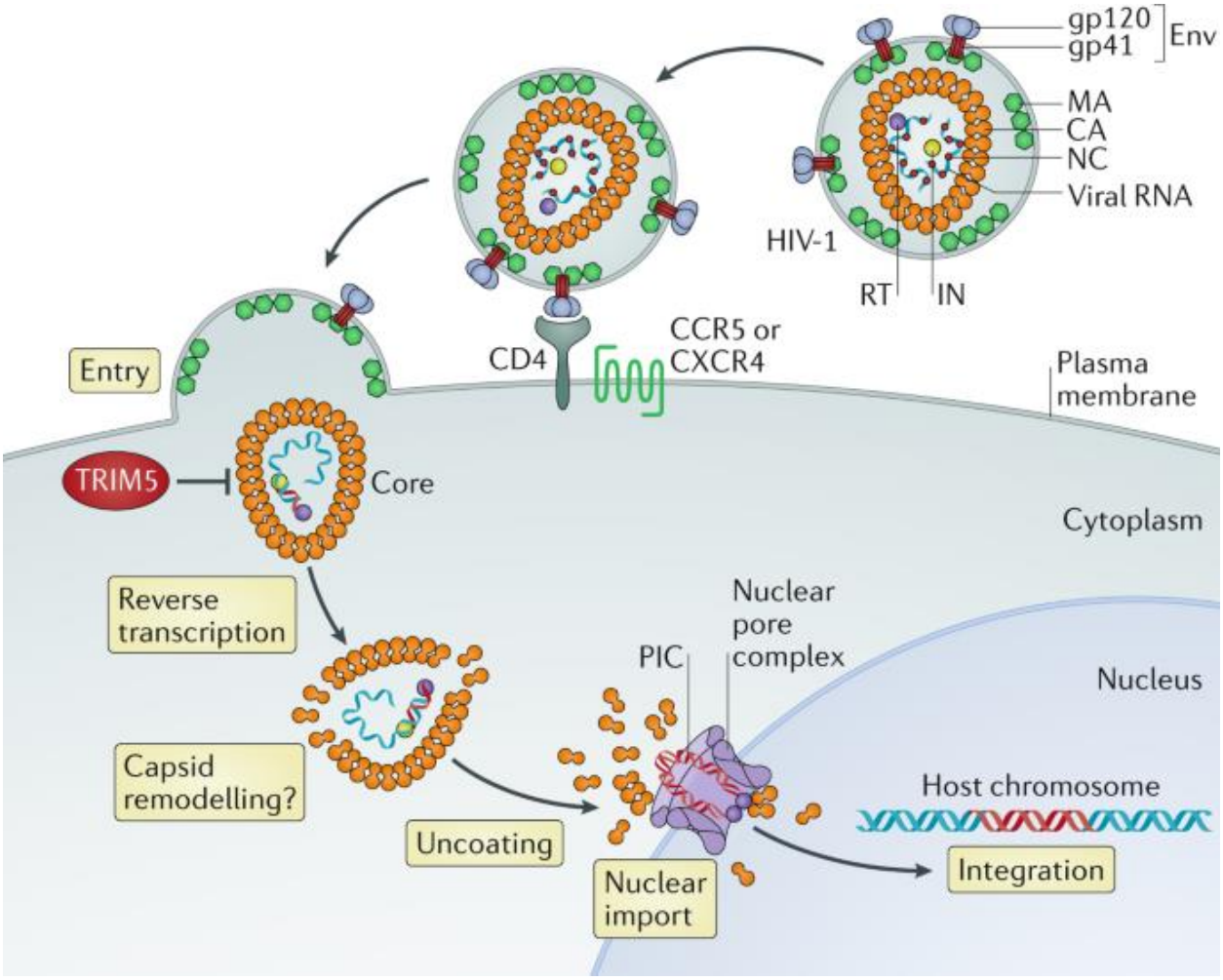

Fig no 2.6 ***Restriction of HIV and other retroviruses by TRIM 5. TRIM 5 recognizes the capsid protein of virus by GPU signaling and prevents uncoating of the virus by capsid remodeling. (Courtesy of Nature Immunology)***

<u>Purinergic Receptors</u>

In the year 1976, purinergic receptors were identified. After two years, the P1 and P2 receptors' base for the two separate families of these receptors was discovered. Two other P1 (adenosine) receptor subtypes were discovered after that. A pharmacological source for the two different P2 receptor subtypes (P2X and P2Y) was proposed in 1985. P1 (adenosine) receptors were discovered, cloned, and classified in the 1990s; four subtypes were identified. The first G protein-coupled P2Y receptors, a different subclass of P2 receptors, were cloned in the early months of 1993.

Purinoceptors should belong to these two significant groups, it was determined in the early part of 1994 based on their structure and transduction methods. As a result, eight P2Y receptor subtypes and seven significant P2X receptor subtypes have been identified, having receptors that can bind both pyrimidines and purines.

Purinergic signalling appears to be a long-standing mechanism of evolution. These play a key role in both neuronal and non-neuronal processes, including inflammation, pain, platelet aggregation and endothelial-mediated vasodilatation, exocrine and endocrine secretion, and immunological responses in mammals. Purinergic receptors also aid in the progression and renewal of cellular differentiation, proliferation, and death.. (Burnstock et al., 2018)

P1 receptors

In 1989, the cDNAs for the two P1 receptor subtypes (A1 and A2) were divided. The A3 subtype was then identified. In the 1990s, four distinct P1 receptor subtypes—A1, A2A, A2B, and A3—were identified, cloned, and described. Both the A1 and A2A receptors have been found to be polymorphic. Adenylate cyclase and P1 receptors pair, indicating that A3 and A1 are negatively connected to the enzyme while A2A and A2B are favourably coupled. Agonists and antagonists that are P1 subtype-selective have been identified. P1 receptor subtypes have a variety of physiological effects, including the modulation of immunological, cardiovascular, and central nervous system (CNS) functions. (Burnstock et al., 2018)

P2X Receptors

P2X receptor

P2X1 Receptors

Rat vas deferens was used to create complementary DNA (cDNA) that encodes P2X1. P2X1 and P2X3 receptors can be distinguished from other homomeric versions by the agonistic effects of ATP as mediated by, -methylene ATP (, -meATP). It has been established that several antagonists are selective for P2X1 receptors.

P2X2

Using PC12 cells and human receptor cDNA from the pituitary gland, P2X2 receptor in rat cDNA was identified and cloned. There are currently no known agonists that are effective against P2X2 receptors. However, P2X2 receptors are potentiated by low levels of zinc, copper, and protons. When compared to the P2X1 and P2X3 receptors, the P2X2 receptor is distinct in that it is sensitizing. (Burnstock et al., 2018)

P2X3

P2X3 receptor subunit cDNAs were extracted from zebrafish, human heart, and rat dorsal root ganglion cDNA libraries. When compared to P2X1 receptors, the antagonist NF023 for P2X receptors is nearly 20 times less potent at P2X3 receptors.

On sensory neurons, which also include nociceptive nerve ends, P2X3 receptors are abundantly visible. There are other known P2X2/3 heteromeric receptors. They are vulnerable to being blocked by 2′(3′)-O-(2,4,6-trinitrophenyl) ATP despite being impacted by low pH like homomeric P2X2 receptors and homomeric P2X3 receptors. Subpopulations of sympathetic ganglion cells, brain neurons, and sensory neurons all have P2X2/3 receptors that are present and expressed.

P2X4

P2X4 receptors were isolated from the brain and superior cervical ganglion (SCG) from cDNAs expressed in rats. Xenopus cDNAs, human, mouse, and chick cDNAs, as well as others, have been discovered. ATP stimulates P2X4 receptors, though -meATP does not. The stimulation of ATP-evoked currents at P2X4 receptors by ivermectin is a significant distinguishing characteristic. The P2X4 receptor in rats has an uncommon activity among the P2X receptors since it is relatively resistant to the conventional non-selective antagonists pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and suramin. Effective P2X4 receptor antagonists have been demanded for derivatives of carbamazepine. Suramin and PPADS, which are likewise members of a class of ectonucleotide inhibitors, may increase the expression of the P2X4 receptor in the mouse, which is currently inhibited by ATP

P2X5

The P2X5 receptor cDNA was first cloned from the rat heart and abdominal ganglia. It was also replicated by bullfrog P2X5 receptors from chicken's early skeletal muscle and larval skin. The human P2X5 receptor cDNA lacks exons 10 (hP2X5a) or exons 3 and 10 (P2X5b). Cells currently stimulated by ATP expressing the rat P2X5 receptor are less important than cells detected with the P2X4, P2X3, P2X2, or P2X1 receptor. P2X5 mRNA is highly expressed in skeletal muscle. The P2X1 / 5 heterodimer is obsolete by α, β, -meATP. Cells that trigger the P2X1 / 5 receptor form a complex with ATP rather than the homomeric receptor. Heteromer P2X2 / 5 receptors have also been discovered. (Burnstock et al., 2018)

P2X6

The rat brain was where the P2X6 receptor was initially discovered. The human P2X6 receptors were purified from peripheral lymphocytes and expressed in both mouse and human skeletal muscle. A heteromultimer is how the P2X6 subunit is expressed.

In HEK293 cells, heteromeric P2X2/6 proteinaceous receptors were discovered. At a specific pH of 6.5, when the current suramin is inhibited, there is a significant difference between oocytes

expressing P2X2/6 receptors and those expressing only P2X2 receptors. In the respiratory neurons of the brain stem cells, P2X2/6 receptors are particularly expressed.

P2X7

Medial habenula was the site of the initial identification of chimaera cDNA encoding the P2X7 receptor identified in rats. Rat brain cDNA was used to create a library of full-length cDNAs. The P2X7 receptor's most significant characteristic is that, in contrast to the normally quick opening of the cation-selective ion channel, it initiates a channel to pores with high exposure to concentrations of ATP and permits big molecules like YO-PRO-1 and ethidium to pass through them. This results in apoptosis, and subsequently, cell death. A possible and non-selective agonist for the P2X7 receptor is an inhibitor like 2′, 3′-O-(benzoyl-4-benzoyl)-ATP (BzATP). (Burnstock et al., 2018)

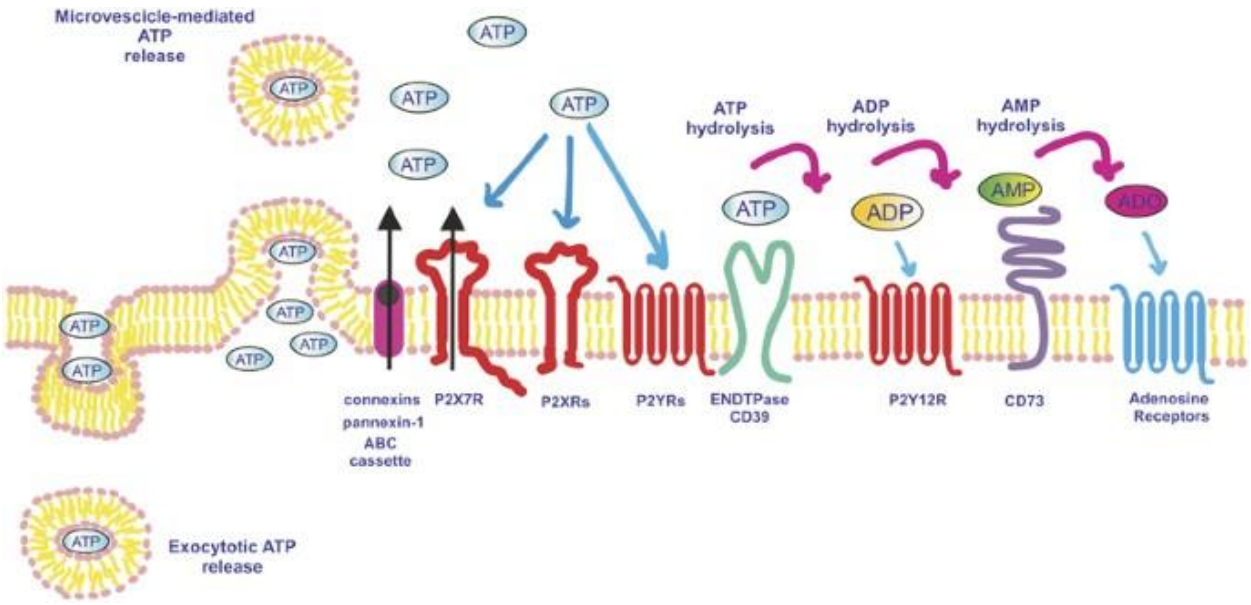

Fig no. 2.7 ***Purinergic Signaling in Viral Infections. ATP is the activator of PR, when a cell is infected by the virus, ATP is released by pannexin 1 and HIV envelop protein interacts with CCR5 and CXCR4 of the host membrane. (Courtesy of Nature)***

Role of Purinergic Receptors in HIV

During HIV infection, macrophages are grave for viral spread in the host. These are the first and foremost cells to become infested and serve as a viral reservoir. Infection in macrophages does not result in their death, and HIV-infected macrophages can continue to survive for longer periods of time in host tissues, even in the incidence of combined ART therapy. These infected macrophages in the central nervous system are mediators of secreting inflammatory mediators, HIV-associated neurocognitive disorders and neurotoxic proteins that function in CNS dysfunction. HIV infests macrophages by forming association to the gp120 envelope protein CD4 and then CCR5 receptors and consequent fusion to the host cellular membrane. Binding to gp120 consequences in enhanced G protein signaling and intracellular calcium which is then involved in opening of calcium activated potassium channels and nonselective cation channels. Though, in addition some specific host proteins that contribute in this process need more assessment. Activation of purinergic receptors is done by extracellular ATP and the byproducts of ATP, which includes ADP and UTP, and are divided into three groups: ATP-gated cation channels (P2X), adenosine receptors (P1) and G protein-coupled receptors (P2Y). Beginning of P2X or P2Y receptors leads to an increase in intracellular calcium. Results from our studies and other investigators have indicated that macrophages principally prompt P2X1, P2X4, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, and P2Y12 receptors.

In macrophages, P2X and P2Y receptors are significantly key mediators of the response to host damage, and identifies eATP as a damaging signal leading to swelling through cytokine release and superoxide formation. In response to infectious agents' studies showed that ATP release and purinergic receptor signaling are activated. We conclude that for efficient viral replication, expressed in primary human macrophages are effectively required for viral replication. More noticeably, we show that P2X1 receptors are required for HIV entry into macrophages.

Research has indicated that data binding of HIV gp120's to macrophages has resulted in ATP release which results in stimulation autocrine P2X and/or P2Y receptors, subsequently leading in HIV entry and subsequent stages of viral life cycle. Our data has provided indication of a novel

function of P2X and P2Y receptors, suggesting new therapeutic targets to limit HIV infection. (Hazleton et al., 2012)

Objectives of the study

- To evaluate the gene expression of host immunological factors during HIV infection in Pakistan population
- To analyze the expression changes in Purinergic receptors, APOBEC3G, tetherin and TRIM 5α in Pakistan
- To analyze how this expression can serve as a means for development of cheap, cost effective and easily available treatment of HIV/AIDS.

Chapter no. 3

Material and Methods

Sampling and Ethical Consent

The patients recruited for this study were well-versed about the design of the study and objective. All patients provided a written consent to participate on their own will in this study and were up to date about the outcomes of this project. The study began after the written approval from the ethical committee of Punjab AIDS Control Program (PACP) from institutional review board (IRB) of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences & Technology (NUST) Islamabad, Pakistan after complete methodical assessment of all research protocols that is involved in this project.

Patients involved in this project involved HIV positive patients from Punjab AIDS Control Program. HIV patients in Punjab were targeted during the study. The study had a particular exclusion criterion. According to the criterion, only those HIV positive patients who had significantly high viral loads been targets of the study. Subjects who retorted to therapy were specifically excluded. Informed consent was acquired.

Patients were also inquired about the systemic effects and possible related comorbidities prior to or during the course of HIV infection. This was attained because HIV-associated co-morbidities could possibly change the results of this project. Additionally, viral load, patient age, gender, and clinical factors were also taken into consideration.

Table 3.1 ***Demographic data of patients obtained for the study. Age, gender, treatment centre, viral load and CD4 count of the patients selected for the study***

NUST ID	**Age**	**Gender**	**Treatment Center**	**PCR Result (IU/mL)**	**CD4 Count (count/uL)**
P1	30	M	AHF - (VCCT Centers)	2864340	187
P2	28	M	Allied Hospital Faisalabad - (VCCT Centers)	1264864	426
P3	65	F	Jinnah Hospital - (Treatment Center)	ND	683
P4	44	M	Jinnah Hospital - (Treatment Center)	ND	976
P5	26	M	Allied Hospital Faisalabad - (VCCT Centers)	88008	530
P6	25	M	Allied Hospital Faisalabad - (VCCT Centers)	687640	301
P7	30	M	Jinnah Hospital - (Treatment Center)	11253304	
P8	28	M	Allied Hospital Faisalabad - (VCCT Centers)	100341	490
P9	33	F	Adult Treatment Center Services Hospital Lahore - (Treatment Center)	8246	332
P10	28	M	Allied Hospital Faisalabad - (VCCT Centers)	30488	199
P11	35	M	Allied Hospital Faisalabad - (VCCT Centers)	527196	334
P12	34	M	Allied Hospital Faisalabad - (VCCT Centers)	7499	250
P13	38	M	Jinnah Hospital - (Treatment Center)	ND	809
P14	34	M	DHQ Hospital Dera Ghazi Khan - (VCCT Centers)	ND	1149
P15	8	F	DHQ Hospital Dera Ghazi Khan - (VCCT Centers)	387412	605

RNA Extraction by Trizol Method

RNA extraction was accomplished using Invitrogen Trizol LS Reagent. With the aid of RBC lysis buffer, the volume was reduced from 50 ml of Falcon tubes containing the contents of the EDTA vial to 25 ml. Ten minutes were given for it to stand at room temperature. The PBMs were then palleted for 10 minutes at room temperature at 600g (14000 rpm). After that, the supernatant was decanted. In 1ul of RBC lysis buffer, the pallet was reconstituted. The mixture was then transferred to a new tube and left to stand at room temperature for 5 minutes. This was once more centrifuged for two minutes at 3000 rpm. The pellet was resuspended in 1ml of PBS after the supernatant was aspirated. To separate the cells, this was once more centrifuged for two minutes at 3000 rpm at ambient temperature. The pallet was once more resuspended in 250ul of PBS after the supernatant was aspirated. Cells were homogenised by vortexing after 800ul of Trizol was added. (Samples may be marked and kept at -20 °C.) It was vortexed for 15 seconds after being given 200 ul of chloroform and 28 ul of glacial acetic acid. Following that, centrifugation was carried out at 4 for 20 minutes at 14000 rpm. An equivalent volume of ice-chilled isopropanol was then poured to the newly transferred, lysate-filled tube. To fully combine it, it was reversed and set for 20 minutes on ice. It was then washed with 75% ethanol and allowed to dry for 20 minutes. RNA was then stored in 20ul NF water.

10X RBC Lysis buffer Recipe

4.495g of NH_4Cl

0.5g of $KHCO_3$

100ul of 0.5M EDTA

The components were dissolved in 30ml of dH2O and pH was maintained to 7.3. The solution was adjusted to 50ml and mixed thoroughly.

cDNA Synthesis

cDNA synthesis was done using RevertAid Reverse Transcriptase, but additional research settled that viral RNA copy number in human plasma is comparatively low as compared to that in cells. Therefore, owing to better speed and resistance to a large number of inhibitors that affect with cDNA synthesis, Superscript III Reverse Transcriptase was put into use and was proven beneficial.

The procedure involves Superscript III Reverse Transcriptase cDNA synthesis, primer annealing to template RNA, an assembly of reaction mixture also addition of reaction mixture to annealed RNA as main steps. It mainly starts with the following components in a reaction tube:

Component	Volume
50 µM random hexamers, 50 µM Oligo(Dt)20 primers, or 2 µM gene specific primers	1 µL
10 mM dNTP mix (10 mM each)	1 µL
Template RNA (10 pg – 5 µg total RNA)	up to 11 µL

Table 3.2: cDNA preparation from RNA extracted from WBCs (I).

After mixing the components, the reaction tube is centrifuged for a short time. The machine was pre-heated to 65°C and after centrifugation, the mixture is then heated at 65°C for 5 minutes and then incubated on ice for 2 minutes. During heating, RT reaction mix was allowed to mix and was then prepared by combination of the following components in other reaction tube:

Component	Volume
5× SSIII buffer	4 µL
DTT	1 µL
RNase Out RNase inhibitor	1 µL
Superscript III Reverse Transcriptase (200 U / µL)	1 µL

Table 3.3: cDNA preparation from RNA extracted from WBCs (II).

The Reverse Transcriptase reaction mix tube was then capped, vortexed and then centrifuged. The next step was adding of RT mix to annealed RNA. Incubation steps followed. They included incubating the collective reaction mix at 50°C for 15 minutes followed by inactivation phase of 10 mins at 80°C. As the probable product size was higher than 1 kb, an optional step of RNA removal was critical.

To remove any RNA present, 1 µL of *E.coli* RNase H was then added to the obtained mixture and was then incubated at 37°C for 20 minutes.

A second reaction tube containing NRTC (non-reverse transcriptase control) was created in addition to the template RNA cDNA to assess the effectiveness of the reaction and rule out the potential of DNA contamination in other reagents.

Primer Designing

To evaluate the host proteins involved in HIV pathogenesis, primers were built for the apobec3g, trim 5, tetherino and a family of purinergic receptors known as P2X receptors of the human

genome. MegaX and the Primer3 web portal (http://bioinfo.ut.ee/primer30.4.0/) were used to create primers manually using online tools and other software. 10 sets of primers were designed. The properties were studied using oligocalc.

Table no 3.2 *Sequence of primers designed for the study, i.e., their annealing temperatures, amplicon size and site of action*

Primer	Sequence	Primer site	Amplicon size	Tm
P2X1				
Forward primer	GTGCTGGTGCGTAATAAGAAGG	304-324	213	59.65
Reverse primer	GAAGACGTAGTCAGCCACATCC	516-495		60.74
P2X2				
Forward primer	CTGGGCTTTATCGTGGAGAAG	762-782	187	58.44
Reverse primer	ACCTGAAGTTGTAGCCTGACG	948-928		60
P2X3				
Forward primer	GATCATCAACCGAGTAGTTCAGC	271-293	175	59.2
Reverse primer	CGTCACGTAATCAGACACATCC	445-424		59.15
P2X4				
Forward primer	CTCTGCTTGCCCAGGTACTC	841-860	355	60.11
Reverse primer	CCAGCTCACTAGCAAGACCC	1195-1176		60.11

P2X5

Forward primer	CCGTGACAAGAAGTACGAGG	1113-1132	193	58.02
Reverse primer	AGATCCGTTCCCCTTCTGAC	1305-1286		58.8

P2X6

Forward primer	CCTGCTCAAGCTCTATGGAATC	870-891	199	58.6
Reverse primer	CTTGGCCTCCTCATACTTTGTC	1068-1047		58.73

P2X7

Forward primer	CGACTTCCTCATCGACACTTACT	1148-1170	220	59.87
Reverse primer	GACATCTTGCAGACTTCTCCCTA	1367-1345		59.55

Apobec3g

Forward primer	TCTGGCTGTGCTACGAAGT	185-203	119	58.65
Reverse primer	GTGGAAGAATCTCATCTCTGG	303-283		55.49

TRIM 5

Forward primer	TGCTGGCTTCCAACCTGAT	1656-1674	165	59.23
Reverse primer	ACAGAGAGGGGCACAATGAA	1820-1801		58.93

Tetherin

Forward primer	TGATCTCATCAGTTCTGAGCGGGT	664-687	89	63.47

Reverse primer	AGACCTGCTCCAGAGGCCCTTCTC	752-729		67.4

GAPDH

Forward primer	CCCACTCCTCCACCTTTGAC	1046-1065	179	59.96
Reverse primer	TCCTCTTGTGCTCTTGCTGG	1224-1205		59.96

Amplification of HIV genes via Polymerase Chain Reaction

PCR was run to optimize all the genes. GAPDH was used as a control because it is a house-keeping gene and is always expressed. P2X family corresponds to 7 components namely P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7. 3 other genes known as apobec3g, tetherin and trim5 were also optimized. The reaction mix included the following components.

Reagents	Volume Added
Buffer	5 µL
MgSO4 (50 mM)	2 µL
dNTP (10 mM)	1 µL
Forward Primer (10 µM)	1 µL
Reverse Primer (10 µM)	1 µL
Taq Polymerase	0.4 µL

Sterile Water	34.6 µL
DNA (template)	5 µL

Table 3.5: Conventional PCR Recipe.

Depending on the number of reactions to produce, all reagents except DNA were combined into a master mix. Finally, each reaction tube received DNA. The entire procedure was carried out while the combination was kept on ice. Normally, three reactions were made for each reaction mix: Experimental, Positive Control, and Negative Control. The positive control used HXB2 plasmid instead of DNA, while the negative control used sterile water. The sample and controls were softly mixed and thoroughly spun or mildly centrifuged after the reaction mixture was prepared so that all of the components would settle down. The following conditions were used to start up PCR

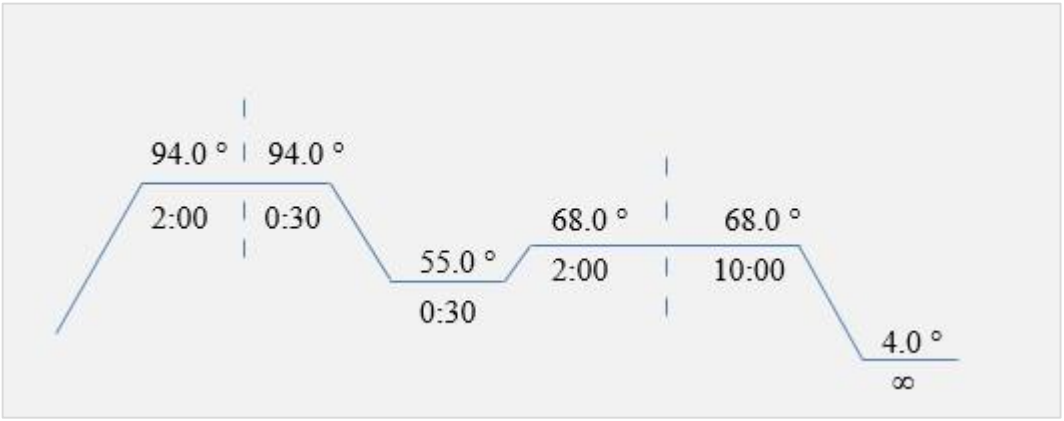

Figure 3.1: Conventional PCR Cycles and Setup.

Real time PCR was done by using HOT FIRPOL DNA Polymerase, with 5X EvaGreen qPCR buffer, 12.5mM of MgCl2, 1X PCR solution (2.5 2.5 mM MgCl2 dNTPs, including dTTP to improve reaction sensitivity). Evagreen dye was used and evagreen is found to be superior than SYBR Green in many cases.

The applications of real time PCR are viral load quantification, microbial detection, gene expression analysis

Component	**Volume**	**Final conc.**
5x HOT FIREPol® EvaGreen® qPCR Mix Plus	4 µl	1x
Primer Forward (10 pmol/µl)	0.16-0.5 µl	80-250 nM
Primer Reverse (10 pmol/µl)	0.16-0.5 µl	80-250 nM
DNA template	1-5 µl	1-50 ng/µl
H_2O PCR grade	up to 20 µl	
Total	**20 µl**	

Table 3.6. qPCR Recipe

Recommended qPCR cycles:

40 cycles of real time PCR were implied. An additional denaturation step was done to actovate the polymerase.

Cycle step	**Temp.**	**Time**	**Cycles**
Initial denaturation	**95ºC**	**15 min**	1
Denaturation	95ºC	15 s	40
Annealing	60º-65ºC	20 s	
Elongation	72ºC	20 s	

Gel Electrophoresis

For the processed cDNA samples, gel electrophoresis was used. TAE buffer (pH 8.0) was utilized in this experiment. In a flask, 100 ml TAE buffer was added, and 2.5 g agarose was weighed and added to it. Buffer was brought to a boil until it was clear. After the solution had cooled, 2.5 liters of ethidium bromide were added to it. The gel was put into a gel caster after it had cooled, and the gel was given a 30-minute setting time. 6 liters of sample were added to each reaction tube, along with 2 liters of 6X dye. Along with the sample/s, a respective ladder (50 bp) was run. The settings for gel electrophoresis were 80V, 500Amp, and 60 minutes. Later, the gel was observed using Dolphin Gel Doc Visualizer.

Chapter no 4

Results

Demographic Details of Patient Data

15 test samples and 15 control samples were obtained. The viral load and CD4 count was brought under investigation. The ages, gender, viral load and CD4 count of the patients was determined and noted as follows.

Fig no 4.1. ***Demographic data of patients along with their CD4 count and viral load***

NUST ID	Age	Gender	Treatment Center	PCR Result (IU/mL)	CD4 Count (count/uL)
P1	30	M	AHF - (VCCT Centers)	2864340	187
P2	28	M	Allied Hospital Faisalabad - (VCCT Centers)	1264864	426
P3	65	F	Jinnah Hospital - (Treatment Center)	ND	683
P4	44	M	Jinnah Hospital - (Treatment Center)	ND	976
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P6	25	M	Allied Hospital Faisalabad - (VCCT Centers)	687640	301
P7	30	M	Jinnah Hospital - (Treatment Center)	11253304	
P8	28	M	Allied Hospital Faisalabad - (VCCT Centers)	100341	490
P9	33	F	Adult Treatment Center Services Hospital Lahore - (Treatment Center)	8246	332
P10	28	M	Allied Hospital Faisalabad - (VCCT Centers)	30488	199
P11	35	M	Allied Hospital Faisalabad - (VCCT Centers)	527196	334
P12	34	M	Allied Hospital Faisalabad - (VCCT Centers)	7499	250
P13	38	M	Jinnah Hospital - (Treatment Center)	ND	809
P14	34	M	DHQ Hospital Dera Ghazi Khan - (VCCT Centers)	ND	1149

P15	8	F	DHQ Hospital Dera Ghazi Khan - (VCCT Centers)	387412	605

RNA Extraction

RNA extraction of PBMCs was done by Invitrogen Trizol LS method. The RNA was extracted and quantified using nanodrop. The extracted RNA was run on ethidium bromide gel and 2 subsequent RNAs were run on gel. 2 distinct RNA bands were visualized indicating that the RNA extracted is pure and of high-quality.

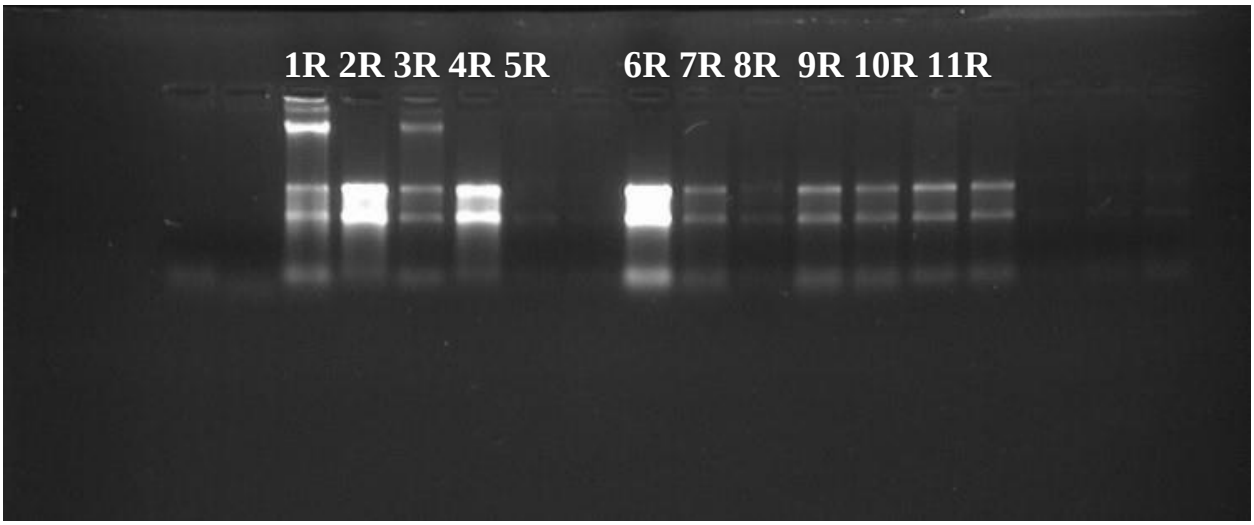

Fig no 4.1 Denaturing gel electrophoresis showing 16S and 28S RNA

cDNA Synthesis

cDNA was synthesized using Revert AID cDNA synthesis kit. The Resulting cDNA was taken 1ug and gradient PCR was done for the amplification of genes. The cDNA was then verified by amplifying the cDNA with house-keeping genes GAPDH and run-on gel.

Gradient PCR

Gradient PCR was run, each primer had different annealing temperatures which was adjusted accordingly. GAPDH was used as a house-keeping gene control. All primers were optimized except for P2X3 whose annealing temperature was yet to be found.

Purinergic Receptors Primer Optimization

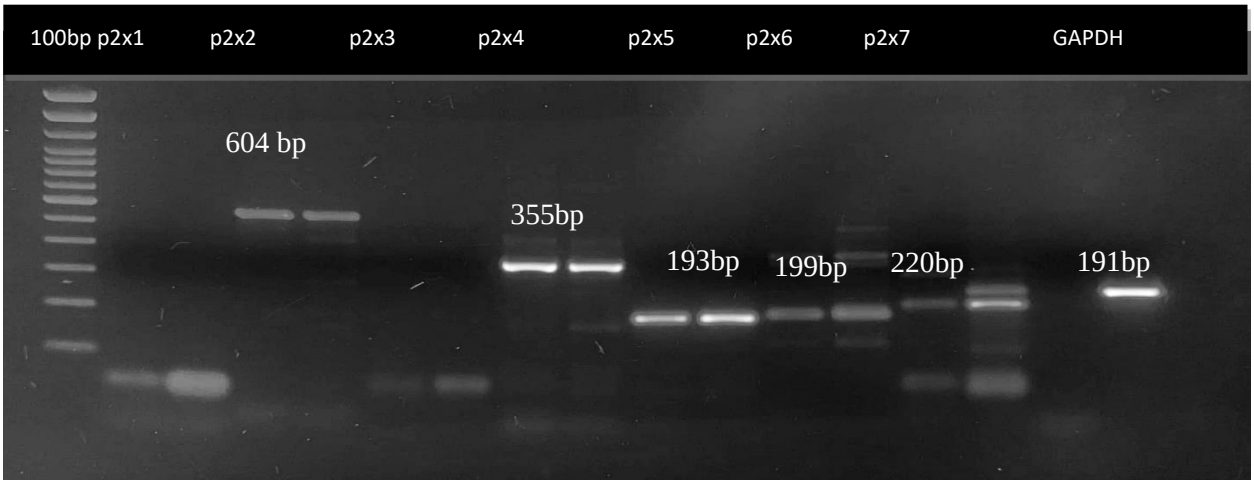

Fig no. 4.2 Gel Electrophoresis showing amplified products i.e., Purinergic receptors

<u>APOBEC3G, TRIM 5 and Tetherin Primer Optimization</u>

Apobec3g, TRIM 5 and tetherin were optimized using gradient PCR and then subsequently run-on gel with a 100bp ladder and GAPDH as a control.

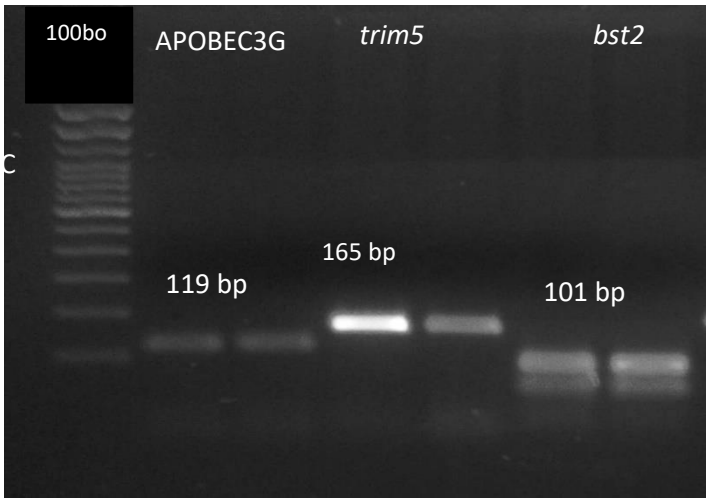

Fig no. 4.3 Gel electrophoresis showing amplified products Tetherin, APOBEC3G, TRIM 5 alpha

Real-Time PCR

Real time PCR was performed by state of the art PCR. Primer efficiency was calculated and cDNA was diluted 1:3 times. 9 sets of primers were present for 9 genes and the results for each of them were calculated and analyzed. T test was also performed to check significance of the data.

APOBEC3G pathway

APOBEC3G was found to be down-regulated in HIV positive individuals as compared to the control samples indicating that vif has bound to it and downregulated it.

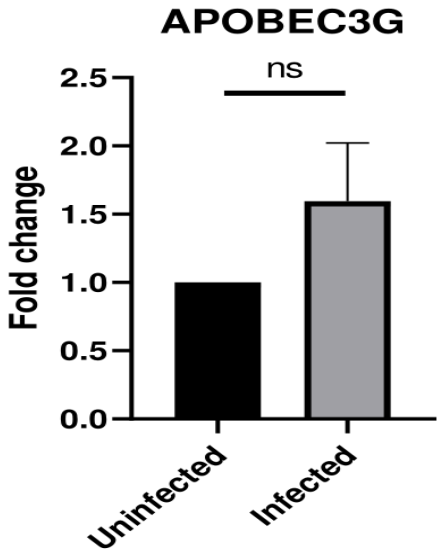

Fig no 4.4 ***Expression of APOBEC3G is upregulated in HIV infected individuals which is exhibited by increase in fold change***

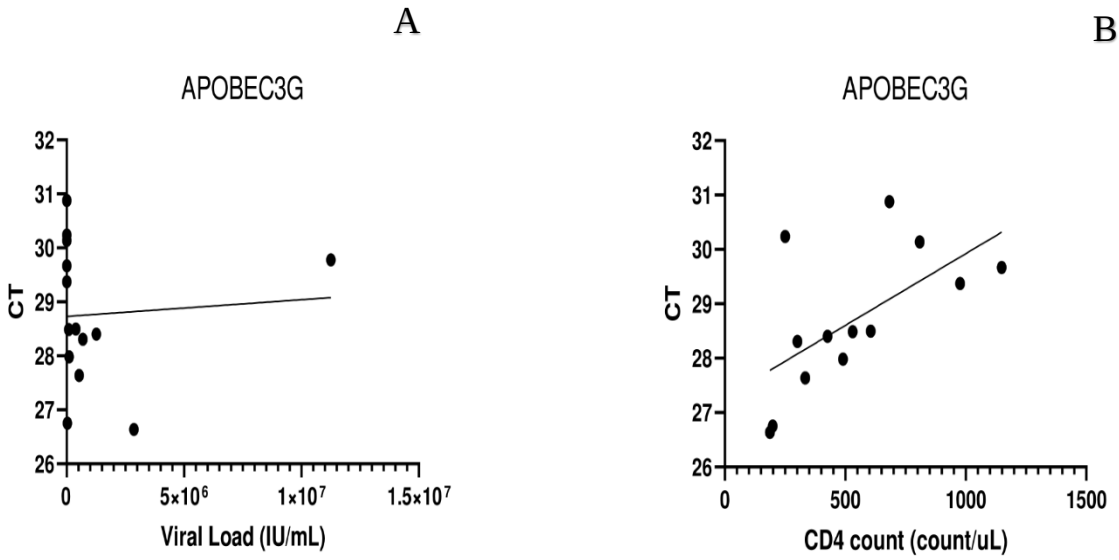

Fig no 4.5 A and B represent linear correlation between CD4 count and CT, and between viral load and CT

Tetherin

Tetherin expression was found to be increased in infected patients. Fold Change was found to be considerably higher in infected patients as compared to control.

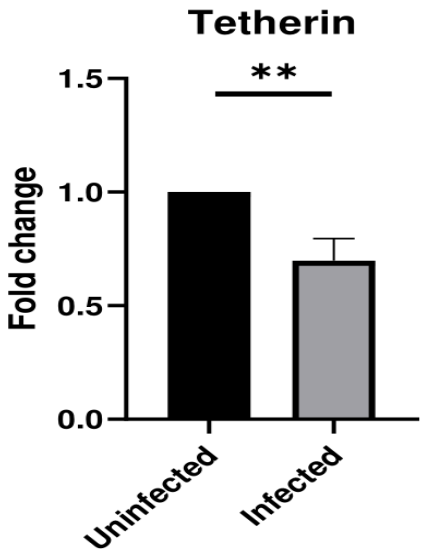

Fig no 3.7 Expression of Tetherin is downregulated in HIV infected individuals which is exhibited by increase in fold change

P =0.0042

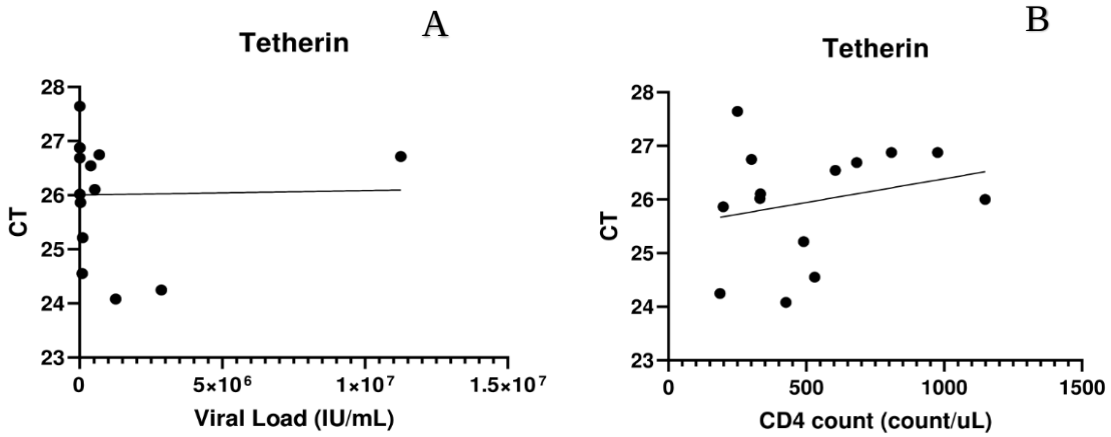

Fig no 3.8 A and B represent linear correlation between CD4 count and CT, and between viral load and CT

TRIM 5α

TRIM 5α expression was found to be increased in infected patients. Fold Change was found to be considerably higher in infected patients as compared to control.

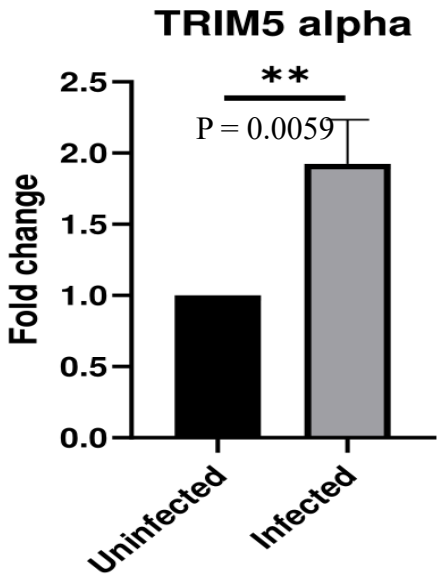

Fig *no 4.9 Expression of TRIM 5α is upregulated in HIV infected individuals which is exhibited by increase in fold change*

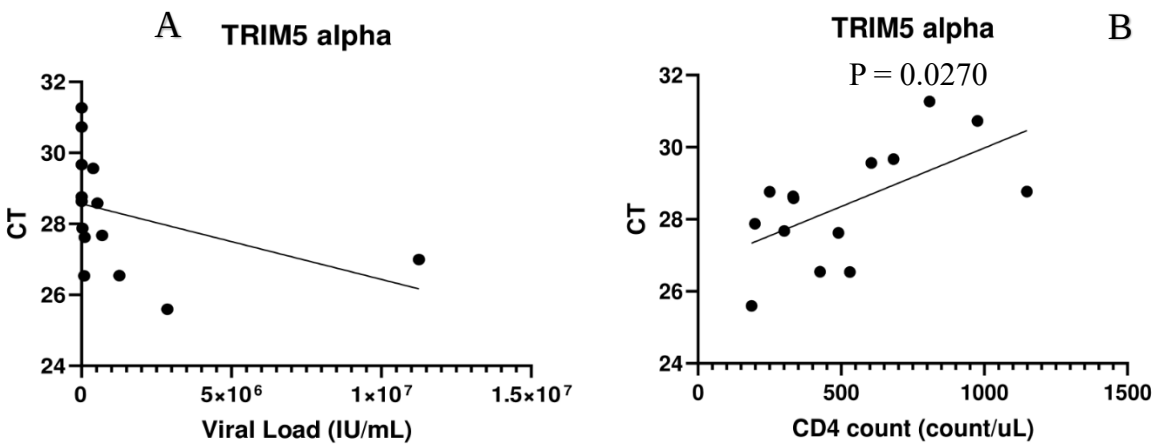

Fig no 3.10 A and B represent linear correlation between CD4 count and CT, and 9between viral load and CT

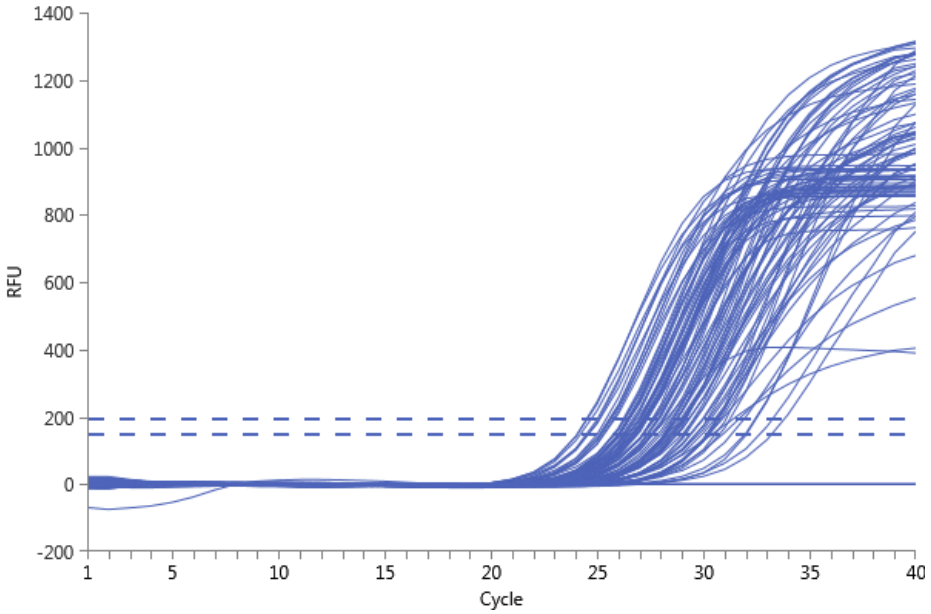

Figure no 4.11 Curve showing expression of TRIM 5, Tetherin and apobec3g

P2X1

Purinergic signaling is found to be important in HIV pathogenesis. P2X1 is the gene responsible for entry of the HIV virus into the host genome. That's why, the expression of p2x1 during HIV infection is increased as compared to control samples.

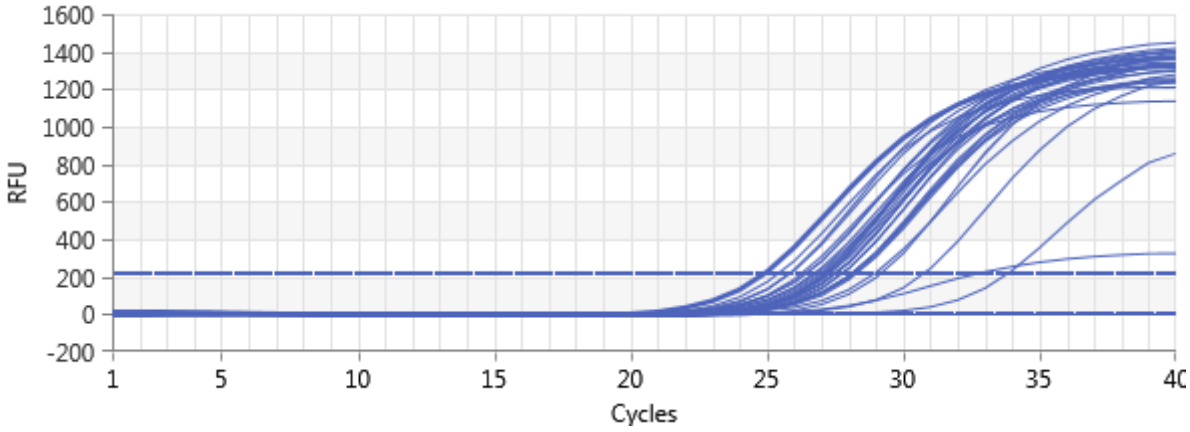

Fig no 4. 15 Curve showing amplification plot P2X receptors

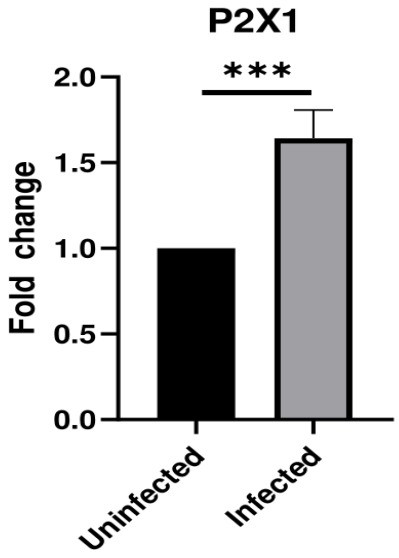

Fig no 4.12 Expression of P2X1 is upregulated in HIV infected individuals which is exhibited by increase in fold change

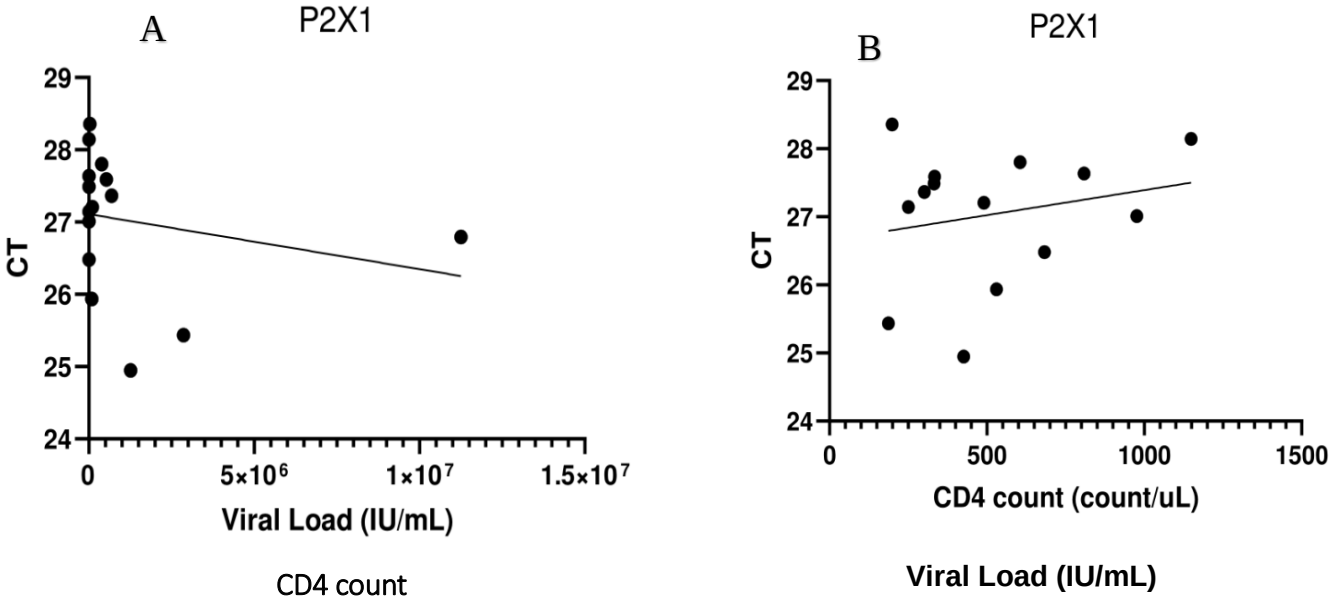

Fig no. 4.13 A and B represent linear correlation between CD4 count and CT, and between viral load and CT

P2X2, P2X4 and P2X5

Purinergic receptors P2X2, P2X4 and P2X5 have not shown much difference between the control samples and the infected ones. The expression of p2x4 is decreased in infected individuals whereas p2x5 expression is increased.

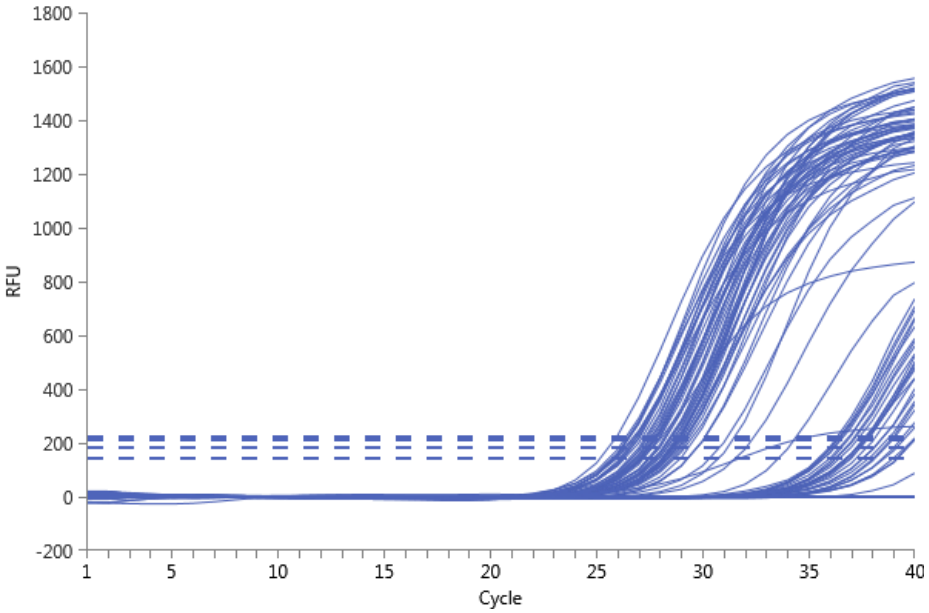

Fig no 4.8 *Real-time curve showing amplification plot p2x2, p2x4 and p2x5*

P2X4

Expression of P2X4 is not changed in HIV infected individuals which is exhibited by stability in fold change.

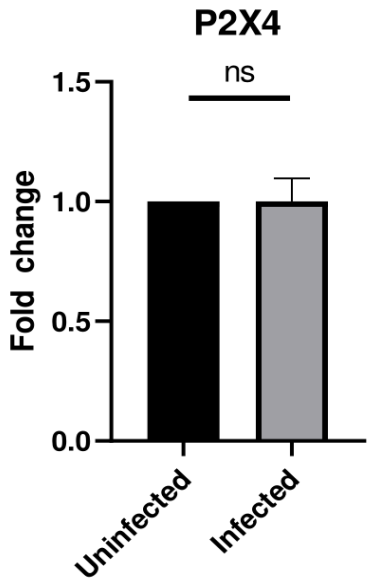

Fig no 4.18 Expression of P2X4 is not changed in HIV infected individuals which is exhibited by stability in fold change

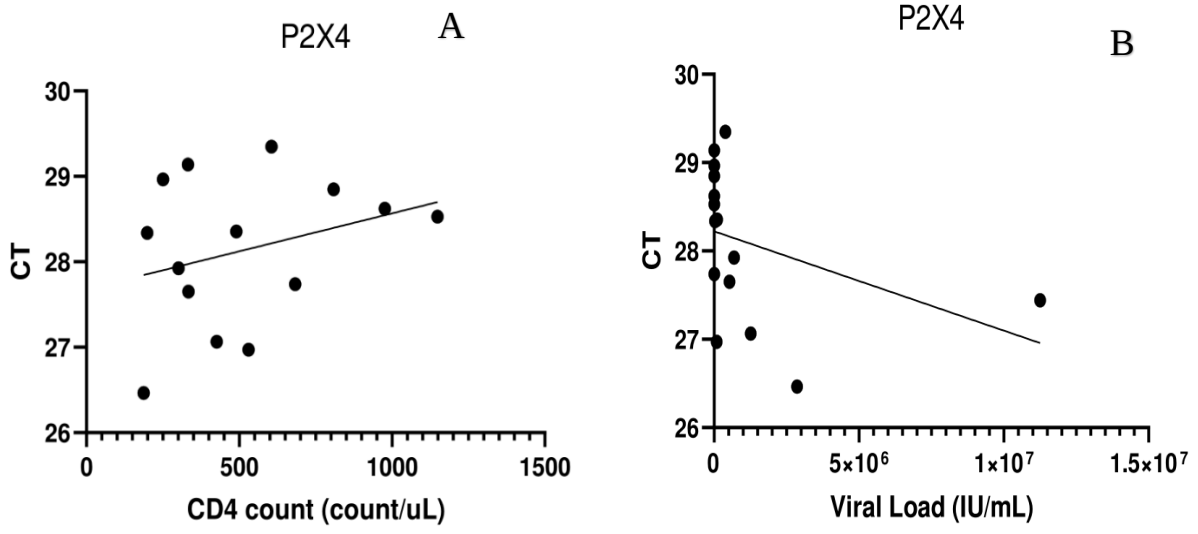

Fig no 4.19 A and B represent linear correlation between CD4 count and fold change, and between viral load and fold change

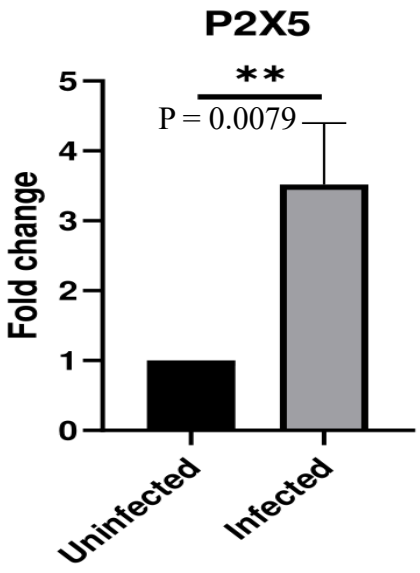

Fig no 4.21 Expression of P2X5 is upregulated in HIV infected individuals which is exhibited by increase in fold change

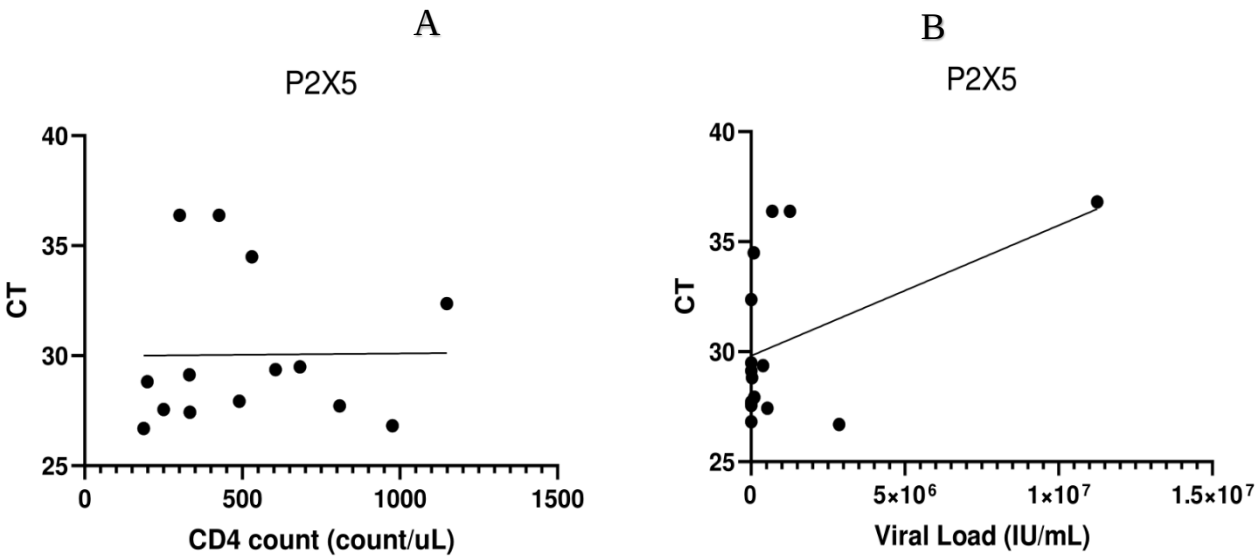

Fig no 4.20 A and B represent linear correlation between CD4 count and CT, and between viral load and CT

P2X6

The receptor gene expression was not found to be considerably different between the patients and control groups.

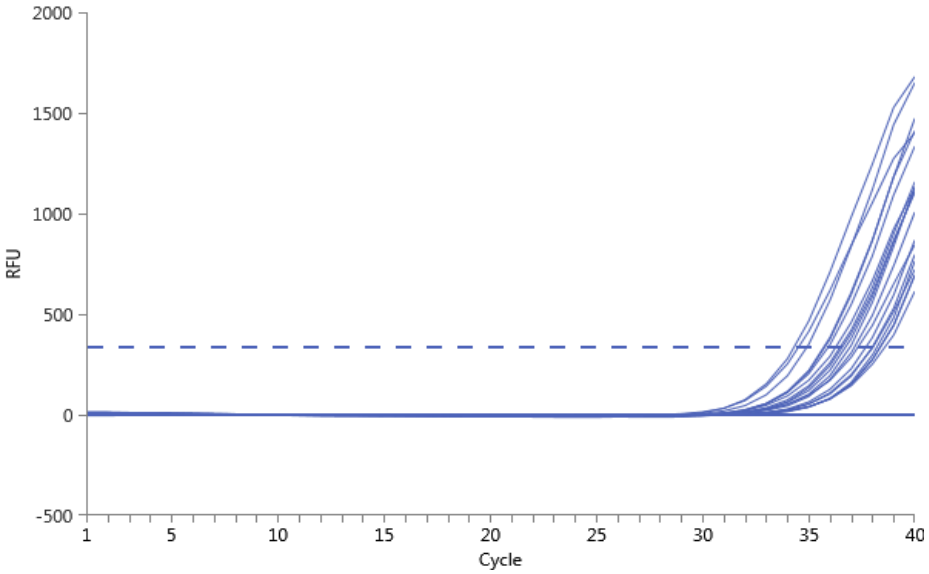

Fig no 4.21 Real-time curve showing amplification plot p2x6

P2X7

Expression of P2X7 was found to be increased in HIV positive individuals. Because purinergic receptors aid in entry of HIV into the host cells and spread of the virus, therefore, their expression is considerably higher in host cells.

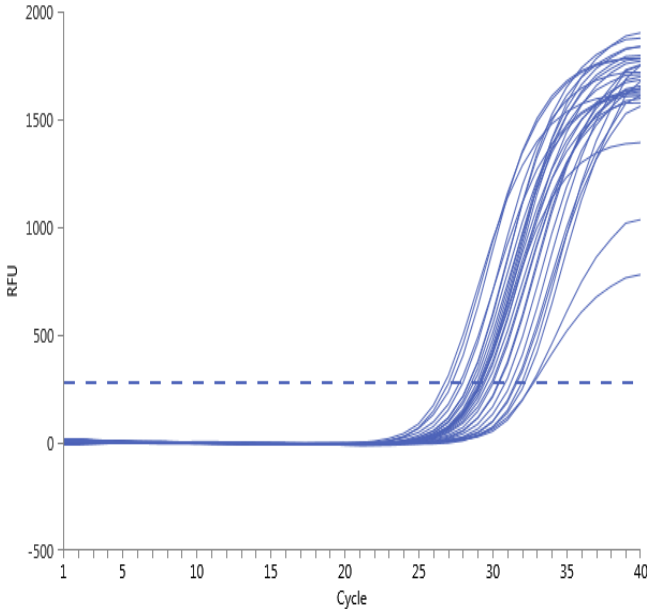

Fig no 4.22 Real-time curve showing amplification plot p2x6

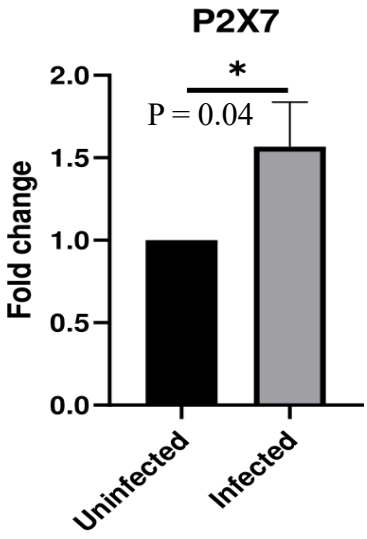

Fig no 4.24 Expression of P2X7 is upregulated in HIV infected individuals which is exhibited by increase in fold change

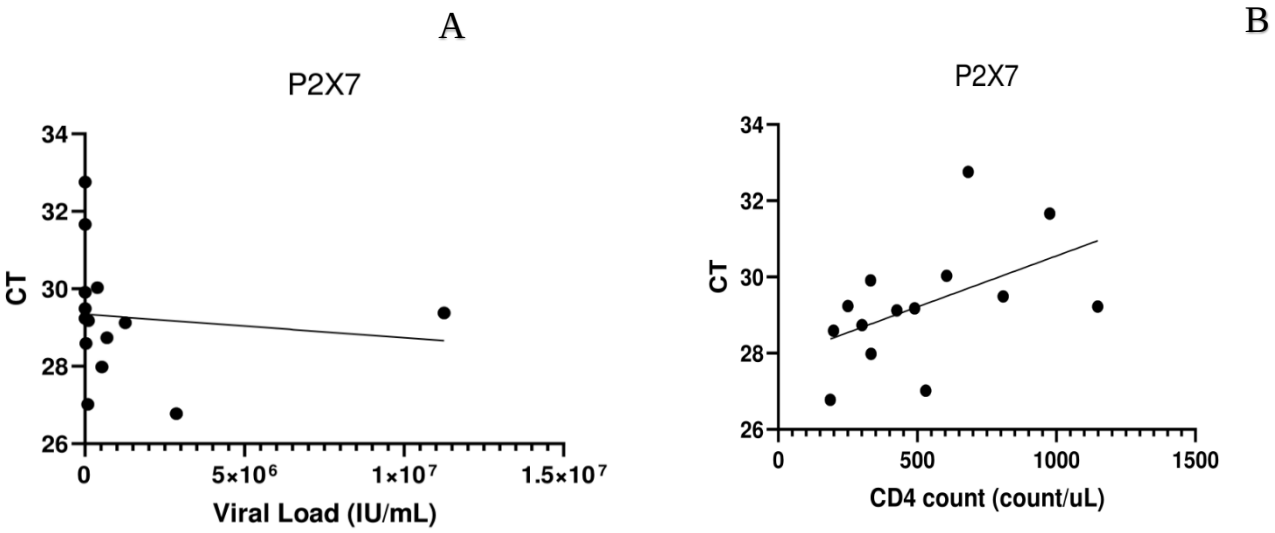

Fig no 4.25 A and B represent linear correlation between CD4 count and fold change, and between viral load and fold change

Conclusion

- The expression of P2X2, P2X3, P2X6 were not expressed on cDNA while expression of P2X1, P2X5, P2X7, TRIM 5α and APOBEC3G was significantly upregulated in HIV infected individuals indicating that they are involved in HIV progression.
- The expression of P2X4 did not change significantly in HIV infected individuals.
- Tetherin expression was significantly downregulated in HIV infected individuals.
- Only TRIM 5α and APOBEC3G has shown significant correlation with between CT values and CD4 count.

Chapter no. 5

Discussion

Since its discovery as a silent killer, HIV has been the center of interest and despite the fact that the whole world is trying to find its cure, attempts have been futile. There is a treatment of HIV, but no cure is available till now. The drugs developed for HIV have their accommodating side-effects and there is still a need for new antivirals against HIV. The virus is of extreme intelligence and mutates its genome rapidly so antivirals against this virus, would become futile. We need new antivirals that possess minimum side-effects and have more potent results. Moreover, antivirals with minimum cost and easy availability need to be designed so that the government is not over-burdened while providing free medications the individuals.

HIV can be silent in the body and remain latent for decades. We may not even now that the virus exists in our body but, however, many of the times the virus exists its lysogenic phase and enters the lytic phase targeting the neighboring CD4 cells for infection. The virus becomes lethal when it infects CD4 cells to a level that they are depleted below 200 and hence leading to the death of the individual. HIV is given so much importance because of the fact that it directly attacks the immune cells of the body and targets the host immune system.

The genes involved in HIV are of specific importance because it completely relies on the host mechanism for its replication. In contrast, there are several of the host genes that assists HIV and several others that oppose its infection. Purinergic signaling is of importance because purinergic receptor P2X1 assists in viral entry to the host cells. In our study, p2x1, p2x4, p2x5 and p2x7 expression has been found to be considerably different in control and infected samples. The expression of purinergic receptors is p2x1 and p2x7 is increased whereas expression of p2x4 and

p2x5 is decreased in infected individuals. Infected hepatocytes generate ATP during hepatitis C virus (HCV) infection, which binds to Purinergic receptors (P2X) on peripheral blood mononuclear cells (PBMCs) and triggers downstream signaling pathways by upsetting the cell's ionic equilibrium. the expression of P2X4 and P2X7 receptors, respectively, increased thrice and 1.8fold in the treatment-naive group. (Manzoor et al., 2016). HIV-1 infection of CD4+ T cells in the presence of a P2X receptor inhibitor significantly and dose-dependently suppressed HIV-1 infection through both cell-free and cell-to-cell contact. (Swartz and others, 2014) Rats given gp120 had higher levels of P2X7 expression. (Wu et al., 2017) The inhibitors designed for purinergic receptors have been found to inhibit the HIV virus in the host cells. Giroud et al., also demonstrated that p2x1 is necessary for viral entry, so its expression is increased in infected cells, however, HIV is inhibited in presence of p2x1 antagonists. (Giroud et al., 2019). Expression of p2x7 and p2x1 is increased in HIV-1 infected cells. These receptors are most widely present on surface of macrophages and CD4 cells i.e., the primary targets of HIV infection. (Soare et al., 2018)

APOBEC3G is an evolutionary protein that induces hypermutations into the HIV ssDNA and ultimately interferes with the viral replication, however, HIV has now evolved vif, which destroys the activity of APOBEC3G. The expression of APOBEC3G is upregulated in HIV infection relative to the housekeeping gene TBH whereas it is degraded in the presence of Vif actor of HIV. Our study suggests that expression of APOBEC3G is downregulated in HIV infection. (Ikeda et al., 2019). It has been demonstrated that the HIV-1 viral infectivity factor (Vif) preferentially inhibits several cellular deaminases from the APOBEC3 or A3 family (A3A to A3H) by enlisting an E3-ubiquitin ligase complex and causing their polyubiquitination and destruction through the proteasome. (Stupfler et al., 2021). Even though this pathway has so far been thoroughly defined,

Vif has also been demonstrated to obstruct A3s through processes that are independent of degradation. APOBEC3G is rapidly reduced in expression upon HIV 1 infection. The gene may be upregulated in early viral attack. However, it is rapidly downregulated when HIV infected individuals. (Reddy et al., 2010)

Tetherin is also an evolutionary protein that is involved in the tethering of the virions to the surface of the cell and doesn't allow the virus genome to be incorporated into the host cell. Even in the presence of the viral antagonist Vpu against BST-2, cells with significantly increased BST-2 expression demonstrated efficient suppression of HIV-1 synthesis and replication. (Zhang et al., 2019) When Vpu was absent, tetherin particularly prevented the release of retrovirus particles, and its expression closely mimicked the distinctive deficiencies in HIV-1 particle release that have previously been linked to the absence of Vpu function. (Neil et al., 2019). HIV-1 viral protein U (Vpu) facilitates viral egress and enables infected cells to avoid host protection by downregulating BST-2/tetherin and CD4. (Umviligihozo et al., 2020). Our studies suggest that tetherin is downregulated in HIV infection because HIV vpu protein destroys it. Downregulation or inhibition of tetherin is necessary for HIV-1 infection. (Kuhl et al., 2008). Inhibition of Tetherin is necessary for HIV 1 infection. (Pickering et al., 2014)

TRIM 5α is induced by INFα and higher levels are responsible for inhibiting the HIV. It is part of our innate immune system and recognizes the virus by its capsid proteins. HIV-1 viral protein U (Vpu) facilitates viral egress and enables infected cells to avoid host protection by downregulating BST-2/tetherin and CD4. (Ciccosanti et al., 2021). TRIM5hu Expression in Human CD4+ T Cells Is Reduced, Reversing HIV-1-P90A Virus Infection (Selyutina et al., 2020). In our studies, TRIM 5 alpha expression is upregulated in HIV individuals. The expression is increased in HIV infected individuals. (Tabah et al., 2014)

Purinergic signaling contributes to HIV signaling and inhibitors to p2x receptors can serve to inhibit the infection. APOBEC3G, tetherin and TRIM 5 are the genes that can inhibit the HIV 1 infection if their expression is increased and in the absence of virus accessory proteins which lead to their degradation.

In our studies, only TRIM 5 has shown significant correlation between fold change, CD4 count and viral load. The rest have shown a trend but the results are not significant.

Future Prospects

The study is important in analyzing the expression of the genes in Pakistan population. The sample size of the study can be increased and more samples can be enrolled to identify the significant fold changes in HIV expression. The study can be further realized by taking into account more host factors that either assist the HIV infection in our body or inhibit it and finding specific inhibitors or inducers of those particular proteins.

Samples from all over Pakistan can be taken into account, analyzed for gene expression and can be used to develop a certain antiviral against HIV with more cost-effectiveness, least side effects and easy availibity.

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