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RUI MANUEL DA COSTA SOARES

CLINICAL IMPLICATIONS OF VIRAL PROTEIN R MUTATIONS IN THE PROGRESSION OF HIV INFECTION

ARTIGO DE REVISÃO

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Clinical Implications of Viral Protein R Mutations in the Progression of HIV Infection

Implicações clínicas das mutações da proteína viral R na progressão da infeção por HIV

ARTIGO DE REVISÃO

Rui Manuel da Costa Soares

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“A simplicidade é o último degrau da sabedoria.”

Khalil Gibran

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List of abbreviations

A3G – ApoEC3G

AIDS – acquired immune deficiency syndrome

AIF – apoptosis-inducing factor

ANT – adenine nucleotide translocator

APCs – antigen presenting cells

ART – antiretroviral treatment

ARV – antiretroviral agents

ATP – adenosine triphosphate

C/EBP – CCAAT-enhancer-binding protein

cART – combination antiretroviral therapy

CBP – CREB binding protein

CDC – US Centers for Disease Control and Prevention

CDKs – cyclin-dependent protein kinases

CNS – central nervous system

CSF – cerebral spinal fluid

CTL – cytotoxic T cells

Cyt c – cytochrome c

DCs – dendritic cells

DNA – deoxyribonucleic acid

FSGS – focal segmental glomerulosclerosis

GHO – Global Health Observatory

HIV – human immunodeficiency virus

HIVAN – HIV associated nephropathy

HIV-D – HIV-1 associated dementia

HIV-E – HIV-1 encephalopathy

hnRNP A1 – RNA binding protein heterogeneous nuclear ribonucleoprotein A1

HSP – heat shock protein

IBB – amino-terminal importin-β-binding

iDCs – immature dendritic cells

IL-12 – interleukin-12

IMM – inner mitochondrial membrane

IN - integrase

LTNPs – long-term non-progressors

LTR – long terminal repeat

MA – matrix protein

MMP – mitochondrial membrane permeabilization

MOI – multiplicity of infection

NE – nuclear envelope

Nef – negative factor

NF-kB – nuclear factor kappa B

NLS – nuclear localization sequence

NMR – nuclear magnetic resonance

NPC – nuclear pore complex

OMM – outer mitochondrial membrane

PCP – *Pneumocystis carinii* pneumonia

PIC – pre-integration complex

PR – protease

RNA – ribonucleic acid

RNAi – interference RNA

RRE – Rev-responsive element

RT – reverse transcriptase

SIV – simian immunodeficiency virus

SP1 – specificity protein 1

SU – surface subunit

TAM – thymidine analog mutations

TAR – transactivation-response element

Th – T helper

TM – transmembrane

Ub - ubiquitin

UPS – ubiquitin proteasome system

Vif – viral infectivity factor

Vpr – viral protein R

VprBP – Vpr binding protein

Vpu – viral protein U

WHO – world health organization

WT – wild type

Clinical implications of viral protein R mutations in the progression of HIV infection

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Abstract

Acquired immune deficiency syndrome (AIDS) is caused by a chronic infection by the human immunodeficiency virus (HIV). Over the last 30 years, research in this area has made many advances on the knowledge of the genetics of the virus and the role of its components in the disease progression, thus giving rise to the development of more efficient therapeutic strategies. HIV-1 viral protein R (Vpr) is an accessory and multifunctional protein that plays an important role at multiple stages of the HIV-1 viral life cycle. This protein interacts with a number of cellular and viral proteins and shows multiple activities including nuclear transport of the pre-integration complex (PIC) to the nucleus, transcriptional activation, cell cycle arrest at G2/M transition phase and induction of cell death via apoptosis. Vpr has been shown to control many host cell functions through a variety of biological processes and by interaction with several cellular pathways. The different functions of Vpr have been linked to enhancement of viral replication and impairment of the immune system in HIV-1 infected patients. Moreover, Vpr is required for efficient viral replication in non-dividing cells, namely macrophages, promoting to some extent, viral replication in proliferating $CD4^+$ T cells. Importantly, Vpr mutations leading to functional defects in this protein have been correlated with slow disease progression of HIV-infected patients and may constitute potential therapeutic targets in future treatments.

This thesis will be mainly focused on HIV-1 Vpr, its functions and the multiple roles of this viral protein during the virus replication cycle. A special attention will be drawn towards the importance of its structural mutations on the progression of HIV infection.

Keywords: AIDS; HIV-1; Vpr; nuclear transport; G2 arrest, apoptosis; mutations

Resumo

O síndrome da imunodeficiência adquirida (SIDA), é causado por uma infeção crónica pelo vírus da imunodeficiência humana (HIV, do inglês "human immunodeficiency vírus"). Ao longo dos últimos 30 anos, a investigação desenvolvida nesta área tem feito inúmeros avanços no conhecimento da genética do vírus e do papel dos seus constituintes na progressão da doença, dando origem ao desenvolvimento de estratégias terapêuticas mais eficazes. A proteína viral R (Vpr, do inglês "viral protein R") do vírus HIV-1 é uma proteína acessória com múltiplas funções e que desempenha um papel importante nas várias fases do ciclo de vida do HIV-1. Esta proteína interage com muitas outras proteínas celulares e virais e é caracterizada pela sua participação em diferentes atividades, nomeadamente no transporte nuclear do complexo de pré-integração (PIC, do inglês "pre-integration complex") para o núcleo, ativação da transcrição, interrupção do ciclo celular na fase de transição G2/M e indução de morte celular por apoptose. A Vpr tem sido ainda descrita por controlar várias funções da célula hospedeira através de diversos mecanismos biológicos e vias celulares. Para além disso, as funções da Vpr têm sido associadas a um aumento da replicação viral e disfunção do sistema imunitário em doentes infetados com HIV-1. Em células que não se dividem, como é o caso dos macrófagos, a Vpr é necessária para que ocorra replicação viral de forma eficiente, promovendo também este processo em células proliferativas, tais como os linfócitos T $CD4^+$. As mutações que ocorrem na Vpr podem originar defeitos funcionais nesta proteína que têm sido correlacionados com uma forma mais lenta de progressão da doença em indivíduos portadores de HIV, pelo que podem constituir um potencial alvo terapêutico em tratamentos futuros.

Esta tese terá como tema principal a proteína Vpr do vírus HIV-1, as suas funções e os vários papéis que desempenha durante o ciclo de replicação do vírus. Será dada uma especial atenção à importância das suas mutações estruturais na progressão da infeção por HIV.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immune deficiency syndrome (AIDS), is a member of the lentivirus sub-family (1).

The HIV-1 genome encodes a 14 kDa accessory protein named viral protein R (Vpr) which is a virion-associated protein composed of 96 amino acids (2). Vpr has a variety of biological functions, including cell cycle G2 arrest, apoptosis induction, nuclear import of the pre-integration complex (PIC), modulation of gene expression and suppression of immune activation. Vpr contains a flexible N-terminal region, three alpha-helical domains and a flexible C-terminal region. Each function of Vpr is attributed to one or more of its domains and seems to correlate with the partners interacting with these domains. The role of Vpr in the pathophysiology of AIDS is of particular interest since it causes many cellular dysfunctions. Furthermore, Vpr is necessary for the efficient infection of non-dividing cells and differentiated macrophages (reviewed in 3), which are important targets during the initial stages of the infection, thus contributing for the establishment of viral reservoirs (4).

Numerous studies have demonstrated that mutations in HIV-1 Vpr could seriously affect its known functions, leading to the expansion of several hypotheses about the role of Vpr in the course of the infection. Indeed, the association of Vpr mutations with long-term non-progressors (LTNPs) gave rise to the interest in this research field, due to the possibility of developing therapeutic approaches directed to this viral protein that could interfere in the virus life cycle.

The main reason for writing about this subject arouse from the knowledge acquired at the Microbiology laboratory from the Faculty of Medicine of the University of Coimbra, while collaborating in a study of HIV-1 vertical transmission. Since then, the growing awareness of the role of Vpr in HIV-1 viral life cycle came as a particular interest in a research field where the knowledge about this viral protein is currently under strong investigation. Moreover, the

theme of this thesis brings new insights and new ideas for further development of research work combining both clinic and basic research, which certainly will constitute a valuable issue in these important areas.

The main objective of this thesis was to gather information underlying the principal functions of Vpr in HIV-1 infection and viral replication. Furthermore, an extensive search through published work about Vpr mutations was carried out in order to understand and correlate their clinical implications with the progression of HIV-1 infection. Importantly, this topic is thought to contribute to increase the ability to draw new lines of investigation directed to the development of therapeutic strategies involving Vpr manipulation and ultimately, work on the improvement of the quality of life of HIV-1 infected patients.

2. Materials and Methods

For the preparation of this work, we started with a brief description of AIDS and went through the analysis of several studies describing the main characteristics of HIV virus, the course of the infection and disease progression. Next we went deeper into the subject of this thesis by searching for published data supporting a description on clinical implications of Vpr mutations and their influence on disease progression under HIV infection. As such, we focused on the structure and possible functions of the protein, its role during HIV cell cycle, its mutations and how they can be involved in HIV disease progression.

A search of scientific articles related to the topics of this review was performed by searching the PubMed database (http:// http://www.ncbi.nlm.nih.gov/pubmed/) using the keywords "HIV and AIDS"; "HIV cell cycle"; "lentiviruses"; "Vpr functions"; "Vpr mutations"; "HIV-1 associated pathologies" and "Vpr as a therapeutic target" to establish a relationship between Vpr mutations and its role on HIV disease progression and the different clinical aspects that may be determined by these protein alterations. Therefore, reviews and original articles published in English were selected, dating from 1985 to the present (2013). Data from the World Health Organization (WHO) available online was also used to refer to the actual numbers related to HIV and AIDS in the world.

3. Overview of AIDS and HIV

Acquired immune deficiency syndrome (AIDS) is caused by a chronic infection with the human immunodeficiency virus (HIV). Considered as an epidemic, the official start of AIDS occurred in 1981 when the US Centres for Disease Control and Prevention (CDC) reported on a cluster of *Pneumocystis carinii* (now *P. jirovecii*) pneumonia (PCP) in five homosexual men (Centres for Disease Control. http://www.cdc.gov/mmwr/preview/mmwrhtmal/june_5.htm). PCP is commonly found in the lungs of healthy people, although it can also be a source of opportunistic infection, causing lung disease in people with a weak immune system. However, there are strong evidences showing that HIV first crossed the simian-human species barrier earlier, possibly in West Africa (5). According to the Global Health Observatory (GHO) from the World Health Organization (WHO), the world number of people living with HIV in 2010 was 34.0 million, compared to 28.6 million in 2001, representing approximately a 17% increase. This reflects the high number of people newly infected with HIV, along with significantly access to antiretroviral therapy, which contributed to reduce the number of people dying from AIDS-related causes, namely since 2004-2005. Nevertheless, the WHO African Region still bears the highest share of the global HIV burden, revealing that the number of people infected with HIV in 2010 reached 22.9 million, 68% of the total global number of infected subjects (http://www.who.int/gho/hiv/epidemic_status/cases_all_text/en/index.html). Despite the improvements on HIV medications, the lower morbidity and death rates in the past decade, there is still great variability in HIV disease progression. Therefore, understanding the basic biology of the virus, the phases of disease progression, the genetic diversity and the cell cycle of HIV, are subjects of crucial interest to find new therapeutic strategies to fight against this fatal disease.

3.1. Phases of HIV disease progression

Over the last three decades, many efforts have been made on HIV research to study the virus behaviour. There is abundant and current information about the impact of the infection on the immune system as well as the dynamics of the acute and chronic infection. During the first weeks of HIV infection, the patients often reveal symptoms of a flu-like illness together with a rash, an illness termed acute HIV-1 infection syndrome (6). This initial phase of HIV infection is then followed by a gradual decline of the immune system defenses. HIV has the capacity to infect $CD4^+$ lymphocytes and a variety of other cells in the organism, including monocytes and thymocytes (7;8). The virus infects target cells via cell surface molecules, including $CD4^+$ and chemokine co-receptors (CXCR4, CCR5) (9). T-helper cells expressing the protein CD4 in the surface, or $CD4^+$ cells, play a central role in the immune response, since they signal other cells, such as the $CD8^+$ cytotoxic T cells and the B cells to execute their functions (10). The progressive decline of $CD4^+$ T cells is a major characteristic of HIV-1 infection and $CD4^+$ count in blood has been used as a marker of disease progression and forthcoming to the development of the syndrome of AIDS. A healthy adult individual has $CD4^+$ counts of 800 to 1200 $CD4^+$ T cells per mm^3 of blood. The $CD4^+$ T-cell depletion induced by HIV infection will compromise many immunological functions essential for effective defenses. Consequently, the immune system becomes weak and vulnerable. Once the $CD4^+$ count falls to numbers below 500 cells/mm^3, it is considered that half of the immune reserve has been destroyed and minor infections including cold stores (herpes simplex), condyloma (warts) and fungal infections, thrush and vaginal candidiasis, frequently occur. These infections are difficult to deal with, but not life threatening. However, as the $CD4^+$ falls to levels lower than 200 cells/mm^3, the patients become particularly vulnerable to the serious opportunistic infections and neoplasic processes typically associated with AIDS, the end-stage of HIV disease. Therefore, AIDS is defined as a $CD4^+$ count of <200 cells/mm^3, or the

presence of a dangerous infection, namely PCP, toxoplasmosis, cytomegalovirus infections of the eye or intestine, as well as debilitating weight loss, diarrhea, HIV dementia, cancers, such as Kaposi's sarcoma and lymphomas (11).

Indeed, monitoring and tracking individuals infected with HIV, together with clinical observations, is currently based on two laboratory criteria: quantification of HIV viral load and $CD4^+$ lymphocyte count. Antiretroviral treatment (ART) should not only be based in laboratory criteria, but also in clinical criteria, namely by testing the patient's resistance to antiretroviral agents. Present criteria are used to apply early therapy in symptomatic patients, in asymptomatic patients older than 50 years with less than $350/mm^3$ $CD4^+$, when viral load is higher than 100,000 copies, when a rapid decline of $CD4^+$ cells occurs, and at the time of HCV (Hepatitis C virus) or HBV (Hepatitis B virus) coinfection takes place or in serodiscordant couples. According to the European standards, treatment should be started as soon as possible, depending on (http://www.aidsmap.com/CD4-cell-counts/page/1327484/):

- Opportunistic infections and $CD4^+$ less than $200/mm^3$;
- $CD4^+$ decrease to less than $350/mm^3$;
- Occurrence of HIV-associated nephropathy or other specific organ failure and the patient is over 50 years old, or in cases of pregnancy or malignancy;
- Individual factors should always be considered, including willingness to adhere to treatment and personal reasons.

However, until present, it was not possible to define a default limit in normal $CD4^+$ count, since this parameter may vary according to race, ethnicity, gender and geographic distribution. Due to mechanisms that are not yet understood, HIV-1 infected patients show different modes of disease progression. For instance, long-term non-progressors (LTNPs), i.e., those in which degradation of immunological status is not observed or there are no evident symptoms of the disease, have the opportunity to delay the start of the treatment. On the other

hand, potential fast progressors can take advantage of an early treatment start. Therefore, knowing the potential of the disease progression may be a factor of high importance as it will help to adjust the start of the therapy.

3.2. HIV transmission

HIV can be transmitted sexually, by contact with infected blood or by mother to child. During sexual contact, the virus can cross the mucosal barrier of the vagina, vulva, penis or rectum, reaching first the immune (dendritic) cells that ultimately carry the virus across the mucosa (12). The immature dendritic cells (iDCs) are present at all mucosal surfaces and come into contact with HIV-1. Once virus contact with iDCs is established, these cells can undergo maturation and migrate to the lymph nodes, where they present processed antigens to T cells and B cells, triggering an adaptive immune response against HIV-1 and the first cycle of infection begins (reviewed in 13). Women are generally more likely to acquire HIV than men during heterosexual intercourse due to female physiologic characteristics, namely the large amount of mucosal surface area that is exposed to the seminal fluid (11). The risk of heterosexual transmission is greatly decreased with consistent and correct condom use.

HIV can also spread by contact with infected blood, which commonly occurs between individuals from risk groups, most often through the practice of reusing or sharing syringes and needles to inject drugs (14). Occupational risk to healthcare workers also exists, mainly through accidental needle stick or mucosal splash with HIV contaminated blood, although the percentage of infection by this type of transmission is very low (11).

There is a risk of vertical HIV transmission from the mother to the fetus or newborn, either during pregnancy or by breast feeding. Optimal interventions, including ART, caesarean delivery and formula feeding have reduced the rate of mother-to-child transmission of HIV-1 from about 25% to 1% to 2% in developed countries (15).

3.3. Factors that may influence viral load and disease progression

Disease progression in HIV infected patients is likely to be dependent on a number of physiological and psychosocial factors. Drug abuse, high-risk sex behaviours and depression may be important elements in the utilization of available HIV prevention and treatment resources (16).

Once HIV enters the organism, it replicates very rapidly, producing a viral burst that infects many $CD4^+$ cells and gets into all lymphatic tissue. In this early infection, viral load can reach millions of virions per ml of blood, which increases the risk of HIV transmission to other recipients. It has been identified a number of key factors with a strong impact on the rate and severity of HIV infection namely, the type of immune response, age, behavioural variables and coinfection with other sexually transmitted diseases (11). In this context, LTNPs are patients who have been infected with the HIV virus for more than 10 years and, yet, are clinically healthy, without any treatment and developed characteristics that demonstrate a beneficial anti-HIV immune response (11;17). Coinfections may also have a role in HIV disease progression. For example, in individuals who were under ART, infection with hepatitis C virus (HCV) was associated with a lower $CD4^+$ cell number (18). Furthermore, in the absence of ART, older individuals have higher rates of disease progression than younger adults. This could be due to fewer naïve cells and more memory $CD4^+$ cells (the preferred target of HIV infection), which occurs with age. Moreover, it seems that either the memory cells are more rapidly depleted or the bone marrow and thymus cannot cope with cell loss in older people (11).

4. HIV- The virus

HIV has two genetically distinct viral types (19). HIV-1 is the type associated with the disease in the USA, Europe, Central Africa and most other areas in the World. HIV-2 has been found mainly in infected individuals in Western Africa. It shares a high degree of similarity with HIV-1, since it shows the same tropism for cells of the immune system and leads to illness resulting in immune deficiency. All HIV types and subtypes are thought to have origin from zoonotic introductions from nonhuman primates (20).

HIV is a member of the viral family *Retroviridae* (retroviruses) and belongs to the sub-family *Retrovirinae*, genus *Lentivirus*, or "slow" viruses (11;21), that are characterized by their use of viral reverse transcriptase (RT) and IN (integrase) for stable insertion of viral genomic information into the host genome. Unlike other retroviruses, lentiviruses can replicate in non-dividing cells (22-24) and cause slowly progressive diseases, including immunodeficiency, anaemia, pneumonitis and encephalitis, in their specific hosts (human, monkey, cat, horse, cow, goat and sheep) (reviewed in 24). Moreover, the course of the infection caused by these viruses is characterized by a long period between the initial infection and the onset of the severe symptoms.

4.1. HIV-1 genome and viral particles

Like all viruses, HIV can only replicate inside the host cells, by sequestering the cell's machinery to reproduce. Retroviruses have their genetic material composed of ribonucleic acid (RNA) molecules. HIV has two copies of a single-stranded RNA genome of approximately 9 kb in length that encodes 15 individual proteins (Figure 1A). The three largest open-reading frames encode its three major structural proteins, namely: Gag, Pol and Env. The *gag* gene encodes viral core proteins [consisting of matrix (MA), capsid, nucleocapsid and p6 proteins], whereas the *pol* gene encodes a set of enzymes required for

viral replication [proteases (PR), RT and IN proteins]. The *env* gene encodes the viral glycoprotein gp160 from the virus surface. In addition to these major proteins, the viral genome also encodes the regulatory proteins Tat and Rev, which are responsible for the activation of the transcription and control of the splicing and nuclear exports of viral transcripts, respectively. Furthermore, there are four other genes that encode the accessory proteins Vif (viral infectivity factor), Vpr, Vpu (viral protein U) and Nef (negative factor). The viral genome is flanked by long terminal repeats (LTRs) that are involved in viral transcription, reverse transcription and integration (Figure 1A).

Gag and *pol* genes encode HIV core proteins that are synthesized from the same transcripts by a ribosomal frameshift (24), which compose the core structure of the infectious viral particle, called the virion. The Gag-Pol precursor protein is also packaged and proteolytically cleaved into their constitutive parts during maturation of the virion, making them available to carry out their respective functions upon infection of a new cell. Env gp160 protein is cleaved in gp120 and gp41, the outer HIV virion's membrane proteins. Gp120 is referred to as the surface subunit (SU) and gp41 as the transmembrane (TM) subunit. Both gp120 and gp41 are essential for normal infection of $CD4^+$ cells by the virus. Once encapsidated, each virion contains Gal, Pol and Env proteins, accessory proteins Vif, Vpr and Nef and two copies of the viral genomic RNA (Figure 1B) (reviewed in 24).

A

B

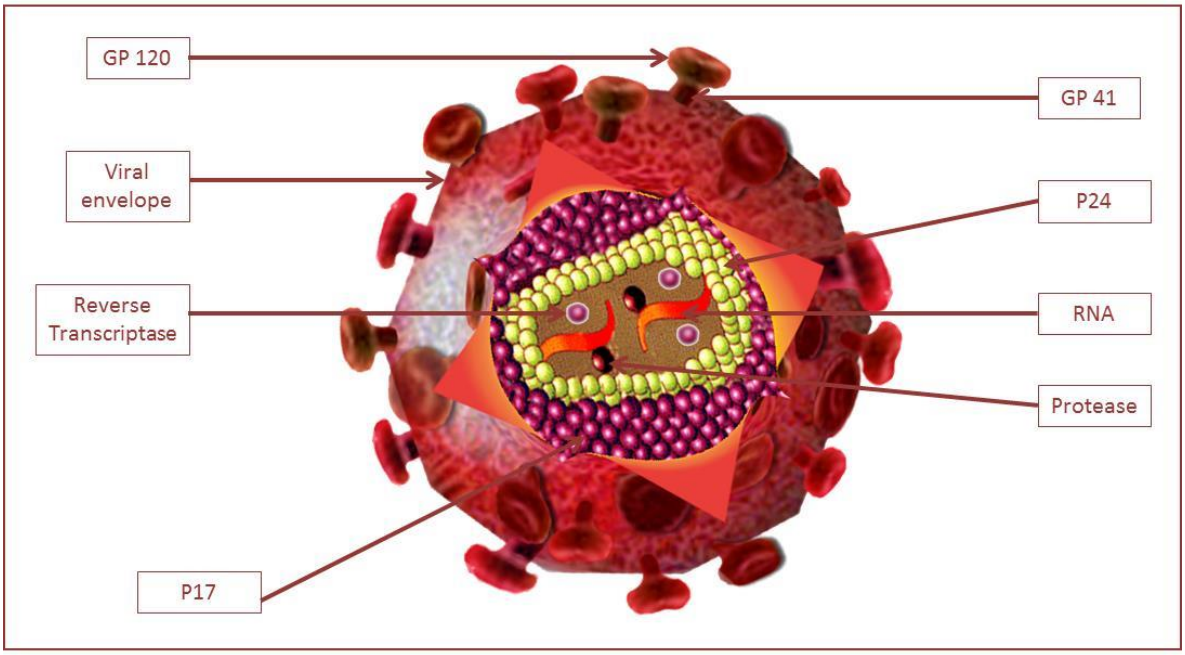

Figure 1 – Schematic representations of the viral genome and structure of HIV-1.

A – The HIV-1 viral genome encodes three structural proteins (Gag, Pol and Env), the regulatory proteins Rev and Tat and the accessory proteins Vif, Vpr, Vpu and Nef. The Gag precursor consists of MA, capsid protein (CA), nucleocapsid protein (NC) and p6. The Gag-Pol precursor protein encodes three essential replication enzymes: RT, IN and PR. The Env gene encodes the viral glycoprotein gp160, which is cleaved in gp120 (referred as SU) and gp41 (referred as TM). The RRE domain is also represented, which is located at the Env region of HIV is also represented. **B –** The viral RNA, RT, PR, IN and the accessory proteins are surrounded by CA (p24). The inner and the outer viral membranes are coated with MA (p17) and Env, respectively.

4.2. HIV-1 infection

The first step in HIV infection cycle is the attachment of a viral particle (SU) to the primary receptor CD4 and to its co-receptor CXCR4 (CXC chemokine receptor 4), expressed on T-lymphocytes or CCR5 on monocytes/macrophages, DCs and activated T-lymphocytes. Upon recognition of the receptor, TM changes its conformation to facilitate HIV membrane fusion with the host cell, leading to viral entry (Figure 2). After fusing the host cell, the capsid proteins from the virus are uncoated, leading to the release of two copies of the viral RNA and matrix protein, RT, IN and Vpr proteins into the cytoplasm. Once there, HIV RT converts the positive sense RNA strand into double-stranded DNA, the nucleic acid form in which the cells carry their genes. The newly synthetized HIV DNA is imported into the nucleus, where the HIV IN helps splicing the viral DNA into the host's DNA. This integrated form of the virus is known as *provirus* (24). Once the viral DNA is integrated into the host cell DNA, the cell, if activated, will produce new viral proteins. This process occurs by copying the DNA back to RNA through a mechanism called transcription, which is controlled by both the host cell and the viral genes. In a more detailed description, after integration of the proviral DNA, the LTRs at the ends of the viral genome will regulate transcription and polyadenylation of viral mRNAs. The LTR at the 5'-end of the genome acts as a combined enhancer and promoter for transcription by host cell RNA polymerase II. The LTR at the 3'-end of the genome helps stabilizing these transcripts by mediating polyadenylation. In the absence of the viral transactivator Tat, the basal promoter activity by the 5'-LTR is minimal. Initial transcription in the absence of Tat is inefficient and produces viral mRNAs that are spliced into short transcripts, which encode the non-structural proteins Tat, Rev and Nef that facilitate subsequent events in the viral life cycle. Newly synthesized Tat binds to transactivation-response element (TAR) on the 5'-end of HIV-1 mRNAs and transactivates and amplifies the transcription process of other structural viral proteins (25). At the same time, Rev binds to the

Rev-responsive element (RRE) on the viral transcripts to facilitate nuclear export of singly spliced or non-spliced viral transcripts and genomes (26). These singly spliced transcripts encode Env, Vif, Vpr and Vpu, whereas non-spliced viral RNAs are used for translation of Gag and Pol and as the genomic RNAs for progeny viruses. Exported viral genomes and proteins are assembled at the plasma membrane. After release from the host cell, multimerization of Gag and Gag-Pol activates the viral PR that converts these immature virions into mature infectious viruses (27;28) (Figure 2). A single cell can make thousands of infectious particles of HIV, either chronically, over weeks, or as a single burst resulting in cell death.

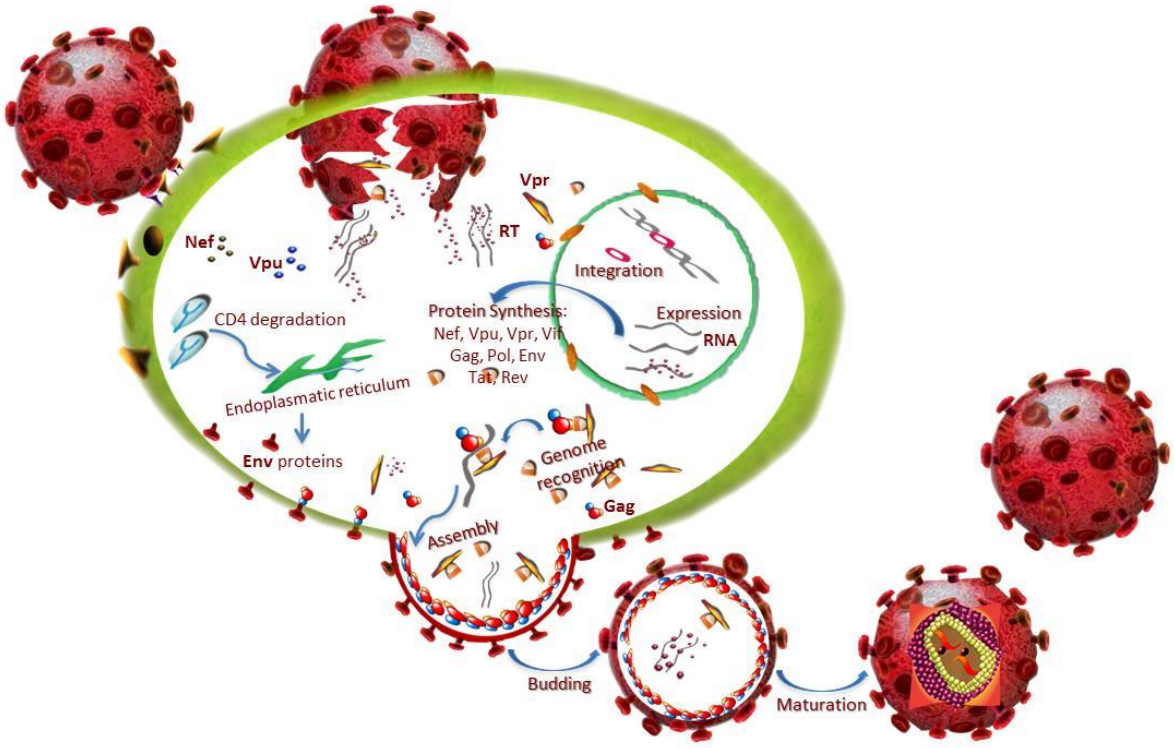

Figure 2 – Schematic view of the HIV-1 life cycle. The HIV envelope glycoproteins interact with CD4 receptors to enter the host cells. HIV outer membrane fuses with the host cell membrane, leading to the virus-host-cell-fusion. The viral capsid core then disassembles and viral nucleic acids enter the cytosol together with virion proteins. RT converts RNA into cDNA which is transported to the host cell nucleus, where IN mediates the integration of viral cDNA into the host cell DNA. Expression of the viral DNA produces a precursor polypeptide that is proteolytically cleaved by HIV

protease to give mature structural and functional proteins. The proteins will then assemble with HIV RNA at the cell membrane, from which they bud to release new mature virions

5. HIV-1 Viral Protein R (Vpr)

The Vpr of HIV-1 is a small basic protein (14 kDa) constituted by 96 amino acids that is highly conserved among primate lentiviruses, namely HIV-1, HIV-2 and SIV (simian immunodeficiency virus) (29). Vpr was isolated almost two decades ago (30;31) and numerous studies along these last 20 years have shown that it is a multifunctional protein. Therefore, Vpr is reported to exhibit numerous biological activities, including modulation of transcription of the virus genome (32;33), induction of T-cell apoptosis and defects in mitosis (34-36), nuclear transport of the HIV-1 pre-integration complex (PIC) (37), facilitation of reverse transcription (38), reduction of the HIV mutation rate (39) and protein transduction through cell membranes (40) (Figure 3).

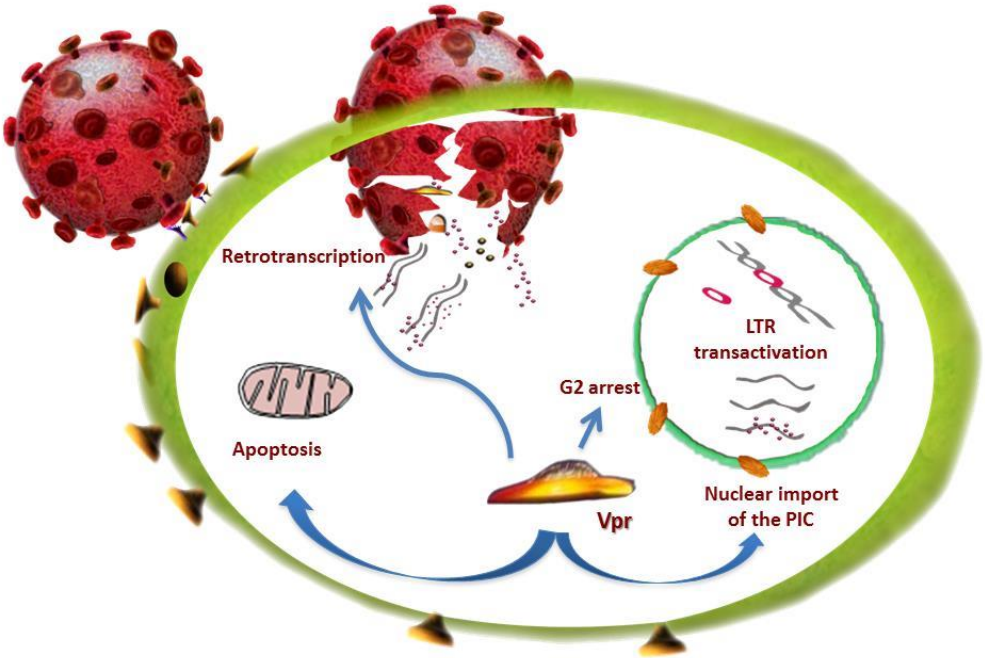

Figure 3 – The role of Vpr in HIV-1 infection. This diagram represents the principal processes in which Vpr is involved during HIV-1 life cycle. Vpr has been shown to be a multifunctional protein

that is involved in the nuclear import of the PIC, reverse-transcription process, HIV-LTR transactivation, cell cycle progression and regulation of apoptosis.

Furthermore, Vpr was shown to mediate many processes that contribute to HIV-1 infection, evasion of the immune system and persistence in the host, thus having a role in the morbidity and mortality of AIDS. Taking this into account and the numerous functions that Vpr plays in the viral life cycle, it has been suggested as an attractive and potential target for therapeutic intervention (41).

Vpr is expressed at the late stage of the virus life cycle, but it is present during the early phases of infection of the target cells, since it is packaged into virions released from the producing cells. Incorporation of Vpr occurs through a direct interaction with the C-terminal p6 region of the Gag precursor (42). While the integrity of the α-helices of Vpr is required for efficient packaging into virions (43), a leucine-rich motif found in the p6 region of the Gag precursor is directly involved in the interaction with Vpr (44;45). Efficient incorporation of Vpr was found to be estimated with a Vpr/Gag ratio of approximately 1:7 (46) that may represent 275 molecules of Vpr per virion. Incorporation of Vpr into HIV-1 virions is blocked by a set of mutations occurring in different regions of the protein, indicating that different domains of Vpr may be involved in its incorporation into virions (section 6).

5.1. Structure of Vpr

The tertiary structure of Vpr was determined by Morellet and colleagues (2003) (47) who performed a nuclear magnetic resonance (NMR) analysis of the full length (1-96) polypeptide. Vpr contains three α-helical domains with amphipathic properties, which are linked by loops and are folded around a hydrophobic core (47) surrounded by a flexible N-terminal domain and a C-terminal arginine-rich region that are negatively and positively charged, respectively (Figure 4).

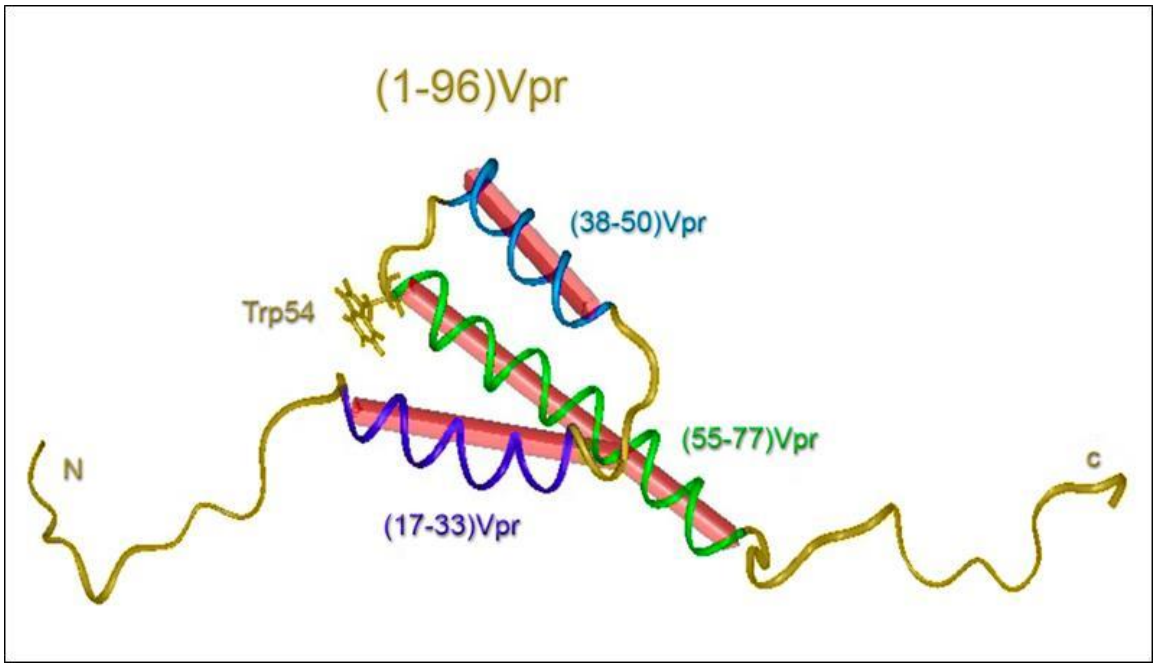

Figure 4 – The three-dimensional structure of Vpr. Representation of the N- and C-terminal domains (in brown), the α-helix 1 (in blue), the α-helix 2 (in light blue) and the α-helix 3 (in green).

The N-terminal domain is characterized by the presence of four conserved prolines in the positions 5, 10, 14 and 35 that show *cis/trans* isomerization (48). Zander and co-workers (2003) (49) reported an interaction between the cellular peptidylprolyl isomerase cyclophilin A and Vpr via prolines in position 14 and 35, which insured the correct folding of the viral protein. Furthermore, the C-terminal domain of Vpr contains six arginines between residues 73 and 96, showing similarity with dose of arginine-rich protein transduction domains and may be the explanation for the transducing properties of Vpr, including its capacity to cross cell membrane lipid bilayers. Indeed, alterations in the cell cycle, including apoptosis, cell-cycle arrest and defects in mitosis, are mostly carried out by the C-terminal domain of Vpr, although alterations in the cell cycle by other regions of Vpr have also been described (reviewed in 50).

Investigation of the structure of Vpr, led to the identification of three α-helical domains, α-H1 [13-33], α-H2 [38-50] and α-H3 [55-77] as well as other structural properties capable of

mediating a variety of biological functions (47). Indeed, Vpr's structure allows the direct binding of many cellular proteins, which seems to enable Vpr to mediate functions related with nuclear import and G_2 arrest. The three α-helices have been implicated in Vpr mediated nuclear localization (reviewed in 41), whereas the G_2 arrest capacity of Vpr has been mainly assigned to the C-terminal region of the protein (51).

5.2. Vpr and nuclear transport of HIV-1 pre-integration complex (PIC)

Resting T cells and terminally differentiated macrophages are important targets for viral replication during the initial steps of infection. Such primary infection of these cell populations contributes to establish virus reservoirs that are determinant for subsequent dissemination of the virus to lymphoid organs and T-helper lymphocytes (52). Lentiviruses such as HIV-1 have developed a strategy to import their own genome through the envelope of the interphasic nucleus via an active mechanism 4-6h after infection of non-dividing cells (53). After virus entry into the cell, the viral capsid is immediately uncoated and the genomic HIV-1 RNA is transformed in the full length double-stranded DNA by RT. This viral DNA then associates with viral and host cell proteins into the so-called PIC. Indeed, the ability of HIV-1 to replicate in non-dividing cells depends on the active nuclear import of the PIC (54). Vpr has been reported to increase the transport of the proviral DNA into the nucleus of non-dividing cells by promoting direct or indirect interactions with the cellular machinery that regulates nucleus-cytoplasm shuttling (reviewed in 50).

In normal cells, nuclear transport of a particular protein is a two-step process, which includes an energy-independent step involving the docking of the cargo protein to the nuclear envelope (NE) and the subsequent energy-dependent translocation and release of the cargo protein from the NE into the nucleoplasm. The imported protein usually carries a nuclear localization

sequence (NLS) domain, consisting of a short region of basic amino acids (lysines and arginines) (55;56).

In addition to Vpr, the viral proteins MA and IN have been reported to take part in nuclear entry. Both proteins hold a functional NLS sequence and the nuclear import function of these proteins requires classical cellular collaborators, namely importin-α and importin-β. Furthermore, the HIV-1 central DNA flap and capsid protein also play a role in the PIC nuclear targeting (57;58), promoting nuclear localization by a linked mechanism that involves PIC uncoating. Thus, HIV-1-PIC nuclear entry can be mediated by multiple and, sometimes, redundant nuclear localization signals (41).

The NLS- and M9-dependent pathways were described as two classical pathways for the transport of proteins across the nuclear pore complex (NPC). The NLS pathway involves the binding of a peptide holding a NLS signal to importin-α via central armadillo repetitive motifs. Importin-α binds to importin-β through and amino-terminal importin-β-binding (IBB) domain (59;60). The binding of the classical NLS to importin-α cannot occur until this IBB binding to importin-β is processed, which leads importin-α to exhibit an internal NLS (60). This multiprotein structure then interacts with the NPC, while importin-β transports this NLS component into the nucleoplasm. The GTPase Ran/TC4 is also involved in NLS mediated nuclear transport. In the M9-dependent pathway, transportin facilitates both nuclear import and export of RNA binding protein heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) by recognizing a M9 signal sequence (reviewed in 41). Like the classical NLS system, M9 nuclear trafficking is also dependent on the function of Ran/TC4 (61).

Localization of Vpr into the nucleus appears to use cellular machinery independent from the classical NLS and M9 pathways. While MA protein was shown to be inhibited by NLS blocking peptides and dominant negative importin-α, Vpr nuclear transport was not affected

by none of the treatments mentioned, which strongly supports the concept that Vpr works in a NLS-independent manner (62). Furthermore, Vpr mediated import was also unaffected by treatment with RanQ69L, a dominant negative form of Ran, that inhibits both M9 and NLS pathways (61;63;64). Studies with GTPγS, a nonhydrolyzable GTP that inhibits Ran function (65-67), demonstrated that this molecule exerted no effect on Vpr localization, further suggesting that Vpr localizes into the nucleus in a non-established, Ran-independent manner (68). In addition, Vpr nuclear localization also seems to be independent of energy, or at least seems to require less energy than the conventional transport. Incubation with adenosine triphosphate (ATP) was shown to impact on the localization of proteins that use the classical NLS, however, it had no effect on Vpr localization (68). Kamata et al. (2005) suggested that Vpr could enter the nucleus via two different mechanisms: one involving importin-α and the other involving energy (69). Nevertheless, it is consensual that Vpr may use importin-α in a non-classical energy-independent manner; even so it is possible that it may also use a yet undetermined mediator in the absence of importin-α in a process involving ATP.

According to Vpr's ability to promote nuclear localization of the PIC, this protein has been shown to be essential for efficient HIV-1 and HIV-2 infection of macrophages (reviewed in 41). However, there is some controversy around this issue. While HIV-1 IN can compensate the loss of Vpr at high multiplicity of infection (MOI) of the virus (62;70), there are other studies showing that HIV-1 deficient for Vpr is non-efficient in macrophages, at least in part due to its inability to cross nuclei of non-dividing mononuclear cells (reviewed in 41). Moreover, Vpr was shown to directly be involved in targeting the HIV-1 PIC to the NE (71). It was also reported that Vpr's nuclear localization and consequent G_2 arrest properties have an important role in HIV-1 infection of primary $CD4^+$ T-cells, no matter the proliferative status (72;73). *In vivo* studies demonstrated that HIV-1 clearly infects resting T cells, where Vpr mediated transport of the PIC into the nucleus was expected to be relevant. However,

Vpr's action seemed to be required for $CD4^+$ T-cell infection, even under promoting proliferation conditions. Therefore, it may be inferred that the transport of the PIC across the NE is important in both proliferating and resting T-cells and macrophages *in vivo*. The karyophilic Vpr properties required for infection of proliferating cells suggest that the targeting of the PIC to the NPC is a characteristic of lentiviral infection, irrespective of cell cycle progression. Analysing these facts from an evolutionary perspective, one can infer that lentiviruses may have evolved to infect non-dividing macrophages and may have expanded later to infect T-cells, although continuing using the infection machinery from the original non-dividing, target cell populations. Indeed, macrophages are a common target of all known naturally occurring lentiviruses (41). T-cell infection is a common process only to lentiviruses that cause immunodeficiency, suggesting once again that these cells may probably be later targets of tropism during evolution of lentiviral viruses. In this context, Vpr may contribute to nuclear localization, while other viral proteins, such as capsid, may help in additional mechanisms necessary for an efficient infection of non-dividing cells. Therefore, Vpr seems to be an important mediator of human lentiviral infection, due to its nuclear localization properties, which may constitute an important effect during periods of low HIV-1 plasma viral load or transmission between individuals.

5.3. Vpr coactivator function in the HIV-1 LTR-mediated transcription

Vpr has been described to promote HIV-1 LTR gene transcription through interactions with several transcription factors in both proliferating T-cells and non-dividing macrophages. Subsequent to the integration of HIV-1 proviral DNA into the human genome, Vpr promotes HIV-1 viral genes transcription by direct interaction with the LTR promoter. LTR gene transcription mediated by Vpr is reached by association of this protein with a variety of transcription factors or co-factors on the LTR promoter. DNA-protein binding assays have

revealed a direct binding of Vpr to the LTR DNA sequences that include a number of transcriptional binding sites on LTR, namely the nuclear factor kappa B (NF-kB); specificity protein 1 (SP1), a Tat responsive RNA element; p300/CBP binding sites and the adjacent CCAAT-enhancer-binding proteins (C/EBP) sites (reviewed in 74). Therefore, in the presence of Vpr, viral production is likely amplified via coactivation of the HIV-1 LTR by a mechanism that seems to be dependent on multiple binding sites within the viral LTR.

Furthermore, it should be mentioned that Vpr also promotes transcription of un-integrated or pre-integrated HIV-1 gene expression (75;76). For example, enhancement of the pre-integration mediated by Vpr is LTR-dependent and selective, which mainly increases the transcription of Gag and Nef genes (75). Thus, Vpr from both virion or newly synthesized, was shown to be essential for un-integrated viral DNA gene expression, in particularly Nef. In the absence of Vpr, the expression of un-integrated viral DNA is decreased 10 to 20-fold (76).

5.4. Vpr and induction of cell cycle G2 arrest

Numerous studies have focused on the ability of Vpr to inhibit host cell proliferation by blocking infected cells in the G2/M phase of the cell cycle, which is usually denominated G_2 arrest (74;77-79). In brief, G_2 phase is the third and final subphase of interphase in the cell cycle, directly preceding mitosis. It follows the successful completion of S phase, during which the cell's DNA is replicated. Vpr's induction of cell cycle G2 arrest is believed to supress human immune capacity and function by preventing T-cell cloning expansion (80). In addition, this process is thought to provide an optimized cellular environment for maximal levels of viral replication since the transcription levels of the provirus is higher during G2 (81;82). Induction of cell cycle G2 arrest by Vpr is a highly conserved activity, since similar effects were observed also in other eukaryotes such as fission yeast (*Schizosaccharomyces pombe*) (reviewed in 74).

Several proteins and genes control the eukaryotic cell cycle. Cyclin-dependent protein kinases (CDKs) initiate the crucial steps of the cell cycle by phosphorylating specific protein targets. This phosphorylation activity is tightly dependent on binding to cyclins, resulting in CDK-cyclin complexes, which are regulated either by inhibitory phosphorylation of the CDK subunit or by binding to inhibitory molecules identified as CDKs inhibitors (83). G2 arrest is characterized by low levels of cyclin B1-p34Cdc2 activity and inhibitory phosphorylation of p34Cdc2. Indeed, Vpr was shown to induce *in vitro* cell cycle G2 arrest specifically through inhibition of the phosphatase Cdc25C, which normally activates cyclin B1-p34Cdc2. In the absence of Cdc25C phosphatase activity, cyclin B1-p34Cdc2 remains in its phosphorylated form, which is inactive, therefore arresting the cell cycle (reviewed in 74).

Another evidence of Vpr-induced G2 arrest is the involvement of ubiquitin (Ub) proteasome system (UPS). There are recent information regarding an interaction between Vpr and a specific Cullin Ub E3 ligase known as Cul4A-DDB1-DCAF1, or VprBD, in the G2 arrest process (74). This E3 ligase association is mediated through direct interaction of Vpr binding protein (VprBP) with Vpr on chromatin (84;85), interfering with the DNA replication machinery of infected cells, resulting in cell cycle arrest (86). As the Cul4A-DDB1-DCAF1 complex is involved in proteasomal degradation, it was suggested that G2 arrest was Vpr-induced through degradation of an unknown protein needed for cell progression from G2 arrest to mitosis (86). Recently, Li et al. (2010), identified Cdc25C to be one of those unidentified proteins as a downstream effector during Vpr-induced cell cycle G2 arrest (87).

5.5. Vpr and apoptosis

HIV infection causes a depletion of $CD4^+$ T cells in AIDS patients, which leads to an impaired immune system and a decrease in its capacity to fight against opportunistic infections. The major mechanism for $CD4^+$ T cell depletion in HIV-1 infection is

programmed cell death, or apoptosis. Vpr is also known to cause cell death; however, its role in this process is still unclear. Even so, the cytotoxic effect is predicted to be associated with clinical symptoms such as dementia and the painful neuropathy of HIV-1 infected patients (88;89). On the other hand, there is evidence that Vpr is a cell-penetrating protein that is present in the fluids of HIV-infected patients, potentially leading to apoptosis of bystander cells (90).

Apoptosis is regulated by one of two recognized pathways: the extrinsic and intrinsic pathways. Both of them share identical features and molecules, namely caspases. The extrinsic pathway is initiated by external stimuli that are detected by cell death receptors localized on the cell membrane. The intrinsic pathway in centred on mitochondria and is characterized by the release of particular apoptosis-triggering molecules (91;92). Several lines of evidence have shown that HIV-1 Vpr is able to induce the intrinsic pathway of apoptosis in a large number of cell lines and promote apoptosis during HIV-1 infection. Furthermore, Vpr not only induced apoptosis in infected cells, but it has also been suggested to trigger this cell death mechanism in uninfected bystander cells (33).

Research studies on apoptosis indicate that mitochondrial intermembrane proteins, such as adenine nucleotide translocator (ANT), apoptosis-inducing factor (AIF), cytochrome c (cyt c), procaspases and heat-shock proteins (HSPs), are released during apoptosis and are essential for the activation of caspases and DNases. A major pathway for Vpr-induced apoptosis is through the mitochondria. This intrinsic pathway is initiated by mitochondrial membrane permeabilization (MMP) (93). Vpr is thought to induce MMP through binding to ANT protein at its C-terminal domain (94;95) possibly upon crossing the outer mitochondrial membrane (OMM). After this process, depolarization and consequent swelling of the inner mitochondrial membrane (IMM) occurs, followed by the release of apoptotic factors. Evidence supporting this model came from studies in fission yeast and mammalian cells, where Vpr depolarized

IMM (96). Furthermore, depolarization of isolated mitochondria by purified Vpr was also demonstrated, together with a strong binding between Vpr and ANT. Reduced cell death in the presence of decreased ANT levels (94;96) and activation of caspases 9 and 3 (97-99) were observed as well. Activation of caspase 9 with no activation of caspase 8 (a hallmark of the extrinsic pathway) gives support to the role of Vpr in mitochondria-dependent induction of apoptosis (98). However, data concerning activated caspase 8 by Vpr have already been published (89;100;101), suggesting that the extrinsic receptor-mediated pathway of apoptosis may also be involved.

While there is strong support for the involvement of mitochondria in Vpr-induced apoptosis, there are some studies that do not fit in this model and increase the possibility that Vpr may induce cell death through other mechanisms. Indeed, Vpr has been consistently demonstrated to be in the nucleus or at the nuclear membrane rather than in the mitochondria (reviewed in 74), which may raise important questions about its localization. Therefore, one could predict that small amounts of Vpr may be localized to the mitochondria and may be sufficient to trigger apoptosis. On the other hand, the predominant nuclear Vpr localization may have some role in initiating cell killing. Consistent with this theory, Roumier and colleagues (2002) revealed that Vpr fragments induced cell death with no caspase activation. Therefore, it is possible that Vpr-induced apoptosis may depend on the protein localization within the cell or cellular compartments (102).

5.6. Vpr and the immune system

HIV-1 has many ways to escape the immune system of infected patients. The high sequence variability of the HIV genome allows virus evasion from both humoral and cell-mediated immune responses. Thus, HIV mutations leading to cytotoxic T cells (CTL) escape have been attributed to the immune system failure to control HIV infection (103). HIV also contributes

to disturb cytokine profiles in the plasma of HIV patients, blocking an effective immune response against the infection (104). T helper (Th) 1 cytokines are detected in the plasma of infected individuals during early HIV infection, whereas at later stages of the disease, the cytokine profile switches to a Th2 profile, indicating a decline in the antiviral immune response (105). Interleukine-12 (IL-12), which is associated with Th1 polarization, was reported to be diminished in HIV-patients comparing to healthy individuals (106). IL-12 is produced by activated antigen presenting cells (APCs), macrophages and DCs and its levels can be regulated by HIV infection, where Vpr is thought to have a role. A study by Majumder et al. (2005) showed that Vpr impaired expression of CD80, CD83 and CD86 costimulatory molecules, together with inhibition of DCs IL-12 production (107). This work supported the idea of the Vpr's relevance as a virulent factor in HIV infection and suggested that the suppressed immune response could be mediated by Vpr through blockade of IL-12 production. In addition, the mechanism of immune dysfunction caused by Vpr seems to trigger apoptosis and G2 arrest of the cell cycle (described above) in bystander T-cells, leading to the reduction of immune cells (reviewed in 41). In general, one may predict that Vpr significantly contributes to the immune deficiency observed in AIDS patients by altering immune cellular function.

6. Implications of Vpr mutations

Vpr is found in HIV-1 virions in infected cells, but has also been shown to be present in sera and cerebro-spinal fluid of AIDS patients, suggesting that it takes part in several mechanisms of HIV-1 biology (108). The role of Vpr *in vivo* has been investigated in rhesus macaques infected with SIVmac. It has been reported that monkeys infected with SIV lacking the Vpr gene displayed a lower virus load and did not developed immunodeficiency disease. As stated

before, diverse functions have been attributed to Vpr *in vitro*, including a role in reverse transcription process, nuclear import of the PIC, cell cycle arrest at G2/M transition, induction of apoptosis and transactivation of the HIV-1 LTR (reviewed in 50).

Although numerous studies have demonstrated that HIV-1 Vpr mutations could dramatically affect its known functions (109-111), these mutations were artificially manipulated and probably did not represent the profile of the naturally occurring mutations over the course of the infection. Indeed, in order to address the correlation between the structural domains and Vpr function, a set of molecular strategies involving deletion, insertion and/or substitution mutagenesis was developed.

Taking into account the three-dimensional structure of Vpr (Figure 4), the effect of mutations on the protein conformation has been being studied by several research groups. Vpr's structure can be modified or denatured by deletion of one or numerous residues, by substitution of residues involved in the helices or loop conformation, or by those that do not allow the formation of the hydrophobic core. Furthermore, numerous mutations that did not modify the secondary or tertiary structure of the protein have been shown to alter Vpr activities, particularly if these residues are directly involved in the interaction of Vpr with cellular or viral components (3). In general, mutations that alter the secondary and tertiary structure of Vpr are involved in the abrogation of several Vpr activities, namely localization in the nucleus, incorporation into the virion, oligomerization and cell cycle arrest function.

Vpr intermolecular interactions seem to be biologically important due to their role in nuclear localization (112). Vpr oligomerization is described to occur in human cells (112), at the NE (3) and has been demonstrated by different methods for Vpr and its fragments. In this context, SDS-PAGE data suggested that Vpr has a tendency to form oligomeric structures (40) and was even detected in a molecular weight consistent with dimers and trimmers. Furthermore, it

has been demonstrated by dynamic light scattering analysis, that in aqueous solution, Vpr appeared in high order aggregates, namely decamers (40). Point mutations located in the N- and C-terminal domains of Vpr were reported to abolish or decrease the capacity of this protein to form oligomers by altering the secondary structure of each α-helix and consequently the three dimensional structure of Vpr (43;113). Fritz *et al.*, 2008 showed that Vpr forms homo-oligomers at or close to the NE and that these dimers and trimmers were both found in the cytoplasm and in the nucleus. Point mutations outside the helical regions (e.g. Q3R; W54G; R77Q and R90K) led Vpr to present an accumulation at the nuclear margin similar to that of the WT protein. Other mutational studies have been performed in residues from the α-helices, showing Vpr's loss of ability to oligomerize even at or near the NE (113). Thus, some of these mutations localized at the α-helices (e.g. R36W, I64E, L67A and I70S), led to the disruption of the intermolecular hydrophobic core formed by the mutated amino acids and to the impairment of Vpr oligomerization and localization at the NE (reviewed in 3).

The first N-terminal amphipathic helical domain of Vpr has an important function in Vpr packaging into virions and contains the main factors essential for efficient incorporation. Mutational analysis of the α-helices located at the N-terminal domain of Vpr, confirmed the implication of this region in virion incorporation, since the majority of the studied mutations blocked Vpr incorporation into viral particles (3). Furthermore, the capacity of HIV-1 to replicate in non-dividing cells such as macrophages, is dependent on the active import of the PIC (54) and both amino- and carboxy-terminals of Vpr seem to contain the nuclear targeting function (64;114). For example, Vpr mutants localized at the N-terminal α-helix affected Vpr's localization into the nucleus, suggesting that at least, one α-helix is required for nuclear import. In addition, when negative charges of the accessible acidic residues located at the

amino-terminal of Vpr were altered by substitution of uncharged glutamines, the protein showed an intense cytoplasmic expression (115).

Several mutational studies have also been performed regarding the function of Vpr in cell cycle G2/M arrest. The first mutational analysis reported that the C-terminal region of Vpr had an important role on the cell cycle arrest at the G2 phase, since C-terminal deletion, as well as point mutations in various residues of the same region, have been demonstrated to suspend or strongly reduce this activity. The mutated residues were all located at the third α-helix and accessible for inter-molecular interactions. Furthermore, the ^{71}HFRIGCRHSRIG^{82} Vpr sequence is highly conserved in HIV-1, HIV-2 and SIV isolates and has been identified as critical for cell cycle arrest in the G2/M phase in yeast and mammals. Deletion of the SRIG sequence (between amino acids 79 and 82) or mutation of all arginines in the C-terminal domain, revealed a prevention of the G2 arrest by Vpr (reviewed in 3).

Vpr was shown to disrupt the mitochondrial membrane (116) leading to the subsequent release of cyt c, later activation of caspase 9 and cleavage of caspase 3, thus playing a role in the apoptotic process. Point mutations in residues from the N-terminal domain, in the middle region and in the C-terminal of Vpr sequence, led to the reduction or even abrogation of its apoptosis-inducing capacity (3). Interestingly, a single amino acid substitution in the position 64 (L64P, A, R), was demonstrated to dramatically enhance the pro-apoptotic activity of Vpr (117). Moreover, Jacotot and colleagues (2001) demonstrated that the C-terminal half of Vpr (Vpr 52-96) was more efficient than the full protein in inducing apoptosis (118).

The extensive mutational analysis performed by several groups has provided a strong basis to understand the relationship between the structure and the main functions of Vpr. However, studies in isolated Vpr from HIV-1 infected patients are of crucial importance to correlate naturally occurring mutations in this protein and its role on disease progression. An initial study by Lum et al. (2003) demonstrated the presence of a point mutation at position 77 of

HIV-1 Vpr that led to a substitution of an arginine (R) by a glutamine (Q) (R77Q), with a higher frequency (80%) in Vpr alleles coming from LTNPs compared to patients developing progressive disease (89). This mutation localized at the C-terminal region of Vpr was shown to abolish the interaction of Vpr with the adenine nucleotide translocator component of the mitochondrial permeability transition pore complex, impairing Vpr's ability to induce apoptosis of T lymphocytes. While other authors confirmed this observation (119), several other groups were not able to find any association between the R77Q substitution and disease progression (120;121). A more recent study from Jacquot et al. (2009) aimed to analyse the molecular and functional properties of primary Vpr proteins isolated from two HIV-1 infected patients (classified as LTNP and fast progressors) and their binding to cellular components, namely docking to NE and cytostatic and apoptotic activities (108). The authors verified that Vpr alleles from the LTNP patient samples (collected in different years) all contained R77Q substitution. This patient also revealed a Q65R (glutamine by an arginine) substitution within the leucine-rich domain of Vpr and failed to interact with DCAF1 subunit from the Cul4A/DBB1 E3 ligase, confirming the important role of this residue in mediating Vpr/DCAF1 interaction. Interestingly, Vpr protein containing this Q65R substitution failed to accumulate at the NE, suggesting that this residue may be involved in the correct localization of Vpr. In contrast, residue 77 had no impact on Vpr binding to other cell partners, including the nucleoporin hCG1 and DCAF1. Furthermore, LTNP patient containing Q65R mutation failed to induce G2-arrest. Surprisingly, Vpr variants carrying the R77Q substitution were even slightly more efficient for G2 arrest and pro-apoptotic activities than those containing an arginine at residue 77, which runs against a deleterious impact of the R77Q mutation on Vpr functions (89). In contrast with these latter results, at the Microbiology laboratory from the Faculty of Medicine of the University of Coimbra, we isolated Vpr genes from the HIV-1 viruses isolated from the blood of two infected children who were respectively classified as

LTNP and fast progressor. Consistently with these observations, we were able to detect a mutation in residue 77 (R77Q) in the vpr gene of the virus carried by the LTNP child, whereas the other did not present alterations in Vpr structure at this specific residue (annex 1). Another study from Caly and co-workers (2008) on Vpr sequence derived from a LTNP patient revealed a significant reduction in Vpr nuclear import and virion incorporation, establishing a link between this Vpr function and HIV disease progression. These authors identified a naturally occurring mutation in Vpr protein at the amino acid 72, F72L (phenylalanine by a leucine), which was responsible for decreasing Vpr nuclear import in viral infectivity (122). The explanation for this great reduced infectivity lies probably on the lack of virion incorporation of the F72L mutant Vpr resulting in absence of PIC nuclear import and consequent less efficient HIV-1 replication in non-dividing cells. In more detail, Caly et al. (2008) demonstrated that wild type (WT) Vpr was able to bind to human importin-β3 with high affinity and that F72L mutation led to a reduction of Vpr nuclear import and consequently to a reduced virion incorporation due to a less efficient binding to importin-β3, corroborating the previous theory (37;123).

Recently, the group of Fourati et al. (2012) evaluated the impact of primary Vpr variants from infected patients as a mechanism underlying resistance to antiretroviral agents (ARV) (124). The authors based their hypothesis on the fact that patients harbouring viral strains encoding partially active Vif proteins are more likely to develop resistance to certain ARVs as these strains are able to promote the selection of some G-to-A substitutions leading to drug-resistance mutations (125). Based on these observations, they investigated Vpr genetic variability in patients failing ARV to assess whether their polymorphisms could have contributed to viral escape to ARV. Interestingly, the authors identified one mutation in Vpr, E17A (glutamic acid by alanine), located at the first α-helix, which was associated with ART failure. However, besides the role of the first α-helix in several Vpr properties and functions,

the results indicated that this mutant had no impact on Vpr activity regarding the binding to its cellular partners, subcellular localization and incorporation into virions. By analysing treatment histories of the patients, Fourati et al. (2012) (124) observed an association between E17A and didanosine (a RT inhibitor). In the population under combination antiretroviral therapy (cART), Vpr E17A was associated with 3 thymidine analog mutations (TAMs) in RT. Furthermore, viruses holding a high number of TAMs have been related to didanosine resistance (126-128). The authors found that the presence of 3 TAMs within RT did not affect didanosine susceptibility; however, when combined with mutant Vpr E17A, these viruses demonstrated a significant reduction in susceptibility to didanosine. The results led the team to conclude that association between TAMs and Vpr E17A in cART-treated patients may be related to a potentiating effect of viruses with TAMs in increasing resistance to didanosine. Therefore, one may infer that the use of didanosine in viruses holding TAMs may select for Vpr mutations that in turn, enhance resistance to didanosine. Other explanation is that HIV-1 infected patients with E17A mutant Vpr before therapy application may be more likely to spring up resistance to didanosine when this drug is introduced and when holding viruses containing TAMs.

As stated before, Vpr has been shown to negatively regulate the function of APCs, namely macrophages and DCs (107;129) and to suppress IL-12 secretion. Based on the hypothesis that Vpr R77Q mutation is associated to LTNPs, Tcherepanova and co-workers (2009), analysed the effect of this mutation in the preservation of IL-12 expression (130). However, the authors realized that WT R77 Vpr and the mutant Q77 did not differ on IL-12 production. Interestingly, they found that another Vpr mutation localized at the C-terminal, R90K, completely reverted IL-12 suppression, restoring the levels of this interleukin in DCs. Reports on R90K substitution have also been associated with LTNP patients (131) and therefore, it is possible that virus encoding Vpr R90K may relieve the blockade of IL-12 secretion. This

finding reinforces Vpr protein as a virulent factor during HIV-infection and the importance that should be given to the preservation or restoration of IL-12 secretion.

7. Vpr and HIV-1 associated clinical pathologies

Although cART has enabled constant progress in reducing HIV-1 replication, the problem of insufficient immune restoration remains, exposing the patients to the risk of development of immune deficiency-associated pathologies.

7.1. Vpr and HIV dementia

The effects of HIV-1 in the central nervous system (CNS) have been a topic of great interest to investigators and clinicians focused on HIV. HIV-1 infection in macrophages in the CNS as well as its accessory protein Vpr, have been associated with dementia in HIV-1-infected patients. HIV-1 associated dementia (HIV-D) is a disease characterized by motor and cognitive deficits and seems to be caused by the presence of HIV-1 in the brain. HIV encephalopathy (HIV-E) is an associated underlying condition observed in autopsy of patients with HIV-D. The prevalence of HIV associated neurocognitive disorders has been increasing, despite the introduction of cART. The mechanism of HIV-D pathology is not yet understood, however, there is evidence suggesting that mononuclear cells play a crucial role in disease progression. Furthermore, the major sources of HIV-1 replication in the brain seem to be macrophages and microglia (41).

Vpr has been implicated in the development of dementia in HIV-infected patients and may also be involved in the more subtle forms of neurologic diseases. It may work as a direct effector of HIV-1 mediated HIV-E, since elevated levels of this protein have been found in the cerebral spinal fluid (CSF) of patients with HIV related deficits. Thus, Vpr was detected

in the basal ganglia and frontal cortex of brains with HIV-E and is elevated in CSF and sera of seropositive HIV patients (132;133). In HIV-E brains, Vpr was found in neurons and macrophages, where it was shown to be essential for the induction of neuronal apoptosis in striatal and cortical cells (134). In addition, Vpr was shown to increase reactive oxygen species production in microglia cells and in neuroblastoma cell lines, to lower ATP levels and plasma membrane Ca^{2+} ATPase protein levels, which has been linked to cell death signalling in these cells. Vpr produced in HIV-1 infected macrophages was also reported to impair axonal growth of neuronal precursors in a process independent from apoptosis (135). Therefore, Vpr seems to contribute to the development of HIV-D by mediating cell death and neuronal impairment in HIV-1 infected patients, together with a role in the infection and survival of macrophages.

7.2. Vpr and HIV-associated nephropathy (HIVAN)

Kidney diseases have also been described in HIV-infected patients. The most common type of HIV associated nephropathy (HIVAN) is a collapsing focal segmental glomerulosclerosis (FSGS), mainly characterized by heavy proteinuria and a rapid decline in renal function due to infection and subsequent expression of viral genes in renal epithelial cells (136). However, HIVAN is not the only cause of kidney disease in patients with HIV infection. Acute kidney injury is also more frequent in HIV-infected people than in general population and is associated with poor health outcomes. The prevalence of kidney chronic disease is also increasing in the HIV-infected population (137).

Research using animal models and *in vitro* studies has shown that Vpr and Nef proteins are the HIV-1 genes most responsible for prompting the typical clinical and histopathologic features of HIVAN. The generation of a mouse model bearing a c-fms/Vpr transgene, led to the induction of FSGS in these animals, suggesting that specific Vpr expression in

macrophages might be sufficient for kidney disease (138). Furthermore, Zuo et al. (2006) expressed Vpr in FVB/N transgenic mice and verified the development of podocyte damage and glomeruloslerosis (139). These authors also reported that double-transgenic mice for Nef and Vpr expression showed severe podocyte injury and glomerulosclerosis by 4 weeks of age, indicating that these two viral proteins can induce kidney damage in a synergistic interaction. Several host factors deregulation, including inflammatory mediators, apoptosis, transcription, cell to cell interactions, among others, may also be critical factors in determining whether renal epithelium infection will result in HIVAN. Additional research is required to determine the mechanisms of HIVAN pathology, whereby Vpr/Nef nephrotoxicity targeting could be a promising area of intervention to prevent and treat this HIV-infection associated disease.

8. Vpr as a possible therapeutic target

The role of Vpr in the pathogenesis and life cycle of HIV has raised considerable interest in the development of potential therapeutic targets for the treatment of HIV infection. Many studies based on several key observations have provided additional support to this notion. For example, in tonsilar cultures infected with defective HIV-1 Vpr, a reduction of fifty per cent in HIV- production was observed (140). Moreover, Muthumani and colleagues (2002) demonstrated that Vpr decreased the efficiency of DNA and SIV-Nef vaccination in *rhesus* macaques, suggesting that in the absence of this protein a more effective immune response to HIV would be possible (141). A study conducted in six vertically infected children that were classified as LTNPs, showed that every patient had a mutated Vpr gene together with mutations in other genes that were not shared by all patients (142). The authors from this investigation concluded that these mutations were involved in Vpr's apoptotic effects, giving rise to the theory that Vpr mutations have clinical implications in the course of HIV infection.

However, as mentioned above, there is another report suggesting that the cytotoxic properties of Vpr could be more related to nuclear localization, taking into account the Vpr Q65R mutation (108).

One of the major clinical consequences of Vpr in HIV-1 infected patients is the contribution to viral reservoirs in macrophages. There is no current therapeutic approach for eliminating macrophage reservoirs that also represent drug resistance sources to HIV-1 infection and contribute to the development of AIDS. RT and PT inhibitors make part of the actual established treatments for HIV-infected patients and are described to be effective in macrophages and in $CD4^+$ T-cells in different periods of the infection. However, while this range of inhibitors prevent cell to cell spread of HIV-1 infection, it is not known what is their efficiency in addressing viruses produced from infected macrophages.

Another useful strategy for targeting macrophage reservoirs is to modify the specific host mediators of Vpr function, since Vpr interacts with many cellular proteins and is involved in many biochemical pathways. HSPs, which are a family of molecular chaperones involved in innate immunity and protection from environmental stress, have been proposed as cellular targets of Vpr and may be involved in the mechanism of antiviral response (143). For instance, overexpressed HSP70 was shown to reduce the Vpr-dependent G2 arrest and apoptosis and to inhibit HIV-1 replication in fission yeast (144). Suppression of HSP70 expression by RNA interference (RNAi) also resulted in increased apoptosis of cells infected with Vpr-positive HIV-1 together with an increase in HIV-1 replication. Another HSP, HSP27, was shown to inhibit Vpr dependent G2 arrest and cell death in T-lymphocytes when exogenously expressed, however it did not seem to suppress viral replication in macrophages (145). The heat shock response is described as cellular protective. Therefore, increasing heat shock pathways may be a promising tool to promote the survival of chronically infected cells. The involvement of Vpr in the apoptotic process and in G2 arrest, both underlying Vpr

binding to DCAF-1, also led to the suggestion that targeting Vpr ubiquitination pathways may be suitable for clinical intervention (41).

Many other pharmacological approaches have already been proposed to target Vpr pathways. Vpr is required to the PIC entry into the nucleus of non-dividing cells and consequently has become an interesting tool to investigate as a potential treatment. Indeed, CNI-H0294, a specific inhibitor of HIV nuclear localization, was demonstrated to inhibit viral production, by preventing new macrophage infection (146). In addition, Suzuki et al. (2009) demonstrated that hematoxylin is a specific inhibitor of the Vpr/importin α interaction, leading to the prevention of the nuclear import of the HIV PIC complex (147).

Due to its multifunctionality, Vpr is able to cooperate and facilitate several HIV-1 functions. Thus, Vpr can traffic into cells and incorporate into HIV particles. Vpr peptide region from residue 14 to 88 amino acid has been used to introduce other protein products into HIV-1 particles (148). In other words, Vpr has already been tested as a vector system for drug delivery. ApoEC3G (A3G), a deoxycytidine deaminase, is a potent antiviral factor that can prevent HIV-1 infection. During Vif-negative HIV-1 replication, A3G was shown to be incorporated into HIV-1 particles, inducing mutations in reverse transcribed viral DNA and inhibiting reverse transcription. However, in the presence of Vif, A3G's activities are counteracted and it loses the ability to incorporate into virions and its therapeutic efficacy (149). The fusion of Vpr 14-88 to A3G facilitated the packaging into the HIV-1 particles and restored the capacity of A3G to inhibit viral replication, demonstrating the use of Vpr to amplify the effect of antiviral molecules or at least facilitate drug delivery.

Targeting the cellular effects of Vpr as a road to treat the consequences of Vpr function in HIV-1 infection, constitute the base of many research studies in this field. Thus, combining

these approaches with the established cART regimens may be useful in lowering viral loads, increasing immune response and even in contributing to the depletion of viral reservoirs.

9. Conclusion

In the last 30 years there have been outstanding advances in the knowledge of the immunopathogenesis of HIV-1 infection and AIDS. Understanding the biological and molecular mechanisms of the viral replication cycle and the host response to infection is currently a well-documented subject, which has contributed to virus specific interventions and consequently to, at least, the partial recovery of the immune system. Therefore, even individuals in late stages of the infection can now have an expectation of long-term survival, whether they can access to cART and improved healthcare.

Vpr is a short viral soluble protein that is expressed late during viral replication and is detectable in the serum of HIV-1-infected patients. Vpr remains the most enigmatic of the accessory proteins from HIV due to its interference with many distinct cellular pathways along the virus life cycle, together with different functions that contribute to the pathogenesis of HIV-1 infection. Huge progresses have already been done in the comprehension of the role of Vpr in the most important steps of the viral replication, namely incorporation, nuclear localization, implication in the cell cycle arrest, LTR activation and subsequent apoptosis. Thus, the involvement of Vpr in these key processes has stimulated the interest in unravelling its structure and understanding the interaction with cellular partners. Although the molecular details of these Vpr actions are still not fully understood, Vpr has greatly contributed to the knowledge of the available information about HIV-1. The findings show that while not essential for viral replication *per se*, Vpr is biologically crucial in the infection of non-dividing target cells, including macrophages and resting T-cells and also promotes the infection of dividing cells.

Strategies involving deletions, insertions and point mutations have been developed to investigate the functions of the structural domains of Vpr. The biological variations occurring in HIV-1 Vpr sequence may influence the function of the protein, depending on the nature of

the mutation and its localization on Vpr sequence. Furthermore, genotypic analysis of the Vpr alleles from HIV-1-infected patients with naturally occurring mutations in this protein have been essential to highlight its relevance in the modulation of the course of the infection, since these alterations seem to delay the adverse consequences of HIV in infected patients. However, one limitation of this research area is the scarce availability of infected blood and tissue samples.

In conclusion, highlighting the pathophysiologic roles of Vpr-mediated pathogenesis in HIV-induced immune disease and its mechanisms of action may constitute a road of investigation to develop useful therapeutic strategies in the future, contributing to control the spread of AIDS.

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ANNEX 1

Impact of HIV1-Vpr mutation R77Q in disease progression: two paradigmatic clinical cases[1]

Brief summary

A broad study aimed to tackle the naturally occurring HIV1-Vpr mutations at the aa position 77 in a group of vertically infected children, lead to the identification of some cases of LTNPs bearing Vpr variants with the mutation R77Q. Herein, two cases are described, one LTNP with the R77Q mutation and a FP with no mutation at this aa position, as examples of the impact of this mutation R77Q in the progression of HIV1-related disease.

Clinical cases description

In what regards the clinical examination of the two patients (A and B), several parameters were evaluated as seen in Table A1. The patient A was a 8 year old child with clinical signs of disease, including asthenia, weight loss, weakness, recurrent fungal infections, poor performance in school and developmental delay. As to patient B, a 10 year old child, with no specific symptoms of disease, clinically followed for medical control, only because the parents are HIV1 carriers.

[1] The clinical cases here described are part of an ongoing study together with the Hospital Pediátrico de Coimbra aimed to characterise vertically HIV1-infected children.

Table A1. Some clinical parameters of the patients

	Patient A	**Patient B**
Age	8	10
Weight (kg)	21	30,2
HIV RNA (copies/ml)	18467	25705
CD4 T cells/ml	400	482
CD4 T cells (%)	24,1	28,2
CD4/CD8	0,612	0,723
Clinical Classification	2	1
Imunological Classification	B	A
Transmition	Vertical	Vertical

The methodology used in order to characterise the Vpr variant (and currently being used in an ongoing study) involves the collection of blood from HIV1-vertically infected children, followed by leukocyte extraction. From the leukocyte population obtained, DNA was extracted and the vpr gene amplified using a Nested PCR strategy. The DNA obtained was sequenced and the sequences analysed using the Mega4 Program.

In Figure A1 it is possible to observe the alignments of the DNA sequences obtained in the two patients HIV1-vpr genes and the predicted aminoacid sequence of Vpr protein.

Amino acid / Sequence	73	74	75	76	77	78	79	80
Patient A. Nint1	CGC	ATC	GGC	TGC	CGC	CAC	AGC	CGC
Patient A. Nint2	CGC	ATC	GGC	TGC	CGC	CAC	AGC	CGC
Patient B. Nint1	CGC	ATC	GGC	TGC	CAG	CAC	AGC	CGC
Patient B. Nint2	CGC	ATC	GGC	TGC	CAG	CAC	AGC	CGC
Vpr_Wild type	CGC	ATC	GGC	TGC	CGC	CAC	AGC	CGC
	arginine	isoleucine	glycine	cysteine	arginine	histidine	serine	arginine

Figure A1. Alignments of the DNA sequences obtained in the two patients HIV1-vpr genes and the predicted aminoacid sequence of Vpr protein

CONCLUSION

With these data we can conclude that the HIV-1 child, without specific symptoms, is a LTNP.

The other one is clinically compatible with FP.