The role of human SAMHD1 in restricting porcine endogenous retroviruses (PERVs) and the innate immune response to PERV infection in human primary immune cells

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1 Abstract

The release of porcine endogenous retrovirus (PERV) particles from pig cells is a potential risk factor during xenotransplantation by way of productively infecting the human transplant recipient. Potential countermeasures against PERV replication are restriction factors, such as SAMHD1, that block retroviral replication. SAMHD1 is a triphosphohydrolase that depletes the cellular pool of dNTPs in non-cycling cells preventing retroviral reverse transcription. Restriction factors and innate immune responses are the first line of defense against invading pathogens. Sensing of viruses in a cell usually results in the production of type I IFNs that lead to the activation of IFN-stimulated genes.

The first part of this study focuses on the analysis of the antiviral activity of SAMHD1 against PERV-A/C in human immune cells. To study that, PERV-A/Cs originating from activated porcine PBMCs was used to infect human primary cells from healthy human donors. In parallel, primary cells were transduced with virus-like particles (VLPs) lacking or containing the Vpx protein from SIVmac239. The result showed that SAMHD1 potently restricts PERV-A/C reverse transcription in human monocytes, monocyte-derived dendritic cells (MDDC), macrophages (MDM) or resting-$CD4^+$ T-cells and in monocytic THP-1 cells. Degradation of SAMHD1 by SIVmac Vpx or CRISPR/Cas9 knock-out of SAMHD1 allowed for PERV reverse transcription. Addition of deoxynucleosides alleviated the SAMHD1-mediated restriction suggesting that SAMHD1-mediated degradation of dNTPs restricts PERV replication in these human immune cells.

The aims in the second part of this study were to investigate the IFN response by monitoring induction of IFN stimulated genes following infection by PERV-A/Cs, and to identify the signaling pathways, which play a role in the type I IFNs response. The results showed that PERV-A/C increased (over 100-fold) CXCL-10 production in human MDDCs, monocytes and MDMs compared with non-infected cells or heat-inactivated virus. Treatment with VLP+Vpx or empty VLP did not cause more CXCL-10 induction compared with untreatment. The JAK-inhibitor (AT9283) reduced the CXCL-10 induction in infected cells indicating that the JAK/STAT pathway is activated by PERV-A/C infection of primary human myeloid cells.

In conclusion, the findings in this work highlight SAMHD1 as a potential barrier to PERV-A/C transmission from pig transplants to human recipients during xenotransplantation.

1 Zusammenfassung

Die Freisetzung von Porcinen Endogenen Retroviren (PERVs) aus Schweinezellen ist ein potenzieller Risikofaktor während der Xenotransplantation. PERV ist in der Lage, in humanen Zelllinien zu replizieren. Mögliche Abwehrmechanismen gegen die PERV-Replikation sind zelluläre Restriktionsfaktoren. Dazu gehört SAMHD1, eine Triphosphohydrolase, die den zellulären dNTP Pool in sich nicht teilenden Zellen erschöpft und die reverse Transkription damit verhindert. Restriktionsfaktoren und die angeborene Immunität sind die erste Verteidigungslinie gegen eindringende Krankheitserreger. Die Erkennung viraler Strukturen führt oftmals zur Produktion von Typ-I-Interferonen und nachfolgend zur Expression von IFN-stimulierten Genen.

Der erste Teil dieser Arbeit fokussiert auf den Nachweis der antiviralen Aktivität von SAMHD1 gegen rekombinante PERV-A/C in menschlichen Immunzellen. Um dies zu untersuchen, wurden diese Viren aus aktivierten PBMCs von Schweinen gewonnen, um humane Primärzellen von gesunden Spendern zu infizieren. Parallel dazu wurden Primärzellen mit virusähnlichen Partikeln transduziert, welche zum Teil zusätzlich das Vpx-Protein von SIVmac239 enthielten. Das Ergebnis zeigte, dass SAMHD1 die reverse Transkription von PERV-A/C in menschlichen Monozyten, von Monozyten abgeleiteten dendritischen Zellen (MDDC), Makrophagen (MDM) oder ruhenden $CD4^+$ T-Zellen und in monozytischen THP-1-Zellen wirksam einschränkt. Die Degradation von SAMHD1 durch SIVmac Vpx oder CRISPR/Cas9-Knock-out von SAMHD1 ermöglichte die reverse Transkription von PERV. Die Zugabe von Deoxynukleosiden milderte die SAMHD1-vermittelte Restriktion, was darauf hindeutet, dass der SAMHD1-vermittelte Abbau von dNTPs die PERV-Replikation in diesen menschlichen Immunzellen einschränkt.

Im zweiten Teil dieser Studie erfolgte die Untersuchung der IFN-Reaktion durch Analyse der Induktion von IFN-stimulierten Genen in humanen Primärzellen nach Infektion durch PERV-A/C. Die Ergebnisse zeigen, dass PERV-A/C die CXCL-10-Produktion in humanen MDDCs, Monozyten und MDMs über 100-fach erhöht. Aufgrund der profunden Inhibition dieser Aktivierung mit dem JAK-Inhibitor (AT9283) wird daraus geschlossen, dass dieser Signalweg für die CXCL-10 Induktion bei einer PERV-A/C Infektion in humanen myeloischen Zellen verantwortlich ist. Die erhaltenen Ergebnisse belegen dass SAMHD1 ein potenzielles Hindernis für die Übertragung von PERV-A/C von Schweinetransplantaten auf Menschen während der Xenotransplantation darstellt.

2 Introduction

2.1 Retroviruses

The Retroviridae are enveloped single-stranded (+) RNA viruses. Viral RNA is reverse transcribed by reverse transcriptase (RT) during the replication cycle and integrated as provirus into the genome of the host cell.

More than 100 years ago, the two pathologists Vilhelm Ellermann and Oluf Bang succeeded in describing the first retroviruses, as cell-free filtrates triggered mouse and fowl leukemia [1]. In 1911, the american peyton rous discovered that ultra filtrates from sarcomas of the fowl led new tumors in healthy, susceptible fowls [2]. Later, the virus, responsible for these tumors was named as the Rous sarcoma virus (RSV). Mouse mammary tumor virus (MMTV) was discovered by Bittner In the year 1936 as other retroviruses associated with tumor in the mammary glands of mice [3]. The importance find out was the identification of ribonucleic acid in RSV particles in 1961 by Crawford [4]. A few years later, the reverse transcriptase essential for retroviruses and their propagation strategy was discovered and described in parallel by Howard M. Temin and David Baltimore [5, 6]. The first human retrovirus, responsible for tumorigenesis in humans, was described by Gallo in 1980 and is known as the human T-cell leukemia virus (HTLV) [7]. Almost in parallel, Luc Montagnier succeeded in 1983 in isolating Human Immunodeficiency Virus Type 1 (HIV-1) from blood samples of AIDS patients [8, 9].

With the discovery of complex retroviruses, the categorization of retroviruses by morphology was replaced with new criteria for defining the Retroviridae. The International Committee on Taxonomy of Viruses (ICTV) currently classifies retroviridae into two subfamilies: orthoretroviridae and the spuma retroviridae (Fig.2.1, [10]). A taxonomy based on the phylogenetic evolution (sequence relatedness of reverse transcriptase, analysis of virus morphology, its pathogenesis and host specificity) classified the orthoretroviridae in 7 genera: Alpharetroviruses, Betaretroviruses, Deltaretroviruses, Gammaretroviruses, Epsilon-retroviruses and the Lentiviriuses while the Spumaviridae family comprises one genus the Foamiviruses (ICTV). The first three genera are considered simple retroviruses, while the rest are considered complex and harbor small accessory proteins encoded by alternatively spliced transcripts that are absent in simple retroviruses [11].

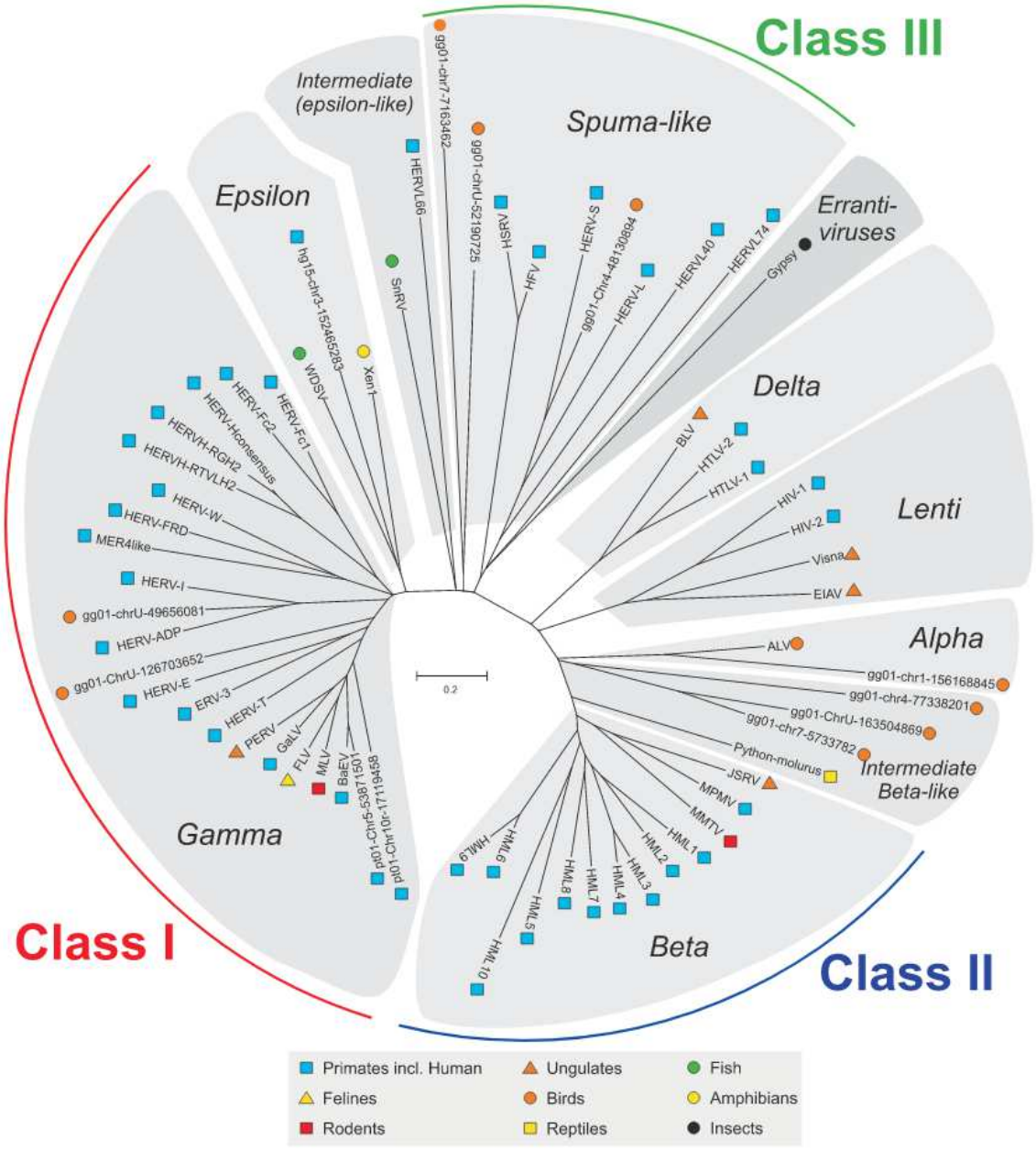


Figure 2. 1 Phylogenetic tree of retroviruses [10].

2.1.1 Endogenous retrovirus

Retroviruses can be exogenous or endogenous (ERV), both consist of the general set of retroviral proteins necessary for replication and assembly, the exogenous retroviruses can be transmitted horizontally from host to host and the endogenous retrovirus proviral integrated into the germline of all vertebrates by old exogenous retroviruses infection and transmitted vertically from generation to generation [12] [13]. In 1977 Van Nie et al. described a previously unknown infection pathway using the mouse mammary tumor virus (MMTV) [14]. Not only can MMTV infect new cells as an infectious exogenous virus particle released by the

cell, but also as an endogenous component of the germline cell genome transmitted to the next generation. Based on the infection of an oocyte, spermatocytes or their progenitor cells by the retrovirus and not a somatic cell as usual, every offspring that develops from the germline cell carries the provirus in every single body cell, at the same chromosomal position and is subject to the Mendelian heredity [15]. Endogenous retroviruses do not undergo a complete replication cycle, but instead remain within the host genome as a provirus. The epigenetic factors like DNA methylation and histone modifications can upregulate the transcription of the ERV, which may impair its ability to complete replication cycle inside the healthy host [16-18].

Human chromosomes integrated with numerous retroviral sequences that form part of the genome. In total, human endogenous retroviruses (HERVs) sequences account for ~8% of human genome [19, 20]. There are about 100 HERV families have been defined for the human genome [20]. HERV-K also referred to as HML-2, is the youngest and the most biologically active human endogenous retrovirus (HERV) family [17, 21]. Various studies have shown that HERV-K (HML-2) proviruses are capable of producing viral proteins and even whole particles [22, 23]. HERV-K (HML-2) particles are observed in some motor neuron disease, infection and cancer, but their involvement in humans disease is still being investigated [24, 25]. In the other side, a wide range of beneficial functions was described for ERVs, for example the env-gene of HERVs encodes for the expression of syncytins (Syncytin-1 and -2, respectively HERV-W and HERV-FRD), which is essential for the formation of syncytiotrophoblast early during embryogenesis [26, 27]. In addition, Env proteins may be play role in immunosuppression to save the embryo in the beginning of pregnant [28].

2-1.1.1 Porcine Endogenous Retroviruses (PERV)

In 1970 was the first description of PERV particles in the supernatant of porcine kidney (PK15) cells and other porcine cells such as peripheral blood mononuclear cells (PBMCs) [29-31]. PERVs morphological and genomic characteristics belong to the genus gammaretrovirus; therefore they are closely related to MLV, feline leukemia virus (FeLV), gibbon ape leukemia virus (GaLV), and koala retrovirus (KoRV) [32-34] (Fig, 2.2).

It was considered that PERVs probably originated from murine retroviruses [35]. The analysis of the mutations in the long terminal repeats (LTRs) of the PERV sequences revealed that the endogenization may have occurred before 7.6 million years [36, 37]. PERVs are

present in the genomes of all pigs and the copy number of PERVs in the porcine genome didn't stop to increase due to re-infection and intracellular transposition [38-40]. Some of PERVs are able to infect human cells *in vitro*, for that PERV may represent a special risk for xenotransplantation [41, 42]. The replication-competent PERVs are further classified in three subtypes according to their env sequence; PERV-A, -B and –C (Fig.2.3) [43, 44]. PERV-A and PERV-B are present in the genomes of all pig strains, at different copy numbers and can infect not only pig cells but also cells of other species like human [45-47]. On the other hand, PERV-C is integrated into the genome in many, but not all pigs and is able to infect only pig cells [48].

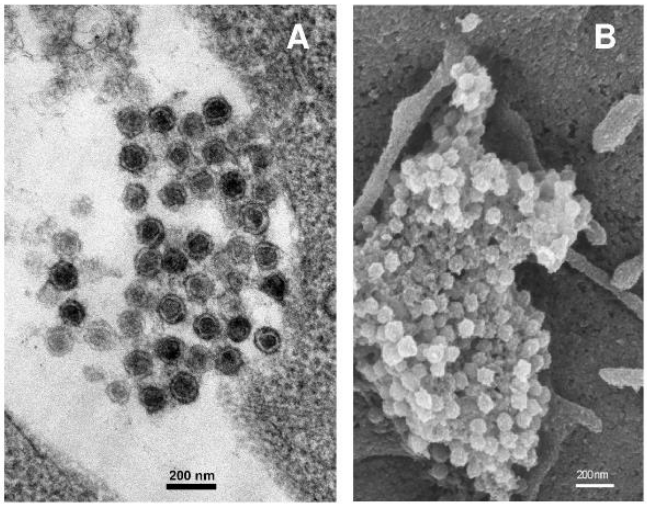


Figure 2. 2 PERVs produced by infected human cells as shown by transmission (A) and scanning (B) electron microscopy. (Klaus Boller, Paul Ehrlich Institute, Langen, Germany.)

A recombination between PERV-A and -C is possible and results in a highly replicating form named PERV-A/-C, which can also infect human primary cells *in vitro* [49]. This increase was associated with some substitutions in the variable region and increase in the length of LTR in the PERV-A/C [50].

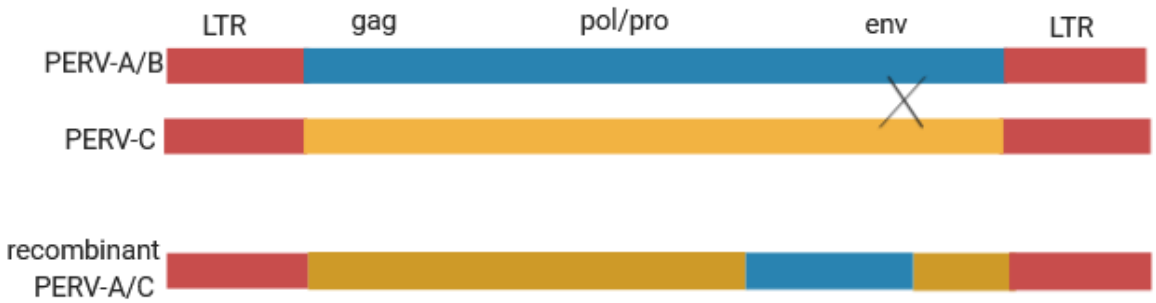


Figure 2. 3 The type of PERVs.

2.1.1.2 Structure of PERV

PERV structures like other retroviruses, the viral particles have a diameter of about 100 nm (Fig.2.4). The outer cellular lipid double membrane of PERV are envelope proteins, which contains the transmembrane glycoprotein (TM), bound to the surface glycoprotein (SU), and are visible in the EM as so called spikes [51-53]. The matrix proteins (MA) are located inside the membrane which are connected to the membrane by myristic acid residues and form a net-like protein layer. Inside the virus a virus capsid protein, which protect a complex of nucleocapsid proteins (NC) and two single-stranded RNA bound to several copies of reverse transcriptase (RT), protease (PR) and integrase (IN), which form together the inner core [51].

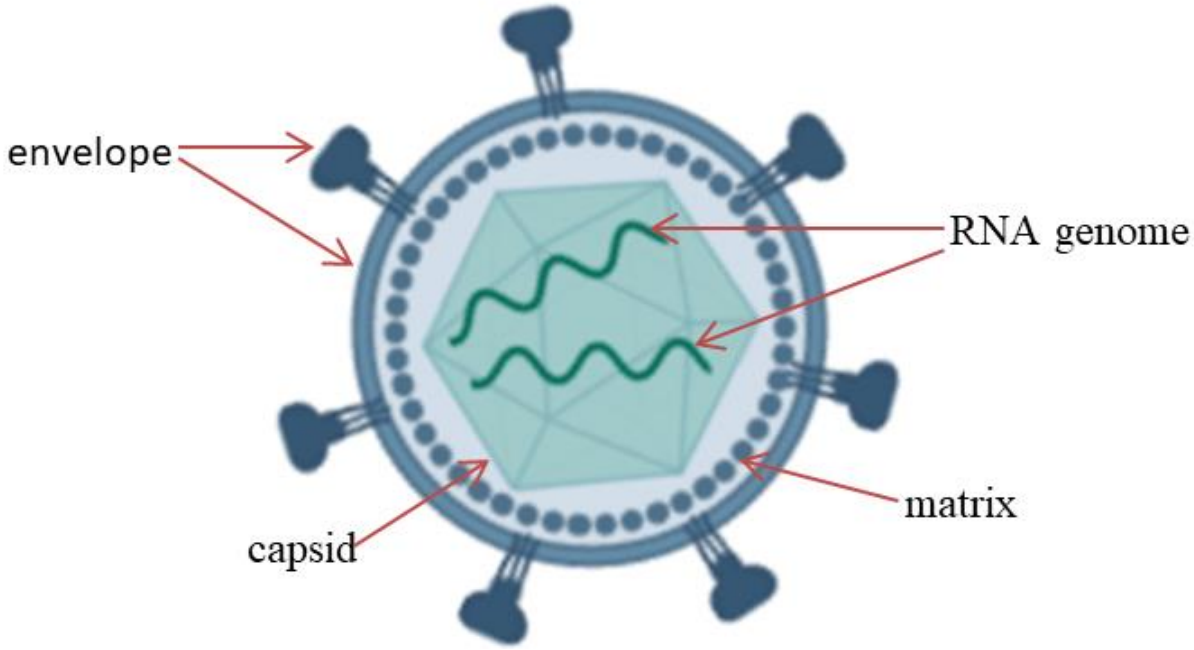


Figure 2. 4 PERV structure (modified from BioRender).

2.1.1.3 Genomic organization and function

The genomic RNA of PERV consists of two identical single strands with positive polarity of about 8100 base pairs and includes both coding and non-coding sequences [41].The non-coding sequences LTR (Long Terminal Repeat) are localized at both ends of the RNA which includes the R (redundant), U5 (unique) regions at the 5′-end and the U3 and R regions at the 3′-end. A primer-binding site (PBS) is located at the end of the U5 element and is important for initiating reverse transcription by annealing to a cellular tRNA Gly [54]. Both LTRs are important for the viral integration and transcription and contain cis-active sequences, promotors and enhancers [55, 56]. Between non-coding sequences, there are sequences

encoding the Gag, Pol, and Env proteins, that is, the gag (group-specific antigen), pol (polymerase gene), and env (envelope gene) genes, respectively (Fig.2.5). The gag gene encodes the structural proteins of the matrix (MA), capsid (CA), and nucleocapsids (NC). CA is the main structural protein of PERV, with a molecular weight of about 27 kDa. NC protein has a molecular weight of about 10 kDa (p10), and it is responsible for the efficient packaging of RNA in the virion [51, 57, 58]. Gammaretroviruses have an additional p12 protein localized in the Gag polyprotein between MA and CA, which play role in the integration of the dsDNA within the genome of the host cell and in the release of new virus particles [59, 60] .

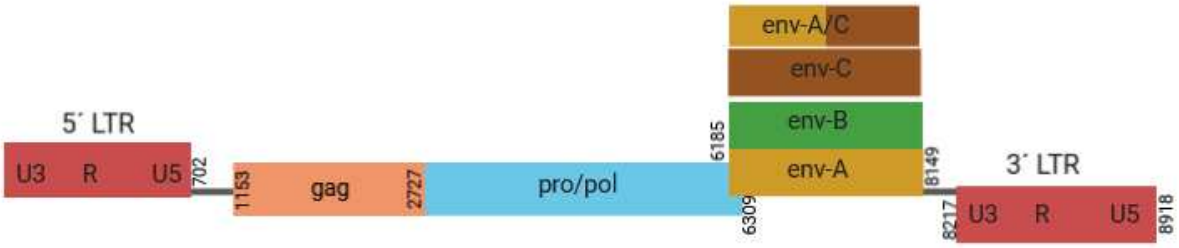


Figure 2. 5 Genome structures of the proviral PERV. LTR; long terminal repeat, Gag; group-specific antigen gene, Pro/Pol; protease/polymerase gene, Env; envelope protein gene. Env subtypes of PERVs and the A/C recombinant are presented.

Protease (PR), reverse transcriptase (RT), and integrase (IN) are encoded by the pol gene. PR is a protein with a molecular weight of 14 kDa (p14) that catalyzes the proteolysis of the Gag and Pol polyproteins into the proteins described above [41, 61]. RT is essential for the transcription of viral ssRNA into dsDNA and IN helps the dsDNA to integrate into the host genome [58].

The Env spikes on the surface of the virion are important for viral attachment and the fusion of viral and cell membranes during the infection process. The *env* gene encodes the precursor proteins of the env as a single polyprotein, which is cleaved by a cellular furin-like protease into two components: the surface envelope protein SU (gp70) and the transmembrane envelope protein TM (p15E) [42, 61]. The env protein is produced only from spliced *env* mRNA [62]. The glycosylation of the env protein is necessary for virus infectivity by influencing the binding to the host receptor; env glycoprotein contains different glycosylation sites: about 10 in PERV-A, 6 in PERV- B, and 8 in PERV-C [52, 63]. The SU protein has the receptor-binding domain (RBD) on the N-terminus; therefore it is the responsible for binding to the host receptor [64, 65]. TM protein doesn't present sequence variations likes SU protein

and the main role of TM protein is to mediate the membrane fusion reaction during infection [66].

Between the U5 region and gag, the primer-binding site (PBS) is located, which is responsible for starting the first DNA strand-reverse transcription (RT). This sequence is complementary with glycine-tRNA in the case of PERV-A and PERV-B, but with proline-tRNA for PERV-C [67]. The genome contains also the splice donor and acceptor sites which result in two splice variants. The first variant sequence coding for Gag and Pol proteins, and the second coding for Env protein [68]. The polypurine tract (PPT) plays role in synthesis the second strand of the DNA copy, PPT is located at the end env region [60]. The 5′-end of the viral genome consists of a cap unit and the 3′-end contains a polyA tail [41, 51, 68]

2.1.1.4 PERV Replication

The replication cycle of PERVs can be divided into early and late phases similar to gammaretroviruses such as MLV. The early phase includes binding to a specific receptor on the cell surface, entry into the cell, reverse transcription, and cDNA integration within the genome of the host cell (Fig.2.6). The late phase includes the expression of retrovirus RNA genes, virus protein particles and releases the maturation virus.

The Early Phase: The viral cycle infection begins with the binding of PERV SU to the suitable receptor on the host cell. Until now, only receptor for PERV-A has been identified. In pigs, it is porcine PERV-A receptor (PoPAR) [69]. Two receptors in human cells were described and named the human PERV-A Receptor (HuPAR1 and HuPAR2), also known as G-protein-coupled receptors 172A and 172B [70]. More recently, these receptors have been renamed as SLC52A2 and SLC52A1, respectively [71]. SLC52A1, 2 receptors contain 11 transmembrane domains and thus a transporter function can be recognized and are encoded by genes located on chromosomes 8 and 17, respectively, which are found in most of the human tissues [69, 72]. The PERV-B receptor(s) are still unknown.

The binding of SU envelope protein with its cell surface receptor activates the transmembrane protein (TM) and induces conformational changes in the gp70 protein. As a consequence, the viral Env fuses with the host cell membrane, and the genetic material of the retrovirus covered by the CA enters into the cytoplasm, where it undergoes partial disassembly, the viral nucleoprotein complex is liberated and the RNA genome is reverse transcribed into a double-stranded DNA by the viral RT using cellular dNTPs [73, 74]. The double-stranded linear

DNA intermediate as well as the reverse transcriptase, matrix proteins and integrase are assembled together to form a subviral particle termed as pre-integration complex. PERV can integrate only the dividing cells, therefore PERV cDNA transfer from cytoplasm into the nucleus during mitosis, when the nuclear membrane has broken down.

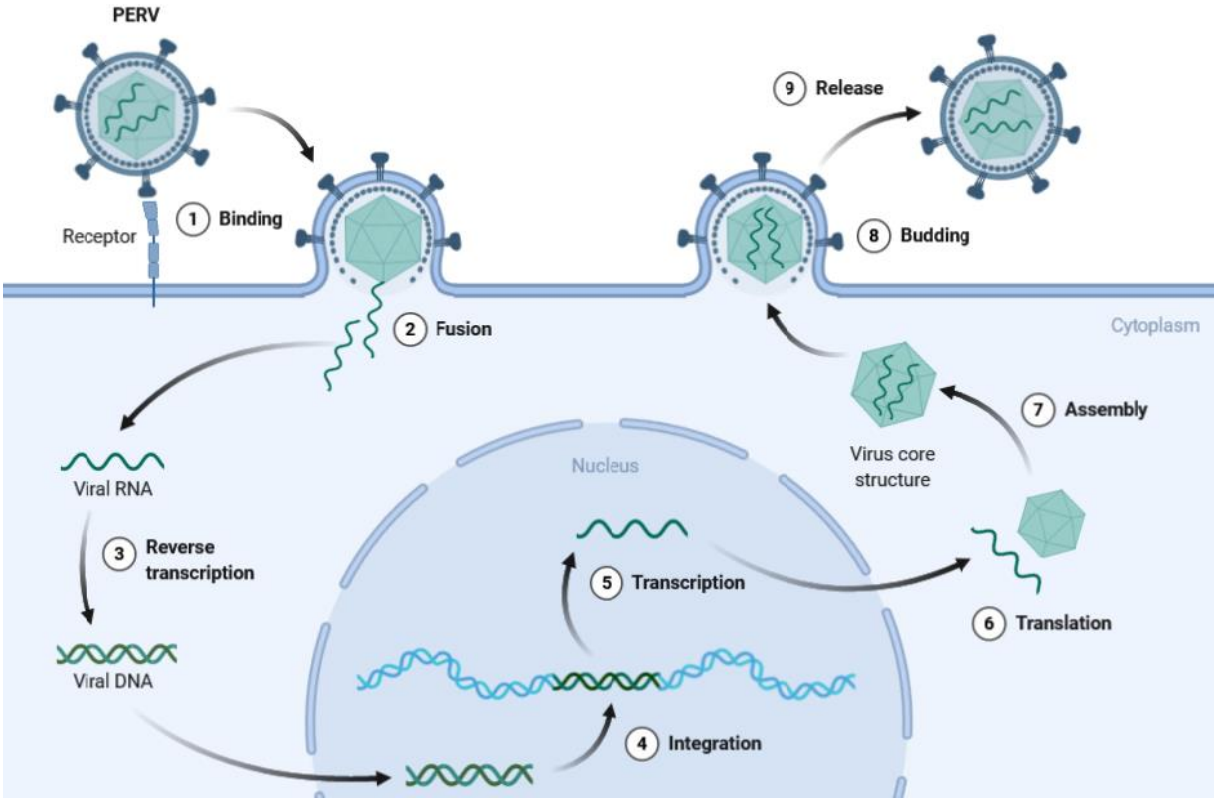


Figure 2. 6 The PERV replication cycle (modified from BioRender).

The Late Phase: After nuclear import the viral DNA becomes integrated into the cell chromosome, the provirus DNA is transcribed by the host machinery to produce descendent retroviruses. The originated mRNA can be used for the production of viral proteins, or become the genomic RNA of new viral particles. Several cellular factors are involved in transcription by the cellular RNA polymerase II. The transcription of the provirus generates spliced and unspliced mRNAs and transported into the cytoplasm. Gag-polyproteins are transported to the cell membrane or aggregate directly at the cell membrane. Env-precursor proteins are synthesized at the membrane of the cytoplasmic reticulum and transported to the cell membrane [75]. The Ψ-signal in unspliced mRNA leads to packaging of viral RNA into virus particles [67]. These capsids are released from the cell by budding of the plasma membrane, which has incorporated also the viral envelope proteins. Budding of viruses is followed by cleavage of the precursor polyproteins by the viral and cellular proteases.

2.2 Xenotransplantation

The definition of xenotransplantation is the transfer of living cells, tissues or organs between different species. Xenotransplantation includes also perfusion of foreign cells by blood of the host or acellular biomaterials (heart valves, blood vessels, tendons) of non-human species [76].

The aim of the xenotransplantation is to overcome the shortage of human donor material for transplantation, because the number of individuals waiting for an allotransplant is always increasing and many patients die before they get any organ. The history of xenotransplantation started in 1964, the chimpanzee kidney has been transplanted into a human with end-stage renal disease, the patient's life was extended for nine months [77]. In 1992 and 1996 two patients received a pig heart in Poland and India as well as the transplantation of two baboons and one pig livers in patients with liver failure [78-80]. Nevertheless, these trials showed limited success.

The major problem of xenotransplantation is the rejection of cells, tissues, and organs; the transplanted material is usually rejected more rapidly and more violently than in the case with allogeneic transplants. The most reason for rejection is the anatomic-physiological incompatibility, which causes immune rejection of the xenografts, due to the phylogenetic distance between humans and animals [81]. Viral and bacterial infections can also transmitted with xenotransplantation and lead to functional loss of the transplant and death of the recipient [82]. In contrast to the transplantation of whole organs, the use of cells and tissues of porcine showed more promising and successful. In the last years, implantations of pig embryonic cells are used in the treatment of Parkinson´s disease, and pig liver cells are transplanted into human patients with liver failure [83]. The most successful xenotransplantation was the transplantation of porcine islet-cells in treating type I-diabetes [84].

2.2.1 Pigs are ideal donors for xenotransplantation

Pigs (*Sus scrofa*) are chosen for several reasons as suitable donor species for xenotransplantation in humans. The physiological and anatomical properties of pigs' organs are similar to human and come in different sizes [85, 86]. Pigs also have a short gestation time about 115 days, reach the reproductive maturity period in 4-8 months, produce high number of large sized litters by multiple births (5-12) and most of their organs reach the maximum

size suitable for human body within only 6 months [87]. Other advantages using pigs as donor animals for xenotransplantation are their suitability for genetic engineering and this was shown by several studies which succeeded to generate pig clones from genetically modified porcine cells, for example, human complement regulating genes or by the knockout of pig cell surface molecules [88]. Moreover, pigs can be easily bred in hygienic environments under control with low costs and the risk of zoonotic transmission is much lower than primates due to their greater phylogenetic distance from humans [89]. Because millions of pigs are slaughtered annually for human consumption the ethical concerns are less restricted and the idea of use of pigs for transplantation is more accepted in the public [90].

To use porcine organs for xenotransplantation, there are several difficulties and barriers remain such as the physiological incompatibilities, the immunological concerns and the major problem is also the infectious risk for human patients by different viruses. More than 20 potentially lethal viruses are known that could be transmitted from animals to humans including hepatitis A and B, Marburg virus, Ebola, herpes B, simian virus 40 (SV40) and simian immunodeficiency virus (SIV), whereas the human immunodeficiency virus (HIV) type -1 and -2 are a result of cross species transmission between the SIV of chimpanzee (SIVcpz) and the sooty mangabey (SIVsm) [91-94]. Human herpes virus 8 (HHV-8), which transmitted from monkey can kill humans in a matter of short time [95].

2.2.2 PERV and xenotransplantation risk

PERVs are widely distributed and integrated within the genome of all pigs. They are present in various proportions depending on the pig breed, tissue type, and retrovirus subtype [39, 47, 96]. There are three replication-competent subtypes of PERVs: PERV-A, and B, replicate in human cells in vitro but PERV-C infects only porcine cells [45]. Recently, recombinants PERV-A/Cs were discovered in different organs and tissues of pigs but not in the germline.

The risk of PERV transmission to various human cells, including peripheral blood mononuclear cells (PBMCs) embryonic kidney cell (HEK-293) line, and normal dermal human fibroblasts (NHDFs) has been confirmed in vitro [97, 98]. Therefore PERVs ability to infection increases the concern, especially in the context of the eventual use of porcine cells, tissues, and organs in xenotransplantation.

Until now, it has not been reported, that PERV can transmit in vivo among patients with type 1 diabetes, after pancreatic islets xenografts [99, 100], recipients of pig's nerve cells [83],

porcine skin graft recipients [101], and butchers exposed to contact with pig tissues. It is possible, that immunological response and restriction factors inhibit PERV activity in *vivo* and in the case of in *vitro* studies, we are not able to reproduce the complicated dependence networks that have a significant impact on the defense of cells against PERV infection in vivo.

To reduce the risk of PERV transmission during human xenotransplantation, donor pigs should be selected depend on the absence of PERV-C to avoid the recombination between PERV-A and PERV-C to produce the high infectivity PERV-A/C. The expression of PERV in organs and Pig strains is different, for that the lowest expression of PERV-A and –B better used for xenotransplantation [96, 102]. For screening biological materials such as animal saliva or blood should be used to determine the PERV expression. However, if the number of PERV copies in the organ for xenotransplantation differs compared to the material used for screening, an investigation of the whole animal or of its sisters or brothers should be performed [103]. Detailed knowledge of the structure and replication cycle of PERVs is a requirement for planning strategies for PERV detection [104-106]. Such detection should be achieved at the genomic and proteomic levels using methods with adequate sensitivity and specificity. Polymerase chain reaction (PCR), with the use of the primers complementary to the conserved regions of the gag and pol genes, permits the detection of PERV proviruses in the analyzed biological material like PBMC or saliva, it was reported that PERV has high expression in PBMC. The virus subtype (PERV-A, - B, or -C) can be detected by the primers complementary to *env* gene. Concomitantly the potential risk of recombination between the subtypes can be determined (PERV-A/C). Primers complementary to the LTRs can serve for the amplification of the whole genome of PERV provirus [107, 108]. In addition, the detection of RT activity can give more confirms for the presence of the virus. Along with the increase of the number of copies of PERV RNA, RT activity can serve as a marker of the active replication cycle. The serology methods; western blot, ELISA, and immunofluorescence can be used to complements the diagnostics and confirm PERV infection [104, 105]. Viral particles can be visualization by transmission and scanning electron microscopy methods to confirm the presence of and their release from infected cells [32]. The genetic material of the virus can also be determined with the use of the *in situ* hybridization technique. This method allows finding the location of the viral nucleic acid and the percentage of infected cells, giving the chance to estimate the viral replication ability and the degree of integration in the host's DNA [109].

There are other viruses can transmissions during xenotransplantation. Hepatitis E virus (HEV) genotypes 3 and 4 can be detected in humans and animals, like pigs and the virus can be transmitted zoonotically by eating uncooked or undercooked pork products or meat [110]. Therefore HEV-GT3 and GT4 might pose a risk in xenotransplantation using pig cells, tissues or organs [111]. The other risk can associated with xenograft these porcine viruses, Porcine cytomegalovirus (PCMV), Porcine lymphotropic herpes viruses and Porcine circoviruses [112-116].

The risk of viral infections due to the use of pigs as donors in xenotransplantation is assessed to be lower than in the case of human allografts. The use of human donors also carries the risk of transmission of viral infections, such as cytomegalovirus (CMV), viral hepatitis B (HBV), C (HCV), E (HEV), Epstein–Barr virus (EBV) or human immunodeficiency virus (HIV) [117-119]. In allotransplantation may prevent complex diagnostics in the search for all possible human pathogens. The immunosuppression can also deplete the immunological barriers of the recipient. In the case of xenotransplantation, pigs devoid of pathogens should be bred to ensure the safety of the procedure.

2.3 Restriction factors

During their replication cycle in human cells, retroviruses interact with multiple replication barriers. As a first-line defense, host restriction factors can limit viral replication, which potently block retroviruses replication by intervening with various replication steps [120-122]. The restriction factors characteristics are: (1) They have the ability to cause a significant decrease in viral infectivity, (2) they are counteracted by some viral accessory proteins, (3) they show signs of rapid evolution, and (4) their expression can be strongly induced by interferons (IFNs), thereby tightly linking their activity with the host's immune system [123]. Complex retroviruses such as HIV and SIV evolved to circumvent these restriction factors allowing infection of human or monkey dividing cells, respectively. Both viruses either encode for accessory proteins that inactivate restriction factors during the replication cycle or carry mutations in those viral genes that encode targets for these restriction factors to avoid recognition [124]. In the last years, several restriction factor proteins with anti-HIV activity have been identified. Interestingly, many of them not only inhibit the replication of exogenous viruses like HIV-1, but were also found to restrict endogenous retroviruses and transposable elements. These factors include apolipoprotein B mRNA-editing enzyme catalytic

polypeptide-like 3 (APOBEC3) family members, Tetherin, Mx2, sterile alpha motif domain and HD domain containing protein 1 (SAMHD1), TRIM5alpha, and others [125-127].

APOBEC3G (A3G) is a single-stranded DNA cytidine deaminase that inactivates the coding capacity of viruses by deamination reactions that convert cytosine to uracil (C to U) during reverse transcription of the genomic HIV-1 RNA leads to G to A hyper-mutations in the newly synthesized in the minus-strand DNA [128, 129]. Human APOBEC3 (A3) proteins include hA3A, hA3B, hA3C, hA3DE, hA3F, hA3G, and hA3H [130]. The hA3F and hA3G are consider the main inhibitors of human immunodeficiency virus (HIV- 1) replication [131, 132], hA3G has the ability to inhibit the replication of other retroviruses, such as simian immunodeficiency virus (SIV), human T-cell lymphotropic virus (HTLV), murine leukemia virus (MLV), and retrotransposons [133, 134]. In several studies, they found that human APOBEC3G (A3G) blocks porcine endogenous retrovirus (PERV) replication by C-to-U deamination porcine endogenous retrovirus and inhibition of DNA strand transfer during PERV reverse transcription [135]. In addition, porcine A3 gene (A3Z2-Z3) can also inhibit PERV replication in a pseudotype assay as well as in a virus-spreading assay [136].

Another restriction factor is tetherin, also known as BST-2, which is an IFN-inducible membrane protein that efficiently blocks release of virion by directly tethering them to the cell surface and prevent the release of virus particles from the virus-producing cells. The antiretroviral activity of Tetherin is counteracted by the accessory protein Vpu of HIV-1 [137]. It has been reported that human and porcine tetherin can inhibit PERV release from producer cells [138]. However, tetherin proteins from different species have different sensitivities to various viral countermeasures [139]. They showed also that a combination of tetherin and APOBEC3 was more efficient in PERV restriction than each individual restriction factor [140].

2.3.1 The restriction factor SAMHD1

Sterile alpha motif domain and HD domain containing protein 1 (SAMHD1) is an IFN inducible host protein acts as a deoxynucleoside triphosphate triphosphohydrolase (dNTPase) that depletes the intracellular pool of dNTPs, which is required for cellular DNA polymerase, by converting dNTPs into the corresponding nucleosides (dNs) and inorganic triphosphate, when dGTP binds to the allosteric binding site in SAMHD1 [141-143]. The discovery of SAMHD1 as a host restriction factor explains the importance of metabolic regulation as an additional mechanism to limit the replication of viral pathogens [144].

SAMHD1 is constituently expressed at various levels in all cell types and highly expressed in myeloid lineage and resting $CD4^+$T-cells [144, 145]. In non-cycling cells, such as macrophages, dendritic cells and resting $CD4^+$ T-cells SAMHD1 is capable to restrict human and non-human retroviral infection by reducing the intracellular dNTP pool below the threshold that is necessary for complete reverse transcription to produce viral cDNA [141, 146]. SAMHD1 is considered an important restriction factor for HIV-1 by preventing virus replication at reverse transcription by degrading the dNTP pool and inhibiting early steps of virus replication cycle, blockade at this step prevents the synthesis of full-length double-strand DNA and disrupts later stages of the viral life cycle, including nuclear translocation and integration of proviral DNA [147, 148]. In addition, SAMHD1 can restrict other human viruses like DNA viruses that rely on dNTPs to replicate their genome such as herpes simplex virus 1 (HSV-1) and vaccinia virus [149, 150].

2.3.1.1 Structure of SAMHD1

Human SAMHD1 comprises 626 amino acids and contains two important domains (Fig.2.7): the sterile alpha motif (SAM) domain, which functions in protein–protein interactions and contributes to SAMHD1 nucleic acid binding, although it is not necessary for its nuclease activity in restriction [151]; the HD domain of SAMHD1 contains the enzymatic sites, which is essential for its triphosphohydrolase activity to hydrolysis the intracellular pool of dNTPs , RNA binding and nuclease activity, for that the expression of HD domain alone is enough to restrict HIV-1 and other retroviruses replication [141, 152]. At the N-terminus of SAMHD1 is the nuclear localization signal, therefore the predominant location of SAMHD1 is in the nucleus [153, 154], but a fraction of SAMHD1 is also found in the cytoplasm [147] . It was reported that SAMHD1 may also contain other nuclear localization signals since deletions of the N-terminal region did not lead to limit cytoplasmic expression of SAMHD1 [155], and SAMHD1 can relocalize in the cytosol if nuclear localization signal mutated [156]. It has been found that, the mutated versions of SAMHD1 that localized in the cytoplasm were still capable of restricting HIV-1 infection [154, 157]. SAMHD1 also contains a domain at the C-terminus, which is the target domain for HIV-2/SIVsm Vpx accessory protein [158, 159]. Prior to the detected of the antiretroviral activity of SAMHD1, it was unclear how Vpx promotes the HIV-2 infection in non-cycling myeloid cells such as monocytes, macrophages and dendritic cells [160-162]. It is now confirm that Vpx counteracts SAMHD1 functions in non-dividing cells and promotes its proteasomal degradation [144].

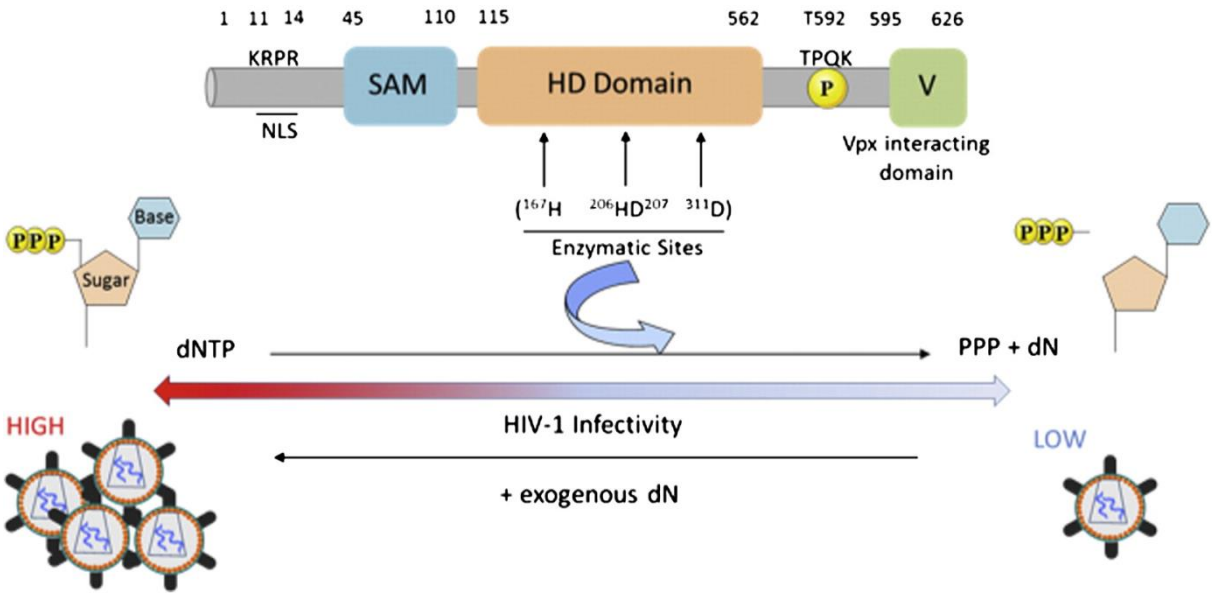


Figure 2. 7 The structure and function of SAMHD1. (Top) Representative schematic of the important domains of SAMHD1, including enzymatic sites, nuclear localization signals and activation/phosphorylation site. (Bottom) How SAMHD1 restricts retroviruses by dNTP depletion [163].

To induce SAMHD1 degradation Vpx should interact with SAMHD1 physically in the nucleus [154, 155]. Vpx interacts with the cullin-4-based E3 ubiquitin ligase CRL4/DCAF1 and recruits SAMHD1 to the Cul4/ CRL4/ DCAF1 complex by binding with the C-terminal domain of SAMHD1, leading to ubiquitination proteasomal degradation and relieving the antiviral activity of SAMHD1 to restricting retroviruses [157, 161, 164]. Following SAMHD1 degradation by Vpx, the level of intracellular dNTP increased and the viral RNA reverse transcribed into cDNA and complete its replication cycle (Fig.2.8). The nuclear Vpx cannot degrade SAMHD1 mutants in the cytoplasm; also the mutant of Vpx that does not localize in the nucleus cannot deplete wild-type SAMHD1, which confirms the Vpx-SAMHD1 reactions occur in the nucleus [154, 165, 166]. In SIVsm and SIVmac, Vpx is packaged through an interaction with amino motifs in the carboxy-terminal p6 region of the Gag precursor polypeptide [167, 168]. Transfer of the P6gag motif of SIV SIVmac to the corresponding location of HIV-1 Gag results in HIV-1 that packages Vpx, which can infect non-dividing cells [168]. Virus-like particles (VLPs) can also deliver Vpx to myeloid cells [144] (Fig.2.8). In addition the C-terminus contains the phosphorylation residue T592; the Phosphorylation of SAMHD1 regulates its capability to block HIV-1 infection in cycling cells [169].

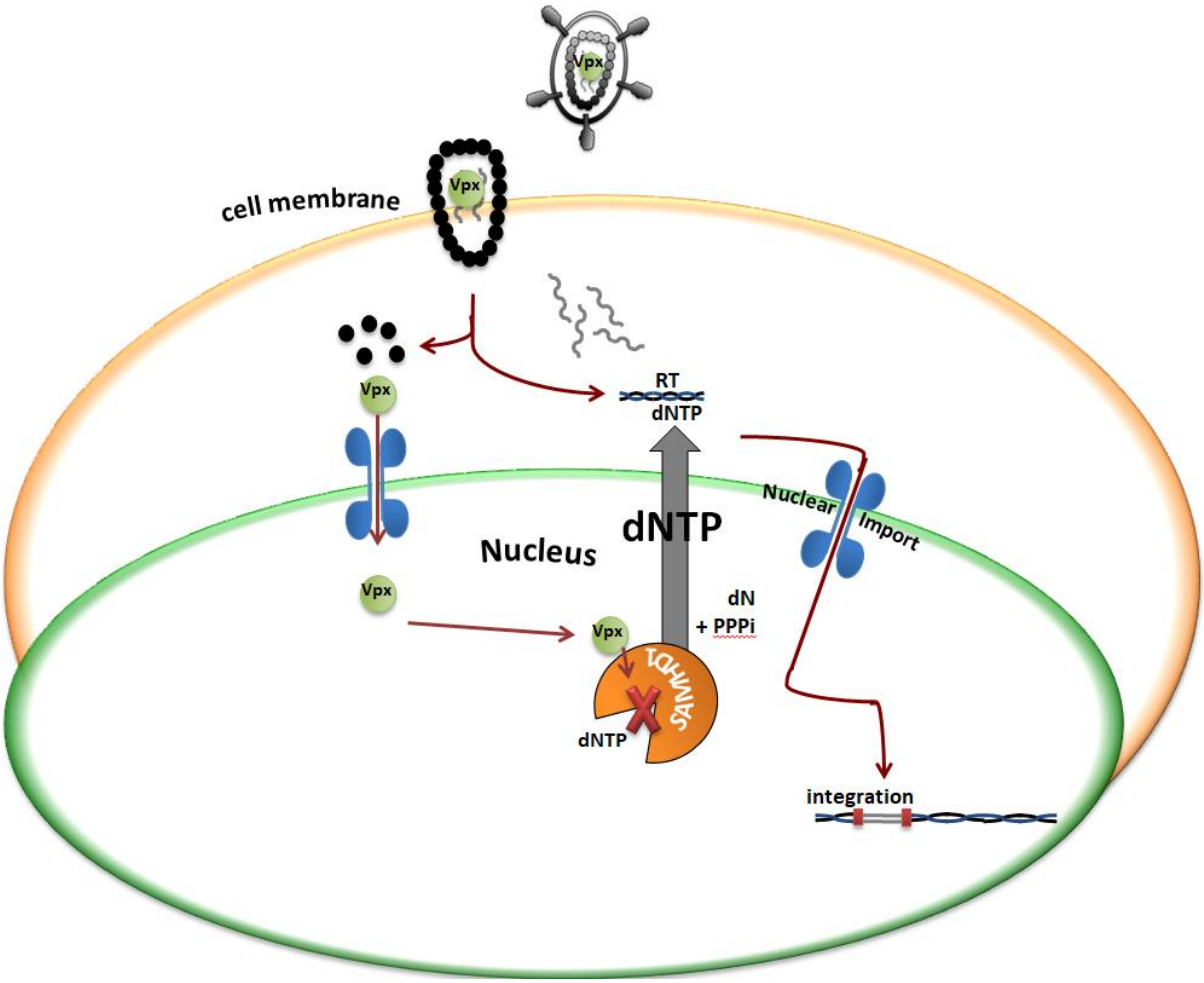


Figure 2. 8 Vpx degrades SAMHD1 and increases the level of dNTP to reverse transcription.

2.3.1.2 Regulation of SAMHD1 activity

SAMHD1 has a crystal structure and the enzymatically active of SAMHD1 forms tetramers. It has been reported that SAMHD1 oligomerizes in a dGTP-dependent manner up to a tetramer and that the tetramerization of SAMHD1 is essential for its dNTPase and antiviral activities, whereas the inactive apo-form interconverts between monomer and dimer [140, 169,170]. There are four allosteric nucleotide binding sites in the active tetramer, each capable of taking two dGTPs that serve to interconnect SAMHD1 chains into the tetrameric state [171]. Several biochemical and structural studies indicate that GTP also acts as allosteric cofactor enhancing the oligomerization. The cofactor-induced tetramerization has been reported to be critical for the role of dNTPase activity and the reverse transcriptase restriction of retroviral infection [172, 173]. The introduced mutation to the allosteric dGTP-binding site D137 does not undergo oligomerization and was mostly monomeric in the presence of dGTP22. The mutation of D137 (SAMHD1D137N) eliminates SAMHD1 dNTPase but not

RNase activity. Unlike the dNTPase activity, the tetramerization of SAMHD1 is not required for its RNase activity [174].

2.3.1.3 SAMHD1 expressed in dividing and non-dividing cells, but the restriction activity of SAMHD1 in both cells types is different

The antiviral activity function of SAMHD1 and its restriction capacity are not exclusively associated with its expression level; but instead are regulated by phosphorylation in cycling cells [145, 169]. SAMHD1 is phosphorylated at numerous residues, but the phosphorylation of threonine 592 was identified as necessary for the negative regulation of its anti-viral restriction activity [144, 169]. Cyclin-dependent kinase 1 (CDK1) and CDK2 in complex with cell cycle regulatory protein cyclin-A, phosphorylate SAMHD1 at T592 in the S-phase during cell cycle [170, 171]. CDK1 and cyclin-A are highly expressed in dividing cells. The amount of SAMHD1 protein may be different during various stages of the cell cycle depending on cell type. It was demonstrated that the highest levels of SAMHD1 happened during quiescence, while SAMHD1 expression was lower in dividing cells [172]. Several studies established that no up-regulated of the expression of human SAMHD1 protein by adding extra IFN-α or IFN-γ into human dendritic cells, macrophages and $CD4^+$ T-cells [145, 153, 173]. On the other hand the expression can be induced in certain cell lines, such as U87-MG, HEK 293T or HeLa cells [148]. It has been indicated in one study that IFN-α can induce SAMHD1 expression in primary monocytes, but in another study did not demonstrate any increase [169, 174].

In monocyte-derived macrophages (MDM) SAMHD1 expression is increased by a combination of IL-12 and IL-18 that lead to cells with more resistant to HIV-1 infection [175]. The mechanism for increase SAMHD1 expression in some cell types by these cytokines is unknown. In recent study it was shown that type I and type II interferons increase activation of SAMHD1 via dephosphorylation at threonine-592 and downregulated Cyclin-dependent kinase 1 (CDK1) at the protein level [176]. In addition SAMHD1 play essential role in the regulation of the cell cycle by preventing accumulation of G1 cells and arrest of the cell cycle [172]

2-3-1-4 The Role of SAMHD1 in the Autoimmune Disorder

SAMHD1 plays a central role in the immunity and nucleotide metabolism therefore the mutations to SAMHD1 can causes immune disease. Different SAMHD1 mutations have been found in several diseases [177, 178]. One of these autoimmune diseases is Aicardi-Goutières

Syndrome (AGS), which is associated with mutations in the enzyme activity site of SAMHD1 [179, 180]. AGS is an autosomal genetic disorder characterized by encephalopathy, leukodystrophy, calcifications of the basal ganglia, psychomotor retardation, cerebrospinal fluid lymphocytosis and in around 40% of the AGS cases, death in the early childhood [181, 182]. The mutation in some proteins, which involved in the nucleic acid metabolism like SAMHD1, TREX1, RNase H2, ADAR1, and MDA5, can cause accumulation in the intracellular dNTP and the endogenous nucleic acid debris that lead to immune sensing and an over-production of type one IFN [183]. Nearly 13% of all documented cases of AGS genetic disease were counted with the mutations in SAMHD1 [177]. LINE-1 is the non-long terminal repeat retrotransposon, which comprises ~17% of the human genome. SAMHD1 can inhibit ORF2p-mediated LINE-1 reverse transcription by deplete the ORF2p level and enhancing stress granule formation [184]. Recently it has been reported that LINE-1 is the potential trigger of systemic autoimmune disease [185]. LINE-1 nucleic acids were accumulated in cells with SAMHD1 mutation, which lose its inhibitory effect on LINE-1 retrotransposition, that lead to nucleic acid sensing by cGAS and IFN signaling activating causing autoimmune AGS. In addition, SAMHD1-deficient monocytes, which are isolated from AGS patients with homozygous SAMHD1 mutations more susceptible for infection by HIV-1 [174], proposing a link between SAMHD1 function in both autoimmunity and HIV-1 restriction.

The mutation of SAMHD1 associated with AGS has several novel features not detected in other AGS associated genotypes, including cerebral vascular hemostasis, arthropathy, and mitochondrial DNA deletions [186]. The clinical presentation of SAMHD1 induced AGS may therefore provide evidence to the mechanism through which SAMHD1 functions in nucleotide homeostasis and negatively regulates innate immunity [187]. It has been reported that interferon levels in SAMHD1 knockout mice are increased**,** but do not developed to autoimmune disorder [188]. On other hand zebra fish showed similar interferon overexpression and cerebrovascular pathologies associated with SAMHD1 deletion, like the symptoms observed in AGS patients [189].

2.4 Innate Immune responses to viral infection

Innate immune responses consider the first line of defense against invading pathogens. They are induced by activated pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) [190]. Identification and characterization of the molecular basis of DNA sensing represents a very important strategy employed by the innate

immune system to discover the presence of pathogens. More than 10 cytosolic receptors of DNA have been identified in recent years, including the cGAS, PYHIN (Pyrin and HIN200 domain) family proteins AIM2 (absent in melanoma) and IFI16 (interferon-γ (IFNγ)-inducible protein 16) [191], which are activate different signaling pathways to expression of interferons and pro-inflammatory cytokines. Pro-inflammatory cytokines are essential for chemical-mediator to recruitment the particular immune cells to the site of infection. Over 20 forms of IFN have been identified, and these have been further classified into three families; types I, type II, and type III depend on their different structures, receptor ligation, and biological activities [192, 193]. Type I IFNs are the largest family of IFN and include five classes in humans, namely IFN-α IFN-β, IFN-ε, and IFN-κ, and IFN-ω [194]. The target receptor for all type I IFNs to bind a common cell-surface is known as the type I IFN receptor and the genes that encode type I IFNs are located on chromosome 9 in humans [193, 195]. On the other hand, IFN-γ is the only type of IFN-II and the gene that encodes this cytokine is placed on chromosome 12 [193, 196]. IFN-γ binds another cell-surface receptor, which is identified as the type II IFN receptor [197]. Type III are identified recently and contain four types IFN-λ1-4 [198], its receptors are heterodimeric receptors [199].

The IFNs are induced following viral infection by pathogen-associated molecular patterns (PAMPs), consist of evolutionarily conserved molecules like nucleic acids, proteins and carbohydrates, which are sensed by corresponding pattern recognition receptors (PRRs) [190]. In general these innate immune sensors can be classified into two major classes depending on their subcellular location. PRRs that recognize extracellular PAMPs are normally found on the plasma membrane or endosomal membranes, and contain the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs) [200, 201]. Usually these membrane-bound PRRs are mostly expressed in immune cells, such as macrophages and dendritic cells. In another side, intracellular PRRs are located in the cytoplasm or nucleus of mammalian cells, and include the NOD-like receptors (NLRs; also known as nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) containing proteins), RIG-I-like receptors (RLRs), and a group of intracellular DNA sensors that contains interferon-γ (IFNγ)-inducible protein 16 (IFI16) and cyclic guanosine monophosphate-adenosine monophosphate (GMP-AMP) synthase cyclic GMP-AMP synthase (cGAS) [202, 203]. The innate immune system identifies the type of viral PAMP and triggers mechanisms which stimulate genes responsible for bringing the defending cell into an anti-viral state. After sensing viral nucleic acids, the (cGAS) and other innate immune sensors are a highly conserved sensor for cytosolic nucleic acid and structural

proteins, create the second messenger, cyclic guanosine monophosphate-adenosine monophosphate, that activate the stimulator of IFN genes (STING), mitochondrial antiviral signaling protein (MAVS) or MYD88, as well as a common set of well-studied serine/ threonine kinases and restriction factors [204, 205]. STING activation induce the phosphorylation of TANK binding kinase 1 (TBK1) and the following phosphorylation and dimerization of interferon-regulatory transcription factors IRF3 and IRF7. Nuclear translocation of the IRF3/IRF7 homo-or-hetero dimers will activate IFN-I gene expression. In addition, STING activates the IκB kinase (IKK), which phosphorylates IκB family of NF-κB

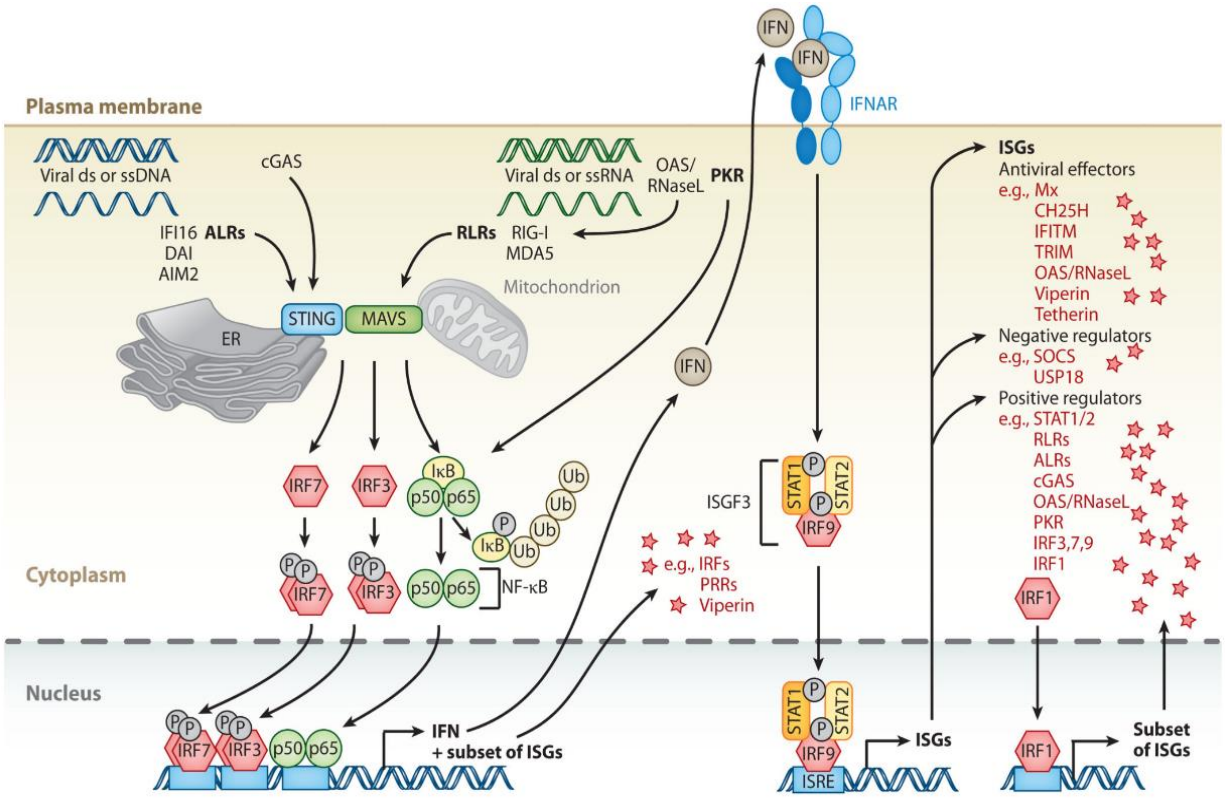


Figure 2. 9 The activation and recognition of ISGs by cytosolic nucleic acid pattern. Viral double-stranded (ds) or single-stranded (ss) DNA or RNA is recognized by pattern-recognition receptors (PRRs). The foreign DNA sensing goes through AIM2-like receptors (ALRs), such as IFI16, DAI, or AIM2 itself, while RIG-I-like receptors (RLR)-RIG-I and MDA5 specialize in RNA detection. cGAS is considered the major DNA sensor. 2′-5′-oligoadenylate synthetase (OAS) is an additional sensor for foreign RNA. The stimulator of IFN genes (STING) and mitochondrial antiviral-signaling protein (MAVS) activate by the DNA or RNA sense, that lead to phosphorylation of interferon (IFN) response factors 3 or 7 (IRF3/7), or to phosphorylation and ubiquitin-mediated degradation of IκB. After phosphorylation of IRF3/7 or NF-κB, they translocate to the nucleus and activate specific promoters, causing expression of IFN as well as a subset of ISGs. Through the JAK-STAT pathway, IFNs induce

large expression of ISGs that can be acted as antiviral effectors and negative or positive regulators of IFN signaling [206].

inhibitors [207]. Phosphorylated IκB proteins are degraded, that lead to releasing NF-κB, which enters the nucleus and induces pro-inflammatory cytokines. This signaling cascade results in an upregulation of IFN-I against viral infection [208, 209]. After PRRs have been activated in cells, IFN-I and IFN-III are secreted and bind, in an autocrine or paracrine manner, to the membrane receptor IFNAR (IFN-α/β receptor chain) to induce JAK-STAT signal transduction driving the transcription of more than hundreds interferon-stimulated genes (ISGs), which encode antiviral proteins [206]. Proteins that are encoded by ISGs play important role in targeting and inhibiting the viral life cycle and also regulate innate immune sensing and induce the production of cytokines, thereby creating an antiviral state. Cytokines and chemokines that are produced following the activation of PRR signals are also essential for determining an effective adaptive immune response [203].

Furthermore, cGAS and STING activity are regulated under normal conditions by post-translational modifications, such as phosphorylation, ubiquitination, SUMOylation, and glutamylation, to prevent sustained innate immune activation [210-213]. The activation of intracellular sensing pathways by endogenous DNA is associated with the pathogenesis of autoimmune and inflammatory disorders [214, 215].

3 Objectives of this work

Porcine organs are of significant importance for xenotransplantation. However, porcine cells contain numerous Porcine Endogenous Retroviruses (PERVs) that can form replication competent particles. The risk of PERV transmission to various human cells is relevant during xenotransplantation, because PERVs are integrated within the genome of all pigs and the ability of PERVs to infect human cells has been shown in vitro.

The first objective of the work is to investigate the restriction activity of SAMHD1 against PERV-A/Cs infection of human cells at the reverse transcription step. For this purpose, replication competent PERV-A/C viruses should be used. Furthermore, selected human immune primary cells should be used as recipient cells, as these are known to be particularly obstructive for HIV-1 and other retroviruses by restriction factors like SAMHD1. The analysis should include an investigation into the relation between the amount of the expressed SAMHD1 and its restriction function for PERV-A/C infection. Another topic should be the analysis of the mode of restriction by SAMHD1. It has to be determined whether it's the presumed depletion of the dNTP pool in the cytoplasm. This could be analyzed by adding exogenous deoxynucleosides to the human primary cells. Moreover, the investigation of the SAMHD1 restriction should be extended to resting and activated human $CD4^+$ T-cells.

Since no experiments had yet been performed with the innate immune response to PERVs infection, the second general aim is to study the IFN response through monitoring induction of IFN stimulated genes (e.g. CXCL/IP-10, IFN-β, and ISG-54) of human immune primary cells infected by PERV-A/Cs. Monocytes, MDMs and MDDCs should be used to determine the induction of an innate immune response against PERV-A/C infection. In case of a response, the additional aim is to identify the signaling pathways, which play a role in the type I IFNs response in human primary cells infected by PERV-A/Cs. To investigate that, cells might be pretreated with different inhibitors including a JAK-inhibitor (AT9283), TBK1 (BX-795), NF-κB (Parthenolide) and at least one TLR7/8 antagonist.

4 Materials and Methods

4.1 Materials

4.1. 1 List of Human cells

Type of cells	Origin	Medium
Hek 293T	Kidney	MDEM
THP1	Acute monocytic leukemia	RPME 1640
Monocyte	PBMC	DC
MDDC	PBMC	DC
MDM	PBMC	DC
CD4+T-Cells	PBMC	RPME-1640

4.1. 2 List of bacterial strain

Bacteria Strain	Genotype	Manufacturer
OneShot® Top10 chemically competent cells	E-coli	Invitrogen
OneShot® Top10 electrical competent cells	E-coli	Invitrogen

4.1. 3 List of plasmids and vector backbones

Constract/ plasmid	Description	Manufacturer
VSV-g	Functional envelope plasmid, containing promoter and env gene of the vesicular stomatitis virus glycoprotein	Addgene
Gag-Pol	Precursor HIV-1 proteins	Addgene
Rev	RNA-transporter	Addgene
VPX	Accessory protein find in HIV-2 and SIV-mac239	
pcDNA	Empty vector	Invitrogen

4.1. 4 List of enzyme

Name	Manufacturer
Proteinase K	Biolab
Benzonase	Sigma
DNase	Ambion
RNase inhibitor	Thermofisher
Reverse transcriptase	Thermofisher
TaqMan hot-start Polymerase	Bioline
Trypsin	Biochrom

4.1. 5 List of Antibodies

Name	Manufacturer
α- SAMHD1 (mouse)	Origene
α- P-SAMHD1 (rabbit)	Cell-Signaling
α- MYC (mouse)	Sigma
α- Tubulin (mouse)	Sigma
α- GAPDH (mouse and rabbit)	Sigma
α- IRDye® 680LT Goat anti-Mouse	Li-Cor
α- IRDye® 800CW Goat anti-Rabbit	Li-Cor
α- P24 HIV-1 (mouse)	Abecam
α- $CD4^+$ T-cells biotin cocktail (human)	Milteny Biotec
α- CD4+ T-cells microbead cocktail (human)	Milteny Biotec
α- CD4- FITC (human)	BD Biosciences Pharmingen

4.1. 6 List of cytokinase activators and inhibitors

Name	Manufacturer
Human GM-CSF	Peprotech
Human IL-4	Peprotech
Human PHA	Sigma
PMA	Sigma
AZT	Sigma

4.1. 7 List of Primers and Probes

Gene detection	Sequencing	Reference
PERV-Gag -fw	TCCAGGGCTCATAATTTGTC	[216]
PERV-Gag -rev	TGGCCATCCAACATCGA	[216]
PERV-Gag -probe	FAMAGAAGGGACCTTGGCAGACTTTCT-BHQ	[216]
pGAPDH -fw	ACATGGCCTCCAAGGAGTAAGA	[58, 217]
pGAPDH-rev	GATCGAGTTGGGGCTGTGACT	[58, 217]
pGAPDH-probe	HEX-CCACCAACCCCAGCAAGAGBHQ1	[58, 217]
IFNB,	(ID): Hs01077958_s1	Thermo scientific
CXCL-10	(ID): Hs00171042_m1	Thermo scientific
ISG-54	(ID): Hs00533665_m1	Thermo scientific
GAPDH	(ID): Hs02786624_g1	Thermo scientific

4.1. 8 List of medium

Name	Composition
DMEM	Medium were supplemented with 10 % fetal calf serum (FCS, Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin.
RPMI-1640	Medium were supplemented with 10 % fetal calf serum (FCS, Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin.
DC-medium	RPME-1640 medium were supplemented with 5 % human serum, 100 U/ml penicillin, 100 µg/ml streptomycin,1mM HEPES and filtration with sterile filter.
LB-medium	10 g/l Bacto Tryptone, 5 g/l Bacto Yeast Extract, 8 g/l NaCl, 1 g/l glucose; pH 7.2; supplemented with 100 µg/ml ampicillin
LB-agar	15 g/l Bacto Agar in LB medium; supplemented with 50 µg/ml ampicillin.
S.O.C-medium	20 g/l Bacto Tryptone, 5 g/l Bacto Yeast Extract, 2.5 mM NaCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose in H2O.

4.1. 9 List of reagents and buffers

Name of Buffer	Composition
Phosphate-buffered saline (PBS)	138 mM NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4
HBS buffer (2x)	280 mM NaCl, 50 mM HEPES, 1.5 mM Na2HPO4; pH 7.07-7.15; sterilized by filtration.
Running buffer (10x)	30,3 g/l Tris, 144,1 g/l Glycin, 10 g/l SDS in 1 l aq. dest
Transfer buffer (10x)	100 ml 10x Tris-Glycin Puffer, 200 ml Methanol in 1 l aq. dest.
RBC lysis buffer	NH4Cl 8.02gm,NaHCO3 0.84gm,EDTA 0.37gm, QS to 100ml with Millipore water
NP 40 lysis buffer	50 mM Hepes, 150 mM KCl, 2 mM EDTA, 0,5 % NP40
Washing buffer	10% 10x PBS and 0.1% tween20 in aq. dest
Blocking buffer	5 % [w/v] milk powder and 0.05% tween20 in PBS.
MACS buffer	0.5%FBS and 2mM EDTA in PBS
Carbonate buffer	8.4 g of Sodium bicarbonate, 10.6 g sodium carbonate in 500ml distilled water.
Phosphat-Citrat buffer (0.15 M, pH 5.0)	8.15 g of Na2HPO4-2H2O, 9.336 g of Citric acid in 800 mL of distilled water.

4.1. 10 List of kits

Kit	Manufacturer
QIAGEN Plasmid Maxi Kit	QIAGEN
RNeasy Mini Kit	QIAGEN
RevertAid H Minus First Strand cDNA Synthesis	Thermo Scientific™

4.1. 11 List of reagents and chemicals

Name	Manufacturer
Ethanol	Roth
DMSO	Sigma
Ethidium bromide	Sigma
Sodium Dodecyl Sulfate	Sigma
Tris-HCl 0,5 M	Sigma
Tris-HCl 1,5 M	Sigma
TEMED	Sigma
Polyethylenimin (PEI)	Polysciences
Tween 20	Sigma
Milchpulver	Sigma
Sulfuric acid H2SO4	Merck
SensiFASTTM Probe No-ROX Master Mix	Bioline
Loading Dye 4x	Novex
PageRuler™ prestained protein DNA Ladder	Thermo Fisher Scientific
GeneRuler™ 1 kb+ DNA Ladder	Thermo Fisher Scientific
Nitrocellulose Membrane	Bio-Rad
Tryban blue	Sigma
APS	Sigma

4.1. 12 List of instruments

Name	Manufacturer
CFX96 Touch™	Bio-Rad
Odyssey	LI-COR
Nanodrop 1000	Thermo Fisher Scientific
Stratagene MX3000P	Agilent Technologies
Centrifuge 5415 R	Eppendorf
Microscope Eclipse TS100	Nikon
Centrifuge 4K15	Sigma

Thermomixer compact	Eppendorf
Vortex Mixer	VWR™international
LUMIstar Omega	BMG LABTECH GmbH
Gel-reader	PHASE
FACS-Calibur	Becton-Dickinson
MJ Mini™Thermalcycler	Bio-Rad
Water bath	Köttermann
ELISA Reader Sunrise	Tecan Group
Centrifuge 5804R	Eppendorf
MACS Multi-Stand	Miltenyi Biotec
HB-500 Minidizer™Hybridization Oven	UVP
GFL 3016	Theoder Karom
POWERPAC$_{HC}$	Bio-Rad
Magnetic stirrer Ikamag	IKA
Balance PR803	METTLER TOLEDO
Pipettes	Eppendorf
Vacusafe compfort	IBS Integra Bioscience
CO2 Incubator	Thermo scientific
Safety Cabinets HERAsafe HSP	Kendro laboratory products
MACS holder	Milteny Biotec

4.1. 13 List of consumables

Name	Manufacturer
Cell culture flasks	TPP
Cell culture plates (6-well, 12-well, 96well)	Thermo Fisher Scientific
Centrifuge tubes (1,5ml, 15 ml, 50 ml)	TPP
Petri dishes	TPP
Multiplate® PCR PlatesTM	Bio-Rad
Glass-pipets (5ml, 10ml, 25ml)	TPP
Pipet tips	Thermo Fisher Scientific
Cell Scrapers	TPP
Minisart single use filter unit.	Sartorius stedim biotech

Rapid filter max	Tpp
Syringes	Braun
Gloves	Microflex NeoTouch
LS column	Milteny Biotec

4.1. 14 List of software

Name	Manufacturer
CFX Maestro	Bio-Rad
Easy Reader (ELISA)	Developed by Dr. S. Norley, RKI
Geneious R11	Bio matters
NanoDrop® ND-1000	Thermo Fisher Scientific
MxPro QPCR Software	Stratagene
MS Office 2010	Microsoft

4.2 Methods

4.2.1 Cell culture

4.2.1.1 Cultivation of HEK-293T cells

“HEK cells” is the short term for human embryonic kidney cells, a cell line derived from human embryonic kidney cells. HEK-293T cells are often used in the development of virus vaccines, chemotherapeutics and for the production of recombinant adenovirus-vectors and are a comparably easy-to-handle cell line. For this work, HEK-293T cells were used for virus production and were grown in 75 cm^2 or 150 cm^2 cell culture flasks at 37°C, 5% CO_2 and 98% humidity.

HEK-293T cells form an adherent monolayer in the cell culture flask and should hence not exceed 90% confluency. If the bottom of the flask is completely covered, cells are not able to grow anymore due to contact inhibition. Therefore, HEK-293T cell were split 3 times per week at a 1:10 ratio. First the old media was aspirated and the cells were washed twice with 10 ml (75 cm^2) or 20 ml (150 cm^2) PBS. It is important that all FCS is removed, because it diminishes the activity of Trypsin, which is used for cell detachment. After washing cells were incubated with 1 ml or 2 ml Trypsin/EDTA, respectively, for three minutes at 37°C. Too long incubation leads to toxic effects of Trypsin. After incubation trypsin activity was stopped by addition of 10 ml or 20 ml media containing FCS, respectively. The cells were singularized and split 1:10. At last, 9 ml or 18 ml, respectively, pre-warmed fresh media was added to the cells. Cells were then again incubated at 37°C, 5% CO2 and 95% humidity. All works were performed under a sterile bench.

4.2.1.2 Preparation of viral stocks

In order to infect the human cells, viral stocks should be prepared. For this purpose uninfected HEK-293 T-cells were seeded in T25 cell culture flasks. When cell confluency reached 50%, medium, PERV A/C was added to the cells. The endogenous PERV A/C was initially isolated from porcine PBMC. Cells were then incubated for three days with the viruses afterwards they were trypsinized and further cultured in larger flasks for three days. After the 10th day post infection cell culture supernatant were harvested five times every 3rd day. Supernatants were centrifuged (2000 x rpm, 5 min) and filtered (0.2 µm), and aliquots of 1 ml were freeze down in -80C°. Titration of supernatants by using real-time PCR and the relative cDNA levels were determined using the ΔΔCT method, normalizing to the CT values of the GAPDH gene.

4.2.1.3 Human primary cells isolation and cultivation

Human peripheral blood mononuclear cells (PBMCs) were purified from healthy blood donors (Deutsches Rotes Kreuz Berlin) by ficoll density gradient centrifugation. In tissue culture hood, blood was emptied into a 50 ml canonical tube, 25 ml for each tube, and then completed with RPMI-1640 free medium to 50 ml. 12 ml Ficoll was added to another 50 ml conical tube and carefully the RPMI/ suspension were overlaid onto the ficoll. After that conical tubes with blood were centrifuged (2200 x rpm without braking) for 25 minutes in table top centrifuge. PBMC appeared as a thin white layer above ficoll and below serum, which were removed by pipetting and placed in a new 50 ml conical tube and completed with PBS to 50 ml. PBMC with PBS were centrifuged (1600 x rpm) for eight minutes, supernatants were removed and added 5 ml RBC lysis buffer (ammonium chloride) to the cells pellet for 10 minutes at room temperature to lysis the rest of red blood cells. Cells were washed by PBS and centrifuged (1200 x rpm) for five minutes, then PBS supernatants were removed and PBMC cells pellet were re-suspended in RPMI-1640 medium. PBMC cells were routinely counted using a Neubauer counting chamber and seeded in different cell culture flasks and plats depending on the type of primary cells, which differentiated from PBMC cells.

To obtain monocyte cells, PBMC cells were seeded at a density of $5x10^6$ cells/ well in 12 well-plates for 90 minutes to allow the adherent of cells. DC-medium and suspension cells were removed, the plate was washed with warm RPMI-1640 free medium two times, and then fresh DC-medium was added. Monocyte cells were adherent with the bottom of the well-plate.

Monocyte derived macrophage (MDM) cells were differentiated from Monocyte after isolated monocyte from PBMC cells by using 50 ng ml^{-1} GM-CSF for six days in 12 well-plat. DC-medium was changed each two days and added 50 ng ml^{-1} GM-CSF in the same time.

To differentiated monocyte cells to Monocyte derived dendritic cells (MDDC), PBMC cells were seeded at a density of $8x10^6$ ml^{-1} in cell culture flask (T-150). After 90 minutes cells suspension with DC-medium were removed and washed with warm RPMI medium two times. Fresh DC-medium was added to the monocyte cells. To differentiated monocyte to MDDC, 50 ng ml^{-1} GM-CSF and 20ng ml^{-1} Interleukin-4 were added to the cells each two days for six days. After that floated MDDC were collected and counted. Monocyte derived dendritic cells were seeded at a density of 10^6 cells/ well in 12 well-plates.

4.2.1.4 $CD4^+$ T-cells isolation

Human $CD4^+$ T-cells were isolated from peripheral blood mononuclear cells by negative selection as described by the manufacturer (Milteny Biotec). Briefly, after PBMCs were isolated from human healthy donors as described in (4.2.1.3) and cells were counted, the cells density were selected depending on the type of experiment. Cells were mixed with MACS buffer, biotin-antibody cocktail for 10 minutes at 4°C, and then microbead cocktail for 10 minutes also at 4°C. LS columns were fixed on the magnetic MACS-holder and the mix cells with antibodies were added into the column. In new tube $CD4^+$ T-cells were collected and after that counted. Resting $CD4^+$ T-cells were seeded at a density of 10^6 cells/ well in 24 well-plates. The 10^7 cells of $CD4^+$ T-cells were plated in small culture flask for activated with 5 ug ml^{-1} phytohemagglutinin (PHA) and 50 IU ml^{-1} Interleukin-2. Activated $CD4^+$ T-cells were counted and seeded at a density of 10^6 cells/ well.

4.2.1.5 THP-1 cells cultivation

THP-1 was derived from the blood of a one-year old boy with acute monocytic leukemia. The THP-1 cell line has high expression of the restriction factor SAMHD1, and to find out the role of SAMHD1 in restricting the endogenous retrovirus, THP1 cells were treated with stable CRISPER/ Cas9 to knock-out SAMHD1. THP-1 cells transduced with a vector encoding for a non-targeting guide RNA served as a control [29]. The cells were differentiated with 30 ng ml^{-1} phorbol 12- myristate 13-acetate (PMA) for 72 h. During this time, cells attach to the bottom of the cell culture plates and develop macrophage-like morphology. THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% FBS.

4.2.1.6 Storage, thawing and freezing of THP-1 and 293T cells

Cells were stored in nitrogen. Cells were thawed quickly in a 37ºC-water bath. Using a floating device, the ampules were kept above the water surface to prevented contamination. The thawing content was slightly shaken until only a very small ice-crumb was left in the vial. The vial was then sprayed with 70% ethanol all over and its surface was wiped with clean tissue in the hood. As soon as the last ice crystals were molten, the suspension was aspirated gently and transferred into a culture flask containing 10 ml pre-warmed growth medium. The cells were then incubated in the CO_2 incubator for 3 hours for adherence. After that the medium was replaced with fresh medium. Aliquots of cells were frozen after 10-14 days of cultivation. They were trypsinized, 8 ml medium was added, and the cells were then

transferred into a 15 ml Falcon tube. Subsequently, the cells were centrifuged (300 xg; RT, 10 min). The cell pellet was then carefully re-suspended in culture medium. 1 ml of the cell suspension was combined with one ml freezing medium, transferred into a cryovial and frozen overnight in a freezing container at -80°C. The next day, the cells were transferred in liquid nitrogen for storage.

4.2.2 Nucleic acid methods and transfections

4.2.2.1 Transformation

Transformation of plasmid DNA in bacteria cells, chemically or via electroporation in competent cells serves the amplification of plasmid DNA by the growth of the bacteria. For this work, only chemical transformation was implemented.

For chemical transformation, competent E. coli Top10 was used. Competent cells were stored at -80°C and thawed before the DNA was added. For re-transformations one ng DNA-sample/ 50µl bacteria were used. The sample was chilled on ice for 30 min. Then, a heat shock of 42°C for 45 second followed. After heat shock, the sample was chilled on ice for another 2min. Then, 200 µl S.O.C. medium was added, and the suspension was incubated for one h, 650 rpm at 37°C. Then, the suspension was either spread on two LB-Amp plates, with 200 and 100µl suspension per plate, respectively. Alternatively, the whole suspension was spread on one plate for reactions with low probability of success. For re-transformations, only 50 µl was spread on one plate. Incubation took place at 37°C overnight.

4.2.2.2 Isolation of plasmid DNA

4.2.2.2.1 Mini-preparation

Small-scale production of plasmid DNA was done in competent E. coli Top10 bacteria. For this purpose, a single bacteria clone was picked from an LB plate and put into LB medium with required antibiotics. After incubation over night at 37°C in an orbital shaker at 200 rpm, two ml of the solution were centrifuged for one minute at 14000 rpm. The supernatant was removed and the pellet was resuspended using 100 µl of solution I. Procedure was done according to the manufacturer´s manual (QIAprep Spin Miniprep Kit" from Qiagen) and the DNA was eluted with 50 µl A.bidest. DNA concentration was measured via NanoDrop 1000 and the plasmids were stored at -20°C for further use.

4.2.2.2.2 Maxi-preparation

For higher amounts of DNA with a higher purity and for cell culture transfection experiments, maxi scale plasmid DNA isolation was performed using Endo-free Plasmid Maxi Kit" from Qiagen. Large-scale production of plasmid DNA was done in competent E. coli Top10 bacteria. A single DNA-colonies were incubated in 5 ml LB-Amp medium at 37°C, 200 rpm for ca. 8 h and subsequently 250 µl of the suspension or further cultivated with 250 ml LB-Amp medium (1:1000) in baffled flasks overnight. It was used also small smear picked from plasmid glycerol stock by sterile swab, inserted into 250 ml LB-Amp medium and further cultivated over night at 37°C in an orbital shaker at 200 rpm for plasmid isolation. Purification was done with help of the "Endo-free Plasmid Maxi Kit" from Qiagen. Procedure was done according to the manufacturer´s manual and the DNA eluted with 200 µl A.bidest. DNA concentration was measured via NanoDrop 1000 and the samples were stored at -20°C for further use.

4.2.2.3 DNA measurement

DNA concentration was measured with help of a spectral photometer NanoDrop ND-1000. A. bidest was used as a blank. The measurements were automatically done at a wavelength of 260 nm and 280 nm.

4.2.2.4 DNA agarose gel electrophoresis

DNA agarose gel electrophoresis is used for quality control and for separation of DNA fragments according to their size and charge. The 0.8% agarose gel was solubilized in 1x TAE buffer and completed with 0.5 µg/ml Ethidium bromide (EtBr). EtBr intercalates with the nucleotides of the DNA fragments and renders them visible under UV light at a wavelength of 302 nm. A 6x loading buffer was added to the DNA fragments and the fragments were hence separated in a gel chamber filled with 1x TAE buffer at a voltage of 80V. In order to determine the size of the DNA fragments, a marker suited for the expected fragment sizes was added (GeneRulerTM 1kb). Subsequently a picture of the gel was taken with a gel documentation system or the DNA fragments were extracted.

4.2.3 Transient transfection and virus normalization

4.2.3.1 PEI transfection

HEK-293T cells were used for PEI transfection to produce HIV-1 virus like particles (VLP). The day before transfection, HEK-293T cells were seeded in 10 cm cell plates with appropriate growth medium (10 ml) and incubated overnight at 37°C, 5% CO_2 and 98% humidity. On the day of transfection, the cells growth should be 70-75% confluent. The ratio of µg PEI to µg DNA should be 1:1 considering transfection efficiency and cytotoxicity. Prior to transfection; all reagents were brought to room temperature. Total plasmids of DNA-VLP were diluted in serum-free DMEM, and the equal amount of PEI was added to the diluted DNA, the total volumes were mixed immediately and incubate at room temperature for 15 minutes. PEI and DNA were added dropwise to the cell culture dish and then incubated at 37°C, 5% CO_2 and 98% humidity. After 6 h of incubation, the medium was again renewed. 48 h after transfection the viruses were filtrated and concentrated by ultracentrifugation.

4.2.3.2 Calcium phosphate transfection

Transfection of HEK-293T cells using calcium phosphate is a cheap and used for large-scale virus production in 10 cm cell plates or 15 cm cell plates. The virus produced by calcium phosphate transfection in 293 T-cells is around two-fold higher in titer than virus produced by lipofectamine transfection. Calcium Phosphate Transfection was used to produce HIV-1 virus like particles (VLP) package with the accessory protein Vpx to degrade SAMHD1. The day before transfection HEK-293T cells were seeded in 10 cm cell plates with appropriate growth medium (10 ml) and incubated overnight at 37°C, 5% CO_2 and 98% humidity. On the day of transfection, the cells growth should be 70-80% confluent. In tissue culture hood, dilute 36 µg VLP-DNA mixes in sterile, double deionized H_2O to the final volume appropriate for the size of dish. Then, 75 µl $CaCl_2$ was added and the mixture was well vortexed. For each sample were added 750 µl of 2X HBS slowly and drop wise while “bubbling” $CaCl_2$/ water/ DNA solution with 2 mL pipet. The reaction was then incubated at room temperature for 30 min. In the meantime, the medium in the cell culture flasks was room temperature for five minutes. After incubation, the DNA mixture was added dropwise to the cell culture dish and then incubated at 37°C, 5% CO_2 and 98% humidity. After 6 h of incubation the medium was again renewed. 48 h after transfection the viruses were filtrated and concentrated by ultracentrifugation. A list of plasmid DNA mixes used for production of pseudotype virus particles can be found in a table (4.1.3).

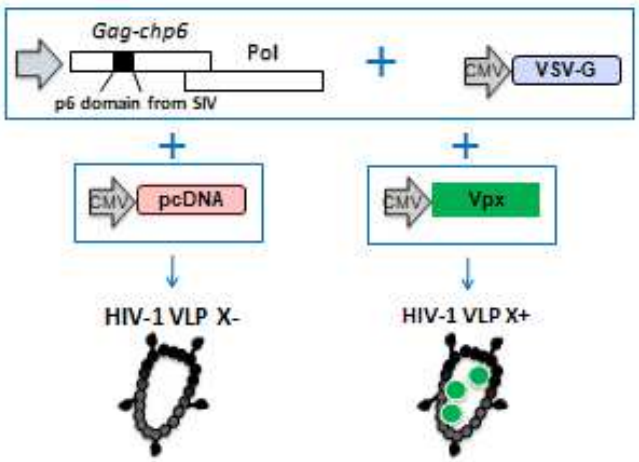


Figure 4. 1 The package of Vpx into HIV-1 VLP (VLP system is based on Sunseri et al.).

4.2.3.3 Purification of virus production by HEK-293T cells after transfection using ultracentrifugation

Purification of transfect cell culture supernatants was carried out via ultracentrifugation. First, the transfect cell culture supernatants were collected, filtrated and transferred to 50 ml falcon tubes. 5 ml of 20% sucrose was added to the centrifuge tubes (Beckman Coulter) and with lowest aid speed of pipet the virus containing supernatant were carefully overlaid onto sucrose. The loaded centrifuge tubes were then inserted into the rotor and calibrated to two decimal points. Work was done entirely under the sterile bench. The samples were then centrifuged at 32'000 rpm, 4°C for 3 h. After centrifugation, the supernatant was discarded, and the virus pellet dissolved in 3 ml (for 30 ml transfection volume) MDEM. The solution was then transferred to a fresh Eppendorf tube and 10 µl was separated for ELISA to normalization. ELISA samples were stored at -20°C, and the vital viruses were stored at -80°C for later infection. Rotor tubes were sterilized in disinfection reagent for 20 min and then washed in 70% EtOH and air-dried afterwards.

4.2.3.4 Enzyme-linked immunosorbent assay (ELISA)

In order to normalize virus particles before infection, an ELISA (Enzyme-linked immunosorbent assay) was performed. Therefore, a 96-well plate was covered with capture antibody and incubated at 4°C overnight. On the next day, the supernatant was discarded and the plate rinsed three times with 0.05% Tween. Then, the plate was blocked with PM and incubated at 37°C for 45-60 min. In the meantime, virus samples were inactivated with 0.2% Tween for 5-10 min at room temperature. After incubation, the PM was discarded and the plate firmly tapped but not rinsed. Then, the samples were applied and incubated at 37°C for

60 min. After incubation, the plate was rinsed three times with 0.05% Tween. Subsequently, the primary antibody was applied and the plate was incubated 60 min at 37°C. Afterwards, the plate was rinsed three times with 0.05% Tween and the secondary antibody was applied and the plate was again incubated at 37°C for 60 min. After this last incubation step, the plate was rinsed three times with 0.05% Tween. In the next step, the stain was added and the plate incubated for 10-15 min at room temperature until staining became clearly visible. The reaction was finally stopped with H_2SO_4. The plate was then read in an ELISA reader system. All reagents used and their volumes are listed in table (4.1.11).

4.2.4 Infection Assay

4.2.4.1 PERV infection of MDDC

Monocyte derived dendritic cells were isolated and differentiated from PBMCs. New fresh DC medium was added before infection procedure. MDDC were treated in two wells in each 12 well-plate by 10 µM azidothymidine (AZT) one hour before PERV and HIV-1 VLP were added to inhibit the virus revere transcription, therefore AZT served as control. Three hours before PERV was added, Vpx-containing or controls VLP were added to delivery Vpx into the MDDC cells in order to degrade SAMHD1. MDDC were spin-infected with $10^{5.7}$ $TCID_{50}$ ml^{-1} of PERV-A/C virus treated for 10 min with 25 U Benzonase ml^{-1} at a room temperature to remove contaminating plasmid DNA. After 40 hours, medium was removed, and cells were washed two times with PBS. Cells were harvested with 500 µl PBS, collected and transferred into two Eppendorf tubes. Cells were centrifuged and stored the pellet at -20°C. Cells in one tube were quantified by qPCR to detection PERV-A/C cDNA. The other cell pellets were used for western blot to detect SAMHD1.

In another experiment, MDDCs were treated with one µM deoxynucleotides (dN) (dA, dG, dC, dT) at a concentration of one mM for 3 hours before infection with PERV-A/C. The exogenous deoxynucleotides increased the reverse transcription products. After that, cells were coinfected with HIV-1 VLP, then infected with PERV-A/C virus treated for 10 min with 25 U Benzonase ml^{-1}. Six hours later, the deoxynucleotides and viruses were washed out and removed with medium and fresh DC-medium was added to the cells. After 40 hours medium was removed, cells were washed with PBS, harvested, collected, and transferred into two Eppendorf tubes. MDDCs were centrifuged and the pellet stored at -20C°. Cells in one tube were quantified by qPCR to detection PERV-A/C cDNA. The other tube cells were used for western blot to detect SAMHD1.

4.2.4.2 PERV infection of monocyte and MDM cells

For differentiated PBMCs to monocytes, cells were plated in a 12-well culture plate at a density of $5x10^6$ cells/ well, after 90 minutes cells were washed two times with warm free RPMI-medium. MDMs were differentiated from monocytes by adding 50 ng ml^{-1} GM-CSF for six days. Monocytes and MDMs were treated with AZT and VLP before infection with $10^{5.7}$ $TCID_{50}$ ml^{-1} of PERV-A/C. After 40 hours, medium was removed, and cells were washed two times with PBS. Cells were harvested with 500 µl PBS, collected and transferred into two Eppendorf tubes. Cells were centrifuged and stored the pellet at -20°C. Cells in one tube were quantified by qPCR to detection PERV-A/C cDNA. The other cell pellets were used for western blot to detect SAMHD1.

4.2.4.3 PERV infection of resting and activated $CD4^+$ T-cells

$CD4^+$ T-cells were isolated from PBMCs by magnetic negative selection. Resting and activated $CD4^+$ T-cells were seeded in the 24-well culture plates at a density of 10^6 cells/ well. Cells were treated with 1uM deoxynucleotides (dN) (dA, dG, dC, dT) at a concentration of one mM for 3 hours before infection with PERV-A/C. All of these cells were spin-infected with PERV A/C treated for 10 min with 25 U benzonase ml^{-1} at a room temperature in the presence or absence of 10 µM azidothymidine (AZT). Six hours later, the deoxynucleotides and PERV-A/C virus were washed out and removed with medium and fresh RPMI medium was added to the cells. After 40 hours medium was removed, cells were washed with PBS, harvested, collected, and transferred into two Eppendorf tubes. Cells were centrifuged and stored the pellet at -20°C. Cells in one tube were quantified by qPCR to detection PERV-A/C cDNA. The other cell pellets were used for western blot to detect SAMHD1.

4.2.4.4 PERV infection of THP-1 cells

THP-1 cells were seeded at a density of $1x10^6$ cells/ well and differentiated with PMA for 72 h into macrophage. PMA was washed out and added fresh RPMI medium into the cells. THP-1 cells were infected with PERV-A/C treated for 10 min with 25 U benzonase ml^{-1} at a room temperature. The PERV cDNA were quantified by RT-PCR after 40 h post-infection.

4.2.5 DNA extraction

In order to prepare DNA samples after infection, the DNA isolation was based on proteinase K lysis. The cells pellets were frozen at -80°C and thawed at 60°C three times. Proteinase K

(dil. 1:100 PBS) was added to the pellet, then pellet was good mixed with proteinase K and incubated for 2 hours at 56°C. The proteinase K activity was inactivated by heating at 98°C for 15 min. Cells were used for quantified PERV cDNA by RT-PCR.

4.2.6 Quantitative RT-PCR

Viral cDNA and cellular DNA were analyzed for the porcine endogenous retrovirus PERV Gag as well as the housekeeping gene GAPDH. For quantification of PERV pro-viral DNA the primers Gag-for and Gag-rev located in the Gag gene and a specific PERV-Gag probe were used in a duplex real-time PCR. The reference gene GAPDH was amplified with the primers GAPDH-for and GAPDH-rev and quantified using an hGAPDH-probe. The RT-PCR primers, probes and the cycling conditions for PERV sequences consisted of as in the table (4.15).

Master mix sensiFAST	10ul
Perv Gag-primer For	0,5ul
PERV Gag-primer Rev	0,5ul
PERV Gag-probe	0,5ul
GAPDH For	0,5ul
GAPDH Rev	0,5ul
GAPDH probe	0,5ul
H2O	2ul
DNA template	5ul

Time	Temperature	Cycles
5:20 min	95°C	1
30 sec	95°C	45
30 sec	56°C	45
72 sec	72°C	45

Table 4.2.1 Reactions mix and cycle conditions for PERV quantitative PCRs.

Quantitative RT- PCR was done using the snsiFAST TM probe No-ROX qRT-PCR Kit see the table of PCR reaction. Levels of cDNA were measured using the ΔΔCT method, normalizing to the CT values of the GAPDH gene. All PCR reactions were carried out using a Real-Time PCR System Stratagene MX3005P and BioRad CFX96.

4.2.7 Fluorescence activated cell sorting (FACS)

Fluorescence activated cell sorting (FACS) was used to measure the purity of isolation $CD4^+$ T-cells from PBMC. After isolated $CD4^+$ T-cells were washed with PBS and counted. The

cells were aliquoted in Eppendorf tubes at a density of $5x10^5$ cells. Cells were incubated with the FITC anti-human CD^4 for 2 h at 4°C. Cells were washed, and added goat anti-mouse IgG-FITC secondary antibody into the cells for 1h at 4°C. Cells were fixed with 1% paraformaldehyde for 1 h at 4°C. Fixation was removed and cells were washed and resuspended in FACS buffer and stored at 4°C. Measurements were performed using a FACS calibur (BD Biosciences) and 10^4 cells of each sample were measured.

4.2.8 RNA isolation

RNA isolation from human primary cells was performed using the RNeasy mini-kit (Qiagen) as described by the manual of manufacturer to measure the relative mRNA interferon response in primary cells after PERV-A/C infection. Depending on the cell number, RNA was eluted in 30-50 µl nuclease-free H2O and concentration was determined using a NanoDrop1000 spectrometer, and stored at -80°C.

4.2.9 cDNA synthesis and qPCR

Reverse transcription of isolated cellular RNA was performed with a RevertAid H minus first strand cDNA synthesis kit. DNase was used to digest the contaminated DNA with RNA, for that RNA was incubated with DNase at 37°C for 30 min. The DNase reaction was stopped by DNase inactivation reagent (dil.1:10) (Turbo DNA free kit), incubated for 5 min at RT. The RNA reverse transcription reaction was started by primer annealing, 1 µl oligo dt primer and 3,2 µl H2O were added to the reaction, then incubated at 65°C for 10 min then transferred the reaction tube on the ice to cold. After that, 4 µl 5x reaction buffer, 1 µl RNase inhibitor, 2 µl dNTP-mix and 1 µl reverse transcriptase were added to the tube. Reaction tubes were incubated at 42°C for 2 h, then heat inactivated at 70°C for 5 min. Quantitative PCR was performed to find out the interferon induction with PERV-A/C infection normalizing to the housekeeping gene GAPDH. For quantification of IFN-beta, ISG54 and CXCL-10 (IP-10), their primers and probes were used in a duplex real-time PCR. The volume qPCR reaction and thermal cycle were as in the table (4.16).

Levels of mRNA were measured using the ΔΔCT method, normalizing to the CT values of the GAPDH gene. All PCR reactions were carried out using a Real-Time PCR System Stratagene MX3005P and BioRad CFX96.

Master mix sensiFAST	7,5ul
Mix Primer and probe: IFN-beta CXCL-10 ISG-54 GAPDH	 0,75ul 0,75ul 0,75ul 0,75ul
H2O	4,75ul
DNA	2ul

Time	Temperature	Cycles
15 min	95°C	1
10 sec	95°C	39
20 sec	60°C	39

Table 4.2.2 Reactions mix and cycle conditions for IFN-response quantitative PCRs.

4.2.10 Protein chemistry

4.2.10.1 Measurement of protein concentration

Protein quantification was performed using the Pierce BCA™ protein Assay kit. The method is based on the reduction of Cu^{2+} ion to Cu^{1+} by proteins in an alkaline medium (The Biuret reaction). The amount of the reduced cuprous ion is proportional to the amount of protein present in the solution. The chelation of bicinhoninic acid (BCA) with Cu^{2+} ions produces a purples-colored complex exhibiting high absorbance at 562 nm. The working reagent was prepared by mixing BCA reagent A with BCA reagent B (50/1). As standard, albumin (BSA) was used with concentrations of 0, 25, 125, 250, 500,750,1000,1500 and 2000 µg/ml. afterwards 25 µl of each sample to measure as well as the standards were added into a 96-well plate and mixed with 200 µl working reagent each. After 30 min incubation at 37°C, absorbance was measured on an ELISA Reader at 560 nm with reference 562 nm. Protein concentrations were calculated using the BSA-standard curve.

4.2.10.2 Preparation of cell lysate

To prepare samples for western blotting, whole cells were lysed to release the proteins of interest using NP-40 lysis buffer and Proteinase inhibitor. Cells were washed 3 times with cold PBS and centrifuged at 2000 rpm for 5 minutes. Lysis followed by adding lysis buffer into the cells and incubating on ice for 10 minutes. Lysates were centrifuged at maximum speed for 10 minutes at 4°C to pellet the cells debris and protein containing supernatant was recovered and frozen down at -20°C till used.

4.2.10.3 Western blot

To analyze the amount of SAMHD1 proteins in the cells, immune blot was performed. Infected human cells pellets were analyzed after 40 h after infection using NP-40 lysis buffer. Twenty micrograms of each protein lysate were boiled in Laemmli buffer, loaded on 12% polyacrylamide gels, subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electro-blotted onto nitrocellulose membranes. After protein transfer, membranes were blocked for 1 h at room temperature in a 5% solution of milk powder (Roth) in 1× PBS-T with 0.05% Tween 20, washed in 3× PBS-T and incubated 1 h at room temperature or overnight with an anti-SAMHD1 mouse MAb and the loading control anti-GAPDH or tubulin at 4°C, after that washed in 3× PBS-T. The secondary antibody IRDye 800 or 680 goat anti-mouse or anti-rabbit were added to the membranes for 1h at room temperature, and then membranes were washed in 3× PBS-T. The proteins were visualized by an Odyssey imaging system (Li-Cor).

5 Result

Since human SAMHD1 limits the replication of a variety of endo- and exogenous retroviruses, we reasoned that the transmission of PERV-A/C from pig organs to human recipients during xenotransplantation may be restricted by SAMHD1. To assess the role of human SAMHD1 in restricting the PERV-A/C virus, we used an assay in which human SAMHD1 expressing cells (primary cells: monocytes, MDMs, MDDCs, $CD4^+$ T-cells and the THP-1 cells line) were incubated with Vpx-containing or Vpx-lacking VLP and then infected with PERV-A/C. In this work, it was also focused on the IFN-I response (IFN- β, ISG-54 and CXCL-10/ IP-10) after stimulation of human primary cells through PERV-A/C infection.

5.1 The role of human SAMHD1 in restricting PERV-A/C in human primary cells and THP-1 macrophages

5.1.1 SAMHD1 restricts PERV-A/C RT-products in monocyte derived dendritic cells (MDDCs)

The antiviral activity of SAMHD1 was found to be regulated by cell cycle-dependent phosphorylation, enabling SAMHD1 to restrict retrovirus infection only in non-dividing cells [218]. To confirm the SAMHD1 restriction for PERV-A/C in non-cycling cells, monocytes were isolated from healthy donor PBMCs (isolated from buffy coats derived from anonymous donors acquired from Deutsches Rotes Kreuz Berlin) and were differentiated to MDDCs using 50 ng ml^{-1} GM-CSF and 20 ng ml^{-1} Interleukin-4. After six days of differentiation the MDDCs were infected with $10^{5.7}$ $TCID_{50}$ ml^{-1} of PERV-A/C. PERV-A/Cs were initially isolated from porcine PBMC, grown on 293T cells and were treated with benzonase before infection. The cells were coinfected in the presence of 100-ng p24 HIV-1 derived virus-like particles (VLP) modified in P6 in order to package or not to package the Vpx protein of SIVmac239. To control the measurement of reverse transcripts, the RT-inhibitor AZT (10 µM) was added in parallel. PERV reverse transcripts were quantified by qPCR 12 h, 24 h and 40 h post infection to find out the suitable time to determine the amount of PERV-A/C cDNA product. The qPCRs have been performed using the primers and probes of PERV Gag, in parallel with the primers and probes of the housekeeping gene GAPDH. Relative cDNA levels were determined using the ΔΔCT method, normalizing to the CT values of the GAPDH gene. The results show that addition of Vpx alleviated reverse transcription in MDDC and the PERV-A/C cDNA products were higher after 40 h post infection in comparison to 12 and 24 h (Fig.5.1). Therefore, 40 h post infection was selected as a time to measure the PERV-A/C

reverse transcription product. As expected, AZT lowered the amount of RT products in the presence of Vpx substantially, confirming the specificity of the qPCR for PERV transcripts.

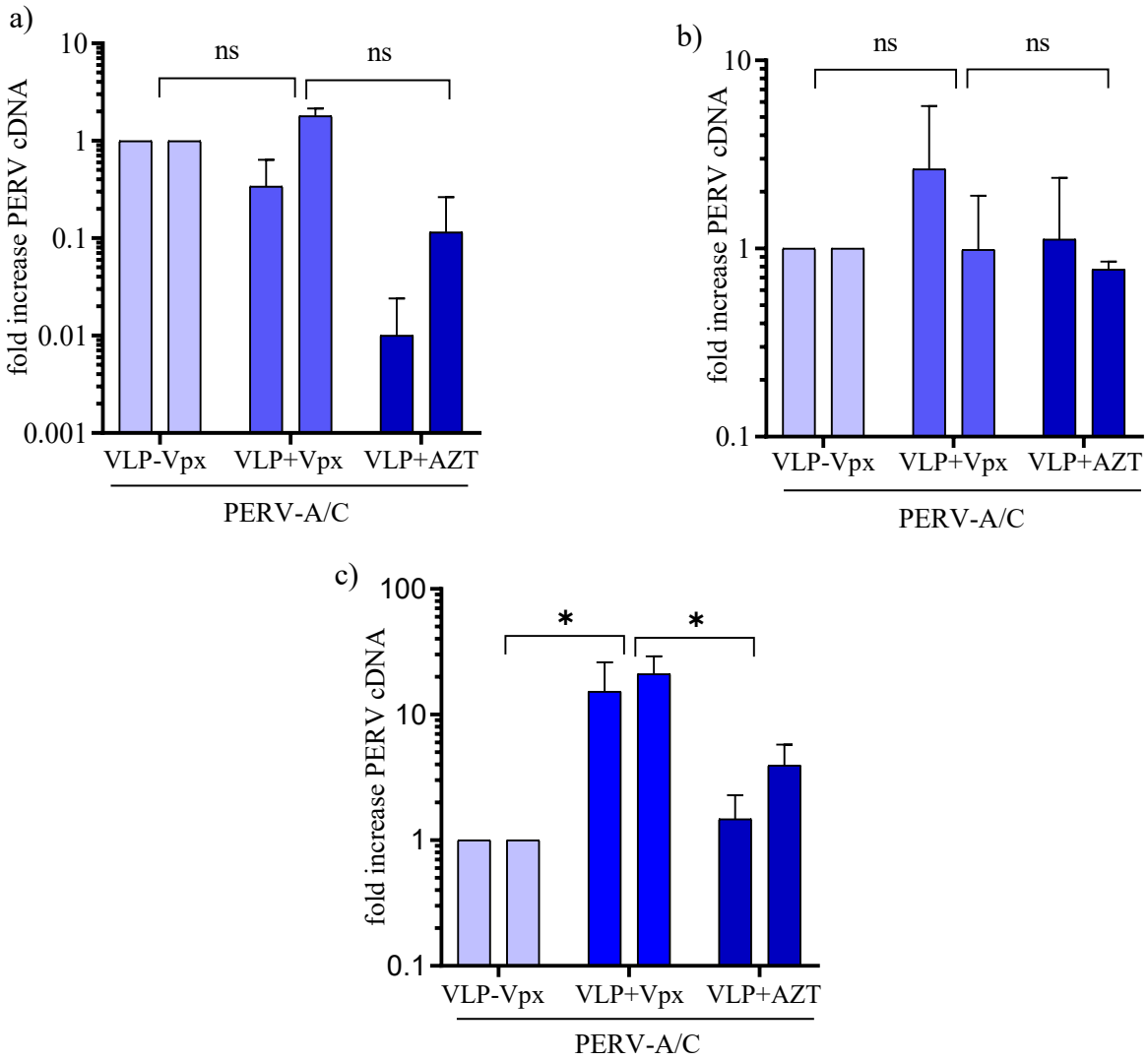


Figure 5. 1 SAMHD1 restricts PERV A/C infection in MDDCs 40 h post-infection. MDDCs derived from two healthy donors were infected with PERV A/C in the presence of Vpx-containing or lacking VLPs. Addition of the reverse transcriptase inhibitor AZT served as control. PERV A/C reverse transcripts were quantified relative to GAPDH 10 h (Fig.5.1a), 20 h (Fig.5.1b) and 40 h (Fig.5.1c) post infection by qPCR. The reverse transcripts were quantified relative to GAPDH using the ΔΔCT method for PERV A/C. The amounts of RT-products quantified in cells infected in the absence of Vpx were set to 1. Mean values and SEM are shown.

To confirm the block of PERV reverse transcription in MDDCs by SAMHD1, the experiment was repeated on six additional donors. After differentiation, MDDCs were pretreated with AZT and HIV-1 VLP containing or lacking the Vpx protein, and then cells were infected with $10^{5.7}$ $TCID_{50}$ of PERV-A/C, treated with benzonase. AZT treated and heat-inactivated PERV-A/C samples were used as a negative control. The qPCRs have been performed using the

primers and probes of PERV Gag, in parallel with the primers and probes of GAPDH. PERV-A/C reverse transcripts were quantified by qPCR 40 h post infection. Relative cDNA levels were determined using the ΔΔCT method, normalizing to the CT values of the GAPDH gene.

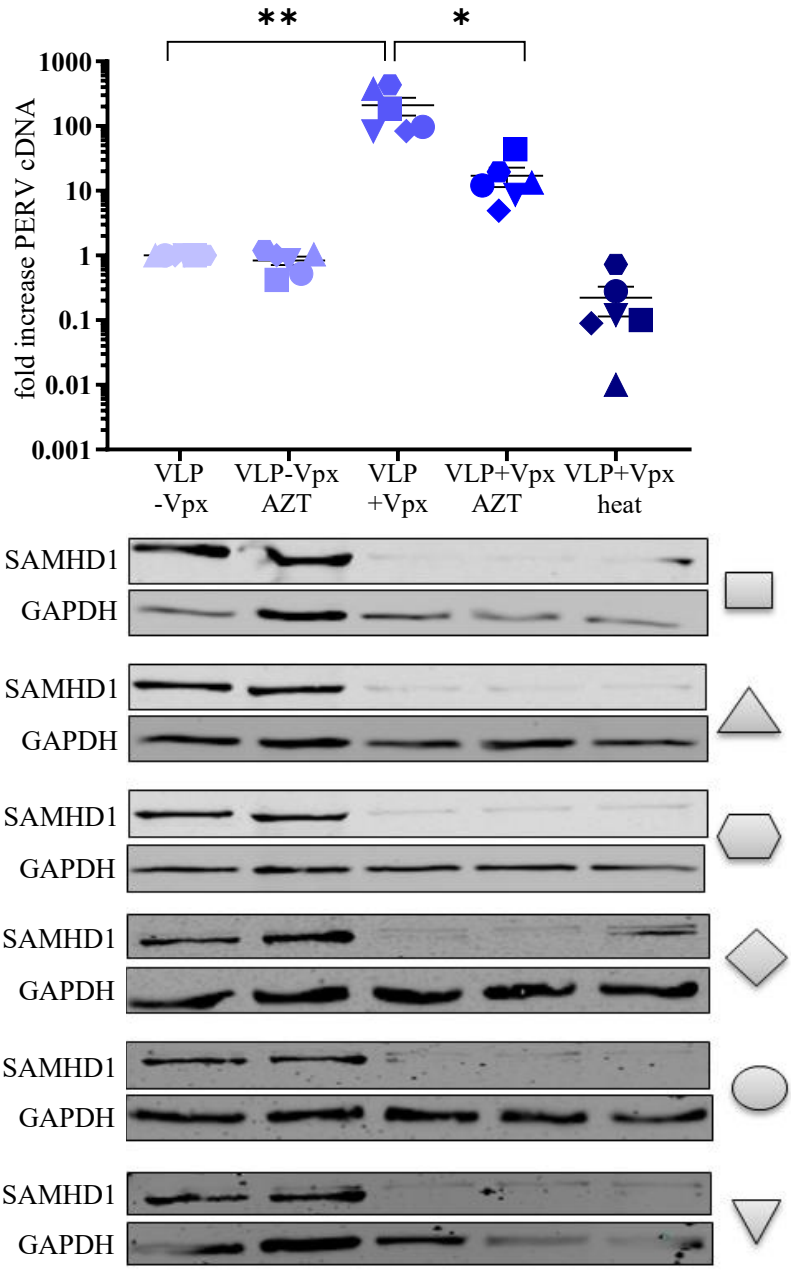


Figure 5. 2 SAMHD1 restricts PERV A/C cDNA production in MDDCs. a) MDDC derived from six healthy donors were infected with PERV A/C in presence of VPX-containing or lacking VLP. Addition of the reverse transcriptase inhibitor AZT or heat inactivation of the virus served as controls. PERV A/C reverse transcripts were quantified relative to GAPDH 40 h post infection by qPCR. The reverse transcripts were quantified relative to GAPDH using the ΔΔCT method for PERV A/C. The amounts of RT-products quantified in cells infected in absence of Vpx were set to 1. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test. b) Immunoblot analysis for six donors to detect

SAMHD1 in MDDCs after treated with VLP packaging Vpx or lacking Vpx and infected with PERV A/C. The cell lysates were collected 40 h post infection, GAPDH served as a loading control. The proteins were visualized by an Odyssey imaging system.

Quantification of PERV-A/Cs cDNA in MDDCs revealed that Vpx lead to about 100 fold more cDNA (Fig.5.2). The PERV cDNA levels in MDDCs pretreated with VLPs containing Vpx and infected with heat inactivated virus were about 200 fold lower than in the same cells infected with regular PERV. AZT lowered the amount of RT products in the presence of Vpx substantially indicating the specificity of the cDNA analysis (Fig.5.2). Immunoblot analyses for six donors using an anti-SAMHD1 MAb were performed to confirm the Vpx-mediated degradation of SAMHD1 in the infected MDDCs with all six donors (Fig. 5.2. lower panel). As secondary antibodies IRDye 800 anti-mouse was used. The proteins were visualized by an Odyssey imaging system. The results show that Vpx caused substantial degradation of SAMHD1 in all six donors compared to control no-Vpx samples. GAPDH served as a loading control.

5.1.2 The restriction of PERV A/C cDNA depends on the amount of SAMHD1

Exposure of differentiated MDDCs to VLP-Vpx relieves restriction to PERV-A/C RT-products (Fig.5.2.a). To investigate if this restriction is based on the expression of SAMHD1, MDDCs of two donors were treated with increasing amounts of VLPs containing Vpx (3.3 ng, 10 ng, 30 ng, and 90 ng) or cells were treated with control VLP with no Vpx. AZT was added to inhibit the reverse transcription. The qPCR results show that increasing amounts of VLPs delivering Vpx lead to increased PERV reverse transcription (Fig.5.3). SAMHD1 degradation in the immune blot of two donors correlated with the amounts of VLP-Vpx added (Fig.5.3, lower panel) and lead to an increase in PERV cDNA products in MDDCs. AZT decreased the amount of PERV-A/C reverse transcription in the present of 90 ng Vpx and in the absence of SAMHD1 (Fig.5.3).

5.1.3 The addition of exogenous deoxynucleosides (dN) to MDDCs alleviated the SAMHD1 - mediated restriction for PERV-A/C

The role of SAMHD1 in regulating the dNTP pool in primary MDDCs was tested. To determine if PERV-A/C is restricted by the low abundance of dNTPs in MDDC, dNTPs were added to the cells. In this case providing dNTP to the MDDCs should prevent the SAMHD1 restrictions to PERV-A/C infection. To increase the level of intracellular dNTP, MDDCs were

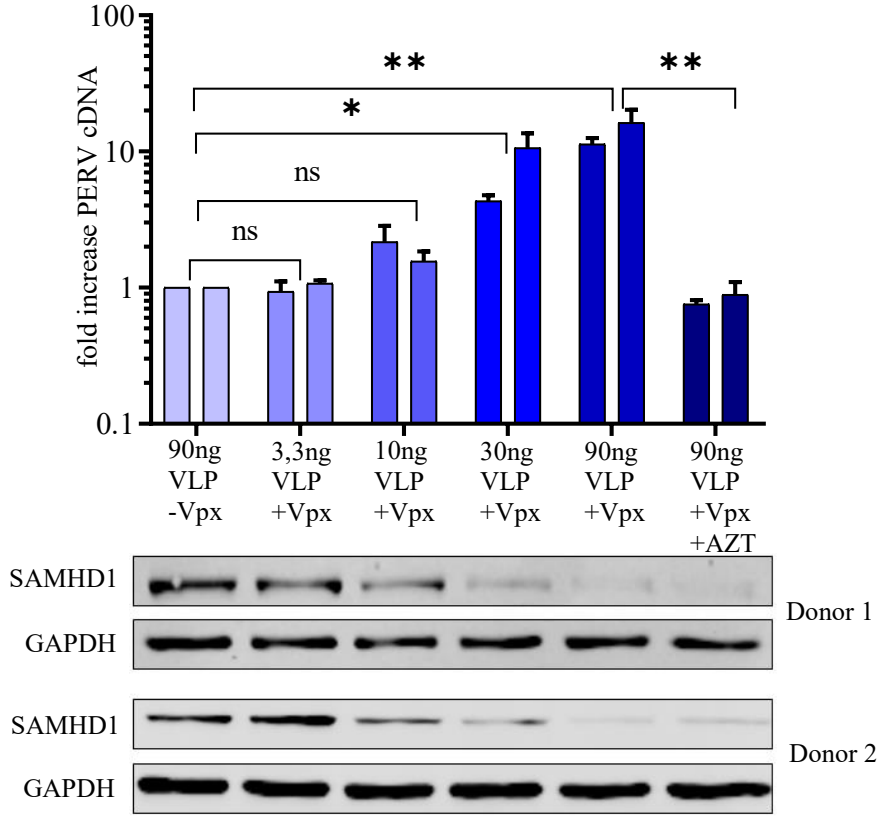


Figure 5. 3 The restriction of PERV-A/C RT-product depends on SAMHD1 expression. MDDCs of two donors were treated with increasing amount of VLP-Vpx or control VLP with no Vpx 3 h before PERV-A/C was added. AZT served as control. PERV A/C reverse transcripts were quantified relative to GAPDH 40 h post infection by qPCR, using the ΔΔCT method. The amounts of RT-products quantified in cells infected in absence of Vpx were set to 1. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test. Immunoblot analysis for two donors to detect the amount of SAMHD1 in MDDCs after treatment with increasing amount of VLP+VPX or lacking Vpx and infected with PERV A/C, GAPDH served as a loading control. The proteins were visualized by an Odyssey imaging system.

treated with exogenous deoxynucleosides (dN) prior to infection with PERV-A/C. These are taken-up and directly converted to dNTP through the salvage pathway of dNTP synthesis, increasing the intracellular dNTP pool. MDDCs of three healthy donors were purified from the peripheral blood mononuclear cells (PBMCs), after that cells were pre-treated with 1 mM dN for 30 min before infection with PERV-A/C and co-infection with VLP+Vpx or empty VLP.

The results show a similar significant increase of PERV reverse transcripts in MDDC treated with 1 mM dN compared to addition of VLP+Vpx (Fig.5.4). A combination of dN supplementation and VLP+Vpx treatment results in a further significant enhancement of

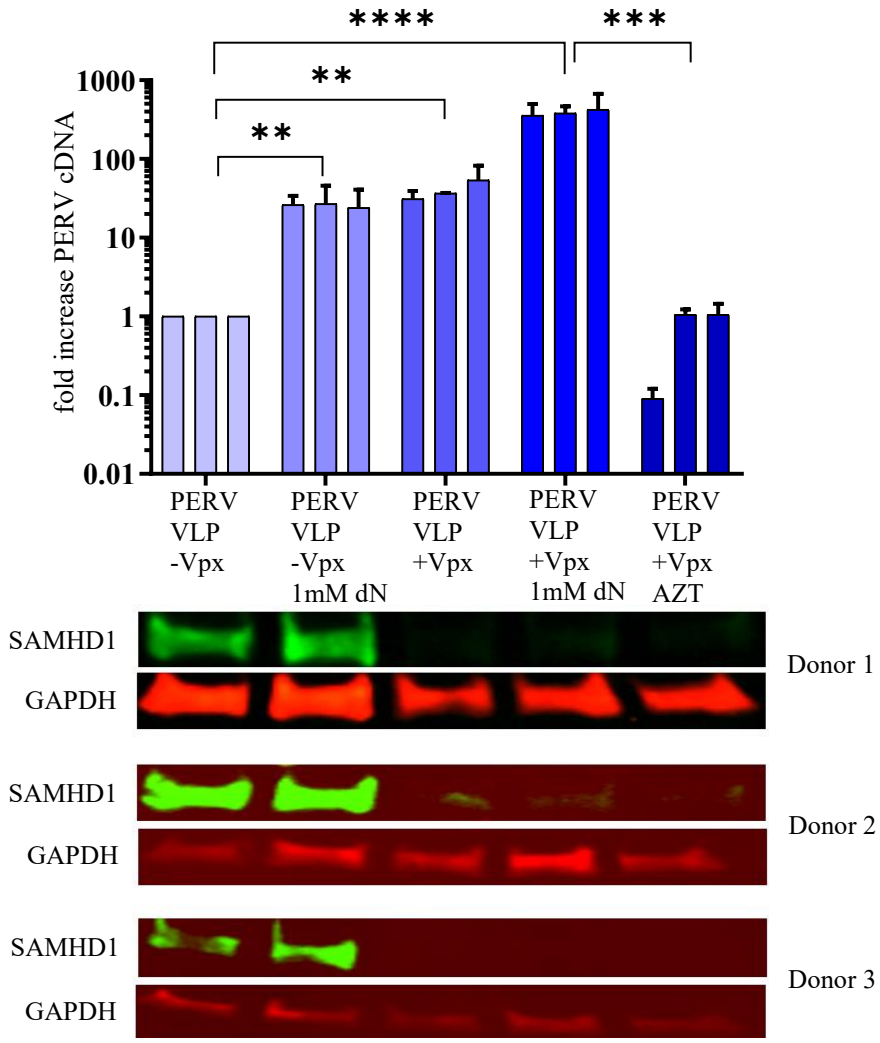


Figure 5. 4 The addition of exogenous dN to the MDDCs alleviated the SAMHD1 restriction. MDDCs were isolated from PBMCs of three healthy donors. Cells were treated with 1 mM dN and control VLP or Vpx-containing VLP for 30 min before PERV infection. AZT served as control. PERV A/C reverse transcripts were quantified relative to GAPDH 40 h post infection by qPCR, using the ΔΔCT method. The amounts of RT-products quantified in cells infected in absence of Vpx were set to 1. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test. Immunoblot analysis for three donors to detect the amount of SAMHD1 in MDDCs after treated VLP+VPX or control VLP and infected with PERV A/C, GAPDH served as a loading control. The proteins were visualized by an Odyssey imaging system.

PERV-A/C reverses transcription. The reverse transcription inhibitor, AZT, prevented the PERV-A/C cDNA production in the absent of SAMHD1. In the immunoblot analysis of three donors, the results demonstrate the degradation of SAMHD1 by the addition of Vpx compared with control VLP and the loading control GAPDH (Fig.5.4 lower panel).

The results demonstrate that PERV inhibition in human MDDC can be overcome by increasing the dNTP level. This further indicates that the restriction at physiological levels of SANHD1 is mediated by SAMHD1.

5.1.4 SAMHD1 restricts PERV-A/C replication in monocytes

To test if PERVs reverse transcription is restricted by SAMHD1 in other myeloid cell types, primary monocytes were infected from six human healthy donors. Similar to the previous experiments, the cells were isolated from PBMCs and infected with normalized PERV-A/C supernatants in presence of VLP containing or lacking Vpx to understand the role of SAMHD1. Heat-inactivated PERV-A/C served as a negative control for cDNA quantification in the real time PCR. The results show that SAMHD1 potently restricts PERV-RT in monocytes and the addition of Vpx to the cells leads to SAMHD1 degradation and to a profound increase of RT products (Fig.5.5). One of the factors contributing to the apparent donor-dependent variability is presumably the efficiency of SAMHD1 downregulation by Vpx+VLPs. The result of the immune blot confirms that SAMHD1 is depleted by Vpx in monocytes (Fig.5.5 lower panel).

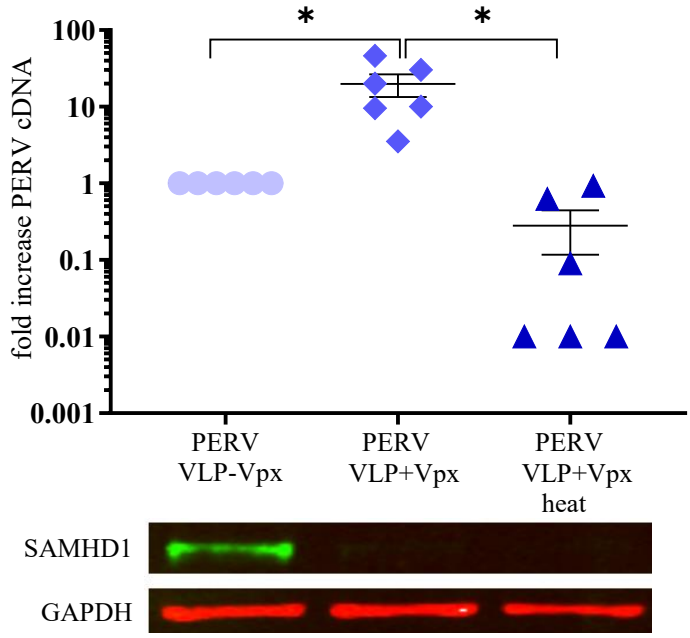


Figure 5. 5 PERV-A/C infection was restricted by SAMHD1 in monocytes. Monocytes were isolated from six healthy donors. Cells were infected with PERV-A/C for 40 h. Heat inactivated PERV served as a control. PERV cDNA was quantified by qPCR and normalizing to the CT value of GAPDH using the ΔΔCT method. The amounts of RT-products quantified in cells infected in absence of Vpx were set to 1. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test. Immunoblot analysis represents one donor to detect the amount of SAMHD1 in MDDCs after

treatment with VLP+Vpx or control VLPs without Vpx and subsequent infection with PERV A/C. GAPDH served as a loading control.

5.1.5 PERV-A/C reverse transcription is restricted in MDM by human SAMHD1

Monocyte derived macrophages from six donors were generated by cultivating freshly isolated monocytes in 50 ng ml^{-1} GM-CSF for six days and changing medium to refresh with cytokine every other day. For monitoring SAMHD1 restriction, MDM were infected with normalized PERV-A/C after cells pre-treated with VLP containing or lacking Vpx. AZT and heat- inactivated PERV was used as a negative control. The delivery of Vpx to the MDM cells by VLP to degrade SAMHD1 resulted in an around 20 to 100 fold increase of PERV cDNA compared to the control VLP (Fig. 5.6). In MDM cells, which express SAMHD1, PERV-A/C is nevertheless reverse transcribed to some extend since AZT treatment resulted in even lower PERV-cDNA levels. That means, SAMHD1 might be does apparently not completely restrict PERV-A/C in MDM cells. AZT treatment or infection with heat- inactivated virus results in a reduced amount of PERV-A/C reverse transcription in the absence of SAMHD1. The immune blot of one donor show the knockdown of SAMHD1 by adding the accessory protein Vpx compared to the control (no Vpx) (Fig. 5.6, lower panel).

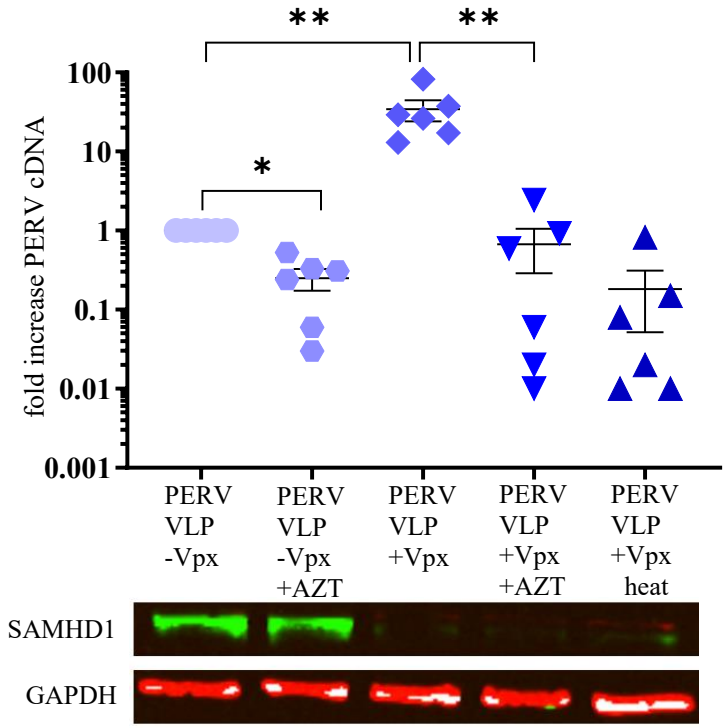


Figure 5. 6 SAMHD1 restricts the PERV-A/C cDNA in monocyte derived macrophage (MDM). Cells from six healthy donors were differentiated from monocyte to MDM by using the cytokine GM-CSF. Cells were infected with benzonase treated PERV A/C. PERV reverse transcripts were

quantified by qPCR relative to GAPDH 40 h post infection, using the ΔΔCT method to calculate the fold difference. The amounts of RT-products quantified in cells infected in absence of Vpx were set to one. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test. Immunoblot analysis represents one donor to detect the amount of SAMHD1 in MDM after treated with VLP and infected with PERV A/C. GAPDH served as a loading control.

5.1.6 The antiviral activity of SAMHD1 restricts the PERV-A/C RT-product in THP-1 cells

THP-1 cells were used with a stable CRISPR/Cas9 knock-out of SAMHD1 to verify the restriction function of SAMHD1 for PERV-A/C. The cells were differentiated with 30 ng ml-[1] phorbol 12- myristate 13-acetate (PMA) for 72 h prior to infection. An immunoblot analysis proves the successful knock-out of SAMHD1 in the two clonal cell lines transduced with a SAMHD1-specific guide RNA and Cas9 (Fig.5.7). THP-1 cells were infected with normalized PERV-A/C supernatants and PERV-A/C cDNA measured 40 h post infection, similar to the previous experiments. Heat-inactivated virus served as a control. The results show up to 10-fold more PERV-A/C cDNA in two clonal cells lacking SAMHD1 compared to the control cells (Fig.5.7). The results confirm the antiviral activity of SAMHD1 in restricting PERV-A/C in THP-1 macrophages.

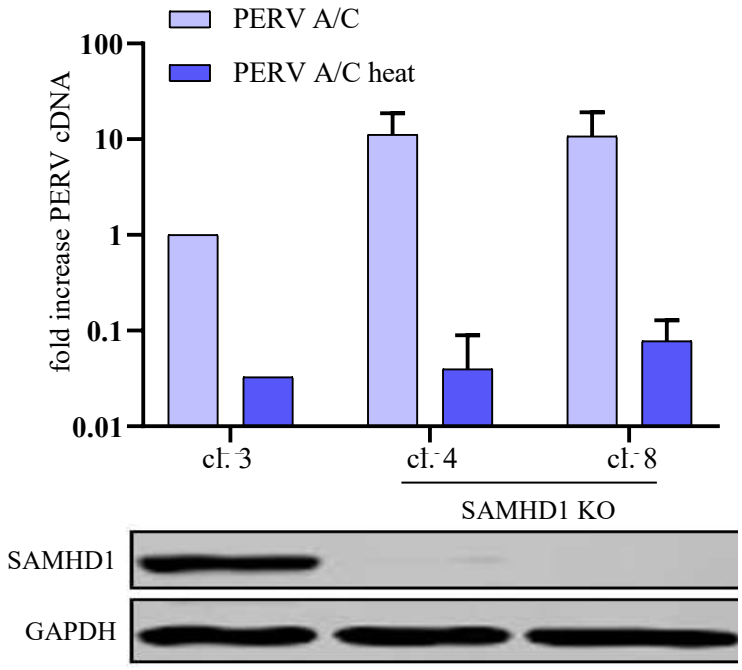
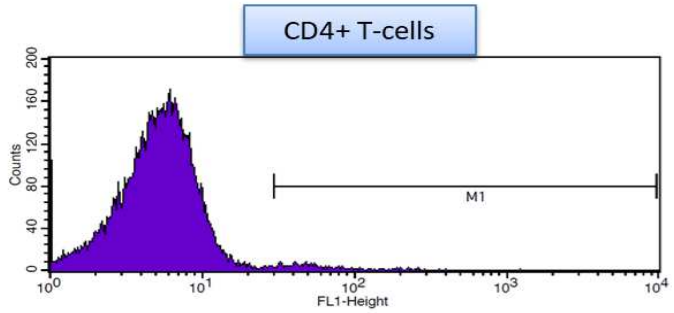


Figure 5. 7 SAMHD1 inhibits the PERV-A/C reverse transcription in THP-1 cells. PMA-differentiated THP-1 cells with a stable CRISPR/Cas9 knock-out of SAMHD1 were infected with PERV A/C and reverse transcripts were quantified relative to GAPDH 40 h post infection. RT-

products quantified in infected THP-1. CRISPR/Cas9 scramble cells were set to 1. Heat inactivated PERV A/C served as a control. SAMHD1 depletion was verified by immunoblot (bottom). GAPDH served as a loading control.

5.1.7 SAMHD1 restricts PERV-A/C replication in resting CD4$^+$ T-cells but not in activated CD4$^+$ T-cells

In addition to myeloid immune cells, SAMHD1 is also expressed to high levels in CD4$^+$ T-lymphocytes. Although expressed to similar levels, SAMHD1 is only antiviral against HIV-1 in resting but not activated CD4$^+$ T-cells [147]. We sought to investigate whether a similar restriction profile is observed in PERV infection. To do this, resting CD4$^+$ T-cells were isolated from PBMCs of six healthy donors by a magnetic negative selection. The purity of the CD4$^+$ T-cells preparation in the different donors was between 85 and 95% as determined by the flow cytometry using a FITC anti-human CD4 antibody (Fig.5.8).



Histogram Statistics

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Log Data Units: Linear Values
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Gate: No Gate
Total Events: 20150

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
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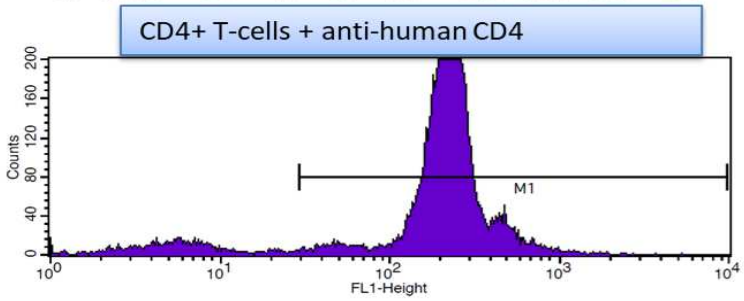

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Acquisition Date: 19-Dec-17
Gated Events: 20080
X Parameter: FL1-Height (Log)

Log Data Units: Linear Values
Patient ID:
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Total Events: 20080

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	20080	100.00	100.00	224.73	162.08	67.88	212.88	239
M1	29, 9734	18471	91.99	91.99	243.70	217.54	59.18	218.70	239

Figure 5. 8 FACS analysis to determine the purity of the CD4$^+$ T-cells after isolation from PBMCs. Anti-human CD4 was added to isolate CD4$^+$ T -cells. Non-treatment CD4$^+$ T-cells with antibody were used as a live gate control. The purity of CD4$^+$ T-cells from the total cells is nearly 92% (see the percent in the fig.5.8, lower).

One half of the resting CD4$^+$ T-cells was infected with PERV-A/C after pretreatment with VLPs containing Vpx or VLPs without Vpx in the presence or absence of 10μM AZT. The other half was activated with 5 ug ml^{-1} phytohemagglutinin and 50 IU ml^{-1} interleukin-2 for two days and infected similarly. Reverse transcripts in the infected cells were quantified by qPCR 40 h post infection. The results indicate that PERV reverse transcription slightly increased between two to four fold in cells treated with VPX compared to resting CD4$^+$ T-cells not treated with Vpx in the cells (Fig. 5.9). AZT and heat inactivation of the virus blocked PERV reverse transcription in cells. Immunoblot analyses for one donor using an anti-SAMHD1 MAb were performed to analyze Vpx-mediated degradation of SAMHD. The results show that Vpx cannot degrade SAMHD1in resting CD4$^+$ T-cells (Fig.5.9, lower).

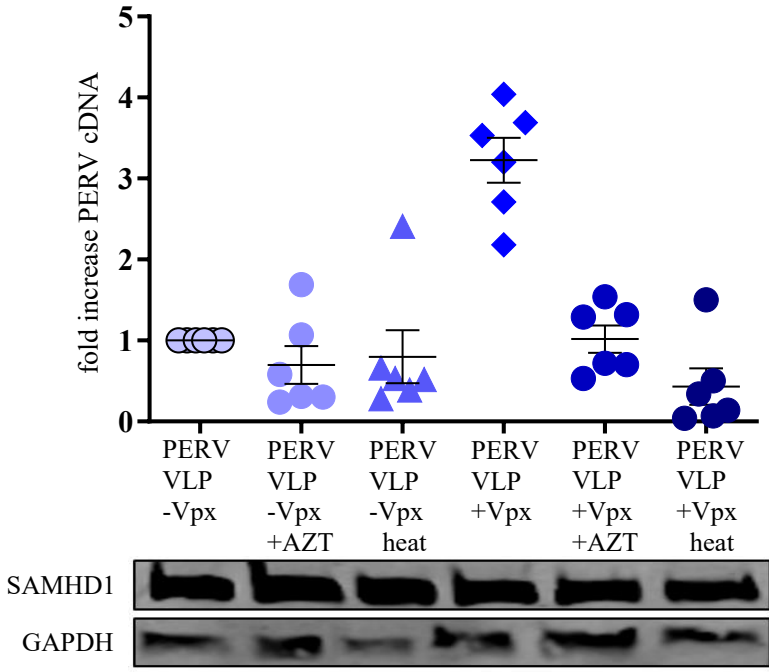

Figure 5. 9 PERV A/C is restricted in resting CD4$^+$ T-cells. Resting CD4$^+$ T-cells from six healthy donors were infected with PERV A/C. Reverse transcripts were quantified relative to GAPDH 40 h post infection. RT-products quantified in infected cells in absence of Vpx were set to one. Mean values and SEM are shown. Immunoblot analysis of one donor (as an example) for SAMHD1 degaradation in resting CD4$^+$ T-cells after treatment with VLP+Vpx or VLPs lacking Vpx is shown in the lower panel. GAPDH served as a loading control. The proteins were visualized by an Odyssey imaging system.

The results with activated CD4$^+$ T-cells show no more difference between cells infected with PERV, treated with VLP contain Vpx and cells treated with control VLP (Fig.5.10). Normally SAMHD1 lost its antiviral activity by phosphorylation in cycling cells like activated CD4$^+$ T-cells, therefore SAMHD1 in activated CD4$^+$ T-cells failed to restrict the PERV-A/C reverse transcription. The results of the SAMHD1 quantity in the immune blot of activated CD4$^+$ T-cells were similar to the results of resting CD4$^+$ T-cells, no degradation for SAMHD1 by addition of Vpx (Fig.5.10, lower panel).

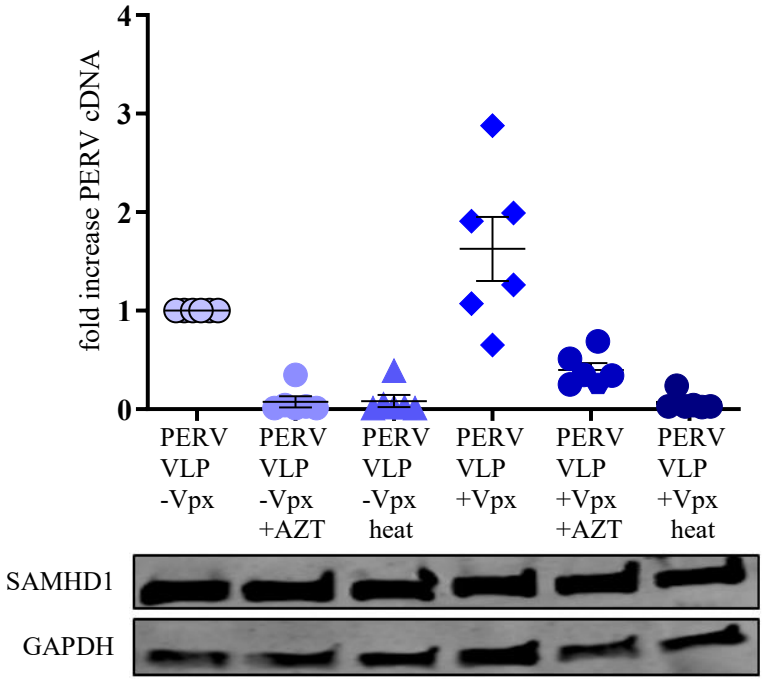

Figure 5. 10 SAMHD1 failed in restricting PERV A/C in activated CD4$^+$ T-cells. Activated CD4$^+$ T-cells from six healthy donors were infected with PERV A/C. Reverse transcripts were quantified relative to GAPDH 40 h post infection. RT-products quantified in infected cells in absence of Vpx were set to one. Mean values and SEM are shown. Immunoblot analysis for one donor to detect the degradation of SAMHD1 in activated CD4$^+$ T-cells after treatment with VLP+Vpx, GAPDH served as a loading control.

To compare resting and activated CD4$^+$ T-cells, the amounts of RT-products quantified in infected resting CD$^+$ T-cells were set to one. As shown in Figure 5.11, the PERV-A/C cDNA product in activated CD4$^+$ T-cells were more with 10 fold higher compared to resting CD4$^+$ T-cells. The results indicate that SAMHD1 blocked PERV RT in resting but not activated CD4$^+$ T-cells, similar to what is described for HIV-1.

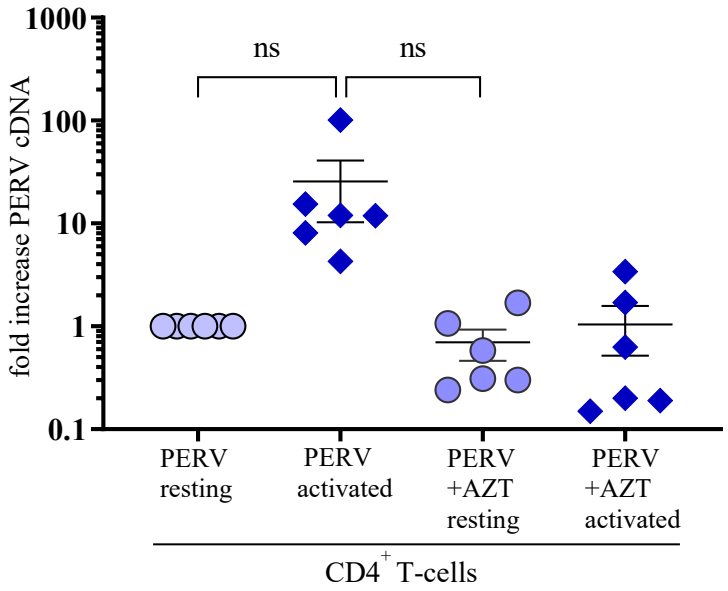

Figure 5. 11 PERV-A/C reverse transcription in activated CD4$^+$ T-cells is significantly higher than in resting CD4$^+$ T-cells. All six donors shown were infected with PERV-A/C and AZT was used as a reverse transcription inhibitor. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test.

5.1.8 The addition of exogenous deoxynucleosides (dN) to resting CD4$^+$ T-cells increased reverse transcription of PERV-A/C

To provide further evidence that the inhibition is exerted by SAMHD1, CD4$^+$ T-cells for three donors were isolated from PBMCs by magnetic negative selection. Cells were seeded and treated with 1 µM deoxynucleosides for 30 min prior infection. Resting CD4$^+$ T-cells were infected with PERV-A/C and 40 h post infection quantified by qPCR. The result indicated that 1 mM dN significantly increased PERV cDNA levels in resting CD4$^+$ T-cells (Fig.5.12a). At this high dN concentration AZT has lost its inhibitory effect for reverse transcription in resting CD4$^+$ T-cells. From this result, it can be deduced that SAMHD1 has the ability to inhibit the PERV-A/C replication cycle at reverse transcription by depletion of the intracellular pool of dNTP. The Western blot shows the amounts of expressed SAMHD1 for three donors in resting CD4$^+$ T-cells (Fig.5.12a, lower panel).

The activated CD4$^+$ T-cells were infected with PERV-A/C after pretreatment with 1mM deoxynucleosides. The results show that in contrast to resting CD4$^+$ T-cells, the addition of 1mM of deoxynucleosides does not induced PERV-A/C reverse transcription (Fig.5.12.b). The Western blot shows the amounts of expressed SAMHD1 for three donors in activated CD4$^+$ T-cells (Fig.5.12b, lower).

In summary the resting CD4$^+$ T-cells can be restricted the PERV-A/C infection and the block could be relieved by addition of 1 mM deoxynucleosides during infection, this intervention is well known to overcome the enzymatic activity of SAMHD1. In contrast, activated CD4$^+$ T-cells can not produce PERV-A/C cDNA in the addition of extra 1 mM deoxynucleosides.

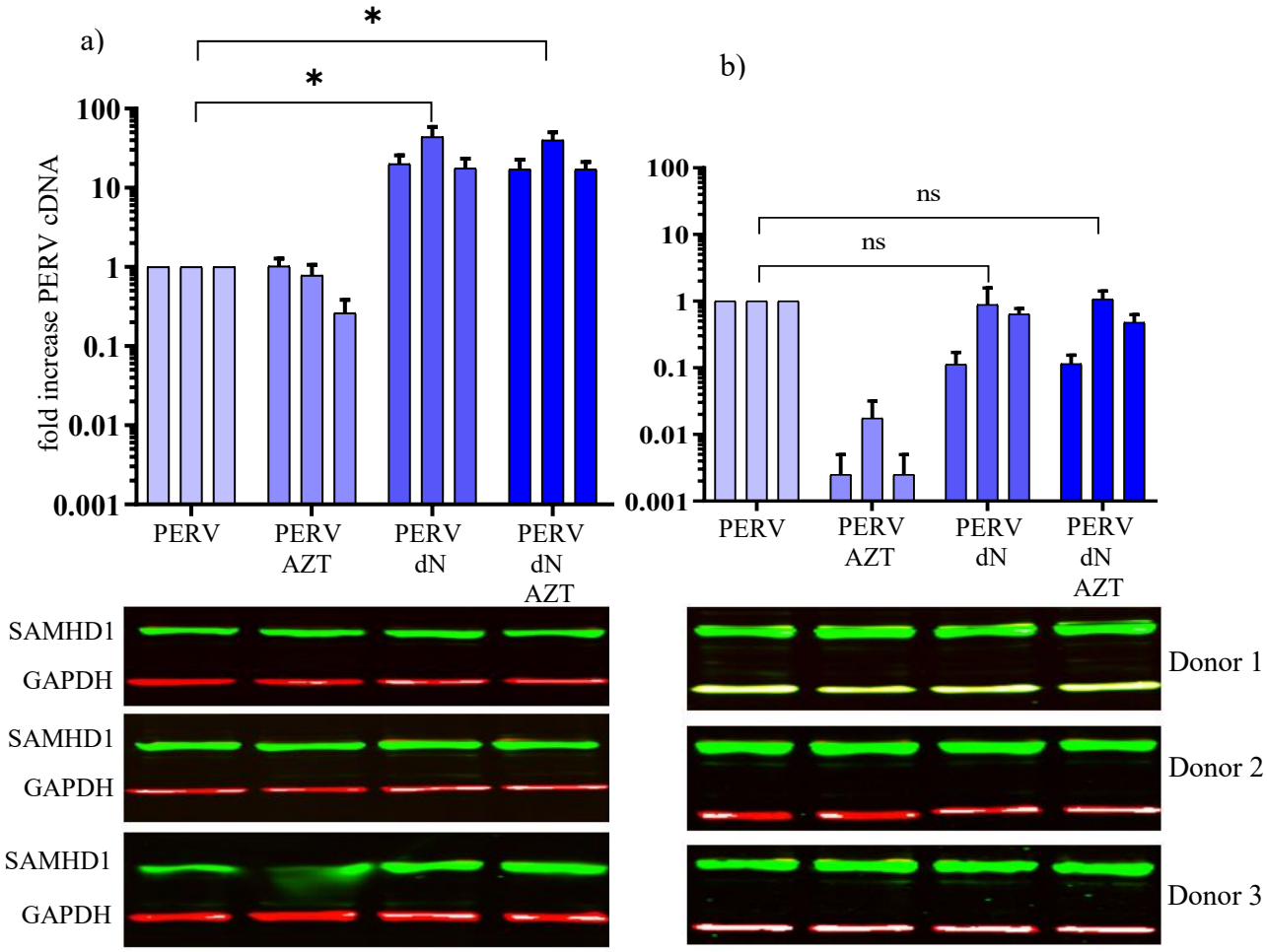

Figure 5. 12 PERV-A/C reverse transcription is increased with increased level of intracellular dNTP in resting CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from PBMCs by magnetic negative selection and either used as resting (a) or activated cells (b). Both types of CD4$^+$ T-cells were treated with 1 mM dN or just medium and infected with PERV-A/C. AZT has been used as a reverse transcription blocker and PERV-heat inactivation served as a control. PERV-A/C RT-products were identified by real time qPCR 40 h post infection. The amounts of RT-products quantified in cells infected with PERV-A/C in absence of dN were set to 1. ΔΔCT method was used to calculate the fold difference to GAPDH. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test. The immunoblot in the figure (a) and (b) at the bottom show the expressed the amount of SAMHD1.

5.2 Innate immune response to PERV

The innate immune response is the first defense line in the human body against a virus and other infections. It is important to know the response from human immune primary cells to PERVs infection, especially in the case of xenotransplantation. To assess the interferon response to PERV infection, PBMCs were isolated from healthy human donors and differentiated into monocytes, MDDCs and MDM cells. Cells were infected with PERV-A/C after pretreatment with virus like particles containing Vpx (VLP+Vpx), control VLP without Vpx or without any pretreatment. In case of a response, we aim to identify the sensors and signaling pathways, which are involved in the type I IFNs response in human primary cells infected by PERV-A/C.

5.2.1 PERV induced the production of CXCL-10 (IP-10) but not INF-B and ISG-54 in MDDCs

CXCL-10 is induced in various cell types during infection by pathogens. Immune primary cells including monocytes, macrophage, T-lymphocytes, natural killer (NK) cells are responsible for the greatest proportion of IP-10 expression [219]. To determine the CXCL-10 response to PERV-A/C infection, MDDCs were differentiated from monocytes of human healthy donors and seeded in 12-well plates. Cells were pretreated with VLP+Vpx or control VLPs and then cells were infected with benzonase treated PERV-A/C. 48h post infections, RNAs were isolated and the relative differences of CXCL-10 mRNA were quantified by real time PCR. Heat-inactivated virus served as a negative control. The results show that PERV-A/C increased the CXCL-10 induction over 100 folds compared with non-infected cells. Treatment with VLP+Vpx or empty VLP alone did not cause significant CXCL-10 induction (Fig.5.13). The expression or lack of SAMHD1 plays no important role in the induction of CXCL-10 by PERV-A/C. Heat-inactivated PERV-A/C lowered the CXCL-10 induction in infected cells, as expected (Fig.5.13).

In contrast to CXCL-10, the response of IFN-β in MDDCs to PERV-A/C infection was not significant compared to the non-infected cells and to the positive control (poly I.C). PERV-A/C induced IFN-β only slightly in one donor (fivefold compared with mock cells). No effect has been seen by addition of VLPs to the cells in induced CXCL-10 (Fig.5.14).

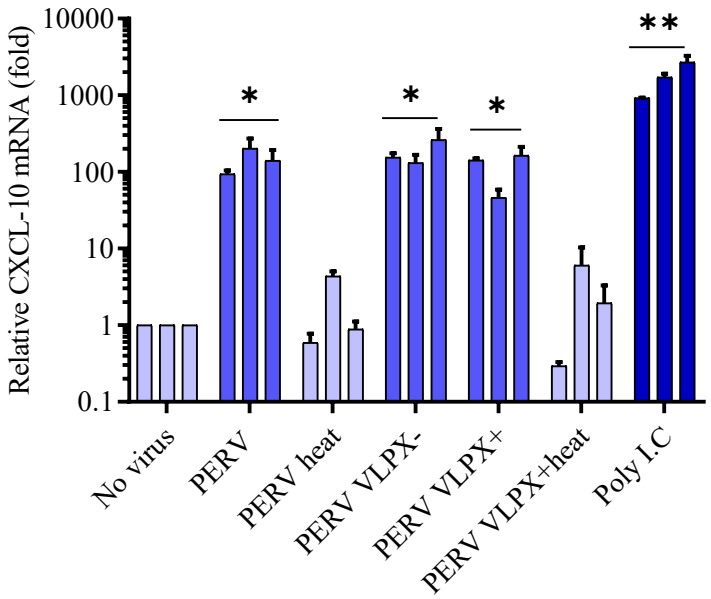

Figure 5. 13 PERV-A/C induces CXCL-10 in MDDCs. MDDCs were infected with a PERV-A/C recombinant virus after pretreatment with virus like particles containing Vpx (VLP+Vpx), control VLP, poly I.C or without pretreatment. The CXCL-10 relative mRNA activity was quantified by real time PCR. Heat-inactivated PERV served as a negative control and poly I.C served as a positive control. The amounts of mRNA quantified in non-infected cells were set to 1. ΔΔCT method was used to calculate the fold difference. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test.

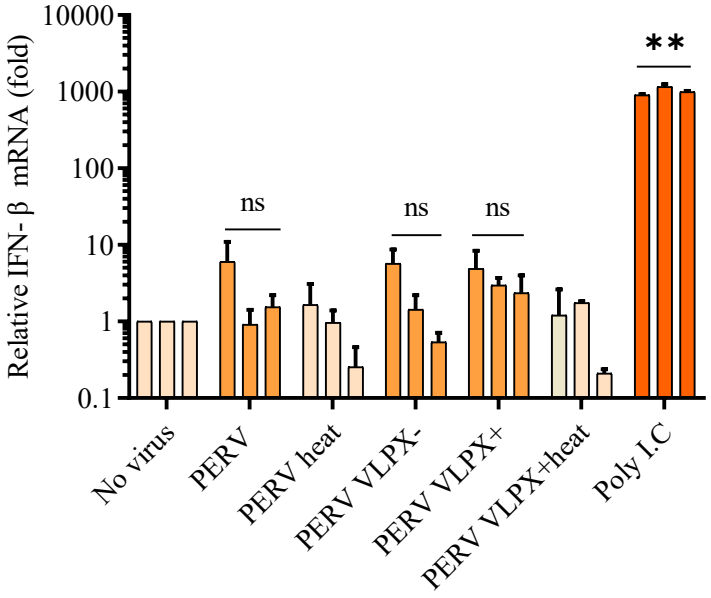

Figure 5. 14 PERV-A/C is incapable to activate IFN-β in MDDCs of three donors. MDDCs were infected with PERV-A/C. Poly I.C has used as a positive control and heat-inactivated PERV was used as a negative control. The amounts of mRNA quantified in non-infected cells were set to 1. ΔΔCT method was used to calculate the fold difference. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test.

The same results were found regarding the ISG-54 response to PERV-A/C infection. Only a weak non-significant two to three fold mRNA increase of ISG-54 was induced after MDDCs infection with PERV-A/C. Addition of VLP did not result in any significant changes (Fig.5.15). Heat-inactivated virus served as a negative control and poly I.C as a positive control.

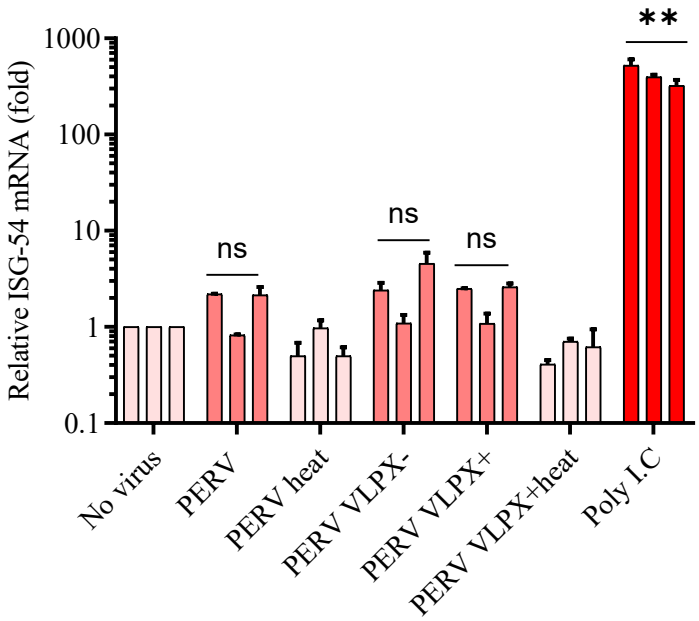

Figure 5. 15 ISG-54 is not induced by PERV-A/C in MDDCs. Amounts of ISG-54 mRNA in non-infected cells were set to 1. ΔΔCT method was used to calculate the fold difference. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test.

5.2.2 CXCL-10 induction by PERV-A/C infection is significant in MDM

To demonstrate that CXCL-10, IFN-β and ISG-54 responses induced to PERV-A/C infection, are also present in other cell types we performed experiments in MDM of three healthy donors. MDM cells were pretreated with virus like particles containing Vpx (VLP+Vpx), control VLP without Vpx and infected with PERV-A/C for 48 h. Cells were collected and washed two times with PBS, after that RNA was isolated; cDNA synthesized and used for qPCR to quantify the relative mRNA induction of CXCL-10, IFN-β and ISG-54. In results presented in Fig.5.16, the result indicated that PERV-A/C infection induced CXCL-10 in MDM of one donor thousand fold and with other two donors to above hundred fold. Knockdown of SAMHD1 by the accessory protein Vpx didn`t change the CXCL-10 induction. Heat inactivated PERV-A/C did not change the amount of CXCL-10 induction significantly.

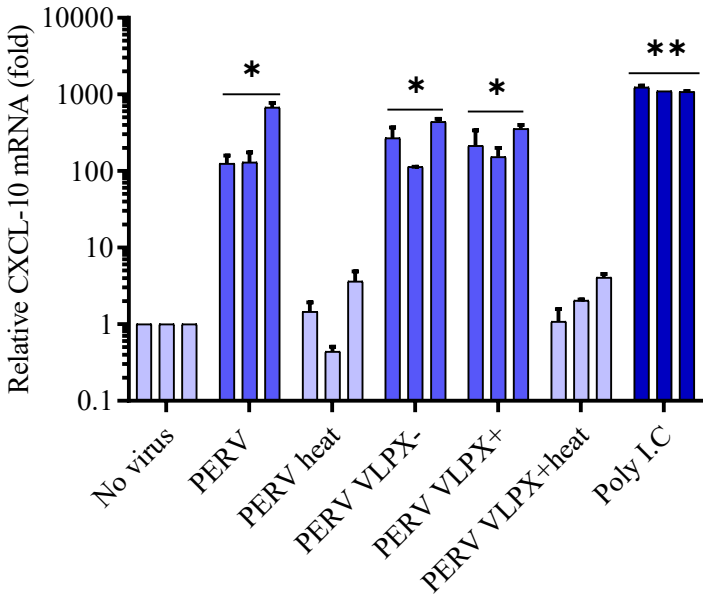

Figure 5. 16 CXCL-10 is significantly induced by PERV-A/C infection in MDMs. MDMs were differentiated for 6 days from PBMCs by GM-CSF. Non infected cells were set to one. ΔΔCT method was used to calculate the fold difference. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test.

As shown in Figure 5.17, PERV-A/C infection failed to induce significant levels of IFN-β in MDM cells in contrast to treatment with poly I.C. IFN-β has been however slightly induced in MDM of one donor without reaching significant levels.

As with the results obtained with IFN-β, the relative mRNA of ISG-54 was not significantly induced by PERV-A/C infection in MDM cells as well (Fig.5.18). From three donors just one donor appeared with slightly induced of ISG-54 response to PERV-A/C infection. Heat inactivated viruses served as a negative control, while poly I.C treatment served as a positive control.

5.2.3 CXCL-10 induction is increased in monocytes by PERV-A/C infection

To confirm the induction of CXCL-10 by PERV-A/C in other cells, monocytes were used. Monocytes were isolated from buffy coats of three healthy human donors and differentiated to monocytes. Monocytes were pretreated with VLP+Vpx or control VLPs without Vpx for 3h, after that, cells were infected with PERV-A/C. RNA was isolated 48h post infection and CXCL-10 mRNA was quantified by qPCR. As with MDDCs and MDM cells, PERV-A/C induced the production of CXCL-10 in monocyte cells (Fig.5.19). The addition of VLP with Vpx to the monocytes played no important role. Heat-inactivated PERV-A/C did not result in significant CXCL-10 induction (Fig.5.19).

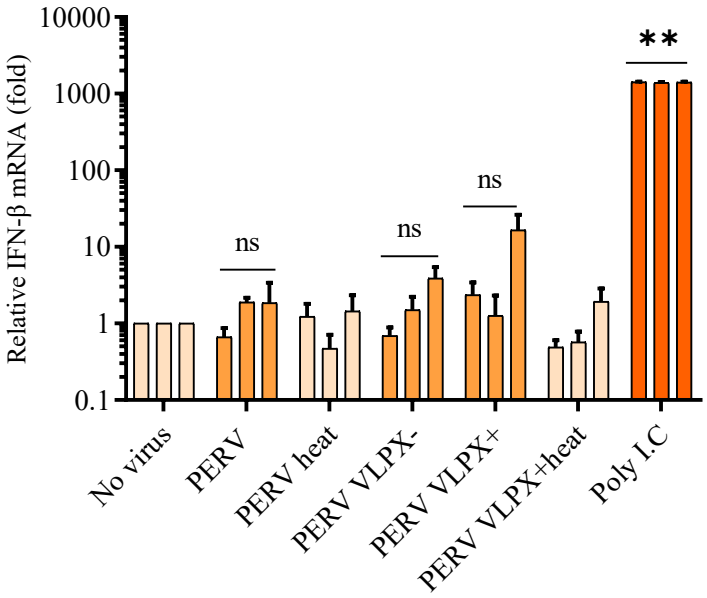

Figure 5. 17 IFN-β failed to be activated by PERV-A/C infection in MDMs. PERV-A/C was used as stimulator for IFN-β and poly I.C as a positive control. The IFN-β mRNA level was quantified by qPCR. Non infected cells were set to one and used as a calibrator for measurement of the fold difference. ΔΔCT method was used to calculate the fold difference for infected and non-infected cells to GAPDH. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test.

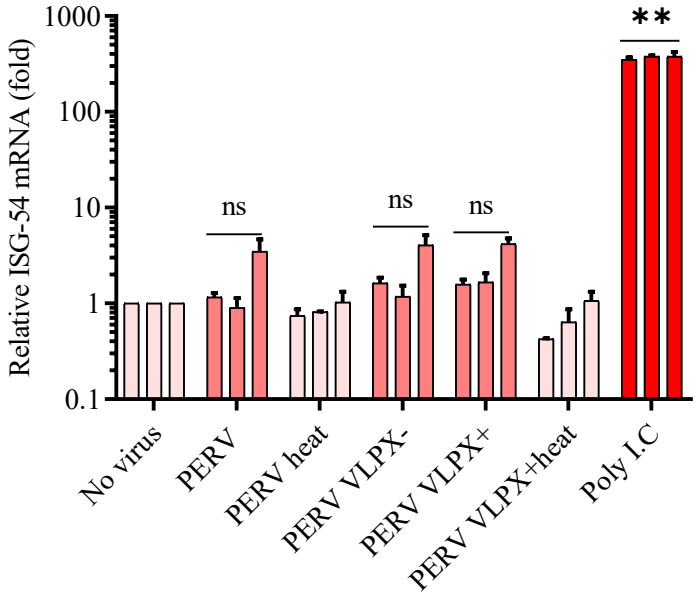

Figure 5. 18 PERV-A/C infection does not induce ISG-54 in MDMs. The cells were infected by PERV-A/C, transfected with poly I.C or not-infected at all. After RNA isolation, the ISG-54 relative mRNA was quantified by qPCR. Non infected cells were set to one and used the ΔΔCT method to calculate the fold difference to GAPDH. Mean values and SEM are shown. *P<0.05; **P<0.005; unpaired t-test.

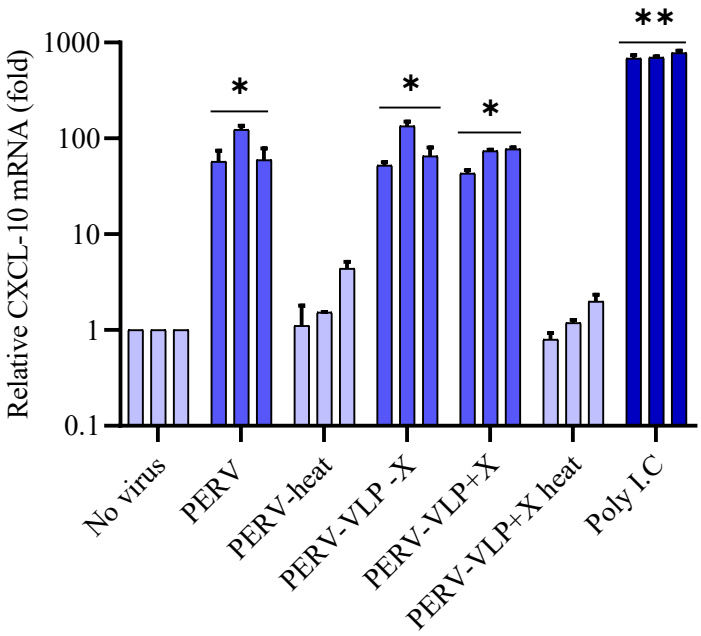

Figure 5. 19 In monocytes the induction of CXCL-10 to PERV-A/C infection was significant. Monocytes were differentiated from PBMCs by adherence. Cells were infected with a PERV-A/C recombinant virus after pretreatment with virus like particles containing Vpx (VLP+Vpx), control VLP or without pretreatment. The CXCL-10 mRNA was quantified by qPCR. Heat-inactivated PERV served as a negative control and the poly I.C transfection served as a positive control. The amounts of mRNA quantified in non-infected cells were set to one and used the ΔΔCT method to calculate the fold difference to GAPDH. Mean values and SEM are shown. *P<0.05; **P<0.005; n.s. not significant, unpaired t-test.

5.2.4 A JAK inhibitor reduces the CXCL-10 inductions in MDM

The previous results demonstrated a significant relationship between the CXCL-10 mRNA induction levels and the PERV-A/C infection. It is therefore necessary to identify the immune sensors and signaling pathways, which play a role in the CXCL-10 response in human immune primary cells infected by PERV-A/C. To investigate that, human primary MDM of six donors were pretreated with 1µM of different inhibitors (NF-κB inhibitor; Parthenolide, JAK inhibitor; AT9283, TBK1 inhibitor; BX-795, and a TLR7/8 antagonist; R837) for one hour before infection with PERV-A/C. 48 h post infection, RNA was isolated; cDNA synthesis of CXCL-10 mRNA was quantified by qPCR. The results show that only the JAK inhibitor reduced the induction of CXCL-10 by PERV-A/C. The pretreatment with inhibitors of NF-κB, TBK1 and TLR7/8 failed to reduce the CXCL-10 induction (Fig.5.20). The results indicate that the JAK-STAT pathway is involved in the CXCL-10 stimulation.

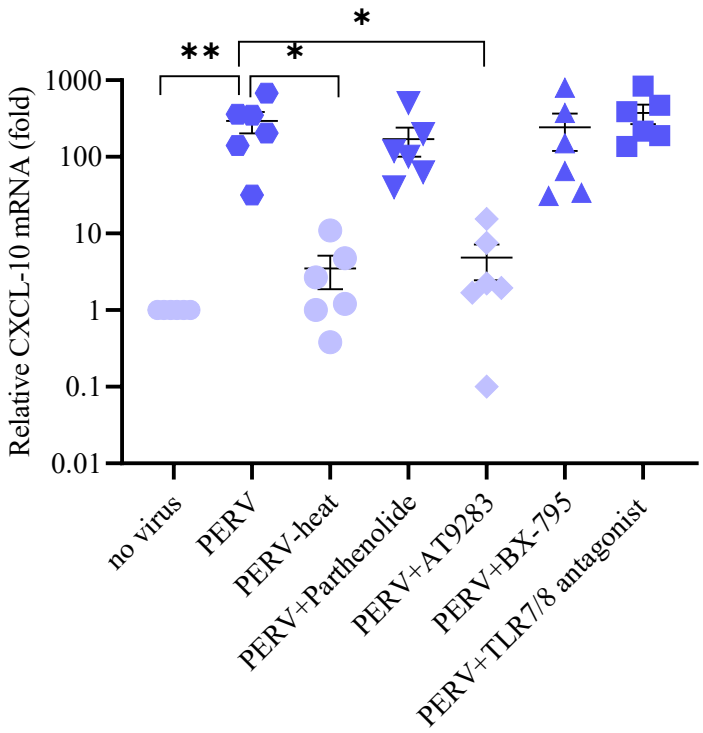

Figure 5. 20 The JAK-STAT pathway is involved in the CXCL-10 response in MDMs to PERV-A/C infection. Cells were seeded and differentiated in 12-well plates. Before infection by PERV-A/C, cells were pretreatment with the indicated inhibitors (JAK (AT9283), TBK1 (BX-795), NF-κB (Parthenolide) and the TLR7/8 antagonist (R837) for 30 min. 48 h post infection, RNA was isolated and CXCL-10 mRNA quantified by qPCR. The results of non-infected cells were set to one and the ΔΔCT method was used to calculate the fold difference to GAPDH. Mean values and SEM are shown. *P<0.05; **P<0.005; n.s. not significant, unpaired t-test.

5.2.5 CXCL-10 induction by PERV-A/C is also inhibited in MDDCs by the JAK-inhibitor AT9283

As above, MDDCs were pretreated with 1 μM of different inhibitors (NF-κB inhibitor; Parthenolide, JAK inhibitor; AT9283, TBK1 inhibitor; BX-795, TLR7/8 antagonist; R837) for one hour before infection with PERV-A/C. RNA was isolated 48 h post infection and CXCL-10 was measured by qRT-PCR. The result in MDDCs provides further evidence that CXCL-10 induction is inhibited by the JAK inhibitor (AT9283). This was the case in all six donors tested. (Fig.5.21). In addition to the JAK inhibitor, the TBK1 inhibitors (BX-795) reduced the CXCL-10 activity in MDDCs as well, but about 10 times less effective than AT9283. Moreover, the NF-κB inhibitor did slightly reduce the amount of CXCL-10 induction as well. However, considerable donor dependence has been noticed (Fig.5.21). The production of CXCL-10 can be induced with different cell signaling pathways depending on

the type of inducers. The JAK-STAT pathway is the main way that PERV-A/C is using to induce CXCL-10 in human primary myeloid cells

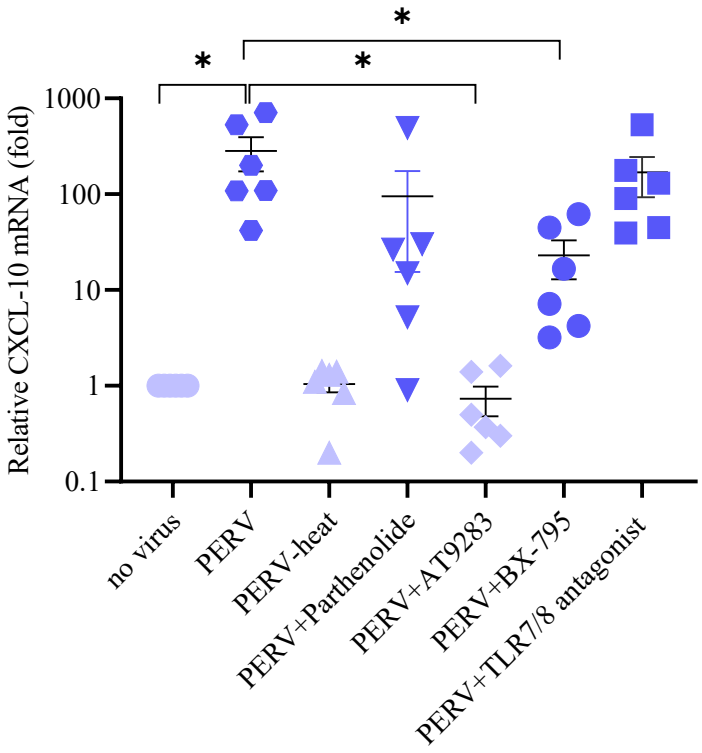

Figure 5. 21 Analysis of inhibitors of innate immune pathways for induction by PERV-A/C in MDDCs. Differentiated cells were pretreated with JAK (AT9283), TBK1 (BX-795), NF-κB (Parthenolide) inhibitors and the TLR7/8 antagonist (R837) for 30 min before infection with PERV-A/C. The relative mRNA of CXCL-10 was quantified by qPCR 48 h post infection. The results of non-infected cells were set to one and the ΔΔCT method was used to calculate the fold difference to GAPDH. Mean values and SEM are shown. *P<0.05; **P<0.005; n.s. not significant, unpaired t-test.

6. Discussion

In human cells, retroviruses replication can be restricted by multiple barriers. Viral replication can either be limited due to missing cellular co-factors, which retroviruses take for efficient replication, or due to the presence of restriction factors such as APOBEC3G, Fv1, TRIM5α, tetherin and SAMHD1, which block viral replication at different steps of the life cycle [120, 121]. Previous studies have investigated the potential of other well-described retroviral restriction factors to block PERV transmission from pigs to humans: various members of the human and porcine family of APOBEC3 cytidine deaminases potently block PERV replication in tissue, thereby reducing the risk of infection of human cells by PERV during xenotransplantation [220, 221]. Tetherin (or BST-2), which prevents retroviral budding, was also found to potently inhibit PERV replication in human cells [138]; in contrast, TRIM5alpha proteins from various species did not restrict PERV replication [222].

The risk of PERV transmission to various human cells is high during xenotransplantation, because PERVs are integrated within the genome of all pigs and the ability of PERVs to infect human cells in vitro was confirmed. Therefore, it is important to investigate the role of human SAMHD1 in restricting the transmission of PERV from pig organs to human recipients during xenotransplantation. The sterile alpha motif domain and HD domain containing protein 1 (SAMHD1) is a restriction factor that potently blocks infection of a variety of human and nonhuman retroviruses independent of the origin of the viruses [144, 223].

In addition, it is important to mention that the zoonotic potential of virus transmissions during xenotransplantation is not limited to PERV. Recent publications identified hepatitis E virus (HEV) and the porcine cytomegalovirus (PCMV) as potential risk factors for the transplant recipient [224, 225]. In transplant experiments where baboons received a heart from HEV positive donor pigs the virus was detected in the recipient as well [226]. For xenotransplantation to become a clinical routine, not only immunological and anatomical hurdles but also the zoonotic risk of PERV, HEV, CMV and other viruses have to be eliminated.

6.1 PERV-A/C life cycle is inhibited by SAMHD1 in human primary cells

6.1.1 SAMHD1 restricts PERV-A/C in MDDCs by depleting cytoplasmic dNTPs

In the present work, the antiviral activities of SAMHD1 to restrict PERV in MDDCs were investigated. The restriction function of SAMHD1 for HIV-1 and other retroviruses was assessed in MDDCs and other immune primary cells previously [223]. In general, the reverse transcription of most retroviruses requires a preexisting pool of dNTPs in the target cell. Therefore, nucleotide pool depletion by SAMHD1 could be a general mechanism to restrict the replication of different genera of retroviruses. The findings in this work confirm that SAMHD1 restricts the replication of PERV-A/C at the reverse transcription in MDDCs. This has been demonstrated in MDDCs of many donors (Fig.5.2). The delivery of Vpx by HIV-1-VLPs into the MDDCs degrades SAMHD1 and the RNA genome of PERV-A/C can be reversed transcribed into cDNA. The results from Sunseri and coworkers also demonstrated that Vpx containing HIV-1 increase the HIV-1 infectivity nearly 100-fold on MDDCs [168]. In previous studies, it has been demonstrated that SAMHD1 can be restricted HIV-1 in MDDCs at the reverse transcriptase step, they found that SAMHD1 restricted infection by depleting intracellular deoxynucleosides triphosphates (dNTPs), reducing their concentrations to below those required for the synthesis of the viral DNA by reverse transcriptase [127, 143].

Normally, to successfully replicate, retroviruses and some DNA viruses, which replicate through a DNA intermediate, they need access to an intracellular supply of dNTP. To understand if PERV-A/C is restricted by the low abundance of dNTPs in MDDC, I supplemented the cell culture with 1 mM dN for 30 min prior to infection with PERV and VLP lacking Vpx or with Vpx. The results show a similar increase of PERV reverse transcripts in MDDCs treated with 1 mM dN compared to the addition of Vpx-VLP, that means the capability of SAMHD1 was not sufficient to deplete the endogenous and exogenous deoxynucleotides (Fig. 5.4). A combination of dN supplementation and Vpx-VLP treatment results in a further enhancement either suggesting an additional restriction mechanism to dNTP pool depletion or resulting from the fact that SAMHD1 protein was not completely degraded by Vpx as seen by the western blot (Fig.5.4). In another study, the authors found that the treatment of cells with deoxynucleosides was less efficient to induced the reverse transcription than was treatment with Vpx-containing VLPs, perhaps because cells treated with deoxynucleosides continue to deplete the dNTPs by SAMHD1, while in cells exposed to Vpx-containing VLPs, SAMHD1 is degraded, which prevents depletion of dNTPs

[127]. The results from an additional previous study suggest that SAMHD1 can restrict retroviruses in the case of low cellular dNTP levels and it lacks its antiviral activity due to the high dNTP levels, like in Hela cells [227]. Moreover, recently, it was demonstrated that the imbalance of dNTP pools in macrophages can significantly inhibit cDNA synthesis by HIV-1 reverse transcriptase and the increased dNTP bias induced the viral mutant rate by about 20–30% per a single cycle infection [228].

6.1.2 The antiviral activity of SAMHD1 inhibits PERV-A/C in monocytes and MDMs

Monocytes and macrophages are at the front line in the immune defense against external invasion by pathogens, providing the first virus-cell contact during infection. In addition, monocytes and macrophages are considered as partially resistant cells for HIV-1 and others virus's infection. The restriction factors SAMHD1 can restrict the PERV-A/C reverse transcription in monocytes and in the absent of SAMHD1 PERV-A/C produced cDNA by reverse transcription (Fig.5.5). The same result was also found in MDM, SAMHD1 blocks the replication cycle for PERV-A/C by depleting the intercellular dNTP at reverse transcription (Fig.5.6). The restriction function of SAMHD1 for PERV-A/C in monocytes and MDMs in this study corresponds to those reported in similar studies [127, 143, 223] showing its antiviral activity for blocking HIV-1 replication cycles in the human primary cells. In agreement with this result, it was reported that monocytes and macrophages can resistant the lentivirus and gammaretrovirus infection under special circumstances [229, 230]. In another side, host restriction factor SAMHD1 inhibits human T-cell leukemia virus type 1 infection of monocytes by inducing cells apoptosis [163]. In another study it was found that the Vpx protein increases the cellular dNTP concentration by degrading SAMHD1 above the threshold values of HIV-1 reverse transcription in macrophages, which can induce the reverse transcription activity and virus infectivity in macrophages [231]. Also in MDM cells vaccinia virus replication in primary human monocyte-derived macrophages was enhanced by Vpx-mediated degradation of SAMHD1 [150].

In (Fig.5.6) the shown result demonstrates that the AZT treatment (PERV+VLP-no Vpx+AZT) lowered significantly the amount of PERV-A/C cDNA compared with untreated (PERV+VLP-noVpx) in MDM cells, that means SAMHD1 can only partial inhibit the PERV-A/C reverse transcription and PERV-A/C can produce cDNA in the present of SAMHD1. The previous result was in agreement with our work, that HIV-1 can partially infect macrophages in the face of SAMHD1 restriction [232, 233]. In contrast with HIV-2 and SIV, which are

incapable to infect macrophages except if SAMHD1 is eliminated by the accessory protein Vpx and the intracellular dNTP concentrations are increased to levels appropriate for viral cDNA production [233]. It was also demonstrated that human cytomegalovirus reduces the steady-state SAMHD1 expression levels and increases SAMHD1 phosphorylation using the viral kinase pUL97 in macrophages [234]. The same results were obtained with other tissue macrophages, which HIV-1 has the ability to infect. These cells are used as a viral reservoir, like microglia in CNS and osteoclast in bone [235-237]. In a recent study, the authors demonstrated that the antiviral activity of SAMHD1 in MDM is sex-dependent; SAMHD1 exists in a hyper phosphorylated and less active state in male-derived macrophages then in female-derived macrophages. In addition, the SAMHD1 phosphorylation by CDK1, presented lower levels of expression in female-derived macrophages in all tested donors [238]. Jauregui et al. found the DNA damage in MDMs by neocarzinostatin, blocking HIV-1 infection by decrease of the phosphorylated SAMHD1 and activating its antiviral activity [239]. In contrast to many studies, it was reported in one other study that the RNase activity of SAMHD1 is responsible for blocking HIV-1 infection by directly degrading the HIV-1 RNA in monocytes and MDM [240].

6.1.3 The replication cycle of PERV-A/C in THP-1 cells can be restricted by SAMHD1 at reverse transcriptase

In the last experiment, SAMHD1 was knocking down by Vpx in MDDCs to find out its antiviral activity. To further verify the restriction function of SAMHD1 for PERV-A/C, THP-1 cells with a stable CRISPR/Cas9 knock-out of SAMHD1 were used. THP-1 cells were differentiated with 30 ng ml^{-1} PMA 72 h prior to infection. The reverse transcription products of PERV-A/C in THP-1 cells with a knock-out SAMHD1 status were 10 fold more susceptible to infection compared with control cells expressing SAMHD1. This indicates that SAMHD1 is able to counteract PERV-A/C infection in non-dividing THP-1 (Fig.5.7). This is consistent with the findings in previous studies which did also use THP-1 lacking SAMHD1 and infected them with HIV-1. They found that HIV-1 infectivity is higher in the absence of SAMHD1 in differentiated or not-differentiated THP-1 cells [241, 242]. It has been also reported that SAMHD1 can be restricted by HCMV in THP-1 cells by comparing the levels of infectious HCMV particles product in Knock-out THP-1 cells with HCMV-infected control cells [234]. In another study, authors used RNA downregulation by shControl and shSAMHD1 vectors to reduce SMAHD1 expression in THP-1 cells to determine whether the restriction to FIV, EIAV and SIV infection was also caused by SAMHD1 in differentiated

cells. They found that the SAMHD1 knock-down relieved the block to infection for all viruses and that Vpx containing VLP had no further effect [223]. In addition, it was demonstrated that SAMHD1 blocks replication of the HSV-1 DNA genome in differentiated macrophage cell lines. Knockdown of SAMHD1 in THP-1 cells enhanced HSV-1 replication [149].

6.1.4 SAMHD1 restriction of PERV-A/C in CD4$^+$ T-cells

I addition to myeloid immune cells, SAMHD1 is also expressed to high levels in CD4$^+$ T-lymphocytes, which are consider the main targets of HIV-1, also important for the virus reservoir and viral latency. The antiviral activity of SAMHD1 against PERV-A/C in CD4$^+$ T-cells was different in activated and resting-CD4$^+$ T-cells. The PERV-A/C cDNA product from reverse transcription in activated CD4$^+$ T-cells was higher with around 10 fold compared with resting CD4$^+$ T-cells. This result indicated that SAMHD1 restricted PERV replication cycle in resting but not activated CD4$^+$ T-cells (Fig.5.11). The report from a previous study is in agreement with this finding, that the deoxynucleoside triphosphate triphosphohydrolase SAMHD1 prevents reverse transcription of HIV-1 RNA in resting CD4$^+$ T-cells [147]. The SAMHD1 role in HIV-1 latency remains unknown; therefore in one study the authors demonstrated that SAMHD1 suppressed not only HIV-1 gene expression but also reactivation of viral latency in primary CD4$^+$ T-cells [243].

In (Fig.5.9) the VLP+Vpx failed to increase PERV RT products significantly in resting CD4$^+$ T-cells; however we were unable to reduce SAMHD1 levels to sufficient amounts using this approach (Fig.5.9, lower). Baldauf *et al.* demonstrated that virion-packaged Vpx proteins from a second SIV lineage, SIV of red-capped mangabeys or mandrills (SIVrcm/mnd-2), enhanced HIV infection in resting CD4$^+$ T-cells without any effect on SAMHD1 degradation, dNTP pool elevation, or changes in SAMHD1 phosphorylation [244]. It has been reported in a different study that SAMHD1 in resting CD4$^+$ T-cells was much less efficiently degraded by VPX compared with primary monocytes. In one study they pretreated resting CD4+ T-cells with VLP-Vpx before they infected cells with HIV-1, after 96 h an average of 14% of resting CD4$^+$ T lymphocytes were expressed HIV-1 EGFP [145]. The divergences in Vpx-mediated loss of SAMHD1 in resting CD4$^+$ T-cells and other human primary cells might result from differences in nuclear/ cytoplasmic exchange [245] and the nuclear Vpx alleles were only capable of diminishing a nuclearly localized SAMHD1 [246].

It was demonstrated that SAMHD1 lost its antiviral function in cycling cells by phosphorylation on residue T592 without affecting its ability to decrease cellular dNTP levels

[247]. In agreement with previous results, in Fig.5.10 the results show no role of SAMHD1 antiviral activity to restrict PERV-A/C in activated $CD4^+$ T-cells and the amounts of SAMHD1 expression were similar in Fig.5.10. The dNTP triphosphohydrolase activity of SAMHD1 continues during phosphorylation at T592 and achieves an important regulatory role to maintain dNTP pool balance for proper DNA replication [248]. In contrast, the result of another study show that SAMHD1 restricts the replication of Line1 and other endogenous retroelements in cycling cells, that means the function of SAMHD1 in antiviral activity and controlling autoimmunity might not only depend on the dNTP hydrolase activity [249].

The addition of 1 mM exogenous deoxynucleosides to the resting $CD4^+$ T-cells increased the PERV-A/C cDNA products significantly (Fig.5.12a). A similar result was achieved with MDDCs (Fig.5.4) that explain the addition of deoxynucleosides overcome the SAMHD1 restrictions to PERV-A/C infection in resting $CD4^+$ T-cells. On the other hand, the addition of deoxynucleosides to the activated $CD4^+$ T-cells failed to induce the RT product of PERV-A/C (Fig.5.12.b). Previously published data indicate that the addition of deoxynucleosides promoted HIV-1 integration and 2LTR formation in resting $CD4^+$ T-cells, but not in activated $CD4^+$ T-cells [250]. In another study the authors reported that dNs enhanced HIV-1 infectivity in resting $CD4^+$ T-cells in the absence of Vpx, while not affecting the activation status of the cells [147].

In conclusion, this study we provide strong evidence that SAMHD1 potently blocks PERV reverse transcription in MDDCs, primary human monocytes and MDMs as well as in the differentiated THP-1 cells line. The block could be relieved by treatment with Vpx or by addition of 1 mM deoxynucleosides during infection, both interventions are well known to overcome the enzymatic activity of SAMHD1. Unlike activated $CD4^+$ T-cells, resting $CD4^+$ T-cells that express SAMHD1 did not support PERV RT and this block could also be alleviated through addition of 1 mM dN. The SAMHD1- mediated PERV-A/C restriction in non-cycling myeloid cells and resting $CD4^+$ T-cells may help develop new therapeutic approaches against PERV-A/C infection by a combination with other available restriction factors and anti-PERV-A/C.

6.2 The interferon response to PERV-A/C infection

Innate immune responses are the first line of defense against invading pathogens. Nucleic acids of many viruses are major structures detected by the innate immune system. Cytosolic DNA sensors activate different signaling pathways leading to activation of transcription

factors such as the IFN regulatory factor 3 (IRF3) or nuclear factor kappa B (NF-κB), which are responsible for the production of type one IFN. The IFNs bind to the IFN-receptor and induce the JAK/STAT pathway that lead to enhanced expression of IFN-stimulated genes, which play an important role in the immune defense against viral infection.

In xenotransplantation, it is necessary to demonstrate the interferon response of human immune primary cells for PERV-A/C infection in order to understand the immune response after xenograft reception and to avoid the immune rejection that can be caused by this response.

6.2.1 PERV-A/C infection induces IP-10 in human primary cells

The results provided within this thesis indicate that CXCL-10 (IP-10) was the most significantly related cytokine in response to PERV-A/C infection in MDDCs, MDMs and monocytes (Fig.5.12, 16, 19). CXCL-10 has been shown to induce chemotaxis in different cell types of the immune system by binding to the CXCR3 receptor [251]. The CXCL-10 expression is inducing in several cells signaling pathways, therefore, the CXCL-10-response is high in most virus infections, inflammation and cancer (Fig.6.1) [252].

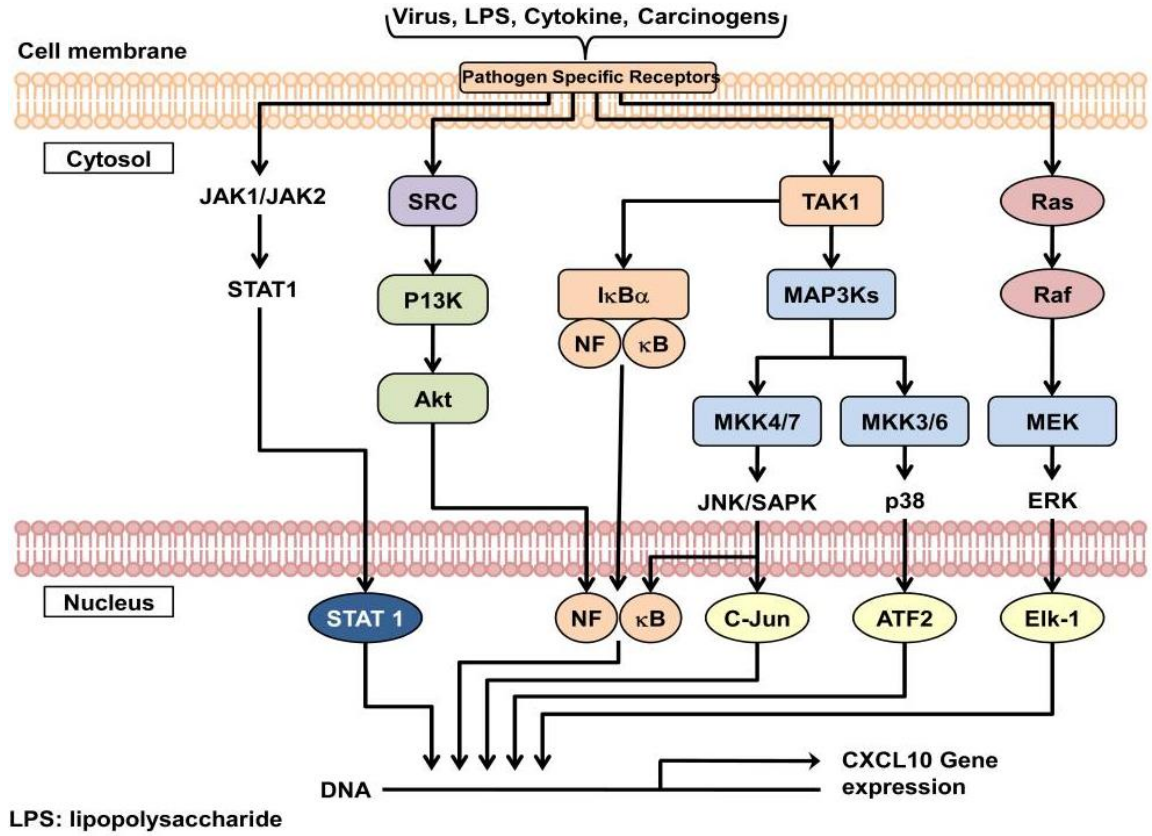

Figure 6. 1 Schematic model of the signaling pathways that play a role in the induction of CXCL-10 in human macrophages, microglia, epithelial and cancer cells [252].

In agreement with our results, one other study has demonstrated that the CXCL-10 mRNA is highly expressed in HIV-1 infected monocytes and MDDCs and the increase level is associated with HIV-1 viral load [253]. Similar to our finding that PERV-A/C potently induces CXCL-10 expression in immune cells, others have reported that IP-10 is induced at higher levels with the acute phase of Zika virus infection in monocyte of pregnant women [254]. The results in this work are in agreement with a recently report by Loevenich et al. showing that human metapneumovirus (HMPV) efficiently stimulated CXCL-10 mRNA in MDMs and MDDCs [255]. This is in line with other previous studies showing that herpes simplex virus type 1 (HSV-1) infection activates CXCL-10 gene expression in these cell types [256]. There is a controversy about the role of CXCL-10 in virus infection, some reports show that CXCL-10 can dominate and drive immune protective response functions against virus infection, like with coronavirus and herpes simplex virus type2 (HSV-2) infection [257, 258]. In contrast, others demonstrated that CXCL-10 enhances HIV-1 infection by activating virus replication in macrophages and lymphocytes [258-260]. It seems that the host immune status and genetic background influences the function of CXCL-10 and decides whether it either protects or promotes infection [261]. Moreover, in one study, it was reported that CXCL-10 acted as an antitumor agent. It stimulates damage in recognized tumor blood vessels and causes tissue necrosis in human Burkitt lymphomas in the mouse model [262].

The IFN- β was a second important innate immune response investigated in this work, which is a cytokine and belongs to a type I interferon produced by immune cells in response to viral infection and other pathogens. In contrast to CXCL-10, the induction of IFN-β in human primary cells MDMs and MDDCs to PERV-A/C infection was not significant compared with uninfected cells and the positive control poly I.C (fig.5.14, 17). Many viruses have developed mechanisms to avoid the IFN response and to facilitate their infection, like HIV-1, which manipulates the induction of IFN response in macrophage and dendritic cells through inhibition of TBK1 autophosphorylation by its accessory proteins Vpr and Vif [263]. These findings completely agreed with results documented in another study showing that single-round pseudotyped HIV-1-based reporter viruses did not activate sensing pathways and failed to induce innate immune responses in cell lines and immune cells that were otherwise responsive to external nucleic acids [264]. On the other hand, in a previous report it has been also observed that herpes simplex virus type 2, Sendai and HIV-2 viruses significantly induced IFNβ [263, 265]. In addition, it has been reported that multiple-round replication of HIV-1 can induce the innate immune responses leading to increased levels of IFN-β secretion and sensing in MDDCs before and after virus integration [266, 267].

Interferon stimulated genes (ISGs) induced predominantly by the activation of JAK/STAT pathway after IFNs bind receptors on surrounding cells. ISGs serve to stand an antiviral state within a host cell, the specific antiviral function of many ISGs has been described and the function of others ISGs remains to be determined. To check the response of ISG-54 to PERV-A/C infection, MDDCs and MDMs were used. The results show no marked response for ISG-54 to PERV-A/C in macrophage and dendritic cells (Fig.5.15, 18). Similar results were reported in previous studies that HIV-1 cannot be recognized in that way that leads to a stimulation of these ISGs by the innate immune response [263, 264]. The results of Nasr et al. demonstrated that infection of human MDMs with HIV-1 induced a number of interferon stimulated genes without stimulation of any type of interferons; this inhibition was mediated by HIV-1 inhibition of IRF3 translocation to the nucleus [268]. In addition, the authors found in another study that Vif and Vpr of HIV-1 degraded IRF3 in MDDCs and prevent the antiviral response during the very early phase of replication [269]. On the other hand, it has been found that several ISGs upregulated during HCMV infection independently of type one IFN-initiated JAK-STAT signaling, but were dependent on the intact IRF3 signaling pathway [270].

In conclusion, our findings demonstrate for the first time that MDMs, MDDCs and monocytes produce significant amounts of CXCL-10 in response to PERV-A/C infection. It has been reported in several studies that the induction of CXCL-10 can be based on different cell signaling pathways; therefore usually the CXCL-10 response is high in many virus and bacterial infections. However, MDDC, MDM cells do not mount efficient IFN-β and ISG-54 response to PERV-A/C infection, mainly due to restriction of virus reverse transcription, which prevents production of viral cDNA [271] and limits its detection through the cGAS-STING pathway. Additionally, the lack of IFN-β and ISG-54 activation by PERV-A/C in these cells might be not due to a lack of sensing pathway components but rather due to the virus not stimulating those pathways.

6.2.2 The JAK-STAT pathway is involved in the CXCL-10 response that follows an infection of human myeloid cells by PERV-A/C

After we confirmed that CXCL-10 can be induce by PERV-A/C infection, the following aim was to identify the sensors and signaling pathways, which play a role in the CXCL-10 response in human primary myeloid cells infected by PERV-A/C. Human immune primary cells were treated with different cell signaling inhibitors. The results show that the JAK-

inhibitor (AT9283) reduced the CXCL-10 induction in infected cells but the other inhibitors TBK1 (BX-795), NF-κB (Parthenolide) and a TLR7/8 antagonist failed to reduce the induction of CXCL-10 in MDM cells (Fig.5.20). The response is sensitive to the JAK-inhibitor indicating that the JAK-STAT pathway is involved in the response. In MDDCs, the JAK (AT9283) and TBK1 (BX-795) inhibitors lowered the amount of CXCL-10 activation compared with untreated cells (Fig.5.21). The JAK/STAT signaling pathway is very important in the innate immune response for transmitting extracellular chemical signals like IFN binding to the nucleus and thereby inducing the transcription of IFN-stimulated genes. The results of this work are in agreement with previous work from Sakar et al, showing that blocking of the JAK-STAT pathway by a JAK inhibitor almost completely inhibited CXCL-10 production by respiratory syncytial virus infection, that means that the JAK-STAT pathway is involved in CXCL-10 induction, while the NF-κB pathway appeared to be not or only slightly involved [272]. In another study, it was reported that human rhinovirus induced CXCL-10 activation in monocytes through JAK/STAT pathway activity [273]. The cofactor of aminoacyl-tRNA synthetase p43 induces both the mRNA and protein expression of CXCL-10 through the JAK-STAT signaling pathway in HMEC-1 cells to inhibit angiogenesis and suppress tumor growth [274]. The production and regulation of CXCL-10 depend on the type of inductions and the target cells. Rabies virus (RV) activate CXCL-10 expression in macrophages by stimulation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) [275], while in microglia in the central nervous system the induction of CXCL-10 was achieved through the activation of p38 and NF-kB pathways [276]. In contrast to the previously mentioned study, the authors found that the induction of CXCL-10 promotes cell proliferation and contributes to the development of tumors by the Raf, PI3K, p38/MAPK, JNK/MAPK and NF-κB signaling [277]. On the other hand, the abrogation of CXCL-10 expression in target cells by inhibiting the JAK/STAT1 signaling pathway might be used as anti-inflammatory effects through reduction of chemokine CXCL-10 activation, like in murine macrophage-like cells [278].

In conclusion, the expression of CXCL-10 can be induced with different cell signaling pathways depending on the type of inducers and the target cells. For PERV-A/C, the JAK-STAT pathway is the main way that PERV-A/C is using to induce CXCL-10 in human primary myeloid cells. Understanding the mechanism of CXCL-10 production and regulation is very important. It might be supportive in the treatment of human diseases, because CXCL-10 is activated and upregulated in different types of infections and diseases.

8 References

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9. Appendix

9.1 Abbreviations:

AIDS:	acquired immune deficiency syndrome
APOBEC3:	apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3
AZT:	azidothymidine
BST-2:	bone marrow stromal cell antigen
CDK1:	cyclin-dependent kinase 1
cGAS:	cyclic GMP–AMP synthase
CXCL-10:	C-X-C motif chemokine 10
CRISPR/Cas9:	clustered regularly interspaced short palindromic repeats
CT:	cycle threshold
DNA:	deoxyribo nucleic acid
dN:	deoxyneucleoside
EBV:	epstein barr virus
Env:	envelope
ERV:	endogenous retroviruses
FAM:	fluorescein amidite
FITC:	fluorescein isothiocyanate
Fw:	forward

Gag:	group-specific antigen
GAPDH:	glyceraldehyde 3- phosphate dehydrogenase
GM-CSF:	granulocyte macrophage colony stimulating factor
GMP-AMP:	guanosine monophosphate-adenosine monophosphate
HERVs:	human endogenous retroviruses
HEX:	hexachloro-fluorescein
HCMV:	human cytomegalovirus
HHV-8:	human herpes virus 8
HEK-293:	human embryonic kidney cell-293
HIV:	human immunodeficiency virus
HSV-1:	herpes simplex virus-1
HTLV:	human T-cell lymphotropic virus
ICTV:	international committee on taxonomy of viruses
IFN:	interferon
IFI16:	interferon-γ inducible protein-16
IRF:	interferon regulator factor
JAK-STAT:	Janus kinases- signal transducer and activator of transcription proteins
IP-10:	interferon gamma-induced protein-10
IRDye:	infrared dye

ISG:	interferon stimulated gene
IU:	international unit
KO:	knockout
LTR:	long terminal repeat
MAB:	monoclonal antibody
MDM:	monocyte derived macrophage
MDDC:	monocyte derived dendritic cell
MMTV:	mouse mammary tumor virus
NF-κB:	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
qPCR:	quantitative polymerase chain reaction
PAMP:	pathogen-associated molecular pattern
PBMC:	peripheral blood mononuclear cell
PBS:	primer-binding site
PERV:	porcine endogenous retrovirus
PK15:	porcine kidney-15
Pol:	polymerase
PRR	pattern recognition receptor
Rev:	reverse
RLR:	RIG-I-like receptors

RNA:	ribonucleic acid
ROX:	6-Carboxyl-X-Rhodamine (dye)
RSV:	rous sarcoma virus
RT:	reverse transcriptase
SAMHD1:	sterile alpha motif domain and HD domain containing protein 1
SIV:	simian immunodeficiency virus
SIVcpz:	SIV of chimpanzee
SIVsm:	SIV of sooty mangabey
STLV:	simian T-cell leukemia virus
TBK1:	TANK binding kinase 1
TCID50:	50% tissue culture infective dose
TLR7/8:	toll-like receptor 7/8
TRIM5:	tripartite motif-containing 5 alpha
VLP:	virus like particles
Vpx:	virus protein x

9.2 Publication and conferences participations:

9.2.1 Publication:

1. **Al-Shehabi H** et al. Human SAMHD1 restricts the xenotransplantation relevant porcine endogenous retrovirus (PERV) in non-dividing cells. J Gen Virol. 2019 Feb 15. doi: 10.1099/jgv.0.001232.

2**. Al-Shehabi H** et al. Porcine endogenous retrovirus infection induces CXCL-10 in human immune primary cells. (in process)

9.2.2 Posters:

1. **Hussein Al-Shehabi**, Uwe Fiebig, Joachim Denner, Norbert Bannert and Henning Hofmann. Human SAMHD1 restricts the xenotransplantation relevant porcine endogenous retrovirus (PERV) in non-dividing cells. The 29th Annual Meeting of the Society for Virology: GFV 20-23 March 2019 in Düsseldorf, Germany.

2. **Hussein Al-Shehabi**, Uwe Fiebig, Juliane Kutzner, Joachim Denner, Torsten Schaller, Norbert Bannert and Henning Hofmann. Human SAMHD1 restricts the xenotransplantation relevant porcine endogenous retrovirus (PERV) in non-dividing cells. International symposium 2018 - spp1923 innate sensing and restriction of retroviruses 21-22 june, 2018 in Heidelberg, Germany.

3. **Hussein Al-Shehabi**, Uwe Fiebig, Joachim Denner, Norbert Bannert and Henning Hofmann. Human SAMHD1 restricts the xenotransplantation relevant porcine endogenous retrovirus (PERV) in non-dividing cells. The 28th Annual Meeting of the Society for Virology: GFV 17-20 March 2018 in Würzburg, Germany.

4. Henning Hofmann, Katharina Tolubaev, **Hussein Al-Shehabi**, Bénédicte Vanwalscappel, Nicolin Bloch, Norbert Bannert and Nathaniel R. Landau. TLR7/8 agonists induce a post-entry SAMHD1 independent restriction to HIV-1 infection of monocytes. The 27th Annual Meeting of the Society for Virology: GFV 14-17 March 2017 in Marburg, Germany.

9.3 Independence declaration

This thesis was based on work that carried out in the period from October 15, 2015 to March 30, 2020 at the Robert Koch Institute in Berlin.

Herewith I declare that the present dissertation has been written independently and with no other sources and aids than quoted. No passages of text have been taken from third parties without having been identified as such and that all tools, personal notifications, and sources used by the applicant have been indicated in this dissertation.

Berlin/ march-2020

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