Human host factors involved in HIV-1 replication

Humane Wirtsfaktoren der HIV-1 Replikation

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Anika Hain

aus Dillenburg

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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis“ an der Heinrich-Heine-Universität Düsseldorf erstellt worden ist. Die Dissertation wurde bei keiner weiteren Fakultät vorgelegt und es wurde bislang noch kein Pomotionsversuch unternommen.

Anika Hain, Düsseldorf den

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Abstract

Like all obligate intracellular pathogens, HIV-1 is dependent on the environment of a host cell which provides suitable conditions for its replication. Cellular molecules that support infection and propagation are called host factors; presence or absence of these factors can severely influence HIV-1 infectivity and disease progression. In this study distinct host factors and their role during HIV-1 infection were examined.

At first, the function of the Il-2 inducible T-cell kinase (ITK) was analyzed. The experiments demonstrated an essential role of ITK for viral replication in Jurkat T-cells. HIV-1 entry into ITK knockdown cells was restricted, but the following viral processes like reverse transcription, integration and protein expression were less effected. Moreover, binding of viral particles to ITK deficient cells was strongly impaired. Analysis of HIV-1 attachment factor expression revealed equal levels of the CXCR4 receptor and increased amounts of CD4 and LFA-1 integrin on ITK deficient cells. Heparan sulfate, which is normally moderately expressed on Jurkat cells, was not detectable after ITK knockdown. Nevertheless, different surface expression of the investigated attachment factors was not the reason for impaired HIV-1 adhesion to ITK deficient cells.

The second part of this thesis focused on the attachment factor lymphocyte function-associated antigen (LFA-1), its ligand intercellular adhesion molecule 1 (ICAM-1) and the importance of their interaction for HIV-1 infection. The results showed ICAM-1 incorporation into HIV-1 particles and thereby enhanced viral infectivity and binding to Jurkat cells. In contrast, knockout of LFA-1 had only little effects on spreading infections with HIV-1 strain NL4.3. Decreased effects in assays using replication competent HIV-1 strains might be explained by Vpu mediated modifications of ICAM-1 by the accessory protein.

At last, APOBEC4 was found to exhibit an enhancing effect on HIV-1 replication; its expression increased production of virus particles and mediates higher viral titer in spreading replication assays. Also reporter gene expression from the LTR and several other viral and cellular promotors were enhanced after APOBEC4 co-transfection. Unlike other APOBEC proteins, APOBEC4 shows no cytidine deaminase activity and only weak interaction with single-stranded DNA *in vitro* as well as no packaging into viral particles.

Zusammenfassung

Wie alle obligat intrazellulären Pathogene, ist HIV-1 abhängig von einer Wirtszelle die geeignete Bedingungen für eine Infektion bietet. Zelluläre Moleküle, die eine virale Replikation unterstützen, haben einen wesentlichen Einfluss auf den Krankheitsverlauf nach einer HIV-1 Infektion und werden Wirtsfaktoren genannt. In dieser Arbeit wurden spezifische Wirtsfaktoren und ihre Rolle während der HIV-1 Replikation untersucht.

Als erstes wurde die Il-2 induzierbare T-Zell Kinase (ITK) analysiert. Ein knockdown von ITK in Jurkat Zellen konnte die HIV-1 Replikation vollständig inhibieren und blockierte hauptsächlich den Zelleintritt von HIV-1. Nachfolgende virale Prozesse wie die Reverse Transkription, Integration und Proteinexpression wurden von dem knockdown weniger beeinflusst. Neben der Fusion war auch die Bindung von viralen Partikeln an die Wirtszelle stark verringert. Die Quantifizierung von Oberflächenmolekülen, die für eine Bindung von HIV-1 bekannt sind, zeigte eine vergleichbare Expression von CXCR4 und erhöhte Mengen von CD4 und LFA-1 auf ITK defizienten Zellen. Die sonst moderate Expression von Heparan Sulfaten wurde durch das Ausschalten von ITK fast vollständig inhibiert. Allerdings konnte die unterschiedliche Expression der Oberflächenmoleküle nicht die verringerte Bindung von HIV-1 an knockout Zellen erklären.

Der zweite Teil dieser Arbeit beschäftigt sich mit dem LFA-1 Integrin (lymphocyte function-associated antigen 1), dessen Ligand ICAM-1 (intercellular adhesion molecule 1) und der Relevanz der Interaktion beider Moleküle für eine HIV-1 Infektion. Es konnte gezeigt werden, dass ICAM-1 in Viruspartikel verpackt wird und dadurch die Bindung und Infektiösität von Reporterviren erhöht. Jedoch war dieser Effekt bei replikationskompetentem HIV-1 über mehrere Infektionsrunden hinweg weniger ausgeprägt. Als mögliche Ursache dafür konnte eine Vpu vermittelte Modifikation von ICAM-1 identifiziert werden.

Im letzten Kapitel dieser Arbeit wird APOBEC4 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 4) als Wirtsfaktor von HIV-1 vorgestellt. Die Expression von APOBEC4 erhöhte die Produktion von viralen Partikeln und bewirkte höhere Virustiter in HIV-1 Replikationskinetiken. Auch die Expression von LTR getriebenen Reportergenen, sowie die Transkription von weiteren viralen und zellulären Promotoren war stark erhöht, wenn APOBEC4

co-transfiziert wurde. Im Gegensatz zu anderen APOBEC Proteinen wurde APOBEC4 nicht in Viruspartikel verpackt, wies keine Cytidindeaminase Aktivität auf und zeigte nur eine schwache Interaktion mit einzelsträngiger DNA.

Introduction

The HIV-1 epidemic

The origin of human immunodeficiency virus (HIV) has been mapped to western Africa, where it was transmitted from primates to human early in the 20 th century [1,2]. Transmission most likely occurred through contact to infected blood during hunting and consumption of monkey meat. The species barrier was crossed several times independently leading to the HIV-1 subgroups M, N, O and P [1]. Group M (major) evolved to the most infectious, pandemic form of HIV-1 [1] and was derived from chimpanzees in southern Cameroon which were infected with SIVcpz [3].

After cross-species transmission HIV adapted to replicate in humans and spread unnoticed until 1983 Luc Montagnier and Françoise Barré-Sinoussi identified the virus as the causative agent of the acquired immunodeficiency syndrome (AIDS) [4,5]. Today around 36.7 million people are living with HIV worldwide. In 2015, 2.1 million people were newly infected and 1.1 million died as a consequence of infection (UNAIDS 2016). Although more than 30 years of HIV research led to the development of potent antiretroviral drugs, which can slow down progression to AIDS, the infection cannot be cured and patients have to live with side effects caused by lifelong medical treatment [6]. Furthermore, emergences of drug resistant virus strains remains an unsolved problem[7].

HIV-1 pathogenesis

AIDS is characterized by a dysfunctional immune system caused by decreased numbers of CD4 T-cells and is accompanied by opportunistic infections with viruses, bacteria or parasites [8]. The most frequent way of HIV transmission is through sexual contact with an infected person or *via* mother-to-child transmission during pregnancy, delivery or breastfeeding. In addition, contaminated body fluids are a source of infection and HIV can be received by needle-sharing during intravenous drug use or by transfusion of contaminated blood [9]. After entering the human body, HIV is usually taken up by dendritic cells located at mucosal surfaces. Using these cells as a ferry, HIV is transported through the body to lymph nodes, where it is presented to T-cells and macrophages, the main target cells of infection [10].

The clinical course of infection can be separated into three stages. At first there is an acute phase three to six weeks after initial infection, which is characterized by flu like symptoms and high titers of HIV in the blood plasma. This period is followed by the chronic, asymptomatic phase were the virus titer is decreasing again because of an HIV specific immune defense and an increase in HIV-specific CD8 T-lymphocytes. Nevertheless the number of CD4 T-lymphocytes is constantly decreasing due to a permanent, low-level virus replication. The final stage of infection and the development of AIDS is specified by T-cell counts less than 200 cells/mm^3 and opportunistic infections due to dysfunction of the immune system [11].

Classification of HIV-1

HIV-1 is a member of the genus *Lentivirus* and belongs to the family of *Retroviridae*, a group of enveloped viruses with single-stranded, positive-sense RNA. Main characteristic of retroviruses is the enzyme reverse transcriptase which uses genomic RNA as a template for reverse transcription into double stranded DNA [12]. Lentiviruses are found in many mammal species, their name *lente*, latin for slow, refers to the long incubation period from infection to disease development [13].

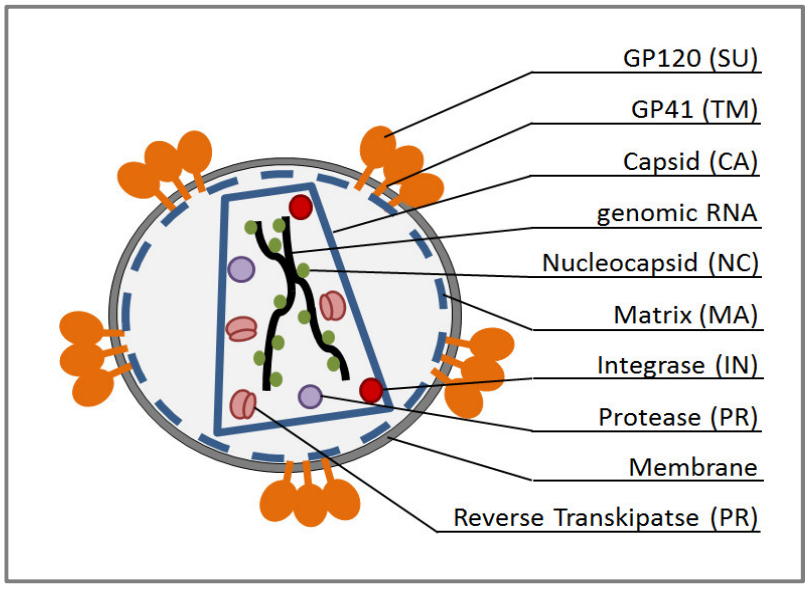

Figure 1: Composition of an HIV-1 particle.

Virion organization

The structural composition of an HIV-1 particle is shown in figure 1. Typical HIV-1 is 100 to 120 nm in size and wrapped by a host derived lipid bilayer. The membrane contains proteins from the host cell and seven to fourteen spikes; glycoprotein complexes each composed of three envelope proteins [14]. Each monomeric envelope protein consists of two non-covalently linked subunits which are encoded by the *env* gene. The outer subunit, Gp120 or surface unit (SU), is highly glycosylated and interacts with CD4 and chemokine receptors on the surface of the host cells. The second subunit, Gp41 or transmembrane unit (TM), is embedded in the cellular membrane. After binding to host cell receptors Gp41 mediates fusion of viral and cellular membranes [15].

A shell of matrix proteins (MA) is located inside the lipid membrane. Matrix proteins are synthesized as a Gag polyprotein composed of matrix (MA), capsid (CA), nucleocapsid (NC) and P6 precursor proteins. After budding from infected cells the polyprotein is cleaved by the viral protease into distinct molecules in a process which is called virion maturation [16]. Capsid proteins arrange in a conical structure and form complexes with nucleocapsid proteins, which are located inside the capsid and mediate packaging of viral RNA. Beside protease (PR) molecules also the enzymes reverse transcriptase (RT) and integrase (IN) are packaged into viral capsids. All of them are encoded by the Pol (polymerase) precursor protein [17]. The genome of HIV-1 consists of two positive single-stranded RNA molecules and measures 9 kb. The dimeric genomic RNA is packaged into particles by binding of the packaging signal Ψ to the nucleocapsid. The genome is flanked by two long terminal repeats (LTRs), repeatable sequences which are important for integration into the host cell genome and transcription of viral genes [18]. The organization of an HIV-1 genome is depicted in figure 2.

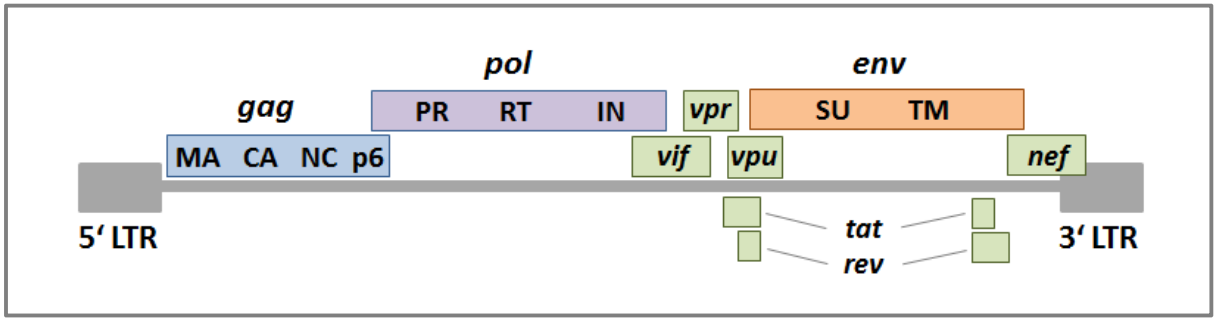

Figure 2: Schematic overview of the HIV-1 genome.

Accessory proteins

HIV-1 belongs to the complex members of retroviruses and encodes six accessory genes in addition to the structural genes listed above. The accessory proteins fulfill regulatory functions like control of gene expression, modulation of immune evasion or counteraction of cellular antiviral proteins [18] and are introduced below.

Tat and Rev

In regulation of gene expression two accessory proteins, Tat and Rev, are involved. Tat stands for transactivator of transcription and is one of the first proteins which is produced during viral replication. It binds to the transactivation-responsive region (TAR) of new transcribed RNA [19] and mediates phosphorylation of the polymerase II c-terminal domain, resulting in enhanced viral transcription and elongation [17,20]. Rev (regulator of expression of virion proteins) is expressed in a later stage during viral replication when higher amounts of unspliced RNAs are needed. It binds to an elongated stem loop structure in the *env* coding region of viral RNA, referred to as rev-responsive element (RRE) [21,22]. After binding Rev facilitates export of unspliced 9 kb and incompletely spliced 4 kb mRNA species, before they are processed by the human spliceosome[23].

Vif

Vif (viral infectivity factor) counteracts cellular cytidine deaminases by targeting them for ubiquitination and cellular degradation, thereby preventing mutation of viral nucleic acids [24].

Vpr

Vpr (viral protein R) is a protein with a broad range of functions. It is involved in nuclear import of HIV-1 pre-integration complexes and replication in non-dividing cells. Furthermore, it can induce apoptosis, regulate gene transcription and induce G2 cell cycle arrest in proliferating cells [25–27]. Novel studies describe the premature activation of the SLX4 complex by Vpr as essential function for mediating cell cycle arrest and escape from innate immune sensing [28].

Nef

Nef (negative factor) has been shown as dispensable for infection, although it elevates the viral titer. The main function of Nef consists in down regulation of cell surface proteins, like CD4 and the major histocompatibility complex I, to prevent lysis of infected cells by cytotoxic T-cells [29]. In addition to this, Nef is also responsible for CTLA-4 down regulation to ensure T-cell activation[30]. It has diverse effects on TCR signaling, like mediating location of LCK to intracellular compartments and thereby enhancing IL2 production [31,32]. Latest reports also discuss a function of Nef in combating anti-viral SERINC proteins [33,34].

Vpu

The last accessory protein, Vpu (viral protein U), is an oligomeric, integral membrane protein which is not packaged into virions. Vpu enhances budding of viruses from the host cell. This effect is achieved by two mechanisms; on the one hand by degradation of newly synthesized CD4, preventing complex formation of CD4 and HIV envelope protein in the endoplasmic reticulum [35]. On the other hand Vpu neutralizes the cellular restriction factor tetherin (BST-2) by downregulation from cell surface, which under normal conditions “tethers” virus particles to the cell surface [36].

The life cycle of HIV-1

Binding

Entry of HIV-1 into host cells requires the interaction of Gp120 envelope protein with CD4 and the chemokine receptor CXCR4 or CCR5. CD4 (cluster of differentiation 4) is a glycoprotein commonly expressed on cells of the human immune system, like T-helper cells, monocytes, macrophages and dendritic cells. Together with the T-cell receptor it recognizes MHC class II molecules on antigen-presenting cells [37]. Although HIV-1 can use a variety of chemokine receptors as co-receptor, only CCR5 and CXCR4 are reported to be used *in vivo* [38]. This related proteins belong to the family of G-Protein coupled receptors and are embedded in the cell membrane by seven trans-membrane helices [39]. CCR5 is expressed on various cell populations including T-cells, dendritic cells and macrophages, but also on endothelial cells and cells of the

nervous system [40]. CXCR4 is expressed in many tissues, including lymphocytes and mediates chemotactic movement as well as homing of hematopoietic stem cells in the bone marrow [41,42].

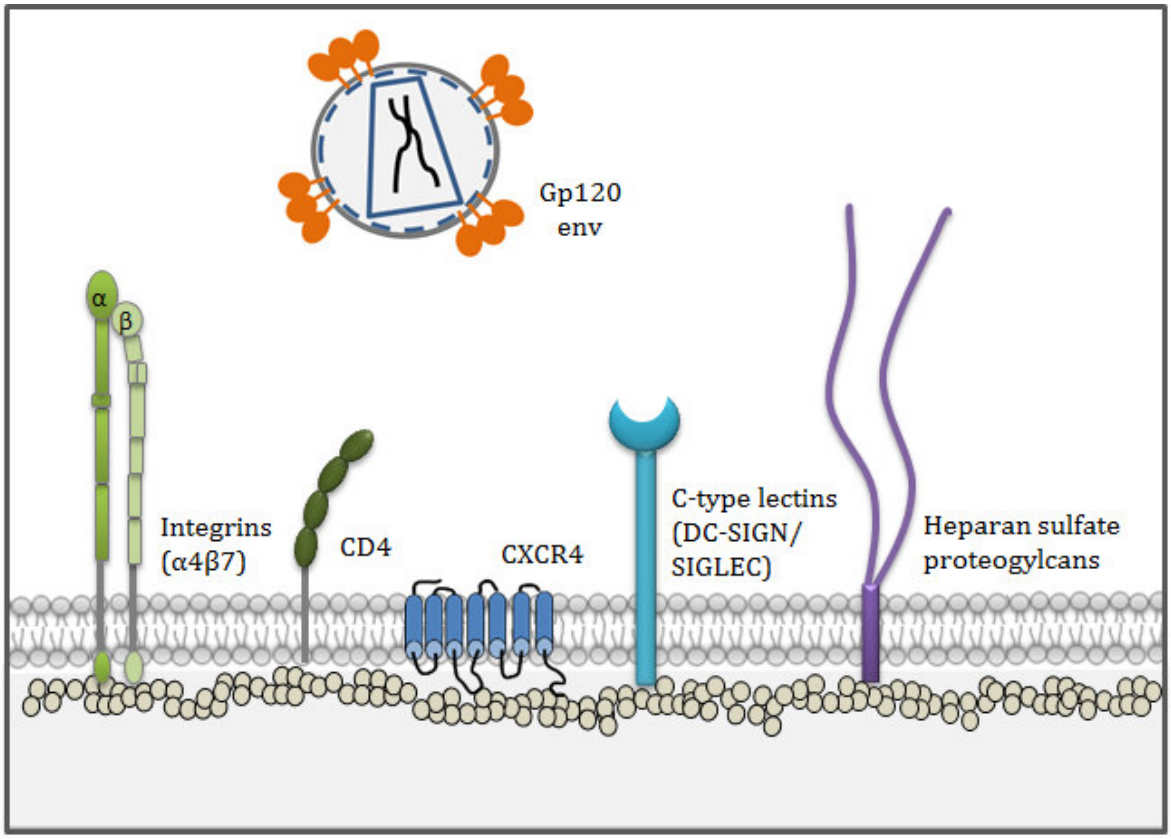

Figure 3: Attachment factors for HIV-1 binding.

Despite this, other molecules have been shown to interact with HIV-1. These molecules do not facilitate fusion or entry but may increase infectivity because of pre-concentration of virion particles at the cell surface. One example is the family of integrins. β1, β2, α4 and β7 integrins are shown to enhance infection [43], especially the α4β7 integrin on gut associated CD4 T-cells has been studied in this context [44].

Another big group of molecules, which is known to interact with HIV-1, are carbohydrate-binding domains known as C-type lectins. They bind to glycosylated motives on HIV-1, preferentially to mannose glycan's [45]. Prominent examples are DC-SIGN on dendritic cells and DC-SIGNR on endothelial cells [46], with DC-SIGN playing a role in efficient transfer of HIV-1 from dendritic cells to T-cells [47]. Also the mannose-binding protein on macrophages belongs to this group, which besides binding to HIV-1 also mediates binding to other pathogens [48].

Electrostatic interactions improving viral attachment are associated with heparan sulfates, anionic polysaccharides expressed on the cell surface of nearly all cells [49]. A schematic overview of the most common molecules involved in HIV-1 attachment is shown in figure 3.

Fusion

After the initial binding of HIV-1 to the cell surface, host cell receptors rapidly polarize to the side of attachment [50]. This process, which is also known as receptor clustering, is dependent on actin re-organization [51]. It allows binding of HIV-1 to multiple receptors at the same time, leading to further stabilization of the virus-host interaction [52,53]. Subsequent viral entry occurs at specific cell surface areas, such as membrane ruffles and microvilli [54,55]. Depending on the cell type membrane fusion takes place either on the cell surface or in endosomal compartments [56]. A mechanistic model of HIV-1 fusion is depicted in figure 4. Trimeric HIV-1 envelope proteins bind to CD4 receptors on the host cell membrane, which mediates conformational changes in the Gp120 surface unit and exposition of the co-receptor binding site. Through interaction with the co-receptor, both membranes come into closer contact. After further conformational changes and extension of the transmembrane subunit, the N-terminal end of the Gp41 fusion-peptide is inserted into the target membrane. Refolding of the peptide and six-helix bundle formation mediates membrane fusion [39].

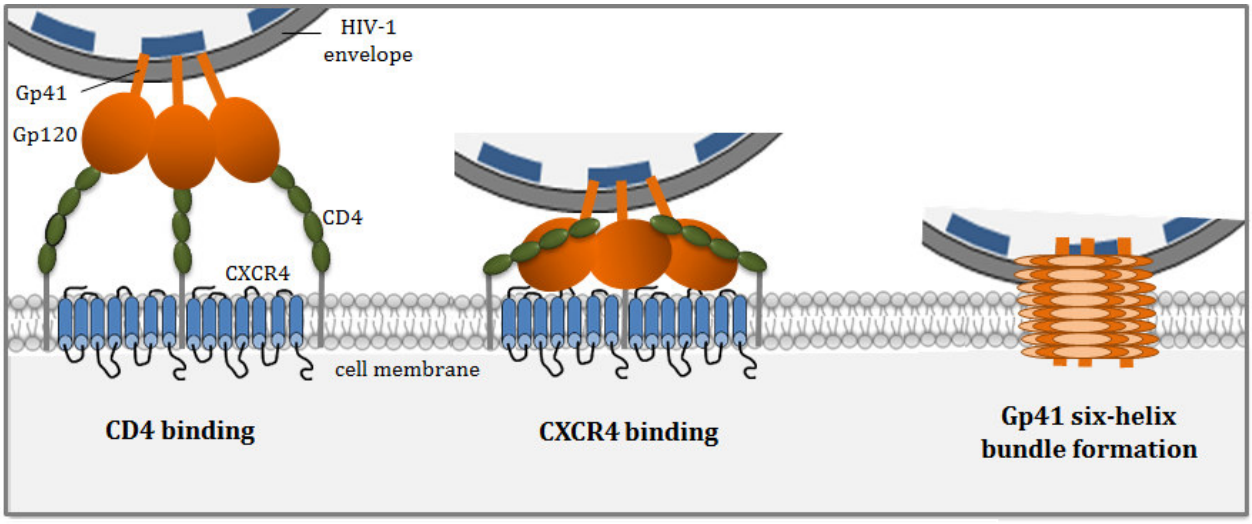

Figure 4: Model of HIV-1 fusion.

Reverse transcription and integration

Fusion of HIV-1 with the host cell membrane delivers the viral capsid into the cytoplasm, where it disassembles in a process called "uncoating". Viral single stranded (+) RNA is reverse transcribed into double stranded DNA by the brought along reverse transcriptase and tRNA primer. Reverse transcription is performed in a transcription-complex (RTC), consisting of viral genetic material as well as viral and host proteins. During transcription the complex is transported to the nucleus *via* microfilaments. Along the way it matures to the pre-integration complex (PIC), which is transported into the nucleus through the nuclear pore complex. The viral integrase joins 3'ends of the viral DNA to 5'ends of the host chromosomal DNA, resulting in the integration of viral DNA into the host cell genome and thereby creation of a provirus [57]. Not integrated viral DNA is degraded by host cell enzymes [24].

Protein expression and formation of new virions

The integrated provirus serves as template for RNA polymerase II driven gene transcription. However, the initial transcription efficiency of the LTR-promotor is low and requires transactivation through the viral Tat protein. Synthesized mRNA resembles host cell mRNA and possesses a 5'cap and 3'polyadenylation. Spliced and unspliced RNAs are transported into the cytoplasm where they are translated into HIV-1 structural and accessory proteins [23,24]. After translation Gag and Pol polyproteins assemble at the cell membrane to form new viral particles. Unspliced 9 kb RNA is used as viral genome and is incorporated into the capsid by recognition of the packaging signal (ψ) [17]. HIV-1 envelope RNA is translated at the endoplasmic reticulum (ER) and Env proteins are transported to the cell surface. Finally complete virions are released by budding from the plasma membrane. To avoid re-infection of producer cells, virions mature in a time delayed manner by cleavage of the precursor polyproteins. Mature virus can infect new target cells by interaction with CD4 and a co-receptor [17]. Picture 5 illustrates the life cycle of HIV-1 and possible restrictions by human, antiviral proteins.

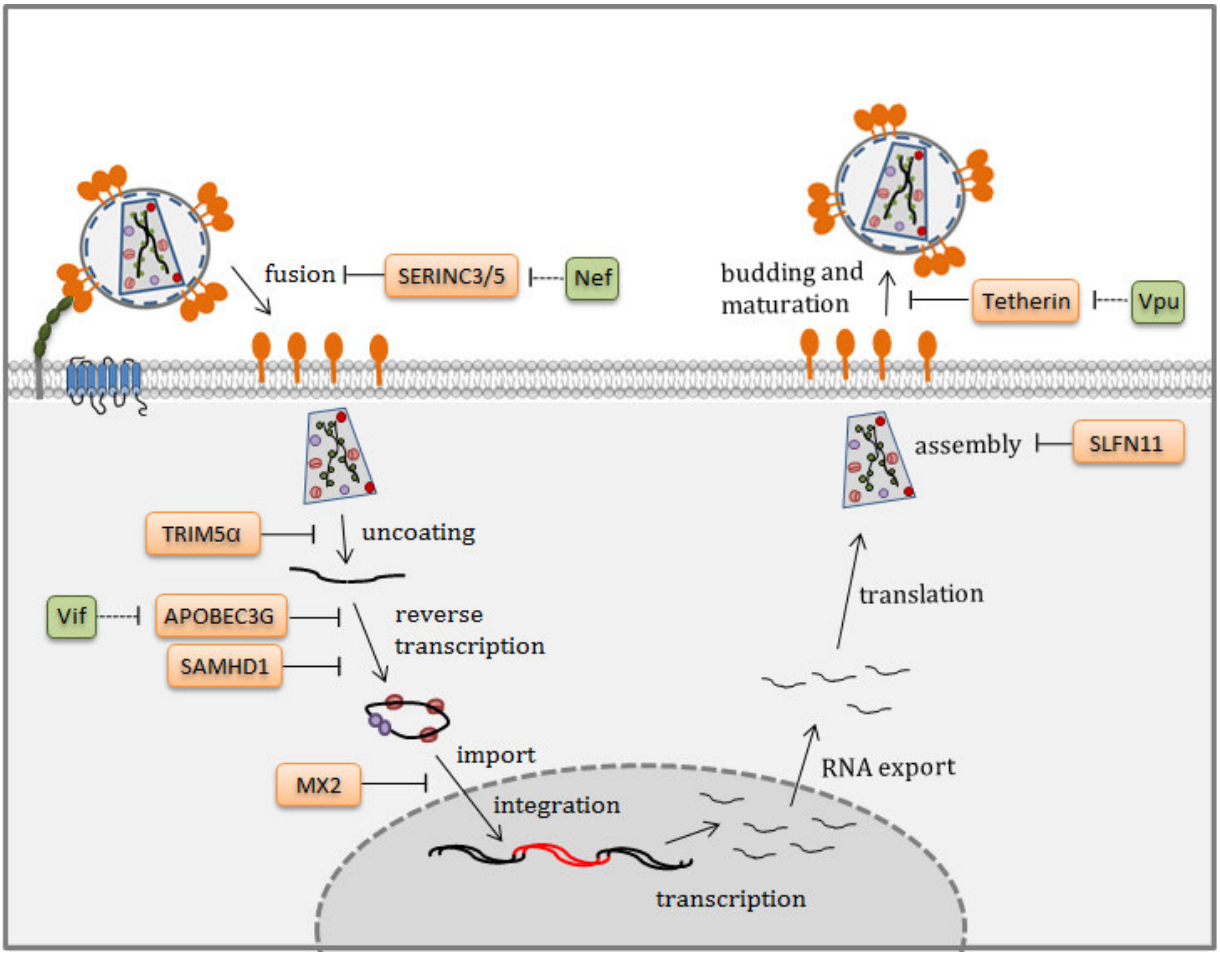

Figure 5: Model of the HIV-1 life cycle and possible restriction factors and their viral antagonists.

Human restriction factors and viral antagonists

In mammals the innate immune system constitutes the first line of defense against pathogens. This defense includes a group of proteins collectively called host restriction factors, which are constitutively expressed but also upregulated in response to interferon. They can block or "restrict" various steps in the viral life cycle of diverse retroviruses [24,58]. In response to the restrictive pressure, retroviruses on their part evolved mechanisms to counteract the host immune defense, leading to an evolutionary arms race between host and pathogen [24]. Human restriction factors involved in the HIV-1 defense are shown in figure 5 and will be discussed in the following passage in more detail.

SERINC3/SERINC5

Two restriction factors were identified in the serine incorporator (SERINC) protein family. This group of proteins consists of five members which are conserved from yeast to mammals and contain 10-12 transmembrane domains [59]. Out of this family only SERINC3 and SERINC5 restrict HIV-1 replication. The proteins are incorporated into viral particles. According to the current model they impair fusion of virus with the target cell and may also influence post entry steps. Nef probably counteracts the antiviral activity of SERINC proteins by triggering their redistribution to an endocytic compartment, which precludes them from being incorporated into viral particles [33,34].

TRIM5α

TRIM5α is a member of the TRIM protein family. TRIM stands for TRIpartite Motif which indicates that proteins of this family consist of three domains; RING, B-box and B30.2 domain [60]. The B30.2 domain of simian TRIM5α, but not of the human orthologue, binds to HIV-1 capsids and mediates a rapid, premature uncoating of the virus, which aborts infection in monkey cells [61].

APOBEC3-Proteins

Cytidine deaminsases of the APOBEC3 family (apolipoprotein B mRNA editing enzyme catalytic polypeptide 3) are active against exogenous and endogenous retroviruses, retroelements and DNA viruses [62–64]. Primates encode seven related but distinct members of APOBEC3 (A3-A, -B, -C, -D, -F, -G and –H), which contain either one or two deaminase domains [65]. APOBEC3 proteins can incorporate into budding virions by interacting with the retroviral Gag polyprotein and RNA. After infection of target cells, during reverse transcription of viral RNA into DNA, the virion-packaged A3s deaminate cytidine to uridine in the viral minus strand DNA, leading to G-to-A hyper-mutations in the plus strand of the viral genome [66]. Deamination often leads to missense mutations or pre-mature stop codons, causing inactivation of viral proteins or DNA degradation by innate immune pathways [67]. Also deaminase independent mechanisms of restriction are reported for APOBEC3 proteins, like inhibition of the reverse transcription process by interfering with tRNA primer annealing, repression of DNA synthesis or integration of viral DNA into the host cell genome [24,68,69]. Also beneficial roles of G-to-A mutations are discussed. For example, higher mutation rates can lead to viral evolution and drug resistance [70].

In CD4 T-cells A3G and A3F are the most potent inhibitors of HIV-1 [71]. To escape restriction by these proteins, HIV-1 uses its accessory protein Vif (viral infectivity factor). Vif targets A3G, A3F, A3C and A3H for ubiquitination, which mediates their proteasomal degradation [24,71,72].

SAMHD1

SAMHD1 is expressed in dendritic and myeloid cells [73] and is composed of the SAM domain, which mediates protein-protein interactions and the HD domain which owns phosphohydrolase activity. The catalytic activity of the HD domain is responsible for depleting the dNTP pool in cells causing inhibition of reverse transcription of viral RNA [74]. Whereas no HIV-1 encoded antagonist of SAMHD1 is known, SIVmac and HIV-2 express Vpx (viral protein X), which mediates degradation of SAMHD1 by ubiquitination and allows infection of macrophages and dendritic cells [75].

MX2

Myxovirus resistance 2 (MX2) together with myxovirus resistance 1 (MX1) belongs to the family of large dynamin-like GTPases. While MX1 was described as restriction factor for viruses like hepatitis B or measles virus [76], recently also MX2 was discovered to prevent nuclear import and integration of HIV-1 cDNA. Although the viral capsid was identified to determine inhibition, the exact mechanism of MX2 mediated restriction is still unknown [77].

SLFN11

Recently several novel interferon-stimulated genes with antiviral activity were discovered. One of this genes is *schlafen11* (SLFN11), it is induced by DNA, RNA and type I interferon. SLFN11 suppresses the translation of HIV-1 mRNA, probably by binding to tRNAs specific for the codon usage of retroviruses, thereby lowering tRNAs available for protein synthesis [78].

Tetherin

Tetherin (also BST2/CD317) is expressed constitutively in mature B-cells, plasma cells and plasmacytoid dendritic cells, but can be induced in response to interferon or cytokines in several other cell types; including lymphocytes and myeloid cells [79,80]. Tetherin has an unusual topology

with both ends embedded in the cellular membrane, connected by an extracellular coiled-coiled structure. Each end possesses a different type of membrane anchor; a transmembrane domain at the N-terminus and a GPI (glycosylphosphatidyl-inositol) anchor at the C-terminus [81]. It inhibits the release of enveloped virus particles by tethering them to the cell surface after budding. It is supposed that for this purpose the GPI anchor is inserted into the viral membrane or that one molecule is packaged in the virion and interacts with one molecule at the host cell membrane. HIV-1 particles sticking at the cell surface are subsequently degraded by the endosomal pathway. Nevertheless HIV-1 can replicate in Tetherin expressing cells because of the expression of the accessory gene *vpu* which mediates Tetherin degradation [24].

Chapter I

IL-2 Inducible Kinase ITK is Critical for HIV-1 Infection of T-cells

The following data are submitted for publication to the Journal of Virology (November, 2016) by
Anika Hain[1], Melanie Krämer[1,2], René M. Linka[2], Saeideh Nakehaei-Rad[3], Mohammad Reza Ahmadian[3], Dieter Häussinger[1], Arndt Borkhardt[2,#], and Carsten Münk[1,#]

[1]Clinic for Gastroenterology, Hepatology and Infectiology, Medical Faculty, Heinrich-Heine University Düsseldorf, Germany; [2]Department of Pediatric Oncology, Hematology and Clinical Immunology, Medical Faculty, Heinrich-Heine University Düsseldorf, Germany; [3]Institute of Biochemistry and Molecular Biology II, Medical Faculty, Heinrich-Heine University Düsseldorf, Germany

Running titel: ITK required for HIV-1 infection

[#]Corresponding authors:
Carsten Münk, carsten.muenk@med.uni-duesseldorf.de

Anika Hain is the first author of this study, performed 90% of the experiments and wrote the manuscript. Melanie Krämer designed and tested all small hairpin RNAs targeting ITK. René M. Linka designed and cloned CRISPR/Cas9 plasmids. Saeideh Nakehaei-Rad performed immunoblots and GST pull-downs with CDC42 and RAC1. Carsten Münk conceived the study, supervised its design and wrote the manuscript. All authors read and approved the final manuscript.

Abstract

Successful replication of *Human immunodeficiency virus type 1* (HIV-1) depends on the expression of various cellular host factors, such as the interleukin-2 inducible T-cell kinase (ITK), a member of the protein family of TEC-tyrosine kinases. ITK is selectively expressed in T-cells and coordinates signaling pathways downstream of the T-cell receptor and chemokine receptors, including PLC-1 activation, Ca^{2+}-release, transcription factor mobilization, and actin rearrangements. The exact role of ITK during HIV-1 infection is still unknown. Therefore, we analyzed the function of ITK during viral replication and showed that the attachment and entry of HIV-1 into Jurkat T-cells was inhibited when ITK was knocked down. In contrast, reverse transcription and provirus expression were not affected. Inhibited ITK expression did not affect CXCR4 receptors on the cell surface, whereas CD4 and LFA-1 integrin levels were slightly enhanced in ITK knockdown cells. Heparan sulfate (HS) expression was completely abolished in ITK depleted T-cells. However, neither HS expression nor other attachment factors could explain the impaired HIV-1 binding to ITK-deficient cells, which suggests that a more complex cellular process is influenced by ITK or that not yet discovered molecules contribute to restriction of HIV-1 binding and entry.

Importance

Although pharmaceutical treatment of patients infected with *Human immunodeficiency virus type 1* (HIV-1) has become increasingly efficient, development of drug-resistant viruses remains an unsolved problem. In this context, targeting of cellular proteins constitutes a promising alternative for medical treatment. We demonstrate here that the interleukin-2 (IL-2) inducible T-cell kinase (ITK) is important for HIV-1 infection. Knockdown of ITK in T-cells causes very early inhibition of attachment and entry of HIV-1 before reverse transcription occurs. As a host factor for HIV-1 infection, ITK constitutes a promising candidate for pharmaceutical treatment.

Keywords: HIV-1, ITK, host factor, heparan sulfate, LFA-1, entry, attachment

Introduction

Although pharmacological approaches to treating *Human immunodeficiency virus type 1* (HIV-1) infection have become increasingly efficient, eradication of the virus in infected individuals remains impossible except in rare circumstances after allogeneic stem-cell transplantation [82]. Antiretroviral therapy does not lead to virus eradication because HIV-1 can establish a highly stable reservoir of latently infected cells [83]. Additionally, highly drug-resistant virus strains are emerging during the treatment of patients, as current treatment approaches target mostly viral proteins only [84]. Thus, the control of HIV-1 by endogenous restriction factors and the dependency on specific host factors have become a focus in modern infectiology [85–87]. Approaches that enhance or reduce such endogenous factors (e.g., by influencing natural cellular processes) may be less prone to treatment failure, at least theoretically. Recently, Readinger *et al.* showed that inhibition of the interleukin-2 (IL-2) inducible T-cell kinase (ITK) influences HIV-1 infection at different levels [88].

ITK is a member of the TEC kinase family, which also includes RLK, BMX, TEC, and Bruton's tyrosine kinase (BTK) [89]. ITK is selectively expressed only in T-cells and mast cells and is absent in all other human cell types. Because HIV-1 infection requires proper T-cell activation, the need for ITK as a host factor likely is related to its role in integrating signaling pathways downstream of the T-cell receptor and chemokine receptors and regulation of processes important for T-cell activation and differentiation [90,91]. These processes include activation of RHO family GTPases RAC1 and CDC42 and actin polarization [91–94], regulation of gene expression [95–97] and intracellular calcium signaling [98]. Additionally, a lack of up-regulation of integrin adhesion was reported for ITK negative cells, probably because of an inability to recruit LFA-1 integrin and other molecules to the site of T-cell receptor stimulation [99]. Although the immune response in ITK deficient mice is impaired, they can still clear viral infections of *vesicular stomatitis virus* (VSV) as well as *vaccinia* and *lymphocyte choriomeningitis virus* [100]. Indeed, ITK is mainly involved in type 2 helper T-cell (T_H2) responses, which can mediate asthma and hypersensitivity [101]. Activation of ITK was shown to cause airway hyper-responsiveness and inflammation in mouse models [102], thus a variety of specific pharmacological ITK inhibitors have been developed and tested in early clinical trials, mostly because of their anti-allergic potential [103–106].

Our goal was to further explore the relevance of ITK for infection of HIV-1 and identify novel interaction partners that could be explored as targets to interfere with viral replication.

Material and Methods

Cell culture. Human embryonic kidney cells (HEK293T, ACC 635; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and TZM-bl cells [107] were cultured in DMEM (Biochrom, Berlin, Germany) containing 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Jurkat clone E6-1 (TIB-152, American Type Culture Collection, Manassas, USA) was cultured in RPMI medium 1640 containing 2 mM Glutamax (Thermo Fisher Scientific, Waltham, United States) supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin. For digestion of cell surface heparan sulfates $1{,}5x10^5$ cells were incubated with 5 U heparinase I and III from *Flavobacterium heparinum* (H3917, Sigma-Aldrich, Taufkirchen, Germany) for 1 h at 37 °C.

Transfections and production of viral particles. Transfections were performed in 6-well plates seeded with $5x10^5$ cells/well using Lipofectamine LTX (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. 48 h post transfection cells were used for further experiments. If cells were transfected to produce viral particles, the virus containing supernatants were collected and filtered (0.45 µm pore size). Virus titer was either determined by using the Cavidi HS lenti RT kit (Cavidi Tech, Uppsala, Sweden) or by infection of TZM-bl reporter cells. Concentrated virus supernatant was obtained by centrifugation through a sucrose cushion (20% sucrose in PBS) at 40000 g for 2 h at 4 °C.

Small hairpin RNA. Small hairpin RNA (shRNA)-mediated ITK knockdown was carried out using a set of lentivirus particles expressing different shRNAs against ITK (MISSION shRNA cat. no.SHGLY-NM_005546; Sigma), or non-target shRNAs (cat. no. SHC002; Sigma). Lentivirus particles were produced by transfecting HEK293T cells with 700 ng shRNA expression plasmid, 280 ng of pMDLg/pRRE [108] packaging construct, 110 ng pRSV-Rev [108] and 60 ng of VSV-G expression plasmid. Jurkat cells were spin-transduced via centrifugation for 1 h at 31 °C at 2000 rpm by lentivirus particles expressing shRNA. Two days after transduction cells were cultured in the presence of 2 µg/ml puromycin (Applichem, Darmstadt, Germany) for a two-week selection period.

Calcium measurements. Calcium flux analyses of human T-cells were performed according to [109]**.** Briefly, $3x10^5$ cells/mL in phenol red-free RPMI 1640 (Life Technologies) containing 10% FCS were loaded with 5 µg/mL Indo-1 AM (MoBiTec, Göttingen, Germany) at 37 °C for 45 min, followed by an additional incubation for 45 min in medium without Indo-1. Cells were kept on ice before equilibration at 37 °C for 5 min, directly before measurement. Changes in intracellular calcium were monitored using a flow cytometer LSRI (BD Biosciences, Heidelberg, Germany). Cells were illuminated using the 325 nm laser-line of a helium-cadmium laser. Fluorescence emissions at 405/30 nm (calcium-bound Indo) and 510/20 nm (free Indo) were detected simultaneously, analyzing the ratio of bound to free Indo over time. After monitoring the baseline activity for 1 min, the cells were stimulated by 10 µg/mL CD3 mAb (clone UCHT1, BD Biosciences) and cells were measured for another 6 min. To confirm proper Indo-1 loading, the cells were then treated with 10 µg/mL ionomycin (Sigma-Aldrich). Kinetics were analyzed using FlowJo v7.6.3 software (Tree Star, Ashland, USA).

q-PCR. Expression levels of ITK transcripts in Jurkat cells stably expressing sh ITK RNA were quantified by q-PCR using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, California, United States). Total RNA was isolated from cells via the RNeasy Mini kit (Qiagen, Hilden, Germany) and 1 µg of total RNA was used to produce cDNA by Quantitect Reverse Transcription Kit (Qiagen). Q-PCR-reactions with cDNA were performed in triplicates using the SYBR green master mix (Applied Biosystems) according to the manufacturer's instructions. ITK was amplified with the primers ITK_Exon1_F1 (5'-TGAACAACTTTATCCTCCTGGAAGA) and ITK_Exon1_R2 (5'-GGTTAACACAAAGAAGCGGACTTTA). After initial incubations at 50 °C for 2 min and 95 °C for 10 min, 40 cycles of amplification were carried out for 15 s at 95 °C, followed by 1 min at 60 °C. ITK levels were normalized to the human reference gene hypoxanthine-guanine phosphoribosyltransferase1 (primers HPRT1_fw 5'-GCTTTCCTTGGTCAGGCAGT and HPRT1_rv 5' GCTTGCGACCTTGACCATCT).

GST pull-down. PAK1 fused GST was expressed in *Escherichia coli* and purified using standard protocols [110,111]. Bacterial lysates were used subsequently to pull-down activated, GTP-bound CDC42 or RAC1 from total cell lysates, as described in [112].

Immunoblot analyses. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCL [pH7.6], 150 mM NaCl, 1% NP40, 1% sodium deoxycholate [SDS], plus protease inhibitor cocktail set III [Calbiochem, Darmstadt, Germany]). Protein concentration of the lysates was quantified by using Bradford reagent (Applichem). Samples were separated by SDS-Page and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with rabbit anti-ITK (ab32113, Abcam, Cambridge, United Kingdom, 1:2000), goat anti-GAPDH (EB06377, Everest Biotech, 1:20,000), rabbit anti-CDC42 (#24645, Cell Signaling, Cambridge, United Kingdom, 1:500) or mouse anti-RAC1 (88751, BD Biosciences, 1:500) followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody or goat anti-rabbit antibody (GE Healthcare, Munich, Germany) or rabbit anti-goat antibody (R131HRP, Acris), and developed with ECL chemiluminescence reagents (GE Healthcare).

Viral replication. Replication competent virus stocks were generated by transfecting HEK293T cells with 2 μg pNL4-3 [113]. Jurkat T-cells were infected with MOI 0.01 and kinetics of viral spreading was analyzed for 14 days. Virus titer in the cell culture supernatant was determined by infecting TZM-bl reporter-cells.

Luciferase assay. To generate luciferase-reporter-virus 500 ng pMDLg/pRRE packaging plasmid together with 250 ng pRSV-Rev [108] and luciferase encoding transfer-vector pSIN luc IRES GFP were co-transfected in 293T cells. For envelope expression either 300 ng VSV-G expression plasmid (pMD.G [114]) or plasmid encoding L102, a variant of the C-terminally truncated (at amino acid 712) HIV-1 Env strain BH10 (pcL102 [115]) was co-transfected. Two days after transfection viral supernatant was harvest and used to infect Jurkat cells. Luciferase activity of Jurkat cells was assayed three days post transduction using the *Steady-Glo Luciferase Assay System* (Promega) according to the manufacturer's instructions in a MicroLumat Plus

luminometer (Berthold Detection Systems, Pforzheim, Germany). Transductions were performed in triplicates; the means and standard deviations of each triplicate are shown.

ß-lactamase based virion fusion assays. ß-lactamase containing viral particles where generated by transfecting 293T cells with 3rd generation lentiviral plasmids; pMDLg/pRRE and pRSV-Rev for packaging, pSIN PPT CMV Luc ires GFP as viral genome, pMD.G or pL102 for pseudotyping and pMM310 for ß-lactamase-Vpr chimeric protein expression [116]. Two days post transfection viral supernatant was removed and concentrated 10 fold by centrifugation through a 20% sucrose gradient. For fusion assays $1x10^6$ Jurkat cells were seeded in a 24 well plate, in a total volume of 500 µl. Then 150 µl of HIV-enveloped or 80 µl VSV-G pseudotyped virus was added. After incubation for 3.5 h at 37 °C cells were washed twice with CO_2 independent, serum-free media. To allow uptake of the fluorescence substrate, cells were resuspended in 500 µl loading solution, which was prepared directly before use and consists of: CO_2 independent, serum-free media, 10 mM HEPES, 1% probenecid, 0.015% solution A (CCF2-AM) and 0.08% solution B (100 mg/ml Pluronic-F127R, 0.1% acetic acid). Solution A and B were obtained from the *GeneBLAzer Detection* Kit from Invitrogen. After incubation for 15 h at 25 °C, cells were washed with PBS and fluorescence was measured with BD FACS canto II (BD Biosciences) analysis were done using the software FlowJo 7.6.3 (Tree Star, Ashland, USA).

Biotinylation. HIV-1 envelope protein was obtained from the NIH AIDS Reagent Program (HIV-1 gp120IIIB (CHO), No. 11784) or purified with Ni-NTA Agarose beads from cell lysate after transfection with pEFgp120-linker-myc-6HIS plasmid. Proteins were labeled with activated NHS-biotins (EZ-link Sulfo-NHS-Biotion, 21326, Thermo Fisher Scientific, Waltham, United States), which covalently binds to primary amines. 5 µg protein and 100-fold molecular excess of biotin were incubated 30 min rotating at room temperature, following manufacturer's recommendations. Unbound biotin was removed by washing using desalting columns (UFC503024, Merck Millipore, Darmstadt, Germany). Lysate of un-transfected 293T cells containing no gp120 protein was processed in parallel as negative control.

Gp120 binding assay. To analyze attachment of biotinylated Gp120 protein to Jurkat cells, $5x10^5$ cells were washed and incubated with 5 µg protein on RT for 1 h in a total volume of 100 µl. After washing cells were re-suspended in 150 µl buffer containing 2 µg streptavidin APC-C7 (554063, BD Bioscience) and incubated for 30 min at RT to stain attached Gp120. After repeated washing 1% paraformaldehyd was used for fixation overnight and fluorescence was analyzed by flow cytometry. For binding and washing $HEPES^{++}$ buffer was used (binding buffer: 50 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5% BSA, 0.1% NaN_3, washing buffer: 50 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM NaCl).

Attachment assay. To study binding of viral particles to Jurkat cells, virus with Gag-EGFP fusion protein was generated by transfection of 293T cells with 800 ng pCHIV.eGFP(Env-) and 800 ng pCHIV(Env-) [117]. Fluorescent virus was incubated with target cells for 2 h at 37 °C. Cells were washed two times with PBS and re-suspended in PBS 2% PFA for flow cytometry analysis.

Expression of cell surface molecules. Amount of cell surface molecules was quantified by immunofluorescence staining and FACS analysis. Therefore, $5x10^5$ Jurkat cells were washed once and re-suspended in 100 µl PBS. For CD4 staining 10 µl mouse IgG anti-hCD4PE (Clone MT310, Dako) was added and cells were incubated 30 min at 4 °C. For CXCR4 staining 10 µl mouse IgG anti-hCXCR4 PE (555974, BD Biosciences) was used and cells were incubated 15 min at 37 °C. LFA-1 was stained with 2 µl FITC-labeled anti-CD18 antibody (Beckman Coulter) and ICAM-1 with 2 µl anti-ICAM-1 (CD54) clone HA58 FITC (eBioscience, San Diego, USA), both for 30 min at room temperature. For labeling of ICAM-1, coupled anti-mouse secondary antibody was used before fixation (Invitrogen). 2 µl anti-human SIGLEC1/CD169 (clone 7-239, eBioscience) and 2 µl anti-CD47-FITC (clone B6H12, eBioscience) were incubated for 45 min at RT. Staining of heparan sulfate (clone F58-10E4, Amsbio, Abingdon, United Kingdom) was performed for 60 min at 4 °C. As Isotype control 10 µl mouse IgG Iso PE, mouse IgG Iso FITC (BD Biosciences) or mouse IgG Iso APC (clone P3.6.2.8.1, eBioscience) was used. After washing twice with PBS, cells were re-suspended in PBS 2% PFA and fluorescence was measured with BD FACS canto II.

Results

ITK knockdown restricts HIV-1 replication in Jurkat T-cells. To investigate the function of ITK in HIV-1 replication, Jurkat T-cells were stably transduced with lentiviral particles expressing different small hairpin RNAs (shRNAs) targeting ITK. After screening nine different shRNAs (data not shown), we identified two constructs, shITK 258 and shITK 614, that reduced the cellular abundance of endogenous ITK protein (Fig. 1A). Knockdown of ITK RNA was further confirmed via quantitative q-PCR analysis and Ca^{2+} flux measurements (data not shown). The expression of CD4 and CXCR4 on Jurkat cells was determined by flow cytometry. ITK knockdown cells showed regular levels of CXCR4 on the cell surface, but levels of CD4 were moderately higher than in ITK wild-type cells (Fig. 1B). The ability of ITK to regulate RHO

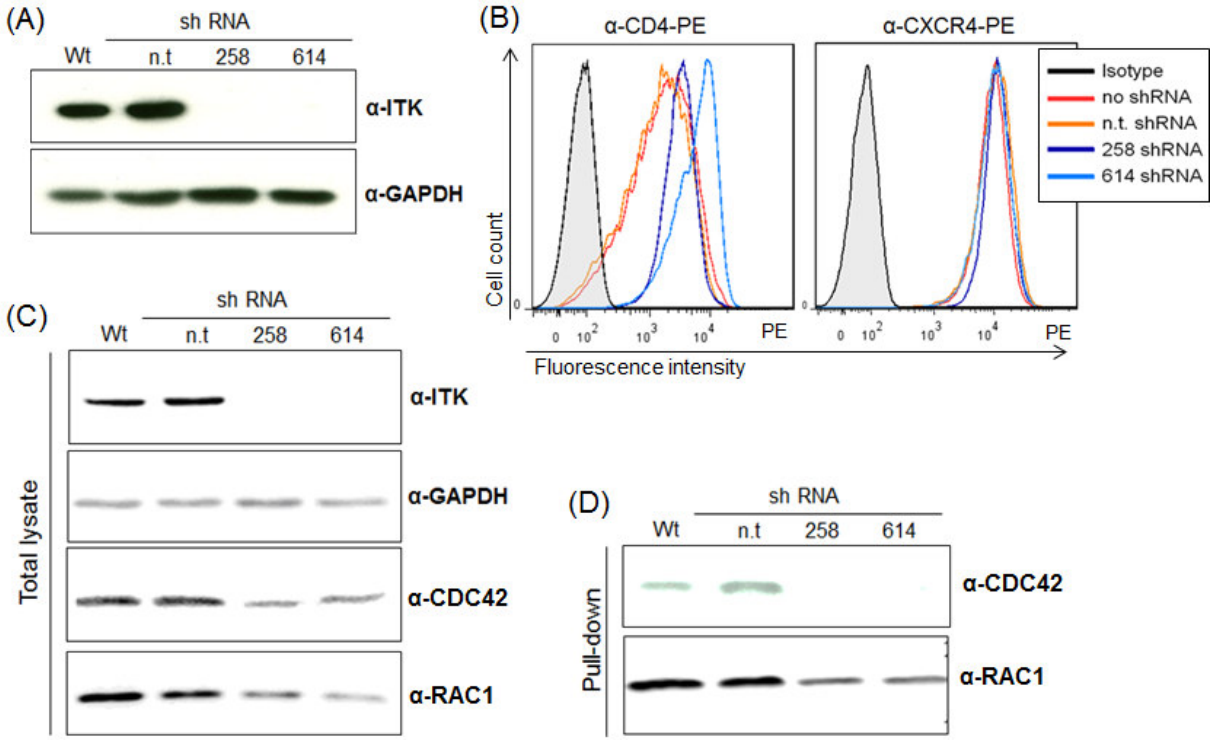

Figure 1: Characterization of ITK knockdown cells. (A) Jurkat cells expressing shRNAs targeting ITK or non-target (n.t.) shRNA were assayed for ITK expression via immunoblots using an ITK-specific antibody. Immunoblots were co-probed using anti-GAPDH antibody to show equal sample concentrations. (B) Expression of HIV-1 receptors CD4 and CXCR4 was analyzed in knockdown and wildtype cells by FACS analysis. (Filled histograms represent isotype control and open histograms staining of CD4 or CXCR4 receptor). (C) Amount of total CDC42 and RAC1 was assayed by immunoblots using CDC42 and RAC1 specific antibodies. Immunoblots were co-probed using anti-GAPDH antibody to show equal sample concentrations. (D) Amount of activated form of CDC42 and RAC1 was assayed by pull-downs, followed by immunoblot detection using CDC42 and RAC1 specific antibodies.

GTPases [91] prompted us to examine the expression and activity of CDC42 and RAC1. Immunoblots of cell lysates and pull-down of activated GTP-bound protein demonstrated higher protein levels of total and activated protein for both GTPases in ITK expressing cells compared to ITK knockdown cells (Fig. 1C), demonstrating physiological consequences of the loss of ITK protein. We also tested several ITK inhibitors *in vitro* (CTA056, BIX02524, Ibrutinib) but failed to replicate the data we obtained with shRNA-modified cells due to toxic effects of the inhibitors on our cultured cells (data not shown).

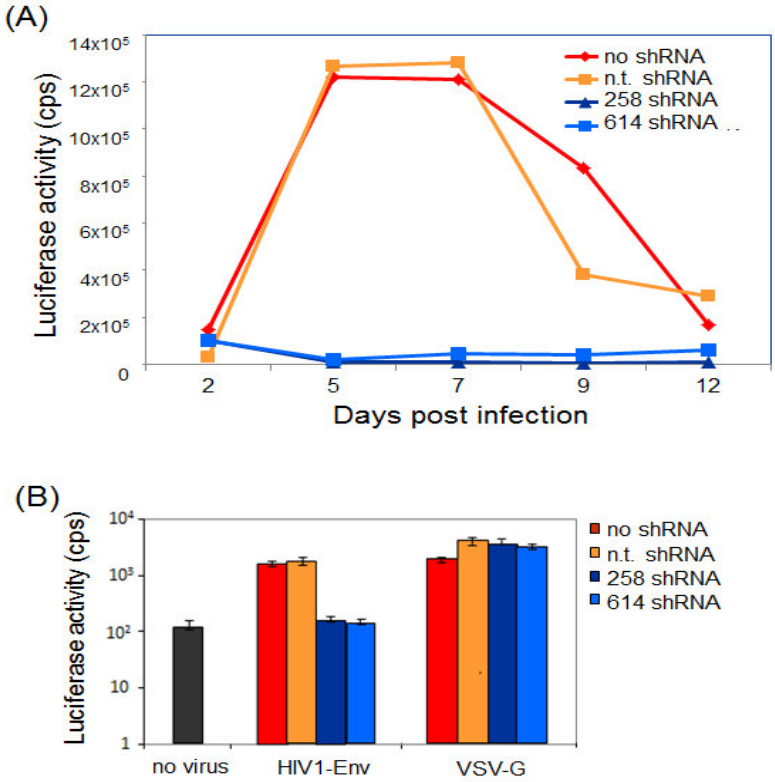

Figure 2: Loss of ITK expression blocks HIV-1 replication in Jurkat cells. (A) Replication of HIV-1 in ITK expressing wild-type and non-target (n.t.) shRNA cells was compared with replication in ITK knockdown cells (258/615 shRNA). Cells were infected with MOI 0.01 and supernatant was collected for 12 days. Virus titer was determined by infecting TZM-bl cells and measuring luciferase activity three days post infection. (B) Jurkat cells expressing ITK or no ITK were infected with single-round HIV-1 luciferase-reporter viruses pseudotyped either with HIV-1 derived envelope protein (HIV1-Env) or VSV-G protein. Luciferase activities of infected cells were determined three days post infection.

To assess HIV-1 replication, cells were infected with replication competent HIV-1 (clone NL4-3) and viral replication was monitored for 12 days. The virus titer was determined by infecting TZM-bl reporter-cells with cell culture supernatant. The results showed that wild-type and non-target (n.t.) shRNA Jurkat cells supported HIV-1 replication, whereas ITK knockdown cell lines

were resistant to viral infection (Fig. 2A). Single-round HIV-1-based luciferase viruses then were used to identify which stage of the viral life cycle was affected by ITK. Infection assays with HIV-1 enveloped particles (from clone L102) showed a strong dependency on ITK expression. HIV-reporter gene activity was strongly reduced in the ITK knockdown cells compared to Jurkat cells that expressed ITK. However, transduction of Jurkat cells with VSV-G enveloped HIV reporter viruses resulted in high luciferase activity independent of the ITK expression (Fig. 2B). This finding indicates that the ITK deficiency caused a specific HIV-1 restriction that involves the viral fusion and entry mechanism and excludes early downstream steps such as reverse transcription, integration, and protein expression.

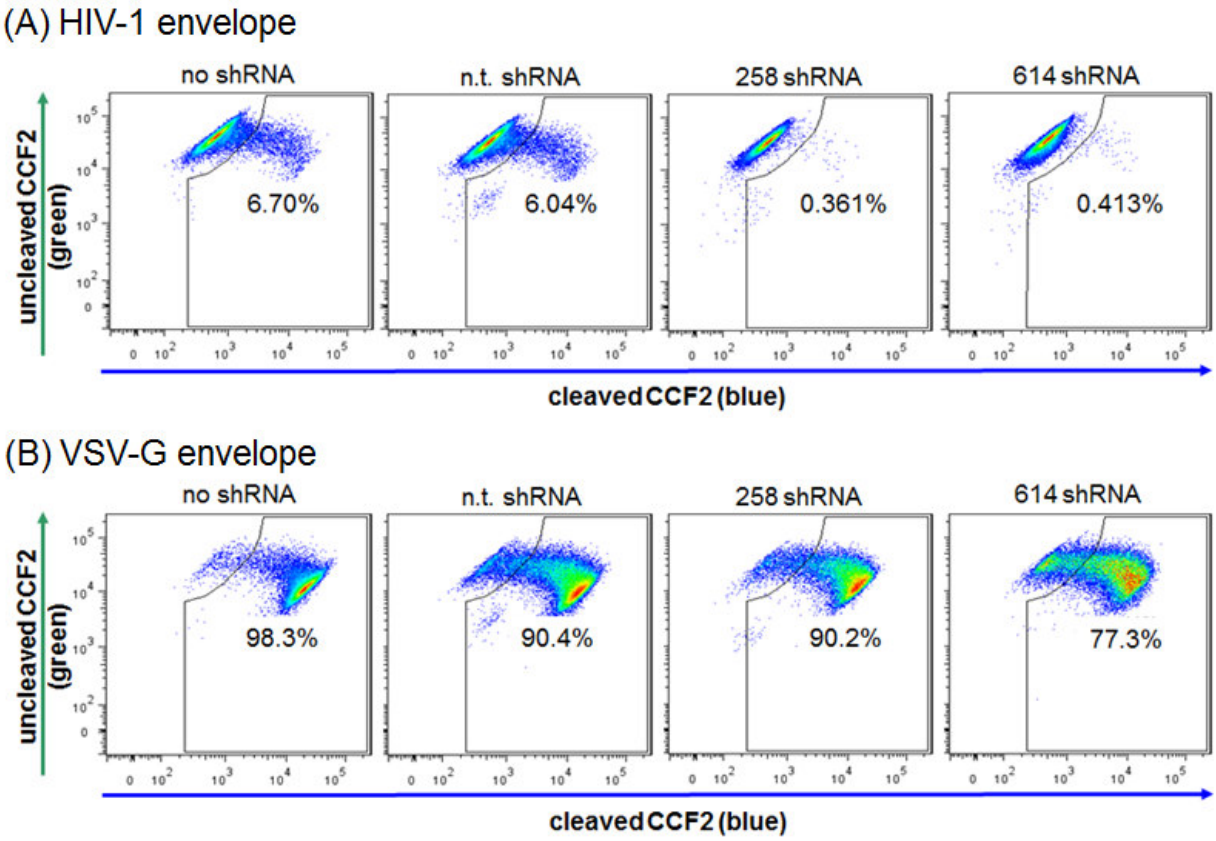

Figure 3 Fusion of HIV-1 enveloped virus with ITK deficient Jurkat cells is inhibited. (A) Jurkat cells expressing ITK (no shRNA/ n.t. shRNA) showed HIV fusion rates of 6.04% to 6.7% with HIV-1 enveloped, ß-lactamase containing viral particles, whereas ITK knockdown cells (258 shRNA/ 614 shRNA) showed strong inhibition of viral fusion. (B) The same cells were tested for HIV fusion using VSV-G pseudotyped viral particles.

Fusion of HIV-1 with the host cell membrane is inhibited in ITK knockdown cells. Because our experiments suggested an ITK-mediated defect in viral entry, virion-based fusion assays were

performed as described previously [116,118]. Viral particles were generated with incorporated ß-lactamase-Vpr chimeric proteins. After successful fusion with the host cell membrane, ß-lactamase protein was delivered to the cytoplasm where it cleaved a fluorescence substrate (CCF2). Cleavage of the dye caused a change in the emission spectrum from green (520 nm) to blue (447 nm). The viral fusion rate was calculated by flow cytometry based on the green to blue ratio of the cell population. For wild-type Jurkat and non-target shRNA expressing cells the fusion rates were 6.7% and 6.04% respectively, when HIV-1 particles with HIV envelope were applied. However, fusion rates of the same virus stock were only 0.38%-0.41% with the ITK deficient cells (Fig. 3A). VSV-G pseudotyped viruses that were used as the positive control showed equally high rates of viral fusion for wild-type, non-target shRNA expressing cells and ITK-deficient cells (Fig. 3B).

Attachment of viral particles to ITK knockdown cells is impaired in a Gp120 independent manner. Because ITK expression influenced the HIV-1 fusion process, we analyzed the interaction of the Gp120 envelope protein of HIV-1 with the host cells. Biotinylated Gp120 protein was incubated with ITK expressing and ITK knockdown Jurkat cells. After 1 h of incubation, unbound protein was removed and cell-associated Gp120 was visualized by staining with APC-Cy-7-coupled avidin and detected by flow cytometry. Compared to the negative control (cells incubated with biotinylated protein lysate without Gp120), approximately 50% of the cells were positive for APC staining. However, no difference in Gp120 binding was detected between ITK expressing and non-expressing cells (Fig. 4A).

We then analyzed the attachment of viral particles to target cells. Fluorescent viral particles were produced using viral vectors expressing a GFP-fused Gag polyprotein [117]. These GFP protein containing viruses were incubated with wild-type and ITK deficient cells. After removing unbound virus, cells were fixed with paraformaldehyde and fluorescence was analyzed by flow cytometry. Green HIV-1 particles with HIV-derived envelope protein were detected on the cell surface of ITK expressing wild-type and non-target shRNA cells, as demonstrated by a 22% and 23% increase in GFP fluorescence, respectively. In contrast, viral association with ITK knockdown cells was reduced, and the virus-associated fluorescence of ITK knockdown cells only reached 3% (Fig. 4B). Replacing the HIV-1 glycoprotein with the VSV-G protein resulted in green virions that strongly bound to all Jurkat cells irrespective of their ITK expression (Fig. 4C).

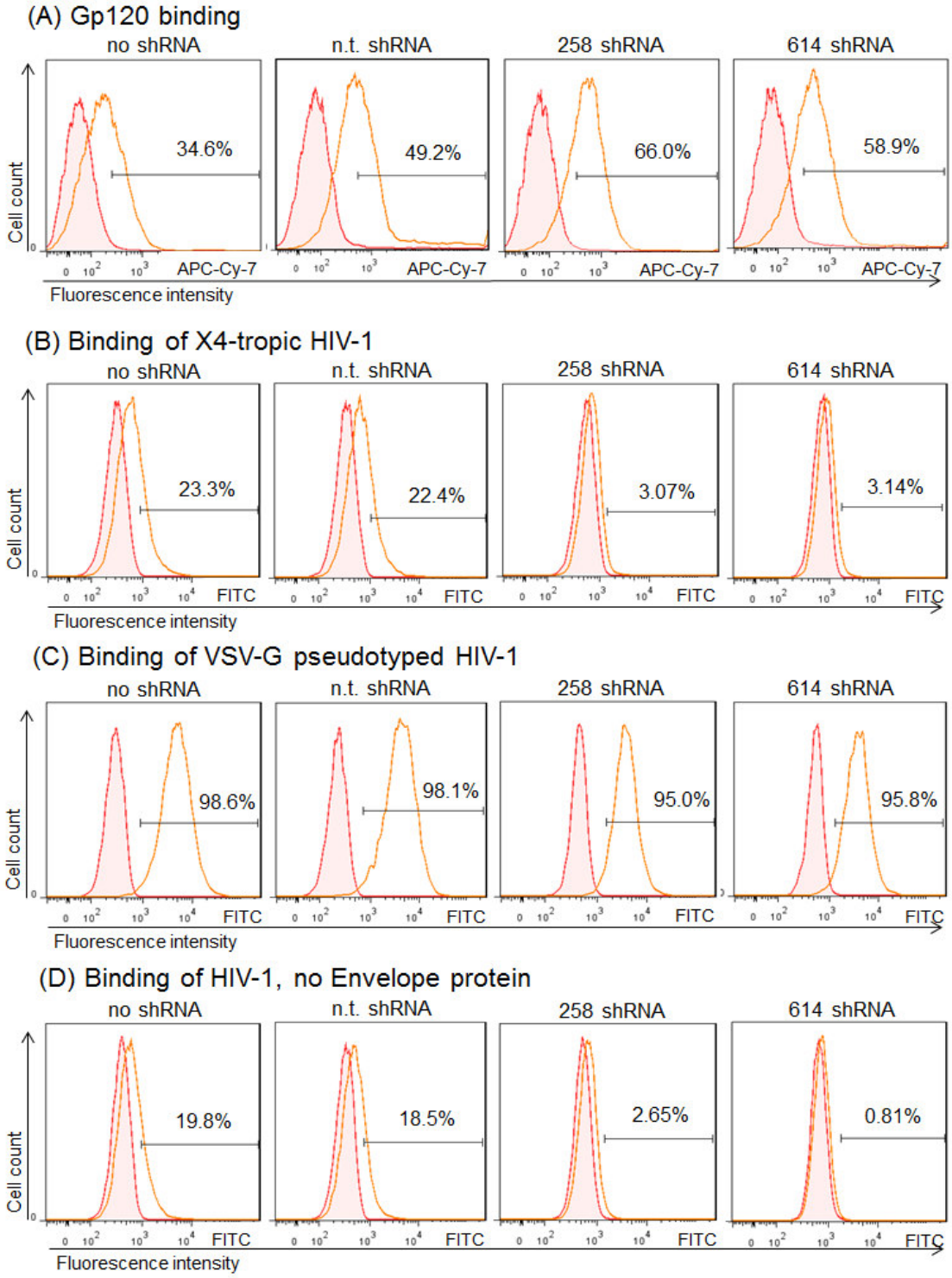

Figure 4: Binding of viral particles but not of Gp120 envelope protein is impaired in ITK knockdown cells. Jurkat cells were incubated either with biotinylated Gp120 protein (A) or GFP labeled viral particles with HIV-1 envelope (B), VSV-G envelope (C) or viral particles that were generated without any viral envelope protein (D). Data were obtained by FACS analysis of cells incubated with particle or protein. Filled histograms represent negative controls composed of cells incubated without Gp120 or virus. All figures show one representative experiment out of three repeats.

Finally, we tested green virus particles that were produced without any viral glycoprotein. These GFP particles weakly bound to ITK knockdown cells, producing a fluorescence of 0.6% and 1.3%, whereas the ITK expressing cells associated strongly with these virions, showing a fluorescence of 13.9% and 16.5% (Fig. 4D).

Cell surface expression of heparan sulfate (HS) is reduced in ITK deficient cells. Our data indicated that ITK knockdown cells have a very early block to HIV-1 infection due to processes affecting viral binding and membrane fusion. Because the expression of HIV receptors CD4 and CXCR4 were comparable in wild-type and ITK knockdown cells, other attachment factors may be affected by ITK. A potential alternative attachment protein is Siglec-1 (CD169) [119]. However, using flow cytometry analysis we found that neither wild-type nor ITK knockdown Jurkat cells expressed Siglec-1 on the cell surface, although it was detected on lipopolysaccharide treated monocyte-derived dendritic cells (data not shown). The presence of lymphocyte function-associated antigen 1 (LFA-1) can increase HIV-1 infection by improving viral attachment [120,121], and ITK was shown to be involved in regulation of integrins such as LFA-1 [99,122]. Therefore, cell surface expression of LFA-1 was analyzed using an anti-CD18 antibody. We also quantified the main interaction partner of LFA-1, ICAM-1, which was reported to enhance the infectivity of HIV-1 by incorporation into viral particles [123,124]. Although the amount of ICAM-1 was only slightly higher, LFA-1 expression was significantly enhanced in ITK knockdown cells

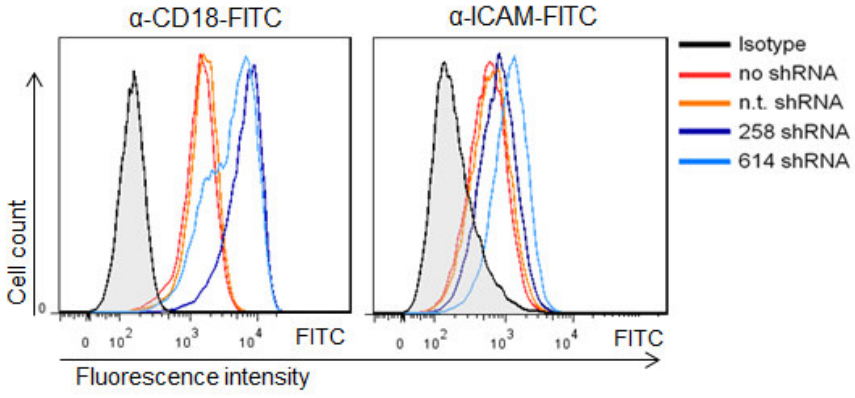

Figure 5: Cell surface expression of LFA-1 and ICAM-1 on Jurkat cells. FACS analysis of CD18 (beta subunit of LFA-1) and ICAM-1 on Jurkat cells expressing ITK (no shRNA, n.t. shRNA) or not expressing ITK (258 shRNA, 614 shRNA), by using anti-CD18-FITC antibody and anti-ICAM-FITC. Filled histograms represent the isotype control. All figures show one representative experiment out of three repeats.

compared to wild-type cells (Fig. 5). The absence of ITK may have caused up-regulation of surface LFA-1, which is, however, unlikely to restrict HIV-1 infection.

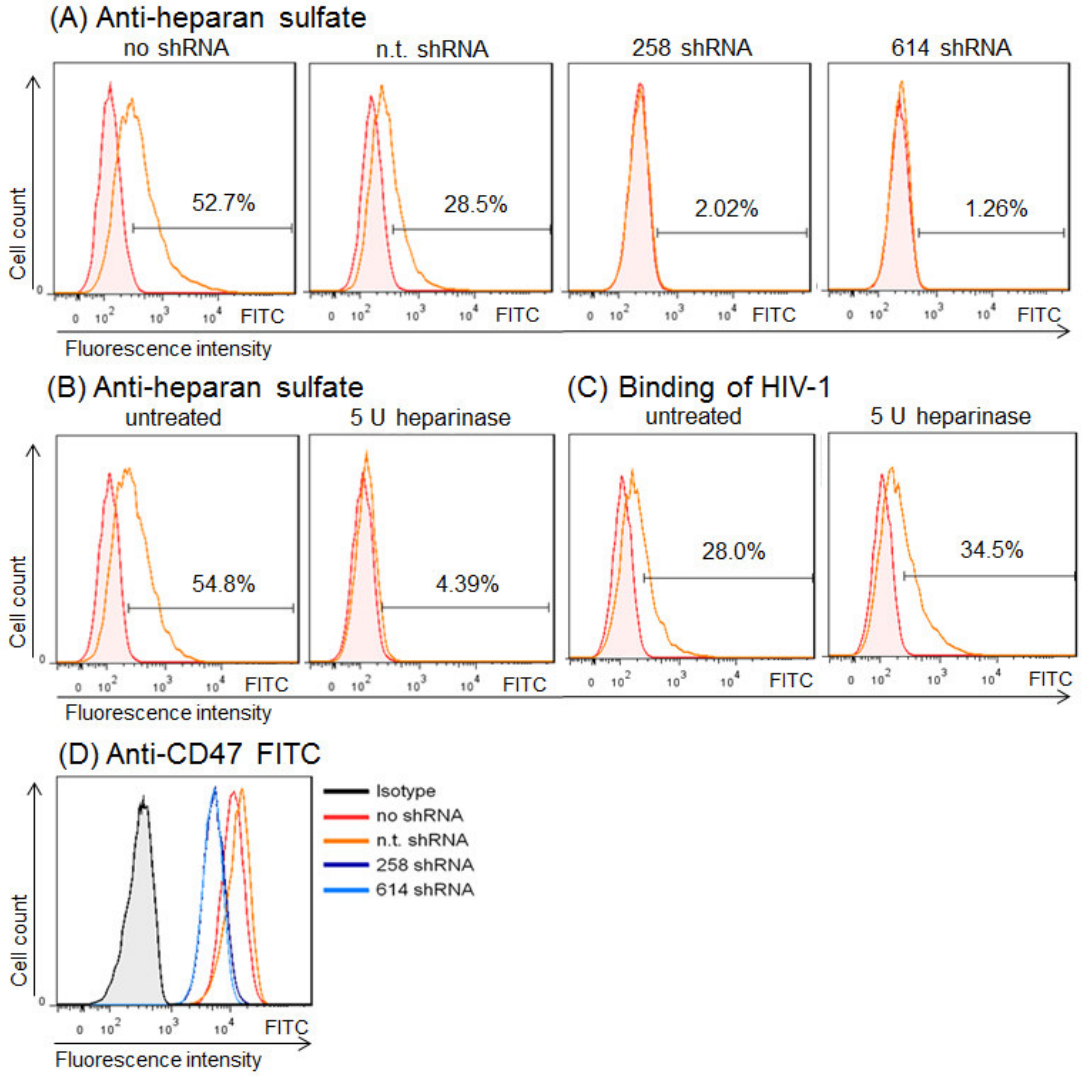

Figure 6: Heparan sulfate expression is missing on ITK deficient cells but is not responsible for reduced viral binding. (A) Jurkat cells expressing ITK (no shRNA/ n.t. shRNA) or not expressing ITK (258 shRNA/ 614 shRNA) were examined by FACS analysis for heparan sulfate on the cell surface using anti heparin sulfate-FITC. (B) Jurkat wildtype cells were treated with a mixture of 5 units (U) heparinase I and III and analyzed by FACS again for heparan sulfate expression using anti heparin sulfate-FITC, proofing the efficiency of enzymatic digestion. Filled histograms represent the isotype control. (C) Jurkat wildtype cells with enzymatically removed heparan sulfate (5 U heparinase I and III mixture) were tested for binding of GFP-labelled HIV-1 virus particles pseudotyped with HIV-1 envelope protein. (D) Jurkat cells expressing ITK (no shRNA/ n.t. shRNA) or not expressing ITK (258 shRNA/ 614 shRNA) were analyzed by FACS analysis for CD47 using anti-CD47-FITC antibody. Filled histograms represent the isotype control.

We also analyzed the presence of HS, a linear sulfated glycosaminoglycan that has been implicated being important for attachment of HIV-1 (for review [49]). HS usually is part of a proteoglycan (HSPG), consisting of a cell surface protein with one or more HS chains covalently attached [125]. Only a small number of HSPGs (< 20) have been identified, whereas hundreds of proteins have the capacity to interact with HS (for review [126]). We measured the HS level on the cell surface of our Jurkat cells with and without ITK expression by flow cytometry using an antibody reacting with the 10E epitope. That epitope is present on many types of HS including N-acetylated and N-sulfated glucosamine residues. Much to our surprise, ITK deficient cells lacked most of the HS that was detected on ITK expressing cells. While wild-type cells showed moderate expression, HS was not detectable on knockdown cells (Fig. 6A). To investigate the importance of HS for HIV-1 attachment, HS was enzymatically removed by a mixture of heparinase I and III. Jurkat wild-type cells, which positively stained for HS expression, had a significantly reduced amount of HS molecules on their cell surface after the enzymatic treatment (Fig. 6B). However, the susceptibility to HIV-1 attachment of HS-stripped cells did not differ from that of not digested cells (Fig. 6C). One of the highly expressed proteins in Jurkat cells that is modified by HS is CD47 [127]. We detected CD47 on all four types of Jurkat cells, with moderately reduced surface levels on ITK knockdown cells (Fig. 6D).

Discussion

The T-cell specific TEC kinase ITK is involved in regulation of various processes, including actin reorganization, Ca^{2+} mobilization, and control of transcription factors. Therefore, ITK plays an important role during cell differentiation and activation (for review see [128]). Because proper T-cell activation is required for HIV-1 infection, a function for ITK as a host factor for HIV-1 replication was expected, and a recent study confirmed a block of HIV-1 at various steps of the viral life cycle after ITK inhibition [88]. Here we show that down-regulation of ITK expression *via* RNA interference (RNAi) led to severely reduced HIV-1 expression and spreading in human Jurkat T-cells as well as reduced membrane fusion of HIV-1 caused by an impaired virus particle attachment. The lower capacity for virus binding might be explained by defects in actin reorganization and receptor clustering, as ITK was shown to be important for control of the actin cytoskeleton and local enrichment of adapter molecules as well as gp120-induced cytoskeleton reorganization [88,101]. In our experiments the typical F-actin depolymerization in Jurkat cells incubated with HIV-1 was not observed in ITK knockdown cells (data not shown). This observation is in agreement with the reduced expression of active RHO GTPases in knockdown cells (Fig. 1C), but the absence of attached viral particles likely contributed to the missing actin depolymerization. In contrast, HIV-1 particles that were pseudotyped with VSV-G were able to undergo membrane fusion and infection in ITK deficient cells. These observations indicate that the step of viral attachment and fusion is the impaired function in ITK knockdown cells. Although VSV entry also requires actin reorganization mediated by RHO GTPase signaling, the route of entry and the involved molecules differ. HIV-1 enters cells in a pH independent way at the plasma membrane, whereas VSV-G mediates pH dependent fusion via endocytosis in clathrin coated vesicles [129]. Thus, for HIV-1 entry the RHO GTPases RAC1, CDC42 and RHOA play a dominant role [87,130–132], whereas RHOB and RHOC seem to be more important for VSV entry [133].

The expression levels of the HIV-1 receptors CD4 and CXCR4 in wild-type and ITK knockdown cells were similar, with slightly higher CD4 levels in the ITK deficient cells. We conclude that the attachment of HIV is not much affected by gp120 binding to CD4/CXCR4, which appears to be needed only to induce membrane fusion. By analyzing alternative attachment factors on Jurkat cells, we found that ITK knockdown cells expressed higher levels of the integrin LFA-1, slightly reduced levels of CD47, and no HS compared to wild-type cells. The anti-HS antibody used in our study recognized the 10E4 epitope that is present in many types of HS. Thus, it is likely that

ITK knockdown cells do not express HS on the cell surface. Alternatively, ITK knockdown cells could express HS variants that do not react with the anti-HS antibody. HS moieties on cell surfaces are known to provide attachment sites for many viruses, including foamy retroviruses [134,135]. In line with the importance of HS for foamy infection, infection of ITK knockdown cells by prototype foamy viruses revealed a 10-fold restriction compared to infection of ITK-expressing Jurkat cells (data not shown). Studies have also shown that HS is important for attachment of HIV-1 to target cells [49,136–141]. Most of these studies concluded that HS interacts with the viral envelope protein. Other studies also reported that envelope protein-free HIV particles bind to cells efficiently via attachment caused at least in part by HS [142,143]. In these studies, treatment of HeLa cells with heparinase I reduced binding of envelope protein free HIV particles by 25%, and the highest heparinase concentrations reduced binding by 50% [142]. We found that HIV-1 particles without a viral glycoprotein bound to Jurkat wild-type cells but not to ITK knockdown cells and that the enzymatic removal of HS from the cell surface of Jurkat cells by a mixture of heparinase I and III did not reduce this binding. These data indicate that Jurkat T-cells contain an HS-independent attachment factor that does not interact with the HIV-1 envelope protein. This factor, however, has yet to be identified. The striking correlation between very low HS expression in ITK knockdown cells and the absence of attached HIV-1 particles may point to a more complex mechanism that cannot be completely described by cell surface staining using the anti-HS antibody. Finally, binding of the HIV particles in Jurkat T-cells might be regulated by a HS-modified protein, and the observed lack of HS may be the result of down-regulation of such a protein. Altogether, our results support the premise that ITK is an important protein that modulates the permissivity of Jurkat T-cells for HIV-1 infection that involves an unknown mechanism for HIV-1 attachment.

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Chapter II

ICAM-1 Incorporation Enhances Binding of HIV-1 but not it's Infectivity

The following data are designated for publication to Retrovirlogy by Anika Hain[a], René Martin Linka[b], Zeli Zhang[a], Arndt Borkhardt[b], Dieter Häussinger[a] and Carsten Münk[a, *]

[a]Clinic for Gastroenterology, Hepatology and Infectiology, Medical Faculty, Heinrich-Heine-University Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, Germany
[b]Department of Pediatric Oncology, Hematology and Clinical Immunology, Center of Child and Adolescent Health, Medical Faculty, Heinrich-Heine-University Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany

[#]Corresponding authors:
Carsten Münk, carsten.muenk@med.uni-duesseldorf.de

Anika Hain is the first author of this study and performed 90% of the experiments and wrote the manuscript. René M. Linka designed and cloned all CRISPR/Cas9 plasmids. Zeli Zhang cloned the pSIN Nan-Luc IRES GFP plasmid and helped writing the manuscript. Carsten Münk conceived the study, supervised its design and helped writing the manuscript.

Abstract

Infection of host cells with HIV-1 is influenced to a large extent by the efficiency of viral attachment. Beside the entry receptors CD4 and CXCR4/CCR5, several additional factors are discussed to enhance HIV-1 binding. One example is the lymphocyte function-associated antigen-1 (LFA-1), which stabilizes virus-cell and cell-cell contact by interacting with its ligand ICAM-1. In our study we show induction of ICAM-1 expression in 293T cells in response to transfections with any nucleic acids, as well as incorporation of ICAM-1 into produced virus particles. ICAM-1 incorporation increases binding of GFP-labeled viral particles to Jurkat T-cells and infectivity of luciferase-reporter viruses. Knockout of LFA-1 abolishes the effect of ICAM-1 on binding but has no influence on infectivity and only minor effects on spreading replication. HIV-1 accessory protein Vpu, which is known for influencing expression of cell surface receptors, was shown here to modify ICAM-1 expression and might explain less relevance of LFA-1/ICAM-1 interactions in spreading replication assays.

Keywords. LFA-1, ICAM-1, HIV-1, Binding, vpu

Background

HIV-1 enters host cells by binding to CD4 and a co-receptor; CXCR4 or CCR5. Beside these principal receptors several other molecules have been identified and are discussed to be involved in HIV-1 attachment and infection [49,144–147]. One example is the membrane glycoprotein lymphocyte function-associated antigen (LFA-1), an integrin which is composed of the integrin alpha L chain ITGAL (CD11) and the beta 2 chain ITGB2 (CD18). It is expressed on T-cells, the main targets of HIV-1 infection, where it functions in recruitment of the cells to sites of infection and interacts with antigen-presenting cells [148]. For binding to one of the interaction partners ICAM-1 (CD54), ICAM-2 (CD102) or ICAM-3 (CD50), LFA-1 needs to be activated first. Activation is either mediated by outside-in signaling through ligand binding to LFA-1 [149,150] or inside-out signaling through signals received by other receptors like chemokine receptors, the T-cell receptor or selectin ligands [148]. Subsequent binding is mediated by conformational changes (affinity), characterized by opening of the headpiece and exposure of the ligand binding side [148,151] as well as lateral diffusion [152] and spatial reorganization [153] of the molecule (avidity). Expression of ICAM-1, ICAM-2 and ICAM-3 is reported for T-cells [120], with ICAM-1 being upregulated after T-cell activation [154]. Also endothelial cells constitutively express ICAM-1 at low levels, which can be significantly upregulated after stimulation with pro-inflammatory cytokines like TNFα, IL-1, IFNγ [155]. During production of HIV-1 cellular ICAM-1 is selectively packaged into new particles by interaction with the viral matrix protein [123,156]. In addition, elevated attachment and entry of “cell free” HIV-1 to CD4 T-cells is mediated by incorporated ICAM-1 [124,157–159]. Since LFA-1 and ICAM-1 are also integral components of the HIV-1 virological synapse, a supramolecular structure which mediates efficient viral transmission between infected and uninfected T-cells, it is speculated that interactions of this adhesion molecules are not only important for infection with cell-free virus particles but also for transmission of cell-associated viruses by direct cell to cell contact [120,160]. While studies on HIV-1 transfer from dendritic cells to CD4 T-cells support the relevance of LFA-1/ICAM-1 interactions [161,162], the importance for virus transmission among T-cells remains controversial [163–166]. In this study we further characterized the role of LFA-1 and ICAM-1 during HIV-1 infection and replication in T-cells.

Methods

Cell culture. Human embryonic kidney cells (HEK293T, ACC 635; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and TZM-bl cells [107] were cultured in DMEM (Biochrom, Berlin, Germany) containing 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Jurkat clone E6-1 (TIB-152, American Type Culture Collection, Manassas, USA) was cultured in RPMI medium 1640 containing 2 mM Glutamax (Thermo Fisher Scientific, Waltham, United States) supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin. For proteasome inhibition cells were incubated with 25 µM MG132 (474790, Calbiochem, Darmstadt, Germany) eight hours before preparing cell lysates.

Transfections and production of viral particles. Transfections were performed in 6-well plates seeded with $5x10^5$ cells/well using Lipofectamine LTX (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. 48 h post transfection cells were used for further experiments. If cells were transfected to produce viral particles, the virus containing supernatants were collected and filtered (0.45 µm pore size). Virus titer was either determined by using the Cavidi HS lenti RT kit (Cavidi Tech, Uppsala, Sweden) or by infection of TZM-bl reporter cells. Concentrated virus supernatant was obtained by centrifugation through a sucrose cushion (20% sucrose in PBS) at 40,000 g for 2 h at 4 °C.

Plasmids. To produce HIV-1 luciferase-reporter virus (HIV-Luc) pMDLg/pRRE packaging plasmid together with pRSV-Rev [108] and luciferase encoding transfer-vector pSIN Nano-Luc IRES GFP were co-transfected in 293T cells. Luciferase plasmid was generated by cloning Nano-Luc luciferase gene from Promega (vector NL1.1) in pSIN.PPT.CMV.IRES.GFP (Addgene plasmid #17448) [167] vector using BamHI and NheI restriction sites. For envelope expression either VSV-G expression plasmid (pMD.G [168]) or plasmid encoding L102, a variant of the C-terminally truncated HIV-1 Env strain BH10 [115] (kindly provided by Barbara Schnierle, Paul-Ehrlich-Institut, Langen, Germany), was co-transfected. For targeted gene knockout the lentiviral packaging plasmid psPAX2 (Addgene plasmid #12260) together with the VSV-G envelope expression plasmid were used to deliver pLenti-ITGAL vector into Jurkat cells. pLenti-ITGAL

expresses Cas9 and sgRNA targeting ITGAL gene and was generated by cloning TGCCCGACTGGCACTGATAGAGG oligo into pLentiCRISPRv1 vector [169]. pcDNA3.1(+) plasmid (Thermo Fisher Scientific) expressing full-length human ICAM-1 cDNA was kindly provided by Michel Tremblay [123]. pcDNA3.1-ICAM-1-HA expressing ICAM fused to three c-terminal HA-tags was generated by PCR using primers ICAM1 fw (gcggatccaccatggctcccagcagcccc) and ICAM_HA rev (ccgaattctcaagcgtaatctggaacatcgtatgggtagggaggcgtggcttg) and pcDNA3.1-ICAM-1 as template. The PCR product was cloned via BamHI/ EcoRI into pcDNA3.1(+) (Thermo Fisher Scientific). pcDNA-Vphu plasmid expressing the Vpu protein of HIV-1 NL4-3 was obtained from NIH Aidsreagents [170].

Viral replication. Replication competent virus stocks were generated by transfecting HEK293T cells with 2 µg pNL4-3 [113]. Jurkat T-cells were infected with MOI 0.01 or MOI 0.1 (MOI based on titration in TZM-bl cells), washed 24 h later and cultured up to 18 days. Cell culture supernatant was collected every 2 or 3 days, stored at -80 °C and used to infect TZM-bl cells.

LFA-1 knockout. Lentiviral particles were produced by transfecting HEK293T cells with 800 ng pLenti-ITGAL, 800 ng packaging construct psPAX2 and 300 ng VSV-G expression plasmid. Jurkat cells were spin-transduced via centrifugation for 90 min at 1200 rpm at room temperature. Three days after transduction medium was replaced with medium containing 2 µg/ml puromycin (Applichem, Darmstadt, Germany).

Luciferase assay. To generate luciferase-reporter virus 500 ng pMDLg/pRRE packaging plasmid together with 250 ng pRSV-Rev and 500 ng luciferase encoding transfer-vector pSIN Nan-Luc IRES GFP and 300 ng envelope expression plasmid pL102 were co-transfected in 293T cells. For virus with additional ICAM-1 500 ng pcDNA3.1-ICAM-1 was co-transfected. Two days after transfection viral supernatant was harvested and used to transduce Jurkat cells. Luciferase activity was assayed three days after transduction using the Nano-Glo Luciferase Assay System (Promega, Madison, United States) according to manufacturer's instructions in a MicroLumat

Plus luminometer (Berthold Detection Systems, Pforzheim, Germany). Transductions were performed in triplicates; the means and standard deviations of each triplicate are shown.

Attachment assay. To study binding of viral particles to Jurkat cells, virus with Gag-EGFP fusion protein was generated by transfection of 293T cells with 800 ng pCHIV.eGFP(Env-) and 800 ng pCHIV(Env-) [117]. Fluorescent virus was incubated with target cells for 2 h at 37 °C. Cells were washed two times with PBS and re-suspended in PBS 2% PFA for flow cytometry analysis.

Expression of cell surface receptors. LFA-1 and ICAM-1 proteins were quantified by immunofluorescence staining and flow cytometry analysis. For LFA-1, $5x10^5$ Jurkat cells were incubated with FITC-labeled anti-CD18 antibody (Beckman Coulter, 1:50 dilution) for 30 min at room temperature, washed with PBS and stored in PBS 2% PFA. For ICAM-1 staining cells were also incubated for 30 min at room temperature with anti-ICAM-1 (CD54) clone HA58 FITC (eBioscience, San Diego, USA, 1:50 dilution). Coupled anti-mouse secondary antibody was used for labeling before fixation (Thermo Fisher Scientific). Fluorescence was measured with BD FACS canto II (BD Biosciences, Heidelberg, Germany) and analysis was done using the software FlowJo 7.6.3 (Tree Star, Ashland, USA).

Proliferation assay. To measure proliferation rates, cells were loaded with CellTrace CFSE dye (Thermo Fisher Scientific) following manufacturer`s recommendations. Cells were grown under regular culture conditions and fluorescence of CFSE was measured in a time interval of 24 hours, beginning directly after staining and ending after 96 hours. As negative control cells were incubated with 100 µg/ml Cisplatin (Accord Healthcare, Freilassing, Germany) 1 h before CFSE staining.

Immunoblot analyses. Transfected cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCL [pH7.6], 150 mM NaCl, 1% NP40, 1% sodium deoxycholate [SDS], plus protease inhibitor cocktail set III [Calbiochem]). Protein concentration of the lysates was quantified by using Bradford reagent (Applichem, Darmstadt, Germany). Samples were separated

by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with anti-p24/p27 Mab AG3.0 (1:50, NIH Aidsreagents [171]), anti-GAPDH C-terminus (1:16000, Everest Biotech, Oxshire, UK), anti-ICAM-1 (1:1000 overnight at 4 °C, eBioscience) or anti-vpu (1:20000, NIH Aidsreagents [172]) followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody or goat anti-rabbit antibody (GE Healthcare, Munich, Germany) and developed with ECL chemiluminescence reagents (GE Healthcare).

Immunoprecipitation To determine ICAM-1 and Vpu binding, 293T cells were co-transfected with 1 µg ICAM-1-HA and 1 µg Vpu HIV wt. 48 h later, the cells were lysed in IP-lysis buffer (50 mM Tris/HCl pH 8, 1 mM PMSF, 10% Glycerol, 0.8% NP-40, 150 mM NaCl, and protease inhibitor cocktail set III (Calbiochem). The lysates were cleared by centrifugation. The supernatant was incubated with 20 µl α-HA Affinity Matrix Beads (Roche) at 4 °C for 2 h. The samples were washed 5 times with lysate buffer on ice. Bound proteins were eluted by boiling the beads for 5 min at 95 °C in SDS loading buffer. Immunoblot analysis and detection were done as described.

Results

Expression of adhesion molecules on Jurkat and 293T cells. To study the importance of ICAM-1/LFA-1 interactions for HIV-1 infection, the expression of both molecules on Jurkat T-cells were analyzed by flow cytometry. The results showed that Jurkat cells expressed LFA-1 as well as ICAM-1 (Fig. 1A). ICAM-1 levels on 293T cells were also analyzed, since they were used for virus production. Almost no ICAM-1 was detected on untreated 293T cells, however, when 293T cells were transfected with viral plasmids (HIV-Luc or pcHIV) or even empty plasmids (pcDNA), ICAM-1 protein expression was detected on the cell surface. For enhanced expression of ICAM-1, ICAM-1 expression plasmid [123] was transfected in 293T cells (Fig. 1B).

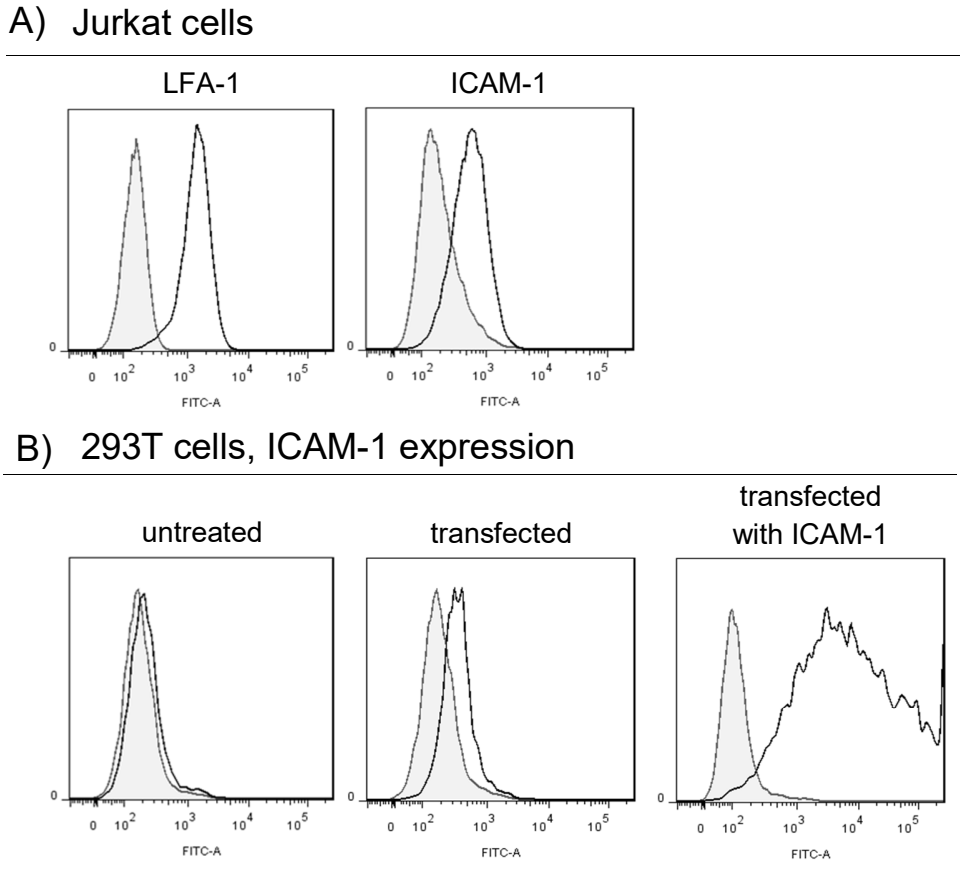

Figure 1 Expression of adhesion molecules on Jurkat and 293T cells. A) Jurkat cells were stained with fluorescent antibodies directed against LFA-1 or ICAM-1 and analyzed by flow cytometry, confirming high expression levels of both molecules. B) ICAM-1 was stained on untreated 293T cells or cells transfected with viral plasmids (NL-Luc or pcHIV) or ICAM-1 expression plasmid (pcDNA ICAM-1). Showing induction of ICAM-1 expression in transfected cells and effective expression of ICAM-1 from plasmid. Filled histograms represent isotype control and open histograms staining of LFA-1 or ICAM-1. (One representative experiment out of three is shown)

ICAM-1 is packaged into viral particles. Several studies demonstrated that ICAM-1 protein is detectable in HIV-1 particles [123,157,173,174]. To confirm this data, 293T cells were co-transfected with empty vector (pcDNA3.1) or ICAM-1 expression plasmids (pcICAM-1) and the virus encoding plasmid NL-Luc or pcHIV. Cell lysates and concentrated viral supernatant was analyzed by immunoblot. ICAM-1 expression was detected in cell lysates and supernatant of cells co-transfected with ICAM-1 plasmid, but was not detected in the absence of such a co-transfection (Fig. 2), likely indicating a low sensitivity of the applied antibody in immunoblots.

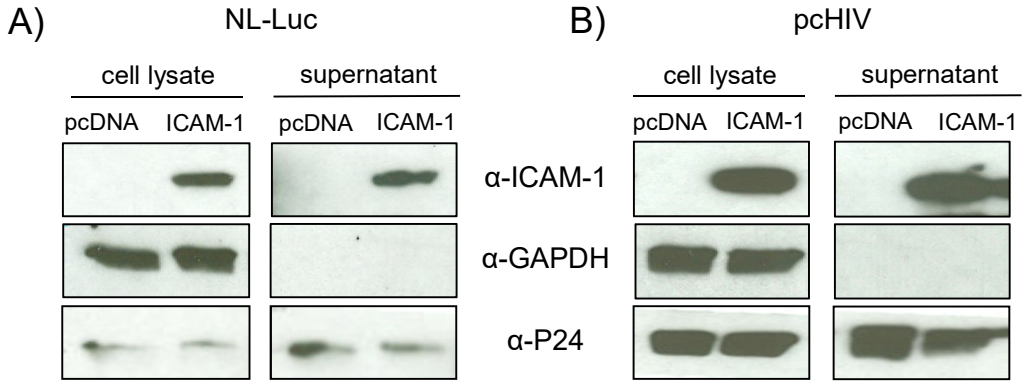

Figure 2: ICAM-1 is packaged into viral particles. 293T cells were transfected with the virus encoding plasmids NL-Luc A) or pcHIV B) and empty vector (pcDNA) or ICAM-1 expression plasmid (ICAM-1). Cell lysate as well as the viral supernatant was analyzed by immunoblot. Anti-ICAM-1 antibody could detect co-transfected ICAM-1, but was not sensitive enough for staining endogenous ICAM-1.

ICAM-1 increases infectivity of HIV-1. Next we asked if ICAM-1 incorporation into viral particles affects infectivity of HIV-1. To this end, HIV-1 luciferase-reporter viruses with CXCR4 tropic envelope (L102) were generated with or without ICAM-1 co-expression. Jurkat T-cells

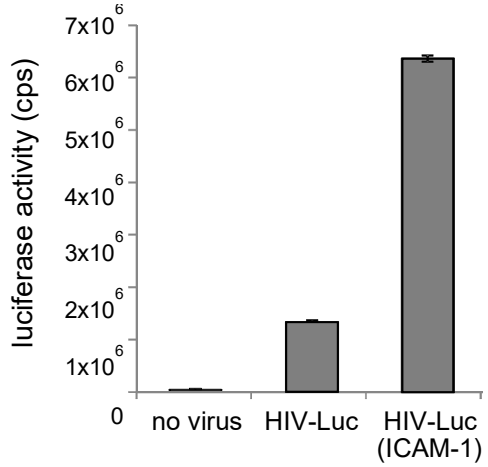

Figure 3: Incorporated ICAM-1 increases infectivity of viral particles. Luciferase-reporter viruses were produced in the absence (HIV-Luc) or presence (HIV-Luc (ICAM-1)) of co-transfected ICAM-1 plasmids and used to infect Jurkat T-cells. Measurement of luciferase activity showed fivefold higher reporter gene activity for cells infected with viral particles with additional ICAM-1.

were infected with normalized viruses to analyze the viral luciferase activity three days post infection. HIV-1 generated in the presence of ICAM-1 displayed a fivefold higher infectivity compared to HIV reporter virus made in the absence of ICAM-1 (Fig. 3). These results are consistent with previous reports describing an enhancing effect of ICAM-1 on the infectivity of HIV-1 [157–159,174,175].

LFA-1 knockout cells show normal growth rates. For specific inhibition of ICAM-1/ LFA-1 interactions, LFA-1 was knocked out in Jurkat T-cells by using the CRISPR/Cas9 system. Lack of LFA-1 expression was confirmed by flow cytometry (Fig. 4A). To exclude an influence of the LFA-1 knockout on cellular viability, the proliferation capacity of LFA-1-deficient cells was analyzed. To measure cell division, LFA-1 knockout and wildtype Jurkat cells were permanently labeled with the cell trace CFSE (carboxyfluorescein diacetate succinimidyl ester) and fluorescence intensity was monitored for up to 96 h by flow cytometry. In this system, fluorescence intensity reflects the cellular proliferation rate with rapidly decreasing intensity in fast dividing cells and a slower decrease in less proliferating cells. Cisplatin knowing to inhibit cell division was applied as a control reagent. The results showed that Jurkat wildtype and LFA-1

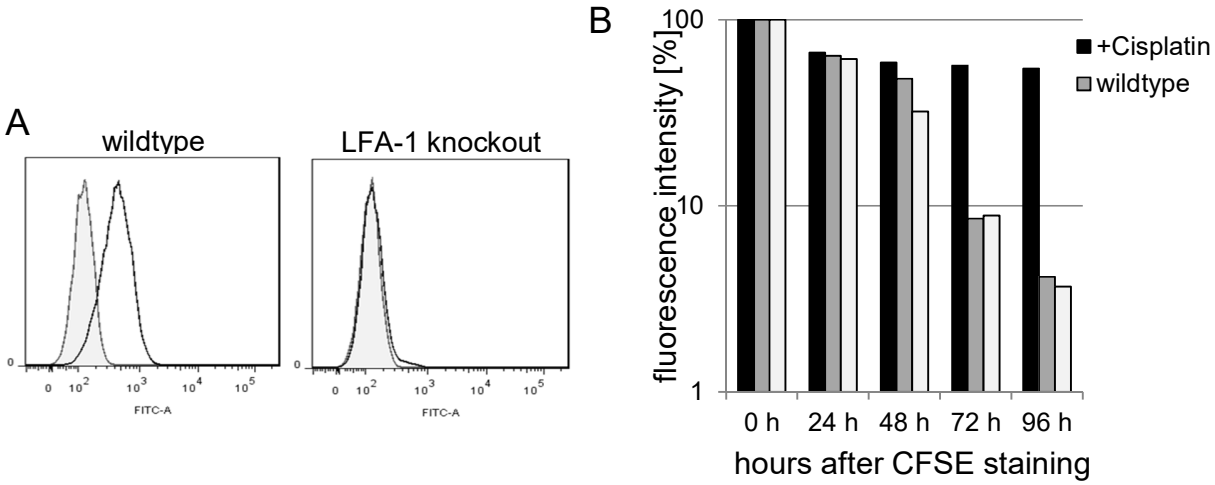

Figure 4: Jurkat cells with LFA-1 knockout show normal growth rates. A) LFA-1 was knocked out in Jurkat cells by using the CRISPR/Cas9 technology, targeting the ITGAL gene. Lack of LFA-1 expression in knockout cells was confirmed by immunofluorescence staining. B) Cells were loaded with cell-tracer violet fluorescence dye and decrease of fluorescence was monitored for 96 hours by flow cytometry, showing comparable growth rates for LFA-1 knockout and wildtype cells. Cisplatin was used to inhibit proliferation and served as negative control.

knockout cells display a very equal decrease in the CFSE signal during the observation period of 96 h (Fig. 4B), indicating similar growth rates and no impact of LFA-1 deficiency on cell proliferation.

LFA-1 deficiency influences virus attachment but not infection. We next analyzed the binding of HIV-1 particles to Jurkat wildtype and LFA-1 knockout cells. A flow cytometry method was used to quantify attachment based on fluorescent particles carrying a Gag-EGFP fusion protein [117]. These green HIV particles were incubated with wildtype and LFA-1 knockout cells, unbound virus was removed by washing and attached virus was quantified by flow cytometry. Compared to cells without virus, cells incubated with labeled particles showed an increase in relative

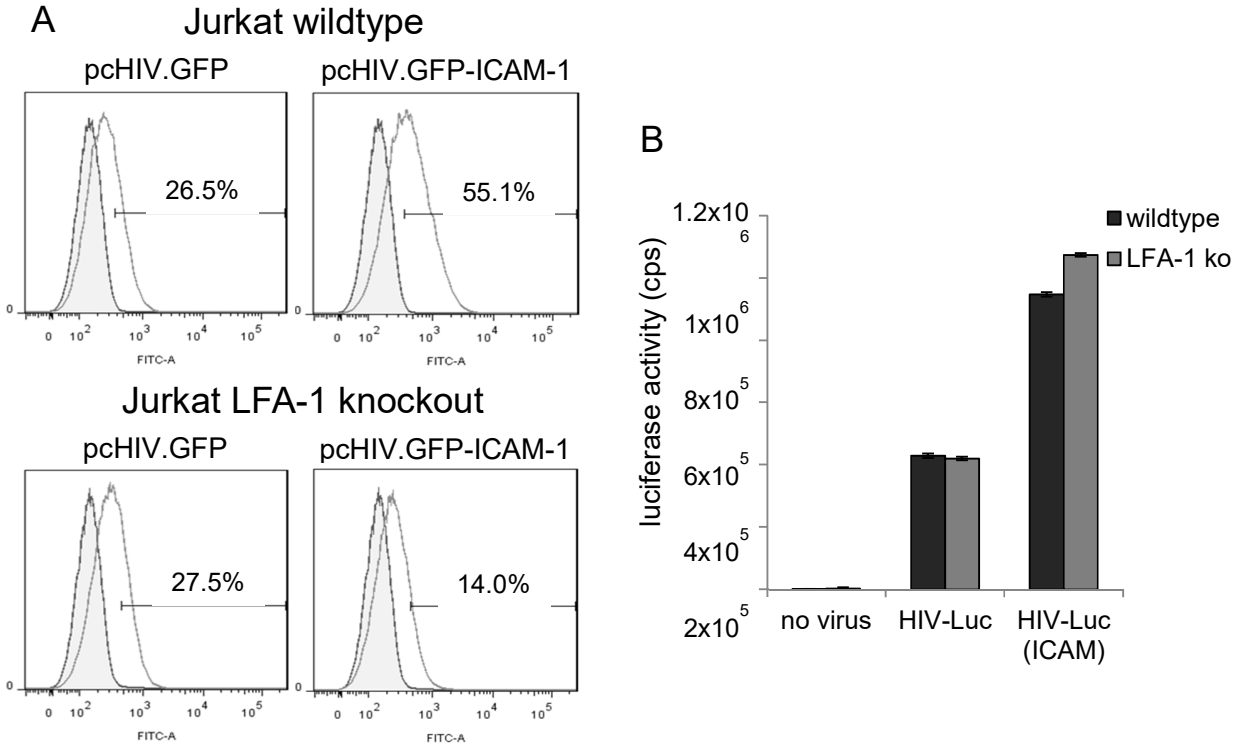

Figure 5: LFA-1 knockout has influence on binding but not infectivity of viral particles. A) Jurkat cells were incubated with GFP-labeled viral particles. After washing, the amount of cell-associated particles was detected by flow cytometry, revealing equal levels of attached virions for wildtype and LFA-1 knock out cells. Incorporation of additional ICAM-1 could increase attachment to wildtype but not LFA-1 deficient cells. (Filled histograms represent fluorescence of cells without virus and open histograms cells incubated with GRP labeled HIV. B) Luciferase-reporter virus was either produced with or without co-transfected ICAM-1 and used for transduction of Jurkat cells. Comparison of reporter gene activity in wildtype and knockout cells showed similar increase of infection by ICAM-1 incorporation.

fluorescence by 26.5% for wildtype and 27.5% for LFA-1 knockout cells, indicating equal binding rates to both cell types to virus without additional ICAM-1 (pcHIV.GFP). If the labeled virus particles were produced with co-transfected ICAM-1 expression plasmid, relative fluorescence increased to 55.1%, reflecting better HIV-1 attachment. However, enhancement of binding by ICAM-1 incorporation was only observed for wildtype but not for LFA-1 deficient cells (Fig. 5A). Furthermore, LFA-1 knockout cells were infected by CXCR4 tropic HIV-1 luciferase reporter virus. Wildtype Jurkat and LFA-1 knockout cells were incubated with the same amount of virus. The results showed enhanced virus infectivity by ICAM-1 co-expression, while the viral luciferase activity was equal in LFA-1 expressing and deficient cells using either virus made with or without ICAM-1, indicating no effect of LFA-1 on infection of Jurkat cells with HIV-1 reporter viruses (Fig. 5B).

HIV-1 replication in Jurkat wildtype and LFA-1 deficient cells. To analyze the impact of LFA-1 on multi-round replication of HIV-1, wildtype and LFA-1 knockout Jurkat T-cells were infected with replication competent HIV-1 clone NL4-3. To this end, wildtype and LFA-1 knockout Jurkat T-cells were infected with MOIs of 0.01 or 0.1. Infected cells were cultivated and virus titer was monitored for up to 18 days. When wildtype cells were infected with MOI

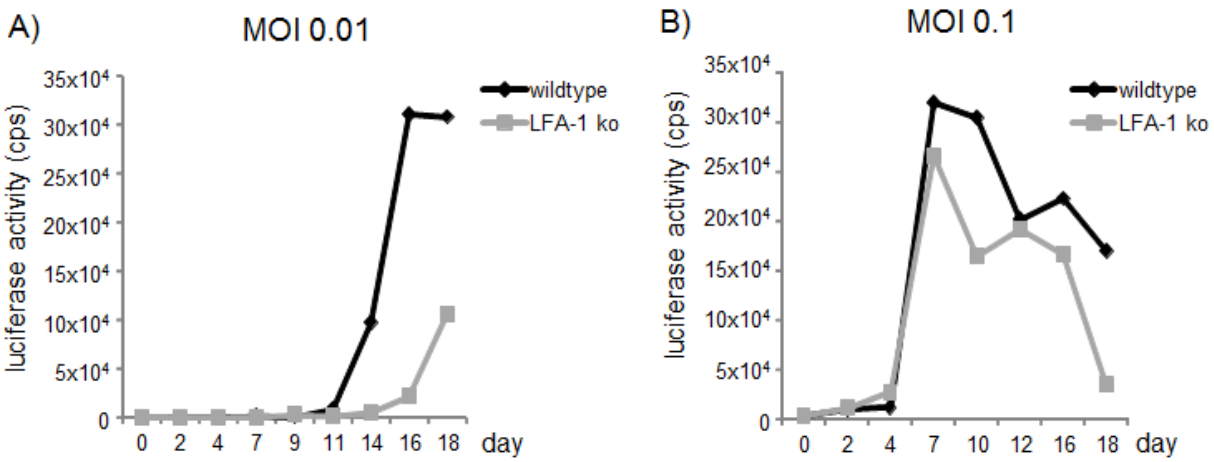

Figure 6: LFA-1 has moderate effects on HIV-1 replication. Wildtype and LFA-1 deficient Jurkat cells were infected with NL4-3 virus and viral spread was monitored by collecting cell culture supernatant and infecting TZM-bl reporter-cells. A) If the multiplicity of infection was low (MOI 0.01), LFA-1 knockout cells showed a delay in viral replication B) For higher viral titer (MOI 0.1), replication kinetics were similar in both cells, with LFA-1 deficient cells showing a slight decrease in the total amount of virus production.

0.01, viral titer reached its maximum at day 14. However, the viral replication was delayed around four days in LFA-1 knockout cells (Fig. 6A). Interestingly, in replication kinetics using HIV-1 with MOI 0.1, viral infection kinetics were similar in both cell lines with little lower overall virus production in LFA-1 deficient cells (Fig. 6B). These results suggest that LFA-1 has only a slight effect on spread of HIV-1 in T-cells.

Vpu expression leads to ICAM-1 modifications. The HIV-1 accessory protein Viral Protein Unique (Vpu) is well described for influencing the protein composition of host cell membranes in order to evade the immune system [176]. Since up to 105 surface receptors are shown to be downregulated in response to Vpu expression [177], we wanted to know whether ICAM-1 expression is affected. To analyze the impact of Vpu on endogenous ICAM-1, 293T cells were

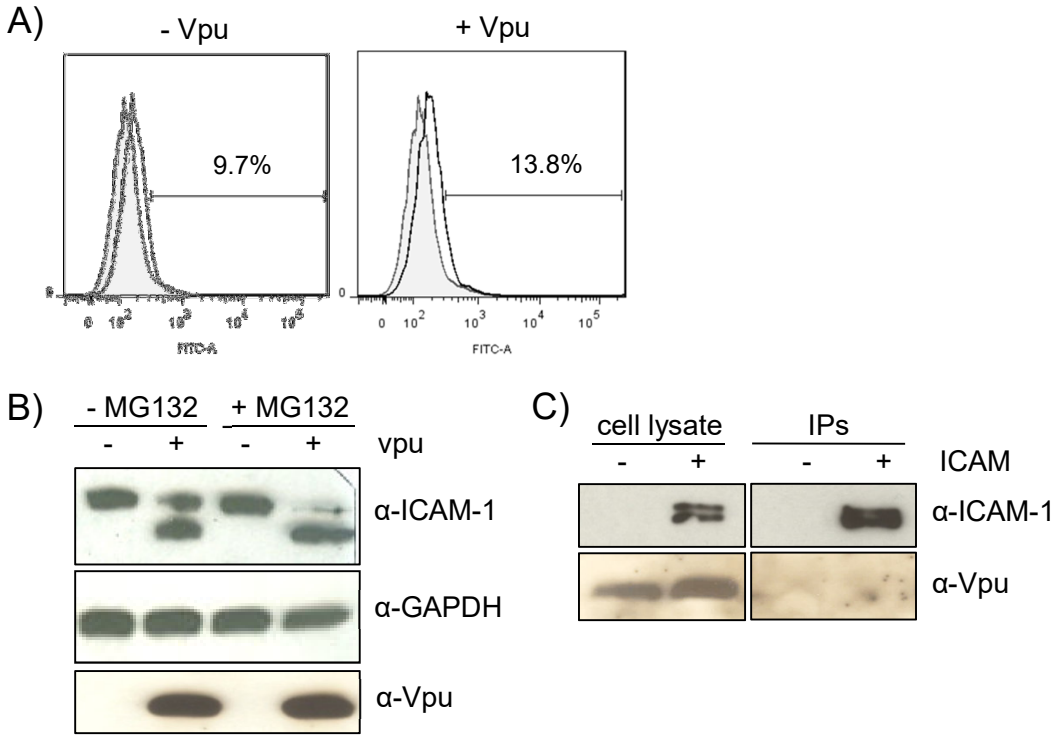

Figure 7: Influence of Vpu on ICAM-1 expression. A) 293T cells were transfected wit empty pcDNA vector (-Vpu) or Vpu expression plasmid pcDNA-Vphu (+Vpu). Cells were stained with fluorescent antibodies directed against ICAM-1 and analyzed by flow cytometry, showing no Vpu mediated reduction of endogenous ICAM-1 expression. (Filled histograms represent isotype control and open histograms staining of LFA-1 or ICAM-1) B) 293T cells were transfected with *icam-1* and empty vector or *vpu*. Immunoblots of cell lysates show degradation of ICAM-1 in presence of Vpu, even when cells were treated with the proteasome inhibitor MG132. C) Co-Immunoprecipitations were performed with lysates of cells transfected with *vpu* alone or *vpu* and *icam-1*, indicating no direct association of these two proteins.

transfected with Vpu expression plasmid (pcDNA-Vphu) or empty vector (pcDNA). Staining of ICAM-1 and flow cytometry analysis showed ICAM-1 expression did not decrease in the prescence of vpu (Fig. 7A). Next, ICAM-1 expression was also studied by immunoblots. 293T cells were transfected with ICAM-1 plasmid together with Vpu or empty vector. In contrast to the results obtained by flow cytometry, immunoblots revealed a reduction of the molecular mass of ICAM-1 when Vpu was co-transfected. This shift in size was also observed when cells were treated with proteasome inhibitor MG132 prior to lysis (Fig. 7B). Additionally co-Immunoprecipitations were performed, the result showed no direct association of both proteins (Fig. 7C).

Discussion

In this study we evaluated the role of ICAM-1/ LFA-1 interactions for infection of T-cells with HIV-1. *In vivo* ICAM-1 is upregulated in response to inflammatory processes [155]. Here we show that it is also upregulated *in vitro* after transfection of viral and non-viral plasmids. Our data also confirm specific packaging of ICAM-1 into viral particles and transduction experiments with luciferase-reporter viruses show up to 5-fold increased infectivity by ICAM-1 incorporation. These observations are consistent with publications from Tremblay *et al.* who also reported packaging of ICAM-1 and it`s enhancing effects on infection [157–159,174]. For specific inhibition of ICAM-1/ LFA-1 interactions, we generated a Jurkat knockout cell line, by using the CRIPR/Cas9 technology. In binding studies this knockout cell line showed equal amount of attached virus like wildtype cells, for GFP labeled viral particles without additional ICAM-1. Compared to this, ICAM-1 co-transfection during virus production could double the attachment to wildtype but not to LFA-1 deficient cells. To our surprise even though the LFA-1 knockout could decrease viral binding, it showed no effect on infectivity in luciferase-reporter assays. ICAM-1 incorporation increased infectivity in the same amount for wildtype and knockout cells. These data might indicate that only binding but not infectivity of HIV-1 is improved by ICAM-1 incorporation as reported recently by Melikyan *et al* [173].

In our studies the LFA-1 knockout showed only moderate effects on NL4-3 replication in cell culture. This effect, manifested in a delayed viral spread, could be overcome with higher viral titers used for initial infection. In single-round infections reporter viruses were generated in 293T cells co-transfected with ICAM-1, leading to high amounts of incorporated ICAM-1. In contrast, in spreading replications virus was produced by Jurkat cells where no ICAM-1 was co-transfected and incorporation was limited to protein levels endogenously expressed. Therefore the lower amount of incorporated ICAM-1 in spreading replications could be the reason for minor effects in these assays. In addition to this, although most publications show that LFA-1/ ICAM-1 interactions enhance infectivity of cell-free virus [121,158,173], the role of this interaction for cell to cell transmission is less clear. The requirement of LFA-1 for HIV-1 induced syncytium formation was extensively studied and all reports confirm a strong decrease in syncytia for LFA-1 deficient cells or after treatment of cells with blocking antibodies against LFA-1, ICAM-1, ICAM-2 or ICAM-3 [120,160,163,164,178]. Also a decrease in spreading replication was reported. However, all this studies measured only the total amount of virus based on P24 or HIV-1 DNA

levels, which is not specific for cell to cell transmission and does not exclude cell-free transmission [120,121]. Other studies reporting impaired cell to cell transmission only focus on transmission from dendritic cells to T-cells and not spread between T-cells [119,162]. Furthermore Pantaleo *et al*. found only two days delay in spreading replication in cells from patients with leukocyte adhesion deficiency syndrome (LAD) [164], supported by studies showing no inhibition of blocking antibodies and equal efficient viral transfer to LFA-1 negative cells [144,163,165,166].

The accessory HIV-1 protein Vpu influences multiple surface molecules of infected cells, specially receptors involved in immune response [176]. Therefore we examined if Vpu expression also downregulates ICAM-1, which could impact HIV-1 replication in Jurkat wildtype and knockout cells. In our studies we detected no ICAM-1 downregulation by flow cytometry but immunoblots showed a shift in molecular weight of ICAM-1 when Vpu was present. This modification seems to be an indirect effect which does not require direct interaction of both proteins as indicated by co-Immunoprecipitations. Also when transfected 293T cells were treated with proteasome inhibitor, the cleavage of ICAM-1 could not be inhibited. Together with the fact that the protein was not completely removed, this points to a proteasome independent pathway activated by Vpu. Obviously Vpu mediated protein modifications do not influence the ICAM-1 antibody binding side, which is located in domains mediating interactions to LFA-1 or Mac-1. Still protein modifications are probably located on extracellular parts of ICAM-1, because the intracellular C-terminal HA-tag is not cleaved of. Therefore an aberrant protein glycosylation may be considered to cause the changes in molecular weight. Also cleavage of ICAM-1 *via* proteases could be an explanation, since human leukocyte elastases, as well as cathepsin G are reported to cleave ICAM-1 [179,180] and regulation of protein function by proteolysis is supposed to influence pro-inflammatory pathways mediated by ICAM-1 signaling [181–183] and types of cytokines produced during T cell responses [184]. Whether manipulation of cellular proteases is beneficial for HIV-1 proliferation is less clear and no modulation of these pathways by Vpu were described so far.

Conclusions

Here we show that ICAM-1 is incorporated into HIV-1 particles and increases viral attachment by interacting with its ligand LFA-1. However this interaction is less important for spreading

replication of HIV-1 among T-cells, probably because ICAM-1 is modified by Vpu induced cellular pathways during infection with replication competent HIV-1.

Declarations

Availability of data and material. The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests. The authors declare no competing interests.

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Authors' contribution. AH performed the experiments and drafted the manuscript, RML designed and cloned the CRISPR/Cas9 plasmids and helped writing the manuscript, CM directed the study and wrote the manuscript, all authors read and approved the final manuscript.

Chapter III

APOBEC4 Enhances the Replication of HIV-1

The following data are published in PLOS ONE (April, 2016) by Daniela Marino[1, 2, *], Mario Perković[1, 2, 3, *], Anika Hain[1, *], Ananda A. Jaguva Vasudevan[1, *], Henning Hofmann[1, 2, 4], Kay-Martin Hanschmann[5], Michael D. Mühlebach[2, 6], Gerald G. Schumann[2], Renate König[7, 8], Klaus Cichutek[2], Dieter Häussinger[1], Carsten Münk[1, 2, #]

[1]Clinic for Gastroenterology, Hepatology, and Infectiology, Medical Faculty, Heinrich-Heine-University Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany. [2]Division of Medical Biotechnology, [5]Biostatistics, [6] Product Testing of IVMPs, [7]Host-Pathogen Interactions, Paul-Ehrlich-Institute, Paul-Ehrlich-Str. 51-59, 63225 Langen, Germany. [8]Sanford Burnham Prebys Medical Discovery Institute, Immunity and Pathogenesis Program, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

[3]Present address: TRON (Translational Oncology at the University Medical Center), Johannes Gutenberg University Mainz, Mainz, Germany.

[4]Present address: Robert Koch Institute, Division for HIV and other Retroviruses, Nordufer 20, 13353 Berlin, Germany

[#]Corresponding author: Clinic for Gastroenterology, Hepatology, and Infectiology, Medical Faculty, Heinrich-Heine-University Düsseldorf, Building 23.12.U1.82, Moorenstr. 5, 40225 Düsseldorf, Germany, Tel: +49 (0) 211-81-10887, Fax: +49 (0) 211-81-18752, E-mail: carsten.muenk@med.uni-duesseldorf.de

*D.M., M.P., A.H. and A.A.J.V. contributed equally to this article and are co-first authors.

Anika Hain (A.H.) contributed to this study with the following experiments:

- Analysis of the expression level of A4-HA fusion proteins (Figure 2).
- Performance of immunofluorescence confocal laser scanning microscopy to localize the subcellular localization of A4 in transfected cells (Figure 3).
- Quantification of virus production in 293T cells co-transfected with A4 expression plasmid. For CXCR4 tropic HIV-1 (Figure 4 A-B), CCR5 tropic HIV-1 (Figure 6) and luciferase vectors (Figure 5 A-C).
- Analysis of the active site mutation (C134A) on A4 activity (Figure 8).
- Investigation of the influence of A4 on the reporter gene expression driven by various viral and cellular promotors (Figure 12).

Abstract

APOBEC4 (A4) is a member of the AID/APOBEC family of cytidine deaminases. In this study we found a high mRNA expression of A4 in human testis. In contrast, there were only low levels of A4 mRNA detectable in 293T, HeLa, Jurkat or A3.01 cells. Ectopic expression of A4 in HeLa cells resulted in mostly cytoplasmic localization of the protein. To test whether A4 has antiviral activity similar to that of proteins of the APOBEC3 (A3) subfamily, A4 was co-expressed in 293T cells with wild type HIV-1 and HIV-1 luciferase reporter viruses. We found that A4 did not inhibit the replication of HIV-1 but instead enhanced the production of HIV-1 in a dose-dependent manner and seemed to act on the viral LTR. A4 did not show detectable cytidine deamination activity *in vitro* and weakly interacted with single-stranded DNA. The presence of A4 in virus producer cells enhanced HIV-1 replication by transiently transfected A4 or stably expressed A4 in HIV-susceptible cells. APOBEC4 was capable of similarly enhancing transcription from a broad spectrum of promoters, regardless of whether they were viral or mammalian. We hypothesize that A4 may have a natural role in modulating host promoters or endogenous LTR promoters.

Introduction

The AID/APOBEC (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) polynucleotide (deoxy) cytidine deaminases family consists of AICDA (activation-induced cytidine deaminase, AID), APOBEC1 (A1), APOBEC2 (A2), APOBEC3 (A3), which has the following seven paralogues in humans: A3A–A3D, A3F–A3H, and APOBEC4 (A4) [190–194]. These enzymes have a diverse range of functions and substrate specificities. Cytidine deamination of single-stranded DNA or RNA was shown to be the principal activity of the AID, A1, and A3 proteins in biochemical and cell culture assays, but such evidence is lacking for A2 and A4 proteins.

Cytidine deaminases of the A3 gene family can inhibit long terminal repeat (LTR) - and non-LTR-retrotransposons and have broad antiviral activity against retroviruses such as HIV and murine leukemia virus (MLV), hepadnaviruses, and non-related viruses [195–210]. A3s mainly act by deaminating cytidine into uridine using single-stranded DNA as a substrate (for review, see [211]). DNA editing introduces hypermutations of the viral genome that eventually render the target genome inactive. Conversely, retroviruses have evolved countermeasures to prevent encapsidation of A3s into viral particles. For example, the Vif protein in lentiviruses, the Bet protein in foamyviruses, the glycosylated Gag (glyco-Gag) protein in MLV, and the nucleocapsid protein in Human T-cell lymphotropic virus accomplish this counteraction using different mechanisms [62,206,208,209,211–216].

AID is a B lymphoid protein that deaminates chromosomal DNA, thereby inducing somatic hypermutations and gene conversion. Furthermore, AID stimulates class switch recombination in B cells [217–223]. AID can restrict LINE-1 (L1) retrotransposition [204,224,225], but it is inactive against HIV-1 [226–228]. A1 catalyzes the cytidine-to-uridine editing of apolipoprotein B mRNA in the intestine [229,230]. Editing generates a premature stop codon, which is translated to produce a truncated form of apolipoprotein B protein, termed *apoB48,* that has distinct functions in lipid transport [231]. The editing mechanism is highly specific for residue C6666 and works in conjunction with A1 complementation factor [232]. Other mRNA targets for A1 editing were recently identified [233]. A1s of rabbit and rodents inhibit both MLV and HIV-1 by mutating the viral RNA and DNA; in contrast human A1 does not edit *in vitro* [227,234–237]. In addition, L1 retrotransposons can be restricted by A1s derived from rodents and rabbits, but this effect is weak

for human A1 [204,238]. A2 plays an important role in regulating and maintaining muscle development in mammals [239]. A2 did not exhibit cytidine deaminase activity of DNA substrates in bacterial or yeast mutation assays [240,241]. Human A2 lacks inhibitory activity against retrotransposons [198,242,243] and HIV-1 [226,228], and murine A2 does not inhibit or edit MLV [234].

A4 protein is more closely related to A1 than to the other APOBECs, and the A4 gene is conserved in chimpanzee, rhesus monkey, dog, cow, mouse, rat, chicken, and frog [192]. A4 is considered to be a putative cytidine-to-uridine editing enzyme. However, experiments conducted using A4 overexpression in yeast and bacteria failed to show cytidine deamination activity in DNA [240]. In mice, the A4 gene is expressed primarily in testis [192], which suggests that it may be involved in spermatogenesis. Whether human A4 participates in intrinsic immunity against HIV as demonstrated for A3s and A1 is unknown, but these anti-viral activities of its sister proteins suggest that it might be possible. Therefore we set out to evaluate the effect of human A4 on the replication of HIV-1 *in vitro*.

Material and Methods

Plasmids pA4-HA (pA4-3xHA) expresses APOBEC4 (A4, GenBank NM_203454.2) fused to three C-terminal HA-tags; pMH-A4_3xHA (obtained from Matthias Hamdorf) was used to excise A4-3xHA using EcoRI x NotI, cloned into EcoRI / NotI of pcDNA3.1*zeo*(+) (Life Technologies, Darmstadt, Germany). pA4-HA-E95Q was generated by side-directed mutagenesis of the pA4-HA plasmid, the mutation was confirmed by sequencing. pA4 expresses A4 without an epitope tag. pA4 was cloned by PCR (pA4-HA as template) using primers hA4 5' (5'-CGGATCCCTAGCAATGGAGCCCATATATG) and hA4 3' (5'-GAATTCTTTATTTCTTCCCTTTCTTCTTCTTC), the PCR product was cloned via BamHI / EcoRI into pcDNA3.1*zeo*(+). HA-A4 (p2xHA-A4): expresses A4 with two N-terminal HA-tags, a pcDNA3.1*zeo*(+)-based plasmid with one N-terminal HA-tag of A4 was generated by PCR using primers HA-hA4 5' (5'-CGGATCCCTAGCAATGGGATATCCATACGATGTTCCAGATTACGCTGAGCCCATATA TGAGGAGTACC) and hA4 3' (5'-GAATTCTTTATTTCTTCCCTTTCTTCTTCTTC); this plasmid served as template for a second PCR using primers for_2xHA-A4 (5'-CGGGATCCCTAGCAATGGGATATCCATACGATGTTCCAGATTACGCTGGCTATCCAT ACGATGTTCCAGATTACGCTGGCTATCCATACGATGTTCCAGATTACGCT) and rev rc_hA4 (5'-GCCGGAATTCTTATTTCTTCCCTTTCTTCTTCTTC). The product was cloned via BamHI / EcoRI into pcDNA3.1zeo(+). pGST-A4: A4 with an N-terminal GST-tag in pGEX-6-P1 (GE Healthcare, Munich, Germany), A4 was cloned via BamHI / EcoRI by excising A4 from pA4. Similarly pGST-A4-ΔKK was cloned in pGEX-6-P1 (GE Healthcare, Freiburg, Germany) using forward primer 5'- ATCGGATCCATGGAGCCCATATATGAGGAG and reverse primer 5'-CGGCGAATTCTTATTCATCTGCCTCCTTGCTACT. pMSCV.A4: a murine leukemia virus-based vector to express A4 fused with three C-terminal HA-tags; it was cloned by PCR using template pA4-3xHA and primers A4_fw_RI (5'-TGGAATTCGCCCTTCAGGCGGTACCAGCCTGGAGACAAATTGATG) and A4_rv_RI (5'-TAGAATTCTCAGTTAGCCGGCGTAG)via EcoRI into pMSCV.neo (Clonetech, Takara Bio Europe/SAS, Saint-Germain-en-Laye, France). pLTR-Luc (pGL3-bas-NL43LTR-luc): containing the LTR region of HIV-1 pNL4-3, cloned by PCR of U3, R, and TAR elements using primers NL4-3-U3(+) (5′-CTCGGCAGATCTCTGGAAGGGCTAATTCACTCC) and U3/R/TAR(-) (5′-GCTCGGAAGCTTGGCTTAAGCAGTGGGTTCCCTAG); amplicons were cloned via HindIII

and BglII (partial digest) into pGL3-Basic (Promega, Mannheim, Germany). P850 luciferase plasmid with LINE Promotor (P850 L1) [244] and reporter constructs with androgen responsive promotors probasin (pGL3Eprob) and PSA (pPSA61-luc) [245] were kindly provided by Wolfgang A. Schulz. APOBEC3G (A3G)-HA expression construct was kindly provided by Nathaniel R. Landau [206]. His-tagged huA3G (A3G-Myc-6His) has been described previously [246]. APOBEC3A (A3A)-HA expression plasmid was obtained from Bryan R. Cullen [247]. pTat (pBS-KRSPA-Tat NL4-3), expressing Tat protein of HIV-1 NL4-3 was a gift of Heide Muckenfuss and Egbert Flory. For cloning of pTat, both Tat exons were amplified and fused by PCR using pNL4-3 [113] as template, and cloned into XhoI / SpeI of pBS-kRSPA [248]. pHSV-RLuc (pRG-TK, Promega), *Renilla reniformes* luciferase expressed by the *Herpes simplex virus type 1* thymidine kinase promoter.

Cells, transfections and infections HOS (ATCC CRL-1543), HOS.CD4.CCR5 [249], HeLa (ATCC CCL-2), TZM-bl [250] and 293T (ATCC CRL-3216) cells, were maintained in Dulbecco's modified Eagle's medium complete (PAN-Biotech, Aidenbach, Germany); A3.01 T cells [251] and Jurkat T cells clone E61 (ATCC TIB152) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (PAN-Biotech) supplemented with 10% FBS, 0.29 mg/ml L-glutamine, and 100 U/ml penicillin/streptomycin at 37˚C in a humidified atmosphere of 5% CO_2. Plasmid transfections into 293T cells were performed with Lipofectamine LTX (Life technologies). HIV-1 and reporter lentiviruses were generated by transfection in 6-well plates with 200 ng pNL.Luc R^- E^- (pNL4-3.Luc.R^-.E^-) [252] plus 50 ng vesicular stomatitis virus G glycoprotein (VSV-G) expression plasmid pMD.G or with 1 µg pNL4-3 [113] and different amounts of A4 expression plasmid. Total transfected plasmid DNA was maintained by adding appropriate amounts of pcDNA3.1zeo(+) plasmid DNA where needed. To produce NL4.3 with the *env* BaL, pNL-BaL [253] was transfected in 293T cells. Reverse-transcriptase (RT) activity was determined using the Cavidi HS kit Lenti RT (Cavidi Tech, Uppsala, Sweden). For infectivity assays, $4x10^3$ HOS cells were transduced in 96-well plates in triplicate with a virus amount equivalent to 10 pg of RT for HIV. Three days post infection, luciferase activity was measured using the Steadylite HTS kit (PerkinElmer, Rodgau, Germany). To quantify firefly and *Renilla* luciferase in the same cell lysate, the dual-Luciferase reporter assay (Promega) was applied. All luciferase assays in

transfected cells were performed two days post transfection. To generate stable A4-expressing HOS.CD4.CCR5.A4 and control HOS.CD4.CCR5.neo cells, pMSCV.A4 or pMSCV.neo plasmid was co-transfected together with pHIT60 [254] and pMD.G for generation of murine leukemia viral vector particles. Vector particles were used to transduce HOS.CD4.CCR5 cells; G418 resistant cells were pooled and characterized for CD4 and CCR5 receptor and A4 expression. Spreading virus replication with NL-BaL was quantified over 20 days infecting HOS.CD4.CCR5.neo or HOS.CD4.CCR5.A4 cells using a multiplicity of infection of 0.01 and testing the culture supernatants with the HIV reporter cell line TZM-bl [250]. Transfection efficiency was monitored by cotransfection of 100 ng Monster Green fluorescent protein expression plasmid hMGFP (Promega).

Immunoblot analysis. Cells were lysed in radioimmunoprecipitation assay buffer (RIPA, (25 mM Tris (pH 8.0), 137 mM NaCl, 1% glycerol, 0.1% SDS, 0.5% Na-deoxycholat, 1% Nonidet P-40, 2 mM EDTA, and protease inhibitor cocktail set III [Calbiochem, Darmstadt, Germany].) buffer, 20 min on ice. Lysates were clarified by centrifugation (10 min, 300 g, 4 °C). Samples were boiled in NuPAGE SDS Sample Buffer and NuPAGE Sample Reducing Agent (Life technologies) and subjected to SDS-PAGE followed by transfer to a PVDF membrane. A3G and A4 Proteins were detected using an anti-HA antibody (Ab) (1:10^4 dilution, MMS-101P; Covance, BioLegend, Fell, Germany), HIV-1 p24 Gag was detected applying HIV-1 p24 monoclonal Ab (1:250 dilution, AG3.0, NIH AIDS REAGENTS, Germantown, USA) [171]. Cell lysates were probed with α-tubulin Ab (1:10^4 dilution, B5-1-2; Sigma-Aldrich, Munich, Germany) and virions with α-p24 monoclonal Ab 183-H12-5C. Vif protein was detected with HIV-1 Vif monoclonal antibody (1:5x10^3 dilution, #319, NIH AIDS REAGENTS) [255]. Secondary Abs.: anti-mouse (NA931V) and anti-rabbit (NA934V) horseradish peroxidase (1:10^4 dilution, GE Healthcare). Signals were visualized using ECL reagent (GE Healthcare).

Chemical cross linking 293T cells were transfected with pA4-HA and lysed two days after transfection with RIPA buffer. Soluble fraction was clarified by centrifugation at 13,000 rpm and 4 °C. To chemically cross link the amines of the protein, the lysate was treated with various concentrations of disuccinimidyl suberate (DSS) (Thermo Scientific, Braunschweig, Germany)

dissolved in DMSO to make a final concentration of 50, 100 and 500 µM and the reaction mixture was incubated for 20 min on ice. To quench the reaction, 20 mM of Tris (final concentration) was added and lysates were subjected to immunoblot analysis without addition of reducing reagent. The presence of A4 monomers and dimers were detected by anti HA antibody.

PCR Total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany). Human testis RNA (DNase free, HR-401) was obtained from Zyagen (San Diego, USA). RNA was reverse transcribed with QuantiTect Reverse Transcription (Qiagen). Semi-quantitative PCR analyses of A4 mRNA: The A4 fragments were amplified from cDNA by Dream-*Taq* polymerase (Thermo Scientific) and the primers Origene_for (5‘-CAAGCCTGGAGACAAATTGATGG) x Origene_rev (5‘-GCAATCGAGAGAGAAGCTTAGCC). As a control, β-2-microglobulin cDNA was amplified in the same PCR reaction applying primer β-2-Mikroglobulin A_for (5’-CTCGCTCCGTGGCCTTAGCTGTGCTCGCGC) x β-2-Mikroglobulin A_rev (5’-TAACTTATGCACGCTTAACTATC): Initial denaturation at 95 ºC for 5 min followed by 39 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min and final extension 72 °C for 15 min. Water instead of template served as a background control and a plasmid coding for A4 cDNA (pA4 cDNA) served as a positive control. The identity of the PCR fragments was confirmed by cloning and sequencing. Quantitative real-time PCR analyses of A4 mRNA: The A4 fragments were amplified from cDNA using SYBR green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom) with an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) and primers: A4_909_for (5’-ACCAATGCATATGGGCCAAA) x A4_906_rev rc (5’-GTGCCTTACGATATTCCTGGGt). After initial incubations at 50 °C for 2 min and 95 °C for 10 min, 40 cycles of amplification were carried out for 15 s at 95 °C, followed by 1 min at 60 °C. The amplification product was normalized to that of HPRT1 using PCR Primers HPRT1_for (5’-GCTTTCCTTGGTCAGGCAGT) x HPRT1_rev rc (5’-GCTTGCGACCTTGACCATCT).

Purification of A3 and A4 proteins from E. coli and 293T cells A3G-His was expressed in 293T cells and purified by immobilized metal affinity chromatography (IMAC) using Ni-nitrilotriacetic acid (Ni-NTA) agarose (Life Technologies) as described [256]. GST-A3C, GST-A4,

GST-A4ΔKK and GST proteins were overexpressed in *E. coli* Rosetta (DE3) cells (Millipore, Merck Chemicals, Darmstadt, Germany) and purified by affinity chromatography using Glutathione Sepharose 4B beads (GE healthcare). After the growth of transformants containing pGEX4T2-GST-A4 until 0.6 OD_{600}, cells were induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and 1 µM $ZnSO_4$ and cultured at 18˚C overnight. A4 harboring cells were washed with PBS and lysed with 1X Bug buster protein extraction reagent (Millipore) containing 50 mM Tris (pH 7.0), 10% glycerol, 1 M NaCl and 5 mM 2-mercaptoethanol (2-ME), clarified by centrifugation (14,800 rpm for 20 min at 4ºC) and the soluble fraction was mixed with glutathione sepharose beads. After 3 h incubation at 4˚C in end-over-end rotation, the beads were washed twice with wash buffer containing 50 mM Tris (pH 8.0), 5 mM 2-ME, 10% glycerol and 500 mM NaCl. The bound GST-A4 protein was eluted with wash buffer containing 20 mM reduced glutathione. Purified protein concentration was determined spectrophotometrically by measuring the A_{280}, using their (theoretical) extinction coefficient and molecular mass.

In vitro DNA cytidine deamination assay Deamination reactions were performed as described [256,257] in a 10 µL reaction volume containing 25 mM Tris pH 7.0, and 100 fmol single stranded DNA substrate (CCCA: 5'-GGATTGGTTGGTTATTTGTTTAAGGAAGGTGGATTAAAGGCCCAAGAAGGTGATGGAAGTTATGTTTGGTAGATTGATGG; CCCG: 5'-GGATTGGTTGGTTATTTGTTTAAGGAAGGTGGATTAAAGGCCCGAAGAAGGTGATGGAAGTTATGTTTGGTAGATTGATGG and TTCA: 5'-GGATTGGTTGGTTATTTGTATAAGGAAGGTGGATTGAAGGTTCAAGAAGGTGATGGAAGTTATGTTTGGTAGATTGATGG). Samples were splitted into two halves; in one half 50 µg/ml RNAse A (Thermo Scientific) was added. Reactions were incubated for at least 1 h at 37˚C and the reaction was terminated by boiling at 95˚C for 5 min. One fmol of the reaction mixture was used for PCR amplification (Dream *Taq* polymerase (Thermo Scientific) 95˚C for 3 min, followed by 19 cycles of 61˚C for 30 sec and 94˚C for 30 sec) and the primers forward 5'-GGATTGGTTGGTTATTTGTTTAAGGA, reverse 5'-CCATCAATCTACCAAACATAACTTCCA used to amplify CCC(A/G) substrate, forward primer 5'-GGATTGGTTGGTTATTTGTATAAGGA with the above reverse primer used for

TTCA. PCR products of CCC(A/G) and TTCA were digested with Eco147I (StuI) (Thermo Scientific) and MseI (NEB, Frankfurt/Main, Germany), respectively, resolved on 15% PAGE, stained with ethidium bromide (5 µg/ml). As a positive control substrate oligonucleotides with CCUA and TTUA instead of respective CCCA and TTCA were used to control the restriction enzyme digestion.

APOBEC incorporation into HIV-1: HIV-1 vectors were produced with 250 ng A3 plasmids and 1000 ng A4 constructs. 48 h later virion containing supernatants were concentrated by layering on 20% sucrose cushion and centrifuged for 4 h at 14,800 rpm. Viral particles were re-suspended in mild lysis buffer (50 mM Tris (pH 8), 1 mM PMSF, 10% glycerol, 0.8% NP-40, 150 mM NaCl and 1X complete protease inhibitor) and used as input for the *in vitro* deamination assay.

Deamination assay using immunoprecipitated protein from 293T cells: 293T cells were transfected with expression plasmids encoding A4-HA, 3xHA-A4, A3G-HA or A3F-HA. Cells were lysed 48 h post transfection with mild lysis buffer (50 mM Tris (pH 8.0), 1 mM PMSF, 10% glycerol, 0.8% NP-40, 150 mM NaCl and protease inhibitor (protease inhibitor cocktail set III, Calbiochem). HA-tagged proteins were immunoprecipitated using 20 µl of anti-HA Affinity Matrix Beads (Roche Diagnostics, Mannheim, Germany) by slowly rotating the lysate bead mixture for 2 h at 4 °C. One third of the beads were used for deamination assay and the remaining was used for immunoblot analysis.

Electrophoretic mobility shift assay (EMSA) with GST-A3C and GST-A4 EMSA method is adapted from [258,259]. Proteins were produced as described above, kept in protein buffer (final concentration 50 mM Tris (pH 8.0), 50 mM NaCl, and 10% glycerol). 10 mM 3' biotinylated DNA (30-TTC-Bio-TEG purchased from Eurofins Genomics, Ebersberg Germany) was mixed with 10 mM Tris (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 2% glycerol, and desired amount of recombinant proteins in a 10 µl reaction mixture, and incubated at 25 °C for 30 min. The protein-DNA complex was resolved on a 5% native PAGE gel on ice, and then transferred onto nylon membrane (Amersham Hybond-XL, GE healthcare) by southern blot. After transfer, the molecules on the membrane were crosslinked by UV-radiation using a transilluminator at 312 nm for 15 min. Chemiluminescent detection of biotinylated DNA was carried out according to the manufacturer's instruction (Thermo scientific).

Confocal microscopy 1 x 10^5 HeLa cells grown on coverslips (Marienfeld, Lauda Königshofen, Germany) were transfected with 500 ng A4 expression plasmids by applying Lipofectamine LTX transfection reagent. At day two post transfection, cells were fixed in 4% paraformaldehyde in PBS for 30 min, permeabilized in 0.1% Triton X-100 in PBS for 45 min, incubated in blocking solution (10% donkey antiserum (Sigma-Aldrich) in PBS) for 1 h, and treated with anti-HA Ab (MMS-101P; Covance) in blocking solution for 1 h. Donkey anti-mouse Alexa Fluor 488 (Life Technologies) was used as secondary Ab in a 1:300 dilution in blocking solution for 1 h. Finally, nuclei were stained using DAPI (4', 6'-diamidino-2-phenylindole; 1:1000 in PBS) (Merck Millipore, Darmstadt, Germany) for 5 min. Coverslips were mounted on glass microscope slide (Marienfeld) using Fluorescent Mounting Medium (DAKO, Hamburg, Germany). The images were captured by using a 63x objective on a Zeiss LSM 510 Meta laser scanning confocal microscope. x-z optical sections were acquired from 0.28 µm layers.

Statistics Evaluation of RT or reporter activity data was performed by means of a multifactorial analysis of variance (ANOVA) with fixed factor *plasmid ratio*. Additionally a random factor *day* was included, if more than one determination were obtained from one day in order to model day-to-day variability. The statistical analysis was performed with SAS/STAT software, version 9.3, SAS System for Windows (Cary, USA).

Results

Analysis of A4 expression in cell lines and human testis tissue. Based on information of public repositories (e.g. GenBank) A4 is detectable mainly in human testis, and neither full length A4 mRNAs nor expressed sequence tags (ESTs) have been identified in blood cells, lymphoid tissues, T cells or macrophages. To functionally test A4 in cell culture, we first wanted to

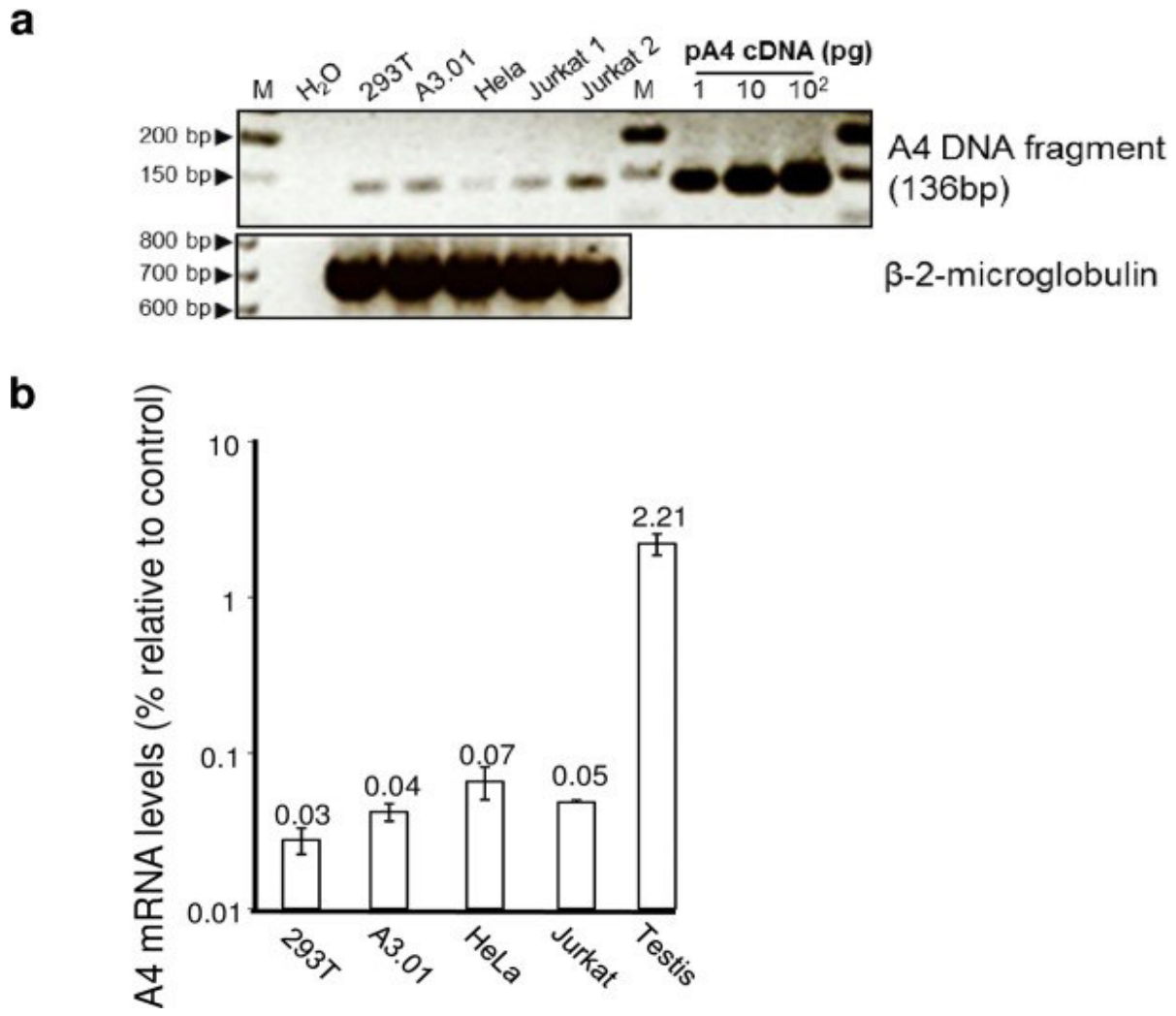

Figure 1: Differential expression of A4. (a) A4 expression was determined by semi-quantitative RT-PCR. Low level A4 amplification by PCR using equal amount of cDNA prepared from total RNA of 293T, A3.01, Hela, and Jurkat cell lines. As a control, β-2-microglobulin (ß-2-M) cDNA was amplified. Water instead of template served as a background control and a plasmid coding for A4 cDNA (pA4 cDNA) served as a positive control. M: 50 bp DNA ladder. (b) Levels of A4 expression were determined by quantitative real-time RT-PCR and measured relative to endogenous HPRT1 RNA levels. A4 is expressed at a high level in human testis tissue, while 293T, HeLa, A3.01 and Jurkat cells exhibit very low A4 expression. Error bars indicate standard deviation.

determine whether widely used human cell lines express A4. To this end, semi-quantitative RT PCRs on total RNA from the 293T, HeLa, A3.01 T, and Jurkat T cell lines were conducted and the weakly detected PCR products were cloned and sequence verified (Fig. 1a). We further compared the A4 expression levels of these cell lines to A4 expression in human testis tissue by quantitative real-time RT PCR on total RNA. Data demonstrate that A4 expression levels in testis are approximately 30- to 50-fold higher than those in the tested cell lines (Fig. 1b). A4 expression plasmids were generated with either N-terminal or C-terminal HA-tags or without any tag (HA-A4, A4-HA, A4, Fig. 2a). A4 constructs expressed 10- to 100-fold less protein than A3 plasmids expressed from the same vector as shown for A4-HA (3xHA-tag) in comparison with A3G-HA

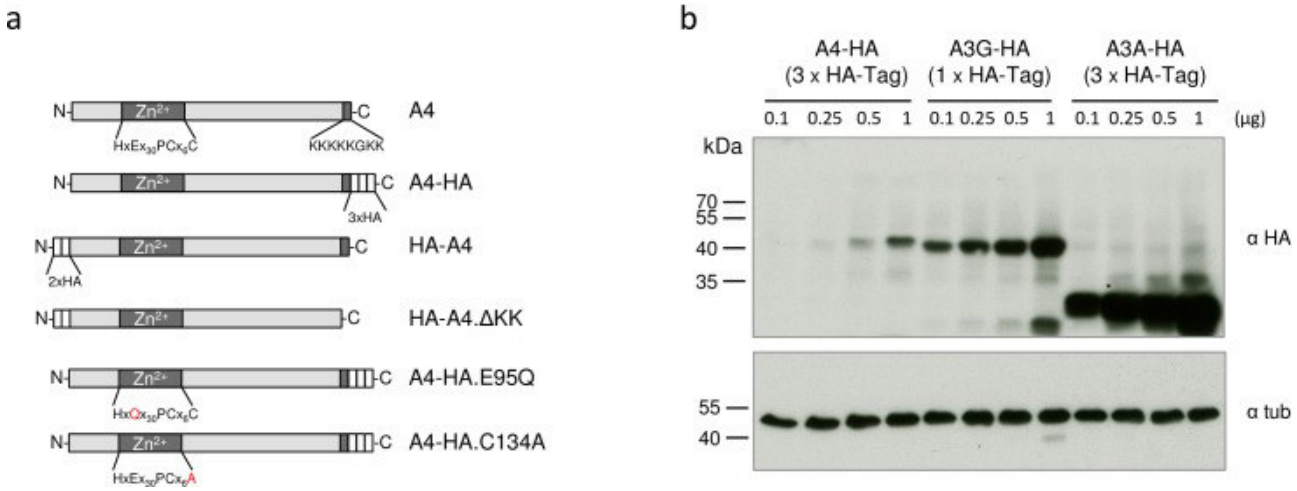

Figure 2: Expression of the A4-HA fusion proteins. (a) Schematic representation of protein domains and motifs found in the human A4 protein and tested variants. Zn^{2+}: presumed zinc-binding domain. HA (white boxes): HA-tag. KKKKKGKK: polylysine domain. (b) Increasing amounts of A4-HA (3xHA-tags), A3G-HA (1xHA-tag) and A3A-HA (3xHA-tags) expression plasmids were transfected into 293T cells followed by immunoblot analysis of the transfected cells using an anti-HA antibody. Immunoblot analysis with anti-tubulin (tub) antibody served as loading control. α, anti.

(1xHA-tag) and A3A-HA (3xHA-tag) (Fig. 2b). To study the subcellular localization of the differently tagged A4 proteins, we analyzed the expression of these proteins in transfected HeLa cells using confocal microscopy. HA-A4 was localized in both cytoplasm and nucleus (Fig. 3a, 3b). A4-HA exhibited a predominantly cytoplasmic distribution (Fig. 3c, 3d). Untagged A4 could not be detected, because there was no A4-specific antibody available. To analyze if the characteristic polylysine stretch (KKKKKGKK) at the C-terminus is important for nuclear localization of A4, an N-terminal HA-tagged mutant lacking the polylysine domain (HA-A4ΔKK, Fig. 2a) was tested. Only few cells showed expression of this protein, however, if

expressed, HA-A4ΔKK was detectable in nucleus and cytoplasm, suggesting that the polylysine stretch does not function as a nuclear localization motif (Fig. 3f).

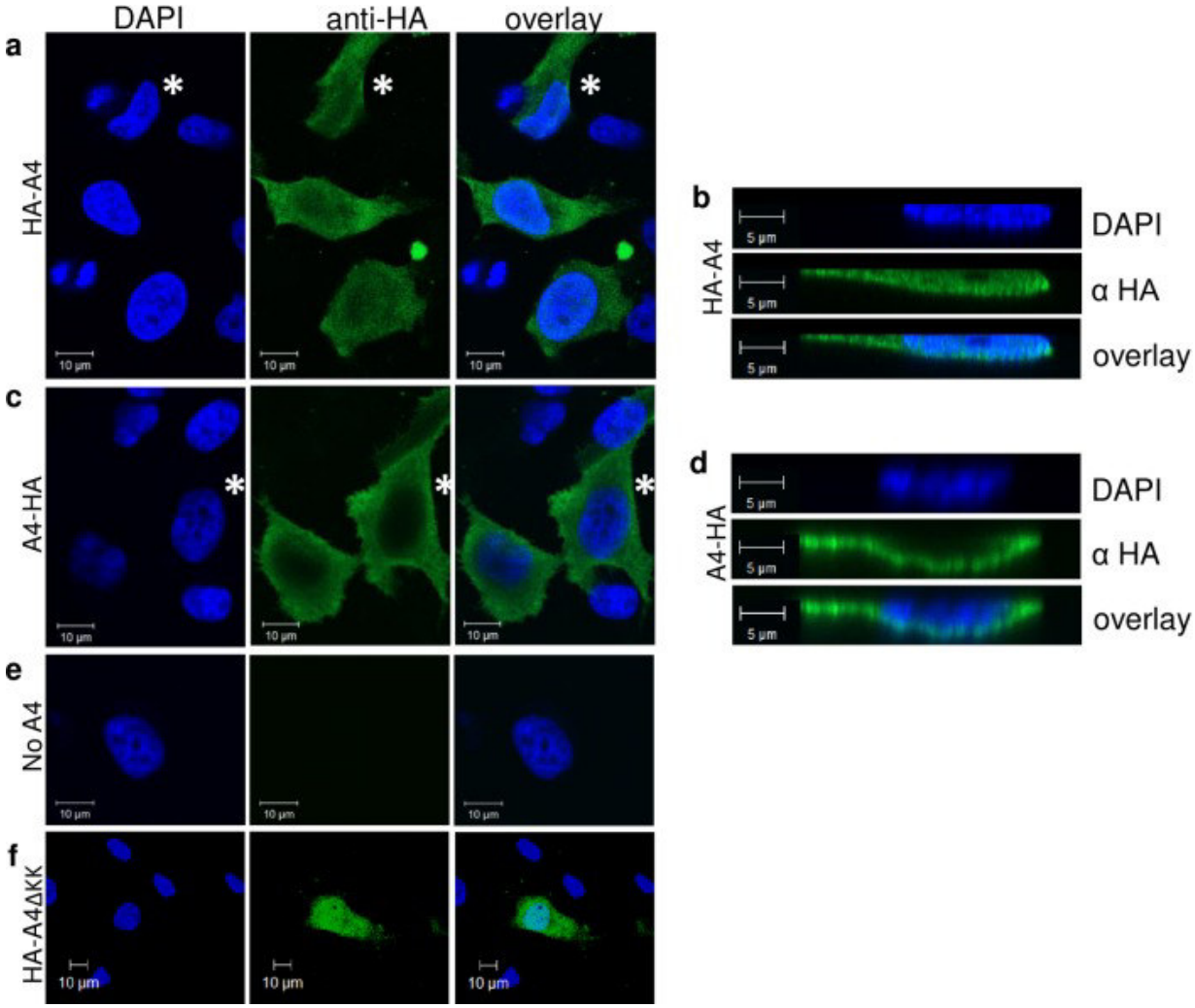

Figure 3: Subcellular localization of A4 in transfected cells. Immunofluorescence confocal laser scanning microscopy images of HeLa cells transfected with N- or C-terminal HA-tagged A4 (HA-A4 and A4-HA). (a, b) HA-A4 proteins show cytoplasmic and nuclear localization. (c, d) A4-HA proteins show cytoplasmic localization. (a, c, e, f) x-y optical sections. (b, d) x-z vertical scanning image of indicated cells (see asterisks). (e) Mock transfected cells (no A4). (f) HA-A4ΔKK transfected cells show cytoplasmic and nuclear localization. To detect A4 (green) immunofluorescence, cells were stained with an anti-HA antibody. Nuclei (blue) were visualized by DAPI staining. α, anti.

A4 expression results in an increased HIV-1 particle yield. To determine the effect of A4 on HIV-1 particle production, we co-transfected increasing amounts of the HA-A4 expression plasmid with a constant amount of HIV-1 expression plasmid (pNL4-3 [113]). The total amount of

transfected DNA was kept constant by replacing HA-A4 with the empty expression plasmid (pcDNA3.1zeo). Two days post transfection, we quantified virus production by measuring viral reverse transcriptase (RT) activity in the cell culture supernatant (Fig. 4a) and tested the cell lysate for expression of HIV-1 Gag (p24) by immunoblot analysis (Fig. 4b). Transfection of incremental amounts of HA-A4 plasmid caused a 2.5-fold increase in the amount of released viral

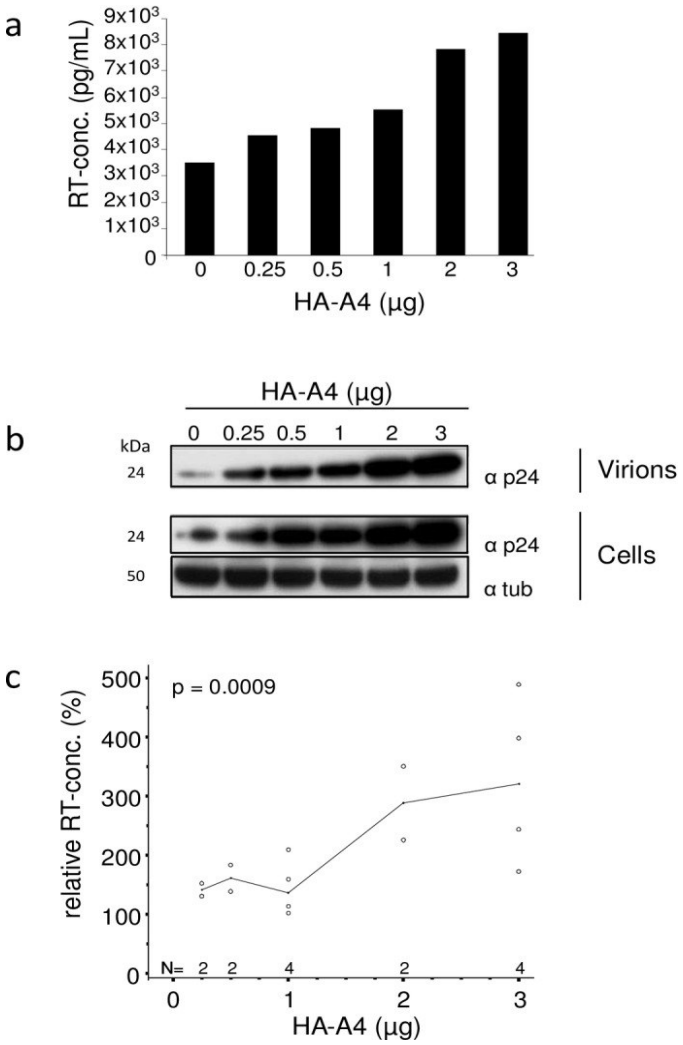

Figure 4: A4 enhances the expression of HIV-1. (a) HIV-1 genome expression plasmid was co-transfected with increasing amounts of HA-A4 expression plasmid, as indicated. A4 increases the production of HIV-1 particles as measured by the RT activity in the supernatant of the transfected cells. (b) Immunoblot analysis of virions and transfected 293T cells (same cells as in (a)). Immunoblots of virions and cell lysates were probed with anti-p24 (capsid) antibody. Anti-tubulin (tub) antibody served as loading control. α, anti. (c) RT concentrations in the supernatant of cells co-transfected with HA-A4 and HIV-1 plasmids relative to supernatant of cells co-transfected with empty vector and HIV-1, as in (a), summary of four independent experiments, median indicated. Evaluation of RT activity data was performed by means of a multifactorial analysis of variance (ANOVA).

particles as reflected by the RT activity detected in cell culture supernatants. Immunoblot analyses of viral lysates concentrated from the cell culture supernatant also demonstrated the A4 stimulating effect on virus expression (Fig. 4b). This positive effect of HA-A4 on late stage HIV-1 particle production was highly reproducible, as demonstrated by data from four independent experiments (Fig. 4c). These results were consistent with experimental findings using untagged A4 protein (data not shown).

Production but not infectivity of HIV-1-luciferase is enhanced by A4 expression. We used VSV-G pseudotyped HIV-1 luciferase virus (NL.Luc $R^-E^-{}^{252}$) to test whether increasing the levels of expressed A4 influences HIV-1 production and infectivity. Co-transfection of A4 expression plasmids (data are shown for A4 and A4-HA plasmids in Fig. 5a) and NL-Luc resulted in a dose-dependent increase of intracellular virus-encoded luciferase activity in transfected 293T cells. Presence or absence of the viral Vif protein (using the *vif*-deficient NL.Luc $R^-E^-\Delta$vif/VSV-G in the same set of experiments) had no detectable effect on the A4-induced stimulation of NL.Luc (data not shown). Immunoblot analysis of lysates isolated from the transfected cells confirmed the A4 dose-dependent expression of viral capsid p24 (Fig. 5b). Figure 5c shows that co-expression of HA-A4 with NL.Luc also caused a similar boost of Gag expression, indicating that the location of the HA tag did not influence virus production enhancement by A4. Results from 28, 16, and seven independent experiments using different amounts of the A4-HA plasmid together with NL.Luc are summarized in Fig. 5d, Fig. 5e, and Fig. 5f, respectively. These results confirmed a significant increase in NL-Luc-mediated luciferase activity in the transfected virus producer cells (Fig. 5d). When testing equal volumes of cell culture supernatants for the presence of infectious HIV reporter virions, we also detected a dose-dependent increase in luciferase activity in infected cells (Fig. 5e). However, when equal concentrations of viral particles normalized for RT activity were used escalating levels of A4-HA did not cause a significant increase in infectivity (Fig. 5f). The summarized individual experiments did not always cover all ranges of applied plasmid concentrations (Fig. 5d), and single virus samples obtained from a subset of experiments were used to study particle infectivity (Fig. 5e, 5f). Taken together, these data indicate that A4 expression enhances the production of HIV-1, but does not change its infectivity.

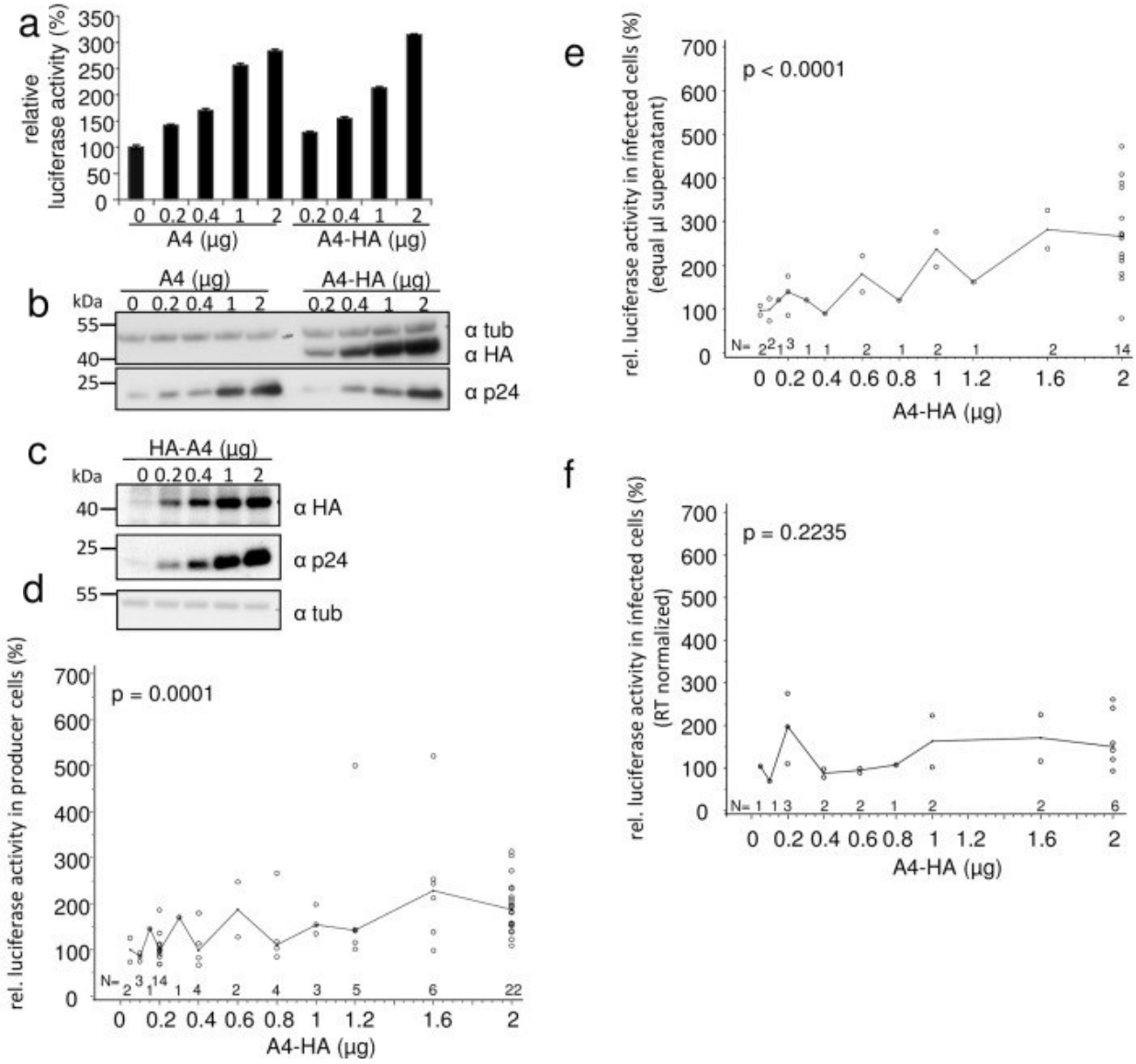

Figure 5: Presence of A4 does not affect HIV-1 infectivity. HIV-1 reporter virus NL-Luc R^-E^- (VSV-G) was produced in 293T cells in the presence of increasing amounts of A4 (no tag) and A4-HA (C-terminal HA-tag). A4 and A4-HA increase in a dose-dependent manner both (a) the virus-encoded luciferase activity and (b) the expression of intracellular viral capsid (p24) in the transfected virus producing cells as demonstrated by immunoblot analysis (same cell lysates used in (a) and (b)). Error bars indicate standard deviation. (c) Immunoblot analysis of intracellular viral p24 (capsid) expression. Similar as in (a) and (b), NL-Luc R^-E^-/VSV-G was co-transfected with increasing amounts of HA-A4 plasmid (N-terminal HA-tag), as indicated. Immunoblots of cells were probed with anti-p24 (capsid) antibody. A4-HA expression in transfected cells was detected by immunoblotting using anti-HA antibody. Anti-tubulin (tub) antibody served as loading control. α, anti. (d) Relative viral luciferase activity in cells co-transfected with A4-HA and HIV-1 plasmids, as in (a). Summary of 28 independent experiments, median indicated. A4-HA was transfected in increasing amounts. (e) Equal volumes of supernatants of cells co-transfected with NL-Luc R^-E^-/VSV-G and increasing amounts of A4-HA were used to infect HOS cells. Intracellular luciferase activities were determined in infected cells; summary of 16 experiments (a subset of the experiments shown in (d)), median is indicated. (f) A subset of samples (seven experiments) used in (e) was quantified

for RT concentrations. RT normalized supernatants of cells co-transfected with NL-Luc R⁻E⁻/VSV-G and increasing amounts of A4-HA were used to infect HOS cells. Intracellular luciferase activities determined in infected cells, median is indicated. (d - f) Statistical evaluation of reporter luciferase activity data was performed by means of a multifactorial ANOVA.

Stable A4 expression enhances multiple-cycle replication of HIV-1. To test whether A4 can also enhance production of CCR5-tropic HIV-1, we co-transfected the replication competent HIV-1 NL-BaL plasmid [253] with A4 expression plasmid and measured the infectivity of RT value normalized particles using the HIV-reporter cell line TZM-bl [250]. The expression of A4-HA resulted in enhanced expression of NL-BaL, as demonstrated by immunoblots probed for viral capsid p24 and Vif proteins (Fig. 6a). The viral particles harvested from this experiment demonstrated similar infectivity after normalization for RT activity (Fig. 6b).

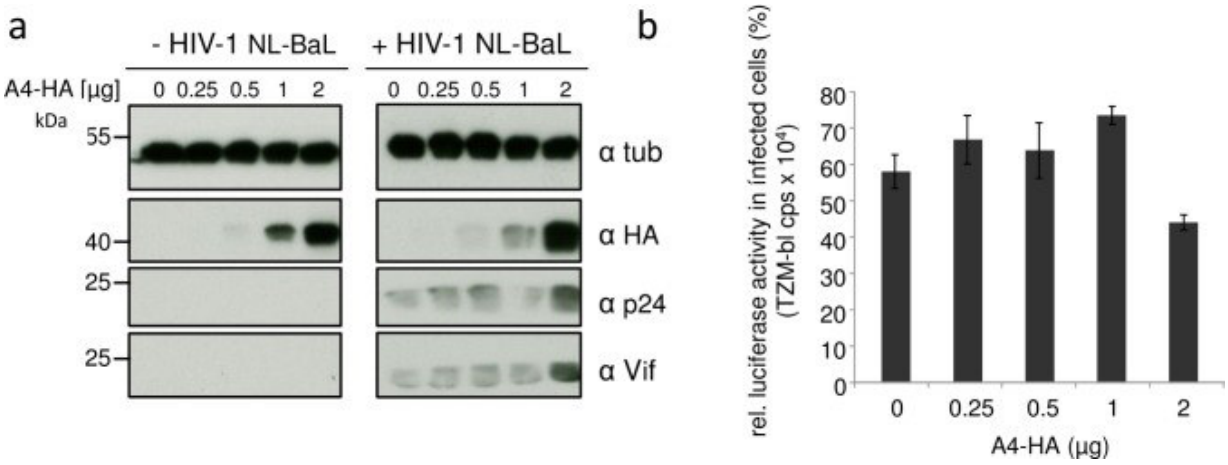

Figure 6: A4 enhances expression of CCR5-tropic HIV-1. (a) Increasing amounts of A4-HA expression plasmid were co-transfected with HIV-1 NL-BaL and immunoblot analysis of co-transfected 293T cells were performed. Immunoblots were probed with anti-p24 (capsid), anti-Vif, anti-HA and anti-tubulin (tub) antibodies. α, anti. (b) Infectivity of RT-normalized viral supernatant of the transfected cells from (a) were used to infect TZM-bl luciferase reporter cells. cps, counts per second. Data are represented as the mean with SD. Statistically significant differences between no A4 and A4 groups were analyzed using the unpaired Student's t-test with GraphPad Prism version 5 (GraphPad software, San Diego, CA, USA). Validity of the null hypothesis was verified with significance level at α value = 0.05. NS: not significant.

To analyze whether A4 expression can also enhance spreading replication of HIV-1, we generated a stable A4-HA expressing cell line derived from HOS.CD4.CCR5 cells [249] using a G418-selectable retroviral A4-expressing vector (Fig. 7a). As a control, we generated HOS.CD4.CCR5.neo cells transduced with a retroviral vector just encoding the G418-resistance gene. The cell lines were infected with NL-BaL, and virus spread was monitored for 20 days

(Fig. 7b). HIV-1 showed comparable overall virus replication kinetics in both cell lines; however, HIV-1 replicated in the A4 expressing cells more efficiently resulting in 2 - 3 fold increased virus titers. These data are consistent with our finding that A4 stimulated HIV expression in the transient transfection experiments, supporting the premise that A4 modulates HIV-1 replication.

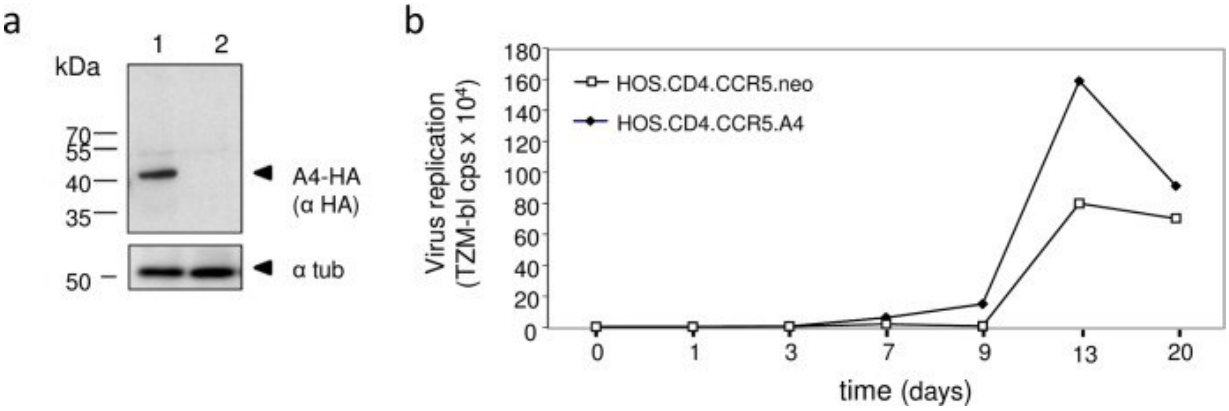

Figure 7: A4 enhances multiple cycle replication of HIV-1. (a) Immunoblot analysis of A4-HA expressing HOS.CD4.CCR5.A4 cells (1) and empty retroviral vector just encoding G418-resistance containing HOS.CD4.CCR5.neo cells (2) using an anti-HA antibody. Cell lysates were also analyzed for equal amounts of total proteins by using anti-tubulin antibody. (b) HOS.CD4.CCR5.A4 and HOS.CD4.CCR5.neo cells were infected with HIV-1 clone NL-BaL, MOI of 0.01. Virus replication was monitored by testing the cell supernatants on TZM-bl cells and measuring luciferase activity.

HIV enhancement is not mediated by cytidine deamination. To test whether cytidine deamination activity is associated with the described A4 effect, we generated the active site mutants E95Q and C134A, in which the zinc-coordinating motif $HxEx_{30}PCx_6C$ (E95 and C134 underlined, x can be any amino acid) was mutated (Fig. 2a). Unexpectedly, only the A4-E95Q construct expressed detectable protein, precluding A4-C134A mutant from functional studies (Fig. 8a). To analyze if the active site mutation has any effect on virus production, HIV-1 luciferase plasmid (NL.Luc R^-E^-) was co-transfected with increasing amounts of A4-HA.E95Q expression plasmid and luciferase activity was measured in virus producer cells (Fig. 8b). 293T cells showed higher virus-encoded luciferase activity after transfection of A4-HA.E95Q in a dose-dependent manner, comparable with the luciferase enhancement after transfection of wildtype A4 (Fig. 5a), indicating that cytidine deamination activity of A4 protein is dispensable for the described HIV enhancing effect.

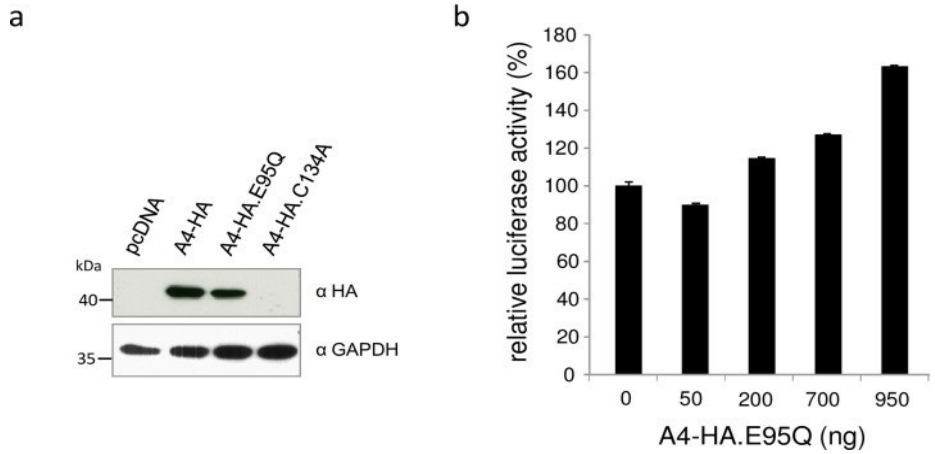

Figure 8: Active site mutation has no influence on A4 activity. (a) Protein expression of A4-HA, A4-HA.E95Q and A4-HA.C134A detected by anti-HA immunoblot analysis, showing equal amounts of A4-HA and A4-HA.E95Q, but lack of A4-HA.C134A expression in transfected cells. (b) HIV-1 reporter virus (NL-Luc R^-E^-) was co-transfected with increasing amounts of expression plasmid for A4-HA.E95Q. Virus encoded luciferase activity in the transfected cells was enhanced by A4-HA.E95Q in a dose-dependent manner.

A4 lacks detectable cytidine deaminase activity, To evaluate the cytidine deaminase activity of A4 directly, we performed *in vitro* cytidine deamination assays as described before [256,257]. We expressed and purified GST-tagged fusion proteins (GST-A3C, GST-A4, GST-A4ΔKK and free GST) from *E. coli* (Fig. 9) and used them for activity assays (Fig. 10) and DNA binding experiments (Fig. 11). In parallel, A3G-His was purified from transfected 293T cells [256] (Fig. 9) and used as a positive control for deamination of CCC to CCU. Because the target preference

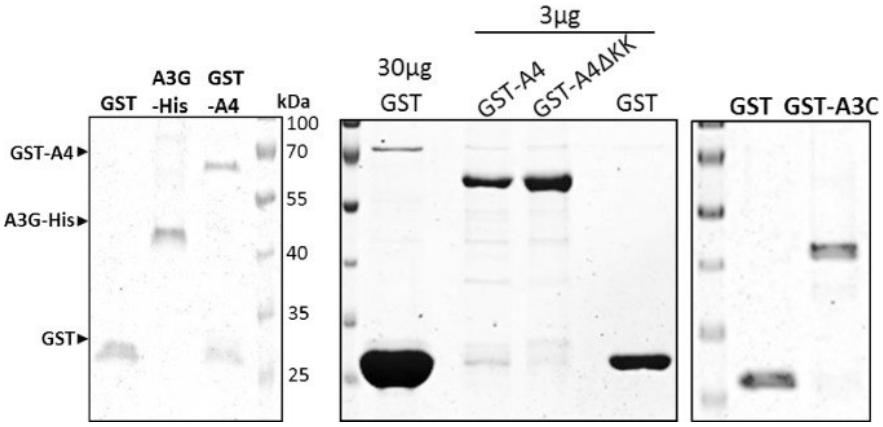

Figure 9: Recombinant produced and affinity purified *E. coli*-derived GST, GST-A3C, GST-A4, GST-A4-ΔKK proteins and 293T cell-derived A3G-His protein were resolved on a 10% SDS gel. Purity of the proteins was determined by staining the gel with Coomassie blue. GST-A4, A3G-His and GST proteins are indicated according to their molecular mass.

for A4 is not known, we used two different oligonucleotide substrates containing either CCCA/G or TTCA in the central region. If deamination of cytidine to uridine occurred, a 40-nt DNA product is generated after restriction enzyme cleavage and detectable after separation of the digested substrate on a polyacrylamide gel. This method demonstrated cytidine deamination of CCC oligonucleotide substrates by A3G-His protein but not by GST-A4 (Fig. 10a, b). Since *E. coli*-derived GST-proteins might not be optimally folded and may differ in deamination activity or DNA binding due to the GST-tag, we additionally tested APOBEC proteins encapsidated in virions, protein lysates of transfected 293T cells or APOBEC proteins immunoprecipitated from transfected cells (Fig. 10c, e) for their deamination activity (Fig. 10d, f). We performed *in vitro* editing experiments with HA-tagged A4, A4-ΔKK, A3F and A3G. In contrast to A3 proteins, A4 were not detected in HIV-1 particles (Fig. 10c). Similarly, only minor amounts of 3xHA-A4 were detectable in lysate of transfected 293T cells, but this could be enhanced by immunoprecipitation with HA affinity beads (Fig. 10e). We saw deamination of CCC to CCU and TTC to TTU only by A3G and A3F, respectively. A4 did not deaminate in any of the above experiments irrespective of its protein source, tags or target DNA (Fig. 10d, f).

A4 weakly interacts with single stranded DNA. For A3 proteins such as A3G, interaction with single-stranded DNA (ss-DNA) and the formation of multiple DNA-protein complexes was shown [257–259]. We purified GST, GST-A4 and as a control, GST-A3C from *E. coli* to characterize whether GST-A4 interacts with ss-DNA. Electrophoretic mobility shift assays (EMSA) were carried out with a biotinylated end-labelled 30 nt DNA oligo (TTCA). GST as a background control protein did not cause any characteristic shift. GST-A3C formed complex I, but a greater proportion shifted on the top of the blot (complex II) at the highest protein concentration (1 µM) (Fig. 11a). However, the addition of detergent NP-40 aided to form the stable complex I at 10 nM GST-A3C and complex II at higher protein concentrations, suggesting a strong GST-A3C interaction with DNA. Importantly, the GST moiety did not affect the binding (Fig. 11a). GST-A4 did not cause a shift at low protein concentration like A3C or A3G [259], but at the highest amount of protein used (500 - 1000 nM) a minor proportion of complex I was formed. All the DNA-protein complexes in the EMSA were disrupted by adding the 80 nt unlabeled competitive DNA in 200-fold molar excess. In contrast, GST-A4ΔKK failed to form any complexes (Fig. 11b).

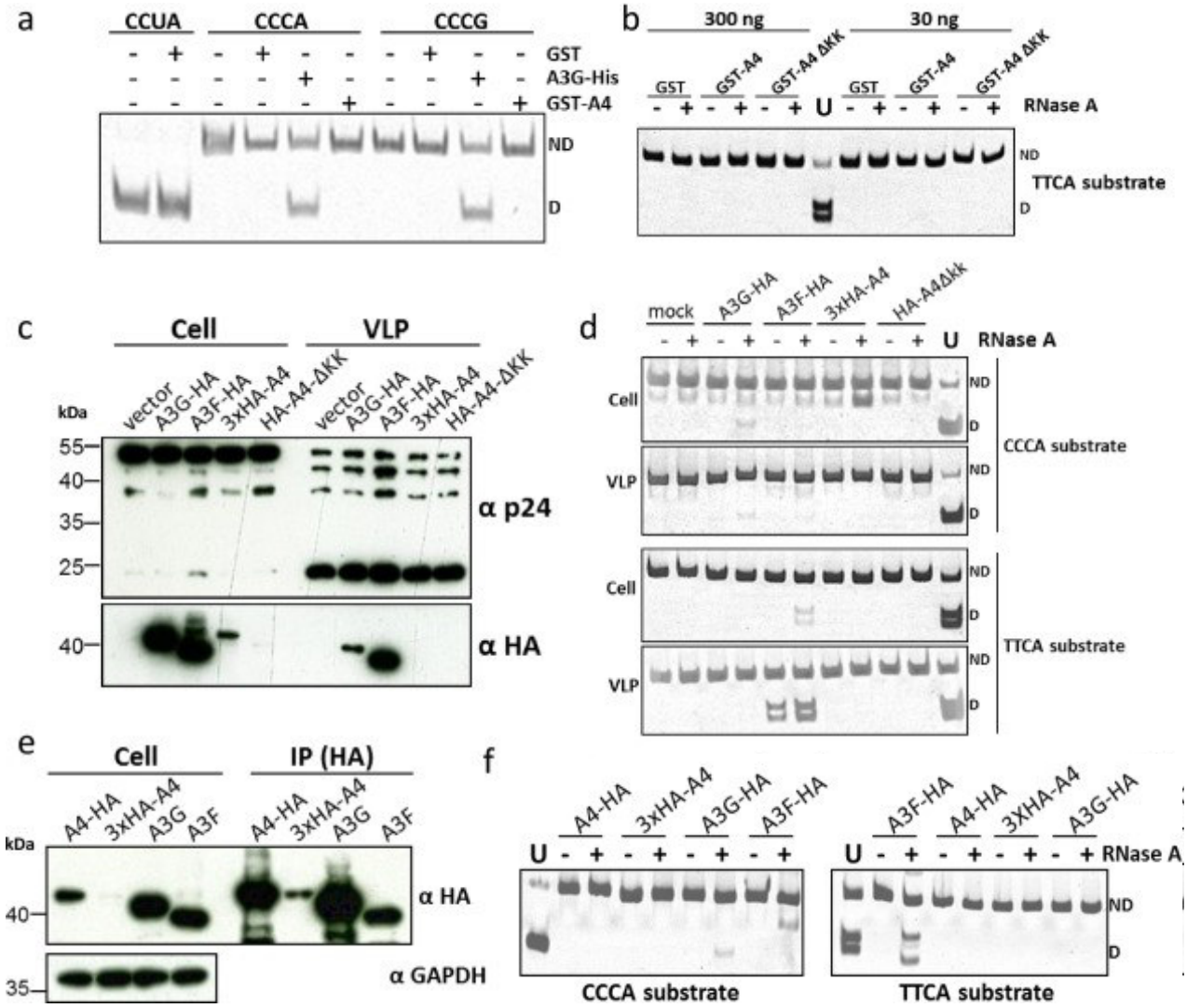

Figure 10: A4 does not deaminate single stranded DNA. (a) Deamination activity of A4 was tested on two different oligonucleotide substrates containing nucleotide sequences CCCA or CCCG. The A3G-His fusion protein was incubated with CCCA and CCCG containing substrates and served as positive control for deamination resulting in 40-bp DNA fragments. Oligonucleotide CCUA served as a marker to denote the deaminated product after Eco147I cleavage; ND: not deaminated; D: deaminated. (b) Deamination experiment using TTCA containing oligonucleotide and GST-purified A4 proteins, RNAse A treatment was included; ND: not deaminated; D: deaminated. (c) Immuno blot analysis of cell lysates and virus lysate of A3G-HA, A3F-HA, 3xHA-A4 and HA-A4-ΔKK expressing cells and HIV virus like particles (VLP), respectively. Anti-HA staining indicates the presence of HA-tagged A3 and A4 proteins, while anti-p24 antibody detects HIV-1 capsid proteins. (d) Deamination assay using transfected 293T cell lysate (from experiment shown in (c)). RNAse A treatment was included; ND: not deaminated; D: deaminated. (e) Immuno blot analysis of cell lysate and immunoprecipitate (IP) fraction of A3 and A4 proteins. (f) Deamination assay using the immunoprecipitated APOBEC proteins (from experiment shown in (e)). RNAse A treatment was included; ND: not deaminated; D: deaminated.

Crosslinking DNA-A3G studies previously showed that the deamination activity on ss-DNA was facilitated when A3G formed dimers and tetramers [260]. These observations suggested analyzing the capacity of A4 to form dimers. To demonstrate that A4 protein multimerizes in human cells, cleared cell lysate was incubated with different concentrations of the cross linking reagent disuccinimidyl suberate (DSS). Immunoblot analysis of cross-linked samples dose-dependently revealed the existence of A4 running at the molecular weight expected for dimers, indicating that primary amines which can be crosslinked with DSS are present within the A4 dimerization interface (Fig. 11c).

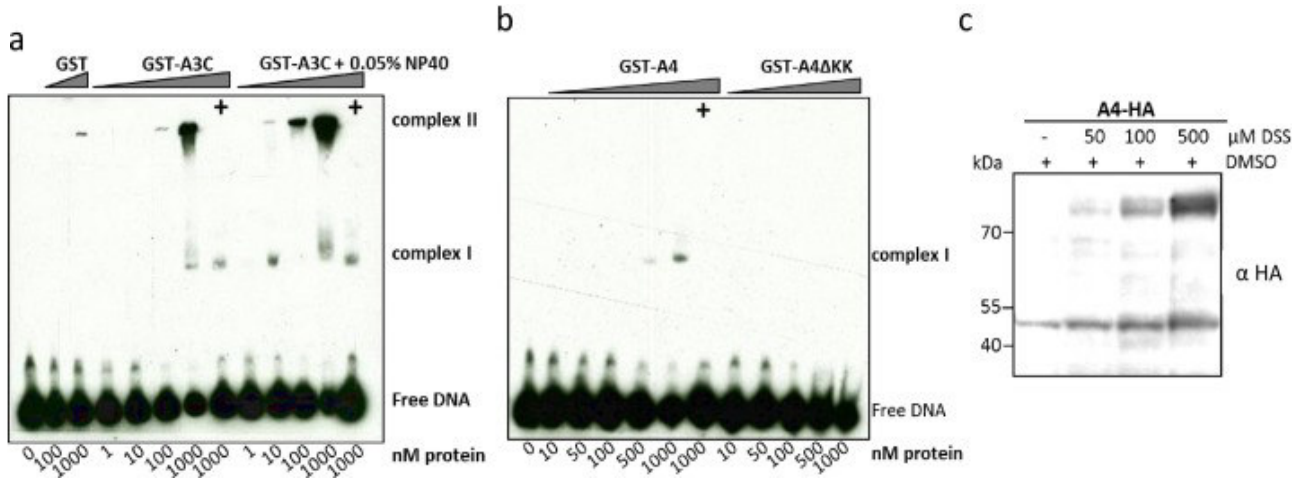

Figure 11: A4 interacts weakly with single-stranded DNA. EMSA with purified, GST-A3C (a), GST-A4 and GST-A4ΔKK (b) performed with 30 nt single stranded target DNA labeled with 3'-labeled with biotin. Indicated amounts of protein (at the bottom of blot) were titrated with 10 nM of DNA. (+) indicates presence of competitor DNA, which is unlabeled 80 nt DNA (200-fold molar excess), as used for deamination assay to demonstrate specific binding of protein to DNA being causative for the shift. For GST-A3C (a) a separate panel was added for reactions containing 0.05% NP-40 detergent. (c) A4-HA crosslinking by DSS. DSS was added to the cleared cell lysates to reach the indicated DSS concentrations. The blot was probed with anti HA antibody to detect monomeric and dimeric forms of A4-HA.

A4 enhances expression of HIV-1 LTR and other promoters. To test whether A4 enhances specifically HIV-1 production, we performed comparative expression analysis of HIV-1 LTR and other viral and cellular promoters with and without A4 in the same cell. To this end, we co-transfected the NL-Luc plasmid expressing the firefly luciferase gene which is located in the *nef* gene together with the Herpes simplex virus (HSV) thymidine kinase (TK) promoter-driven *Renilla* luciferase (HSV-RLuc) reporter plasmid and different amounts of A4-HA expression plasmids. Both luciferases were measured sequentially from single samples. The results revealed

an A4 dose-dependent increase in both luciferase activities (up to 2.5-fold for NL-Luc expression and up to 1.5-fold for the HSV driven luciferase) (Fig. 12a). To test whether A4 affects HIV expression by acting on the viral LTR, luciferase reporter constructs with the HIV-1 LTR (LTR-Luc, firefly luciferase) and HSV-RLuc were co-transfected with increasing amounts of A4-HA expression plasmid with or without addition of an HIV-1 Tat expression plasmid. As expected,

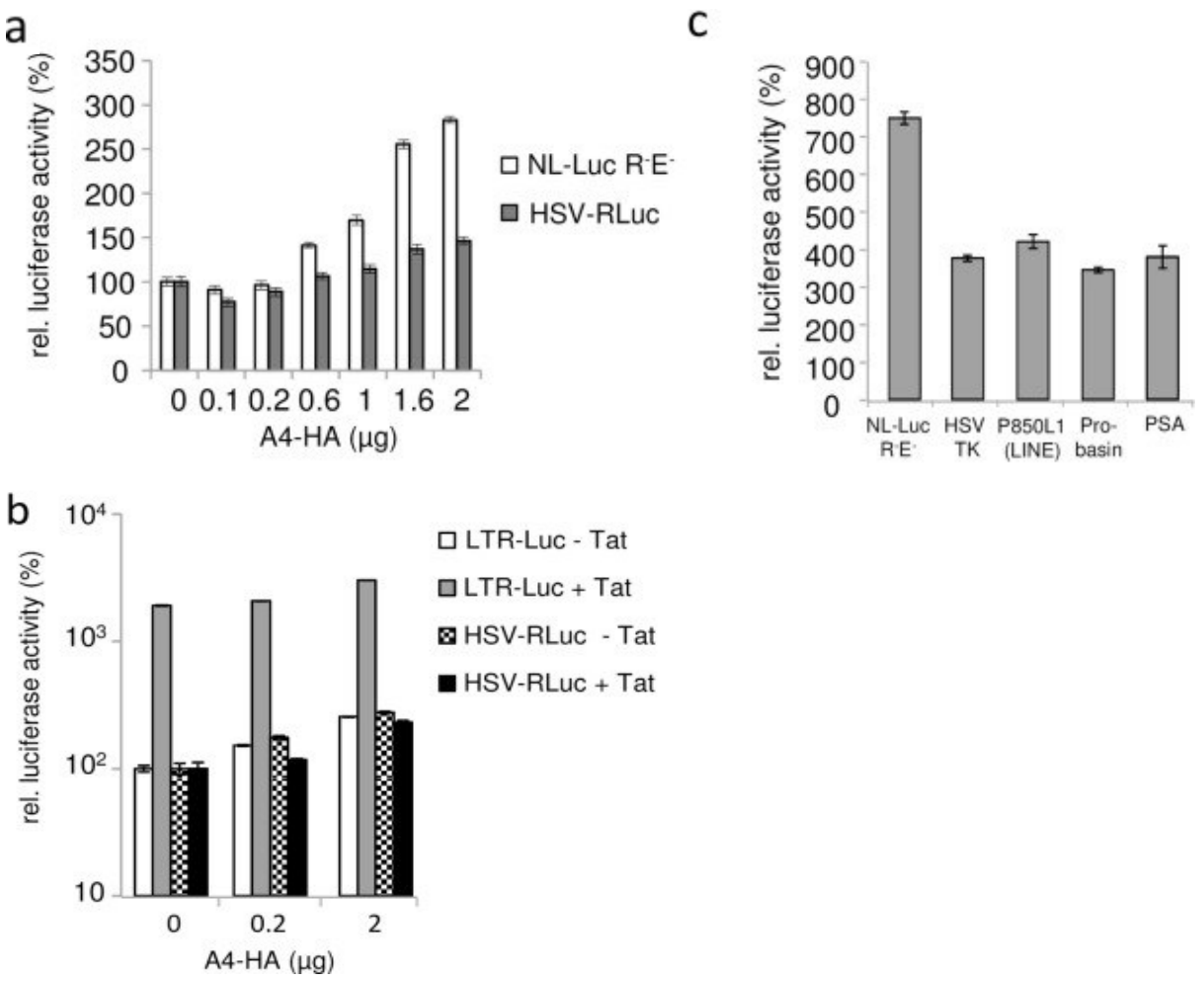

Figure 12: A4 enhances expression of luciferase reporter genes driven by various viral and cellular promoters. (a) Dual luciferase reporter assay was performed two days post co-transfection of NL-Luc R^- E^- and HSV-TK promoter *Renilla* luciferase (HSV-RLuc) with and without A4-HA, relative luciferase activities are shown. (0) indicates transfections in the absence of A4-HA plasmid. A4-HA was transfected in increasing amounts. (b) Relative luciferase activities after co-transfection of LTR-Luc (LTR of HIV-1 driving firefly luciferase) with A4-HA or HSV-TK promoter *Renilla* luciferase (HSV-RLuc) with A4-HA and with and without Tat expression plasmid (c) Luciferase activities driven by various viral (LTR, HSV TK) or cellular promoters (LINE, Probasin, PSA) in presence of the transfected A4-HA expression plasmid, relative to luciferase activity in cells without A4 expression. Total amounts of luciferase expression plasmid and total plasmid DNA was kept constant within all experiments. Error bars indicate standard deviation.

the presence of Tat enhanced the expression of the LTR-Luc construct (by 19-fold) relative to LTR-Luc expression in the absence of the Tat plasmid (Fig. 12b). A4 expression in the absence of Tat stimulated the LTR-Luc expression by up to 2.6-fold and by up to 1.6-fold in the presence of Tat. The thymidine kinase promoter of HSV was not sensitive to the presence of Tat. In contrast, A4 enhanced the HSV-RLuc expression by up to 2.8-fold when Tat was not co-transfected. In the next experiment, we tested firefly luciferase expression constructs driven by promoters of HSV-TK, LINE1 (P850 L1), probasin, or prostate-specific antigen (PSA), together with NL-Luc. Co-transfection with 2 μg A4-HA expression plasmid enhanced the luciferase activity of all these constructs from 3.5-fold to 5-fold, whereas HIV-LTR expression was enhanced by 7-fold (Fig. 12c). Based on these results, we conclude that A4 might directly or indirectly enhance the transcription of HIV and other promoters.

Discussion

Herein we report the first study addressing the potential function of the A4 protein in human cells. A4 is the most recently identified and the least studied APOBEC protein [192,240]. It is more closely related to A1 than to the A3 proteins [192]. Knowledge about the A4 protein is very limited; it is unknown if A4 binds to RNA or DNA or possesses any enzymatic activity, and no biochemical and structural information about A4 is available to date. Our data show for the first time biological activity of A4, which enhances the expression of HIV-1.

As part of this study, we established mammalian expression plasmids for A4 and we also generated bacterially expressed GST-A4 fusion proteins to test for their enzymatic activity. Under experimental conditions that readily detect cytidine deamination by A3G, purified GST-A4 did not carry out any detectable cytidine deamination. We also tested A4 isolated from transfected human cells and similarly found no cytidine deamination activity. These findings are in agreement with the previously reported absence of cytidine deamination of A4 using a cellular mutation assay in bacteria and yeast [240]. In addition, we found that mutating the zinc-coordinating domain of A4 did not abolish the HIV-enhancing activity of A4. Nevertheless, these observations do not mean that A4 is catalytically inactive, A4 may just have different substrate specificity and cytidine deamination may not be the A4 function required for enhancement of HIV expression.

The deamination activity of A3 proteins such as A3G on ss-DNA is facilitated by A3G dimers and tetramers [260]. A4 formed at least dimers, but did weakly bind to ss-DNA only. This weak DNA binding was lost, if the characteristic polylysine stretch (KKKKKGKK) at the C-terminus of A4 was deleted (A4ΔKK), supporting the hypothesis that the net positive charge rendered by polylysines confer some capacity to interact with DNA [261]. Thus, the weak interaction of A4 with ss-DNA may be one reason for the lack of detectable deamination.

We speculated that the polylysine domain would be involved in nuclear localization of A4 and that a C-terminal HA-tag would obstruct this activity, because A4 with an N-terminal HA-tag (HA-A4) localized to both cytoplasm and nucleus of transfected cells, while A4-HA with a C-terminal HA-tag was detected only in the cytoplasm. However, HA-A4ΔKK also localized to both compartments, cytoplasm and nucleus, suggesting that the polylysine domain is not important for nuclear localization. C-terminal stretches of lysines are also found in other proteins unrelated to A4 e.g. in the GTPase KRas (KRAS, K-Ras4B, NP_004976) and FAM133B

(NP_001035146). In KRas, the polylysine region (KKKKKKSK) contributes to the interaction of KRas with Ca^{2+}/calmodulin and strongly influences its binding to the plasma membrane by electrostatic interactions with the membrane anionic lipids [262,263]. Whether A4 specifically interacts with membranes or Ca^{2+}/calmodulin is not known.

We demonstrated that A4 mRNA is highly expressed in human testis, but is barely detectable in 293T, HeLa, A3.01 T and Jurkat T cell lines. Analysis of protein expression of endogenous A4 was precluded, due to the non-availability of any A4-specific antibody. In light of the sexual transmission of HIV-1 and the possibility of sanctuary sites for HIV-1 in the male genital tract, the origin of seminal HIV-1 is a topic of ongoing discussion [264–271]. Human testicular tissue is described to be susceptible to HIV-1 [264,267,272–275] and macaque testis and epididymis are found to be infected by SIV in several studies [265,268,271,276,277]. Since we do not know whether $CD4^+$ cells in testis express A4, we cannot make a statement concerning modulation of HIV infection in testis by A4. A4 also enhanced the expression of firefly luciferase which is controlled by the HIV-1 LTR in a manner similar to that of the unrelated HSV promoter driven *Renilla* luciferase and A4 expression increased the expression of luciferase constructs driven by cellular promoters. However, our results do not clearly demonstrate that A4 is a factor that enhances LTR-mediated transcription. Indeed, it is thus likely that HIV benefits from a broad activity of A4. We hypothesize that A4 creates a cellular/nuclear environment that stimulates for example the expression of HIV-1. A4 may boost expression or activity of a factor important for HIV or reduce the expression or activity of a negative regulator of HIV. It is very well possible that the observed enhancing activity of A4 to HIV is relevant for the expression of cellular promotors and endogenous retroviruses in testis [278]. Future studies investigating the interactome of A4 may help to reveal the A4 pathway and its enhancing activity.

Acknowledgements

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Discussion

After the human immunodeficiency virus (HIV) enters the body, it targets and replicates primary in cells of the human immune system, like CD4 T-cells, dendritic cells and macrophages. Infection of a cell type is determined by the expression of several cellular proteins, which support or restrict the virus. The so called restriction factors exert their activity on various stages of the HIV replication cycle and can significantly influence HIV pathology and disease progression. Host factors which promote infection are often referred to as HIV dependency factors (HDFs), whereas molecules which are employed by the innate immune system and block virus infection are called restriction factors. The research on host factors is ongoing and genome wide RNAi screens still identify new host factors, which were not considered before [85–87,279]. Sometimes there is no distinct separation between dependency and restriction factors. Host proteins which restrict HIV-1 replication on the first sight may help the virus to evade the immune system when a bigger picture is drawn. Like for SAMHD1, which is depleting the dNTP pool in macrophages and thereby inhibits reverse transcription of viral RNA [280,281]. But restriction of reverse transcription also prevents excess production of viral compounds which are recognized by pattern recognition receptors (PRRs) and induce pro-inflammatory cytokines and type I interferon, leading to an antiviral immune response. Beside this, not only presence or absence of a specific molecule influences HIV-1 infectivity, also genetic polymorphisms affect disease outcome. Polymorphisms having an impact on HIV-1 are identified for the MHC region, chemokines and cytokines, IFI16, TREX1, Trim5 and cyclophilin A [282–291]. Targeting host factors with pharmaceutical inhibitors could provide an attractive tool for manipulating infection and lead to the development of new therapeutic strategies. Currently applied “highly active antiretroviral therapy” (HAART) already leads to efficient suppression of HIV-1 replication, but it is limited with regards to side effects, costs and drug resistance. The use of cellular proteins for medical treatment could broaden therapeutic possibilities and minimize the risk for development of drug resistant strains. However, interactions of HIV-1 with human cells are very complex and research leading to a better understanding of the role of host factors is essential for developing new medical approaches.

In the study presented here, we analyzed several host factors and their interaction with HIV-1. In chapter II the Il-2 inducible T-cell kinase (ITK) was introduced as a dependency factor for HIV-1 infection. ITK belongs to the Tec family of non-receptor tyrosine kinases and is mainly expressed in T-cells [89,292]. It is involved in stimulation of the ERK/MAPK pathway [96,122], regulates the amount of Ca^{2+} release and transcription factor activation in response of TCR stimulation [293,294], as well as actin polarization by CDC42 and Rac1 [295,296]. Taken together ITK is a key factor for T-cell activation, which is indispensable for productive HIV-1 infection [88,128,297]. Previous studies already described an influence of ITK on HIV-1 replication by affecting multiple steps of the viral life cycle, like entry, transcription from the LTR and virus spread. Also a decreased actin polarization to gp120 contact sites and defective Gag coordination together with less virus particle release was reported in the absence of ITK [88,298]. Compared to that, our studies indicate that the major block of HIV-1 replication in ITK deficient cells already takes place at the step of cell entry. Inhibition of viral protein expression was not detected when viruses where pseudotyped with VSV-G, indicating that later steps of the HIV-1 lifecycle, like integration and transcription were not affected. Therefore we focused mainly on investigating viral entry and discovered that also HIV-1 attachment was strongly impaired in the absence of ITK. While examining attachment factors we found equal expression of CXCR4, slightly enhanced CD4 and increased LFA-1 expression. Interestingly heparan sulfate was almost completely missing on the cell surface of ITK deficient cells. However, all attachment factors investigated in this study didn`t cause defects in HIV-1 binding, indicating the existence of not yet analyzed or identified attachment factors or the use of a more complex mechanism which involves multiple proteins. Also the influence of ITK on actin reorganization likely contributes to decreased viral attachment in knockout cells, because of their more rigid actin network and stronger barrier of cortical actin. This could affect processes like receptor clustering or “virus surfing”, which probably contribute to firm adhesion.

Nevertheless ITK constitutes an attractive pharmaceutical target for treatment of HIV-1 infected patients. Because ITK is exclusively expressed in T-cells and mast cells [299] side effects on other tissues are limited. In addition, inhibition of ITK leads to an defective T_H2-cell response which impairs but not completely blocks immune functions [300]. ITK deficient mice are still able to defeat viral infections and have even shown to clear infections with *Leishmania* more efficiently, probably because of a more T_H1 than T_H2 based immune response [95,100]. ITK overexpression and

thereby upregulated T_H2 responses are associated with inflammatory disorders, like allergic asthma and atopic dermatitis [102,301–304]. For this reason ITK inhibitors are already available [305–307] and mice with a ITK kinase deleted domain develop no allergic asthma [304]. However, the situation in mice may not necessarily resemble ITK deficiency in humans in detail. Humans with homozygous R335W mutations in the SH2 domain of ITK have been identified. Subjects with this mutation have normal ITK mRNA levels but no detectable protein expression, leading to an highly increased susceptibility to Epstein-Barr-Virus infection, B-cell proliferation and Hodgkin`s lymphoma [308]. Also patients with other inherited ITK mutations were identified; all of them showed high Epstein-Barr-Virus titer and associated symptoms like hepatosplenomegaly, bilineage cytopenia or autoimmune phenomena. Also low amounts of invariant natural killer T-cells are associated with impaired ITK functions [98]. For the use of ITK inhibitors in pharmaceutical therapy it should also be considered, that most inhibitors target the ATP binding pocket located in the kinase domain [305–307]. But for HIV-1 replication also kinase independent functions are important, as for actin reorganization and virus particle formation, where mostly the PH domain is required [309]. Drugs for preventing membrane localization of ITK by PH domain inhibition are also tested *in vitro* [310].

Since binding of HIV-1 to target cells has a strong influence on its infectivity, chapter III focused stronger on analyzation of interactions mediating viral adhesion. Beside the well-studied entry receptors CD4 and CXCR4/CCR5 several additional molecules were identified to support HIV-1 binding [49,144–147], some of them are described in chapter II. In chapter III the attachment factor lymphocyte function-associated antigen (LFA-1) and its interaction partner intercellular adhesion molecule 1 (ICAM-1) were analyzed in more detail. Both molecules are expressed on T-cells [120,148]. ICAM-1 is also expressed on endothelial cells and is upregulated after stimulation with pro-inflammatory cytokines or after T-cell activation [154,155]. In line with previous publications, we could detect specific packaging of ICAM-1 into viral particles and an enhanced infectivity of ICAM-1 bearing reporter-viruses [123,156–159,174]. In addition we show upregulation of ICAM-1 expression in transfected 293T cells. To test the role of LFA-1/ ICAM-1 interactions during spreading replication, a Jurkat cell line with LFA-1 knock out was generated. However, the LFA-1 knockout had only little effects on spreading replication of NL4-3 in cell culture. To find an explanation for this, we examined ICAM-1 expression levels after *vpu* transfection, because the accessory protein Viral Protein Unique (Vpu) was described before to influence the protein

composition of host cell membranes [176]. Indeed co-expression of *vpu* caused a shift in protein size of ICAM-1, detected by immunoblot analysis. If this change in molecular mass of ICAM-1 leads to decreased LFA-1/ICAM-1 interactions during HIV-1 replication needs further investigation. Also reason and nature of the observed modification and if the experimental setting reflects the situation *in vivo* has to be analyzed in future studies. The possibility for therapeutic targeting of LFA-1 or ICAM-1 is probably limited, because of the essential function of these proteins. Nevertheless this study contributes to a better understanding of viral binding and entry. Especially possible interactions of Vpu with ICAM-1 are interesting and could shed more light on how Vpu interferes with the human immune system. Manipulating HIV-1 replication by pharmaceutical targeting of Vpu also seems to be a more feasible goal. The cholesterol-binding compound amphotericin B methyl ester (AME) was shown to inhibit HIV-1 particle production by interfering with the anti-tetherin function of Vpu [311].

The last chapter of this thesis explores the activity of Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 4 (APOBEC4). Knowledge about APOBEC4 is limited; it is primarily expressed in testis and belongs to the family of AID/APOBEC polynucleotide (deoxy) cytidine deaminases, which are known for their catalytic activity to convert cytidine to uridine. In contrast to other APOBEC proteins, like APOBEC3G, which restricts HIV-1 infection by mutating viral genomes [257–259], no antiviral activity of APOBEC4 was detected. In line with this observation only weak interaction with single-stranded DNA and no deaminase activity or packaging of APOBEC4 into viral particles was detected. Chapter IV is the first report describing a biological function of APOBEC4. In contrast to other members of the APOBEC family it does not restrict HIV-1 infection. Instead, presence of APOBEC4 enhances HIV-1 production in a dose dependent manner and increases virus titer in spreading replication assays. Also reporter gene expression from LTR driven promotors was severely enhanced, as well as protein expression from several other promotors, like the Herpes simplex virus (HSV) thymidine kinase (TK), LINE and various cellular promotors. For novel HIV-1 therapies host factors that regulate viral gene transcription are of growing interest, because activation of HIV-1 genes in latently infected cells can reverse latency. This makes infected cells susceptible for cytolytic T-cell killing or cytophatic effects, which can potentially eradicate viral reservoirs. Under this aspect Histone Deacetylase (HDAC) inhibitors are currently of special interest for pharmacological approaches, because of their ability to induce HIV-1 in latently infected cells [312–314].

Taken together the results presented in this thesis contribute to a better understanding of the interplay between HIV-1 and its host cell and may lead to the development of more efficient therapies. However, infection with HIV-1 and the interplay with the human immune system are very complex and host factors influence each step of the viral life cycle, leading to a broad spectrum of potential target proteins. Therefore more research is needed to identify the factors most essential for viral replication and their relevance for infection *in vivo*. Also the suitability of particular molecules for pharmaceutical targeting needs examination. Open questions to be addressed include; does the target protein have an enzymatic domain or interaction site that can be blocked by an inhibitor? And is the inhibitor unspecific, toxic or cause any side effects? Also the bioavailability and how the drug can be delivered into specific cells should be considered [315]. One HIV-1 dependency factor successfully targeted by antiretroviral therapy is the CCR5 receptor. Blockade of CCR5 is well tolerated and subjects with homozygous deletion in the CCR5 allele (Δ32/Δ32), naturally occurring in the Caucasian population, are resistant to R5-tropic HIV-1 infection without significant immunological dysfunctions [316–318]. Patients with heterozygous deletions of CCR5 show delayed disease progression [316,317]. Furthermore, Δ32/Δ32 allogenic stem cell transplantation in a HIV-1 positive leukemia patient led to long term control of HIV-1 without viral rebound for more than 20 month after transplantation in the absence of antiretroviral therapy [82]. A small molecule inhibitor that binds to a hydrophobic pocket in CCR5 is already in clinical use and called maraviroc [319] and similar reagent are tested in clinical trials at the moment, like vicriviroc and anti-CCR5 antibodies [320,321]. Other clinical trials aim to inhibit HIV-1 by removing CCR5 from CD4 T-cells by gene therapy [322]. Finally not only strategies to block viral replication could be useful, new approaches could also reduce HIV-1 inhibition by restriction factors and thereby mediate a stronger immune response to enable the immune system to clear infection or as a tool for vaccine production.

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Publications

The results of the work presented here were in part published in scientific journals or presented at international conferences.

Publications and manuscripts

IL-2 Inducible Kinase ITK is Critical for HIV-1 infetion of T-cells. **Hain A.,** Kämer M., Linka RM., Nakehaei-Rad S., Ahmadian MR., Borkhardt A., Häussinger D., Münk C. (Manuskript in preparation)

ICAM-1 Incorporation Enhances Binding of HIV-1 but not it`s Infectivity. **Hain A.**, Linka RM., Borkhardt A., Häussinger D., Münk C. (Manuskript in preparation)

Determinants of FIV and HIV Vif Sensitivity of Feline APOBEC3 Restriction Factors. Zhang Z, Gu Q, Vasudevan AA, **Hain A**, Kloke BP, Hasheminasab S, Mulnaes D, Sato K, Cichutek K, Häussinger D, Bravo IG, Smits S, Gohlke H, Münk C. Retrovirology (accepted 2016 Jun. RETV-D-16-00031R1)

A novel approach to detect resistance mechanisms reveals FGR as a factor mediating HDAC inhibitor SAHA resistance in B-cell lymphoma. Joosten M, Ginzel S, Blex C, Schmidt D, Gombert M, Chen C, Linka RM, Gräbner O, **Hain A**, Hirsch B, Sommerfeld A, Seegebarth A, Gruber U, Maneck C, Zhang L, Stenin K, Dieks H, Sefkow M, Münk C, Baldus CD, Thiele R, Borkhardt A, Hummel M, Köster H, Fischer U, Dreger M, Seitz V. doi: 10.1016/j.molonc.2016.06.001

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Meeting abstracts

Talks

Hain A, Krämer M, Nakhaei-Rad S, Linka RM, Häussinger D, Ahmadian MR, Borkhardt A, Münk C. ITK plays a central role in integrating signaling pathways required for HIV1 - T-cell fusion 39th annual meeting on Retroviruses, 19.-24. May 2014, Cold Spring Harbor, New York

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Poster

Hain A, Krämer M, Nakhaei-Rad S, Linka RM, Häussinger D, Ahmadian MR, Borkhardt A, Münk C. The IL-2 inducible kinase ITK is critically required for HIV-1 infection of T-cells 25nd Annual Meeting of the Society for Virology, 18.-21. März 2015, Bochum

Hain A, Krämer M, Nakhaei-Rad S, Linka RM, Häussinger D, Ahmadian MR, Borkhardt A, Münk C. The IL-2 inducible kinase ITK is critically required for HIV-1 infection of T-cells 24nd Annual Meeting of the Society for Virology, 26.-29. März 2014, Alpbach

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