APOBEC3-mediated hypermutation of retroviruses: A defensive tool of the innate immune system

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DECLARATION BY CANDIDATE

I, Aikaterini Krikoni, hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged and indicated.

<<In order to create something that functions properly -a container, a chair, a house- its essence has to be explored, for it should serve its purpose to perfection, i.e. it should fulfil its function practically and should be durable, inexpensive and beautiful.>>

WALTER GROPIUS
Neue Arbeiten der Bauhaus Werk Stäten, Bauhaus
Books, no. 7

To my family

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Last but not least, I would like to thank my family for their never-ending encouragement and ongoing support. To them I dedicate this thesis. My very special thanks go to my beloved Thomas for the passion and inspiration in life. I should not also forget all my close friends for finding weekly time to chat and support.

Abstract

The APOBEC3 family of cytidine deaminases (A3-A, -B, -C, -D, -F, -G, and –H) has been identified to restrict the replication and spread of retroviruses, hepadnaviruses and retroelements. In the infected cells, the A3 proteins act as sensors that recognize and bind to the retroviral Gag polyprotein of the assembling viral particles. During the next round of infection, the encapsidated A3s attack and mutate heavily the viral cDNAs synthesised in the reverse transcription step, leading to non-functional viral genomes and reduced number of integrated proviruses.

This work focuses on the broad understanding of the A3 inhibition of two human pathogens: HIV-1 (Human Immunodeficiency Virus type-1) and HTLV-1 (Human T cell Lymphotropic Virus type-1). Both viruses target $CD4^+$ T cells and macrophages. Nevertheless, these viruses evolved independently mechanisms to defeat the A3 immunity barrier. On one side, HIV-1 can escape from the A3 inhibition by its Vif accessory protein, known for promoting A3 proteasomal degradation, and on the other side, HTLV-1 is characterised by the presence of a C-terminal nucleocapsid area that excludes incorporation of A3s from nascent viral particles.

Here, I present that A3A, A3B and A3H haplotype 2 act as potent inhibitors of HTLV-1, while wild-type HIV-1 can be restricted by A3B and A3H haplotype 2, but not by A3A. A3A and A3B exhibit a deaminase-dependent inhibition of HTLV-1, whereas A3H haplotype 2 acts with a deaminase-idependent mechanism of restriction. Analysis of the A3 editing profile of HTLV-1 sequences recovered from T cell lines, which established from HTLV-1 infected patients, shows an extensive hypermuation of the viral genome. The dinucleotide context of substitutions reveals that HTLV-1 may be *in vivo* a target of several A3s, such as A3A, A3B, or A3G.

To further investigate the A3A anti-retroviral activity, I compared the A3A-mediated restriction of a gamma-retrovirus; MLV (Murine Leukemia Virus) and HIV-1. My results demonstrate that MLV tropism is associated with the A3A inhibitory capacity. In particular, A3A can be fully incorporated into the Moloney MLV, but not into the AKV-derived N and B tropic MLV viral particles. These data might indicate that variability of the viral Gag protein determines the viral sensitivity to the A3 molecules. On the other hand, A3A fails to achieve restriction of HIV-1 through incorporation into the produced viral particles. In addition, I present that A3A, but also other A3s expressed in viral target cells; A3B, A3C, A3G, and A3H haplotype 2, can inhibit HIV-1 expressing an inactive integrase (D64V). This finding might suggest that A3 molecules can mediate clearance of the un-integrated HIV-1 genomes in the target cells.

To sum up, my work contributes on the better understanding of the molecular function of the A3 enzymes. A3s can be considered as a potent defensive tool of the innate immunity network against retroviral spread and infection. Strategies to inhibit the viral counteracting mechanisms will allow us to sufficiently control retroviral infections.

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Scope of the thesis

My thesis aims to highlight the role of the human APOBEC3 (A3) family of enzymes as a potent tool to control retroviral infections. To better understand the anti-retroviral function of the A3 proteins, I compared their activity against retroviruses from different phylogenetic groups that include: a) the MLV (Murine Leukemia Virus); a gamma-retrovirus, b) the HTLV-1 (Human T cell Lymphotropic Virus type-1); a delta-retrovirus, and c) the HIV-1 (Human Immunodefieciency Virus type-1); a lentivirus. The above selection is based on the fact that MLV presents a simple genomic organization, while HTLV-1 and HIV-1 encode for various and dissimilar accessory genes in order to accomplish infection and spread in the host target cells.

The specific aims of this study were:

- To compare the A3 activity against HTLV-1 and HIV-1.
- To detect and determine the specific-nucleotide substitutions induced by A3s on HTLV-1 genome *in vitro* and *in vivo* in $CD4^+$ T cells.
- To investigate whether A3s can target MLV, HIV-1, and non-integrating HIV-1 DNA in the infected cells.

Chapter 1
General Introduction

1.1 Viruses

1.1.1 Viruses: Definition

Bacteria, fungi, and protozoan pathogens carry their own machinery for DNA, protein biosynthesis, and energy production. These parameters allow biologists to define them as *cells.* However, viruses are obligatory parasites. In contrast to cells, viruses are not characterized by a growth and division cycle. Their replication cycle depends on the cell host machinery. Within the host cell, viruses redirect the cell's biosynthetic machinery for synthesis of their components. Newly assembled viruses will be transmitted to another host organism or cell for the beginning of a new infectious cycle.

1.1.2 Why to study viruses?

- **Social and economical impact**

Viruses can infect all species of life; mammals, insects, plants (1-6), bacteria, archaea, fungi, and algae (7- 10). Therefore, viral associated infections of humans, domestic animals or agricultural plants can have an enormous personal, social, political and financial impact. Indeed, governments worldwide spend millions of dollars for control and prevention of viral infections. The main area of research includes viral vaccination programs and development of anti-viral therapeutic agents. As a characteristic example, table 1 displays in millions of dollars the annual support level (actual budget for 2008-2011 and predicted needs for 2012-2013) based on grants, contracts, and other funding mechanisms used across the National Institutes of Health (NIH) for the human virus infections in the United States of America.

Research/Disease Areas (Dollars in millions and rounded)	FY 2008 Actual	FY 2009 Actual	FY 2010 Actual	FY 2011 Actual	FY 2012 Estimated	FY 2013 Estimated
Hepatitis - B	$53	$51	$66	$58	$58	$58
Hepatitis - C	$93	$97	$100	$114	$113	$113
HIV/AIDS 6/	$2,928	$3,019	$3,085	$3,059	$3,075	$3,075
HPV and/or Cervical Cancer Vaccines	$19	$25	$25	$24	$24	$24
Influenza	$204	$316	$308	$272	$271	$271
Pediatric AIDS 6/	$241	$227	$216	$228	$228	$229
Pneumonia & Influenza	$295	$392	$396	$382	$381	$381
Vaccine related (AIDS) 6/	$556	$561	$535	$550	$545	$556
West Nile Virus	$39	$59	$46	$65	$65	$65

Table 1 NIH Annual funding for Viral Related Diseases (alphabetical order). Table published: February 13, 2012, source: NIH. -6/Reporting for this category does not follow the standard RCDC (Research, Condition, and Disease Categorization) process. These are project listings only and non-project or other support costs associated with the annual total for the category are not included.

- **Viruses are linked to cancer**

Moreover, viruses are responsible for approximately 20% of the human cancer cases. A viral infection can cause transformation of the host cell, leading to abnormal growth (11-13). Malignancies can occur due to genetic alterations related to the virus integration site in the host cell genome or expression of viral oncogenic proteins. Tumor viruses are sub-classified as either DNA viruses, which mainly include Epstein Barr virus (EBV) (14-15), Kaposi Sarcoma associated Herpes Virus (KSHV) (16-17), Human Papilloma Viruses (HPV) (18-19), Hepatitis B Virus (HBV) (20-22) or RNA viruses such as Hepatitis C Virus (HCV) (21-22), and Human T Lymphotropic Virus (HTLV) (23). Tumor-inducing viruses carry potent oncogenic genes that induce tumorigenesis through manipulation of cellular pathways and disruption of the mitotic checkpoint. The table 2 categorizes the main existing tumor viruses depending on their nucleic acid (DNA or RNA) and presents the basic induced tumor types.

DNA Viruses	Cancer type
Epstein-Barr Virus	Burkitt's lymphoma (B cell Lymphoma)
Hepatitis B Virus	Hepatocellular Cancer
Human papilloma viruses	Cancer of cervix, skin, anus, penis
Human herpes virus-8	Kaposi sarcoma (abnormal tissue development)
RNA Viruses	**Cancer type**
Human T lymphotrophic virus type 1 (HTLV-I)	T-Cell Leukemia or Lymphoma
Hepatitis C virus	Hepatocellular Cancer, Lymphoma

Table 2 Viruses associated with cancer in humans.

1.2 Retroviruses

Retroviruses are probably one of the best-studied categories of viruses. They are adapted to their host species and replicate successfully by hiding themselves from the host immune recognition mechanisms. This is one reason of the failure of scientists to develop efficient approaches for retroviral therapy and prevention. The main examples of retroviral pandemics in the human population are the Human Immunodeficiency Virus type-1 (HIV-1) and the Human T cell Lymphotropic Virus type-1 (HTLV-1).

1.2.1 Structural analysis of retroviruses

Electron microscopy studies reveal that the retroviral mature virions measure about 80-100 nm in diameter, are spherical in shape, and wrapped by a lipid bilayer. The lipid surface is spiked with only 7–14 envelope glycoprotein complexes (Env) per virion. The Env transmembrane (TM) subunit contacts with the inner matrix (MA) shell, while the outer part of the TM component is bound to the surface (SU) Env subunit. The matrix shell forms the outer protein layer, lies under the lipid membrane, and is characterized as the outer shell of the viral core. Inside the matrix coat, the inner shell of the viral core is covered from the capsid (CA) protein. In the centre of the viral core is the nucleocapsid (NC)-RNA complex. Smaller molecules of integrase (IN), reverse transcriptase (RT), and cellular tRNAs are associated with the NC-RNA complex formation (Figure 1). In the immature viral particles protease (Pr) has access to all the cleavage sites of the Gag, Gag-Pol polyproteins, and it is believed that after maturation some Pr molecules are localized inside the inner core and some outside (24-26).

Phylogenetic analysis based on the alignment of the reverse transcriptase or integrase proteins classifies retroviruses into two subfamilies; orthoretrovirinae and spumaretrovirinae, and seven genera (alpharetroviruses, betaretroviruses, gammaretroviruses, epsilonretroviruses, lentiviruses, deltaviruses, and spumaviruses).

Retroviruses can further be distinguished as those with a simple genomic organization; alpha, beta, gamma, and epsilon retroviruses, and those with quite complex genomic organizations; lentivirues, deltaviruses, and spumavirues (27-32).

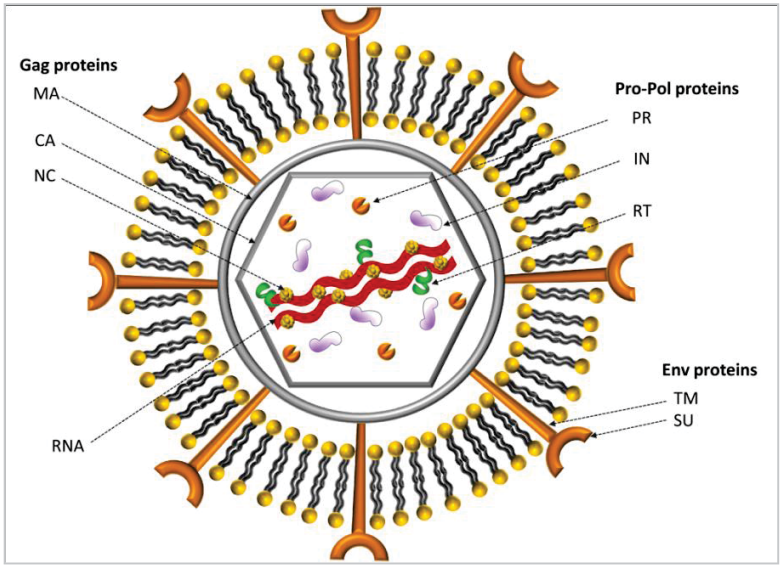

Figure 1 Anatomy model of an infectious HIV particle. The graphic is originally published in http://dx.doi.org/10.5772/18615 under CC BY-NC-SA 3.0 license. (Rodrigues, A. F., P. M. Alves, et al. (2011). Production of Retroviral and Lentiviral Gene Therapy Vectors: Challenges in the Manufacturing of Lipid Enveloped Virus.)

1.2.2 Retroviral genomic organization

Retroviruses are enveloped RNA viruses; belong to the family of *Retroviridae* and characterized by common structure, composition, and replicative properties. The retroviral virions are about 80-100 nm in diameter. The RNA genome is 7-12 kb in size, linear, single stranded and of positive polarity. The hallmark of retroviruses is that their RNA is used as template for a formation of a double-stranded DNA by the reverse transcriptase and the viral DNA integrates into the host cell genome. The basic retroviral genome sequence is 5'LTR-PBS-PSI-*gag-pro-pol-env*-3'LTR (Figure 2).

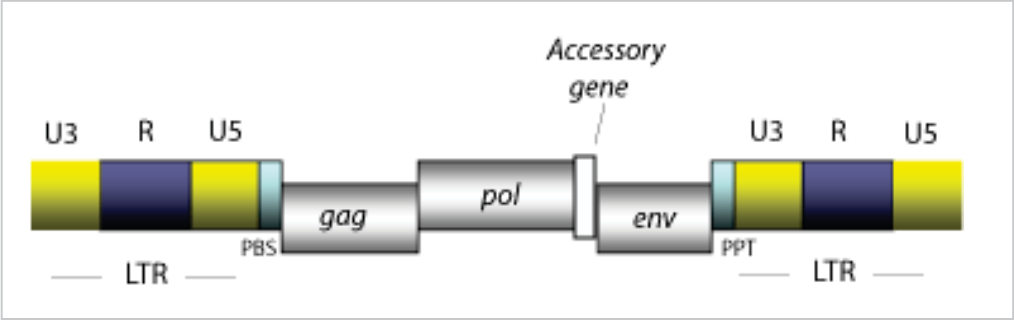

Figure 2 Organization of the retroviral genome. Long terminal repeats (LTRs) composed of U3, R, and U5 elements. The *gag, pro, pol*, and *env* sequences are located between the LTRs. Many retroviruses carry accessory genes that are located between *pol* and *env*. The picture was kindly provided with the permission of Llorens, C., R. Futami, et al. (2011). "The Gypsy Database (GyDB) of mobile genetic elements: release 2.0." Nucleic Acids Res 39(Database issue): D70-74.

- **Long Terminal Repeat (LTR)**

Retroviral genomes and retrotransposons are flanked by two LTRs (Long Terminal Repeats). LTRs are characterized by the presence of repeateatable sequences of DNA and regulate retroviral or retrotransposon gene expression. Retroviral LTRs mimic in function the host cell or tissue specific eukaryotic promoters and transcriptional enhancers. In addition, LTRs offer motifs for the retroviral RNA capping initiation and polyadenylation signaling.

LTRs are subdivided into three distinct elements: U3 (unique 3'), R (repeated) and U5 (unique 5'). The U3 region serves as the transcriptional promoter, whereas the transcription starting point is locted between U3 and R regions. The R area, downstream of the U3, offers the polyadenylation signal. The U5 sequence that follows the R region contains *cis*-and *trans*-regulatory sequences. The *cis*-active elements at the 5' end of the U3 and U5 region are involved in the proviral integration process (33-37).

The U3 region occupies most of the LTRs and in the 5' LTR of the provirus acts as a promoter for the RNA polymerase II. The U3 sequence shows diversity even between related retroviruses and offers binding motifs for transcriptional factors and regulators. Retroviruses may encode their own *trans*-acting transcriptional regulators. Characteristic examples are the Tax protein of HTLV-1 and the Tat protein of HIV-1. Tax stimulates viral transcription after binding to the Tax response element binding-1 (TRE-1) in the U3 5'LTR region of HTLV-1 (38-39). On the other hand, Tat is an RNA binding protein that binds to the *trans*-activation response elements (TARs) of the nascent transcripts and prevents premature transcriptional termination by inducing phosphorylation of the RNA polymerase II (40-42).

The 3' LTR is identical to the 5' LTR, but acts in transcriptional termination and polyadenylation. The synthesis of the poly(A) tail, which is regulated from the R region in the 3'LTR, stabilizes the viral mRNA and influences the steps of mRNA transportation, splicing, and translation (43-44). However, it is not fully clear how the polyadenylation in the 5' LTR from the identical R region is prevented. All the retroviral transcripts are

submitted to polyadenylation, regardless of the fact they are destined for protein synthesis or encapsidation into the newly formed virions.

- **Primer Binding Site (PBS)**

An 18 nucleotide long RNA sequence downstream of the U5 region of the 5'-LTR that servers as the retroviral primer binding site. A cellular tRNA hybridizes to the complementary PBS sequence in the producer cells and serves as the primer for the initiation of the reverse transcription step in the infected cells. In HIV, the tRNA is $tRNA^{Lys3}$ (45-47), but other retroviruses utilize tRNAs of a different sequence. For example, the HTLV uses the $tRNA^{Pro}$ (48), and the avian retroviruses replicate via $tRNA^{Trp}$ (49-50). PBS is a highly conserved sequence within a given virus.

- **Retroviral Psi (Ψ) packaging element**

The final virion should contain a dimer of two identical unspliced copies of the viral genome. Retroviral mRNA must be distinguished during the encapsidation process from both cellular mRNAs and subgenomic viral mRNAs. Moreover, two genomic viral RNA molecules should establish a proper interaction with one another and build a dimer formation. For this reason, the retroviral genomic RNAs carry motifs necessary for packaging that termed psi (Ψ) (51-53). These RNA packaging motifs are recognized by an RNA-binding protein; called nucleocapsid of the Gag polyprotein (54-56). Encapsidation of viral RNA is limited on the total size of the viral nucleic acid. Nucleic acids that are 5 to 10 % larger than the wild type cannot be packaged even when they contain all the appropriate encapsidation motifs. This limitation is critical for the development of viral vectors.

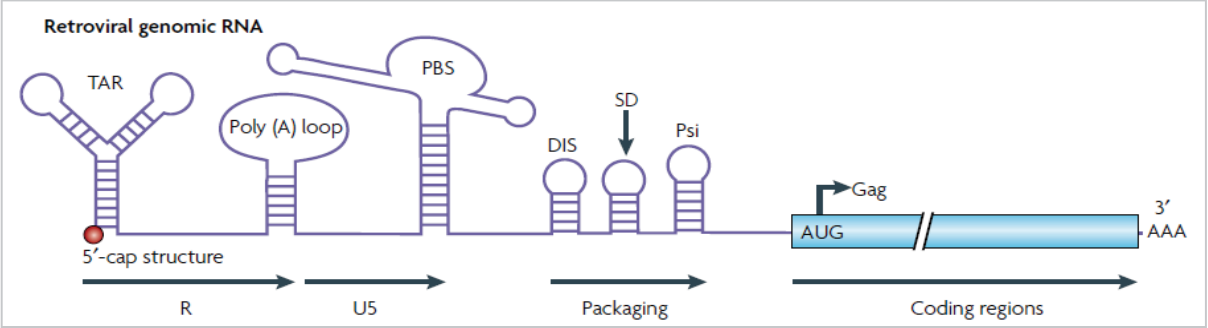

Figure 3 Example of the 5'-untranslated region (UTR) of the retroviral genomic RNA from primate lentiviruses. The 5'-UTR contains the following secondary RNA structures: a) the 5'-cap structure, b) the poly(A) loop, c) the primer-binding site (PBS), d) the dimerization initiation site (DIS), e) the major splice donor (SD), and f) the Psi (Ψ) packaging signals. The figure was adapted from Laurent Balvay et al., Nature Review, 2007 (57).

- **Expression of the *gag* gene**

In all retroviruses the *gag* gene products are translated from unspliced full length mRNAs precursor to internal structural proteins. All Gag precursors contain three distinct domains: the viral matrix (MA), the major capsid domain (CA), and the nucleocapsid (NC) (58-59). These proteins are commonly named after their molecular weight properties (e.g. HIV-1 p24 for the capsid protein of HIV-1).

In the producer cell the retroviral Gag precursor carries all the necessary structural motifs to direct trafficking, assembly and mediate budding of the virions (60). Gag provides not only the principal driving force for recruiting all the viral components that are required to build a fully infectious virus, but also interacts with components from the cellular machinery to build the budding complex (61).

Released retroviruses are infectious only after undergoing necessery structural rearrangements, a process known as viral maturation (62-64). After viral release from the infected cell, the Gag polyprotein is processed into MA, CA, and NC by the viral protease. In mature viral particles, the CA proteins enclose the incorporated viral enzymes (integrase, reverse transcriptase) and the retroviral RNA genome that remains in an interacting formation with the NC protein, while the MA proteins form the viral coat (Figure 4). The proteolytic cleavage of the Gag precursor proteins may also produce spacer peptides of different sizes (65).

NC is known to be a nucleic acid chaperone that stabilizes the dimeric formation of the retroviral RNA genome in the produced particles. In particular, the NC-RNA viral genome interaction is mediated by the presence of one or two zink knuckles (66-68). NC appears to play an important role as an obligatory element during revere transcription. This appears to be mediated by tight interactions between the NC molecules, the viral RNA, the reverse transcriptase, and the primer tRNA. NC is also reported to be involved in proviral integration. Due to the high binding affinity to double-stranded nucleic acids, NC remains anchored on the final DNA viral genome for stabilization and protection from degradation (69).

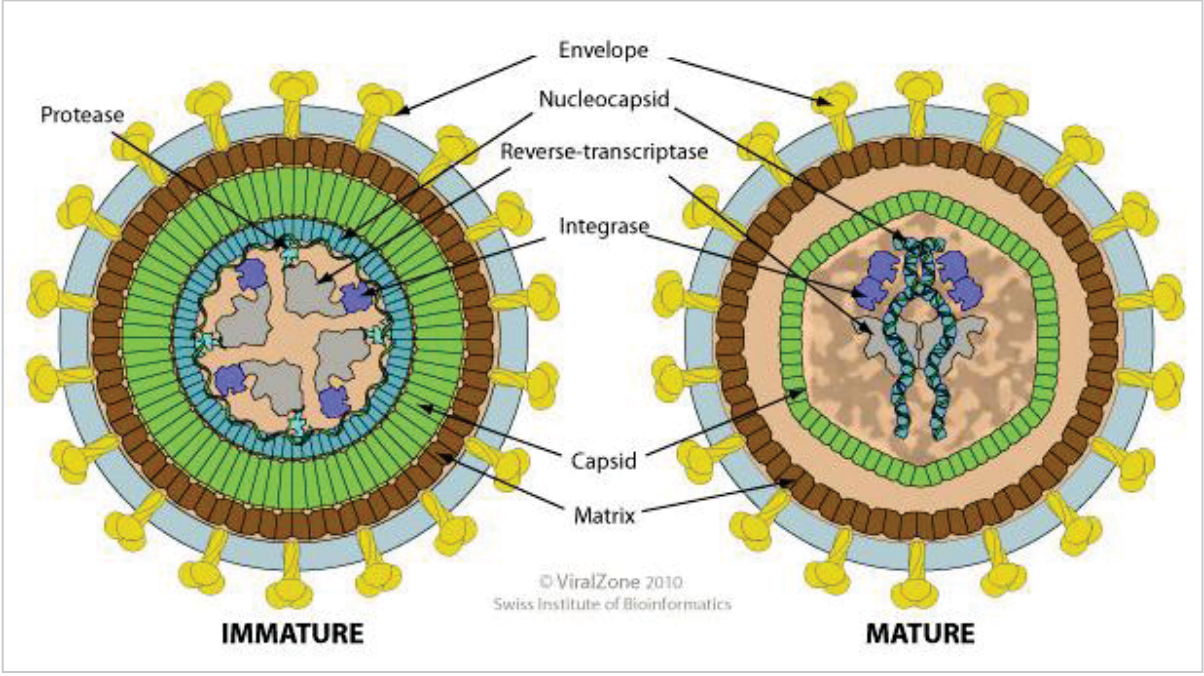

Figure 4 Retroviral maturation. After viral release, the viral protease mediates cleavage of the viral gag polyprotein into matrix, capsid, and nucleocapsid, leading to structural rearrangements of the viral proteins and formation of mature virions. The adapted figure is published online from ViralZone (ExPaSy Bioinformatics resource portal, Swiss Institute of Bioinformatics) and presents the immature and mature forms of an alpharetrovirus.

- **Expression of the *pro* gene**

The retroviral protease (Pr) is a homodimer of 14 kDa that exhibits aspartyl protease activity (70-72). Retroviral Pr is encoded by the *pro* gene that is located between the *gag* gene and the *pol* gene. Compared to the structural proteins, which are produced from the Gag polyprotein, the virus requires in lower amounts the *pro* and *pol*-encoded enzymes. Retroviruses achieve the appropriate translational ratio of these proteins from a single polycistronic full length mRNA by using the mechanisms of RNA ribosomal frameshifting and leaky stops (73-79). Usually, Gag protein ends before the *pro* gene, and only about 5% Gag-Pro fusion polyproteins are synthesized. The general mechanism of frameshifting requires specific mRNA signals such as stem-loops or pseudoknots, named as 'slippery' sequences. These mRNA structures affect the ribosomal movement along the RNA by promoting ribosomal movement one nucleotide upstream. The stop codon after the *gag* locus is no longer in frame, thus the Gag-Pro fusion protein is produced.

During budding, the viral Pr within the Gag-Pol polyprotein cleaves itself out by specifically cutting peptide bonds at either end of its sequence. Afterwards, Pr cleaves additional bonds within the remaining fragment of the Gag-Pol polyprotein to yield reverse transcriptase and integrase.

In the released viral particles, Pr further cleaves the Gag protein to the basic structural components: matrix, capsid, and nucleocapsid to mature infectious particles. The mechanisms of the Pr activation remain unclear. However, it is believed that Pr achieves dimerization in the released particles due to the low pH and high concentration of Pr molecules.

The ability to inhibit the viral Pr activity was always an attractive target for the development of therapeutic agents. In the case of HIV-1, Pr substrate-based inhibitors (analogs) have been commercially approved for use in treatment of HIV-1 infections (80-82).

- **Expression of the *pol* gene**

The *pol* precursor encodes for the reverse transcriptase (RT) and integrase (IN). Retroviral reverse transcription is believed to take place in the cytoplasmic compartment after the viral entry and uncoating. During this step, double-stranded RNA is converted to a double-stranded DNA (cDNA) by the RT activity. Next, the produced retroviral cDNA has to be transferred from the cytoplasmic environment to the nucleus, where it is integrated into the host genome. The cDNA trafficking is mediated toward the microtubule organizing centre (MTOC) to the nuclear periphery.

Reverse transcriptase

The template of the retroviral reverse transcriptase is a single-stranded RNA molecule. The 5' and 3' ends of the RNA molecules are called U5 and U3 respectively. Close to U5 locates the primer binding site (PBS), essential for the DNA synthesis initiation. A cellular tRNA primer anneals to the PBS and primes the synthesis of the minus (-) DNA strand. While extension of the (-) strand to the end continues, the 5' end of the RNA genome is degisted by the RNase H domain of RT. A fragment of 13-15 nucleotides, the polypurine tract (PPT), produced from the RNase H RNA cleavage. PPT acts as the primer for the plus (+) DNA synthesis that begins before the minus strand (-) is completed. The (-) stranded DNA anneals to the complementary sequence of PBS at the 3' end of the (+) strand DNA and forms a circular DNA template that can be polymerized by the RT. The final product is a double-stranded DNA copy of the viral genome with a long terminal repeat signal (LTR) at each end (83). Retroviral reverse transcriptases are characterized by a low fidelity and show no proofreading nuclease capacity (84-85).

Integrase

Retroviral integrase enables the retroviral genetic material to be integrated in the infected cell genome by mediating DNA breaking and joining reactions. The integration sites are random euchromatin regions, usually close to active gene transcription sites and CpG islands (86). Integration of retroviruses can be associated with lethal effects for the infected cells that caused by insertional mutagenesis and include activation of onco-genes and/or disruption of host genes (87-90).

The integration process starts when the synthesised product from the reverse transcription, a blunt-ended viral cDNA, is bound by the IN enzyme to form the pre-integration complex. IN processes the provirus at the 3' ends by removing two nucleotides and exposing recessed 3' hydroxyl groups. In the nucleus, IN targets the integration site and cleaves the host genome's phosphodiester bonds between a 3'-hydroxyl group and 5'-phoshphate group to yield DNA breaks. Subsequently, the 3' ends of the viral DNA are joined together with the genomic DNA gaps. Finally, DNA repair enzymes fill the gaps and form identical stretches of DNA between the two initial breaks flanking the inserted provirus (91-96).

IN is the target of many anti-retroviral drugs. IN inhibitors block the HIV infection by inhibiting the insertion of the viral genome into the host DNA. Raltegravir is the first integrase inhibitor approved to treat HIV infections from the U.S. Food and Drug Administration (FDA) in 2007 (97). Elvitegravir and Dolutegravir are also FDA approved for HIV therapy in 2012 and 2013 respectively.

- **Expression of the *env* gene**

Envelope is the outer 'capsule' of retroviruses. Typically, envelope is derived from the host cell membrane and composed of phospholipids and host cellular proteins. In addition, retroviral envelopes are characterized by the presence of retroviral Env glycoproteins encoded by the *env* gene. Env proteins allow virus to mediate attachment and subsequent entry to the target cells by binding to a specific receptor on the cellular surface.

The *env* gene is localized downstream of the *gag* and *pol* genes and expressed via spliced mRNAs (98). Env proteins are glycosylated in the lumen of the golgi/endoplasmic reticulum, where mannose-rich oligosaccharides are attached to the asparagines in Asn-X-Ser or Asn-X-Thr motifs. Glycosylation is required for the proper folding and stabilization of the Env proteins. Other posttranslational modifications include formation of disulphide bonds, oligomerization and cleavage into two distinct domains: an N-terminal subunit called SU (SUrface) and a C-terminal transmembrane subunit named TM (TransMembrane). The mature Env product anchors on the virion surface. The SU domain motif carries the receptor-binding signal and defines the species and host-specific tropism (99-100).

Env is the only viral protein that is localized on the surface of the produced virions and the main target of neutralizing antibodies. The HIV-1 SU domain binds to the CD4 receptor to gain enty into the target cells. Usually, an additional interaction with a co-receptor molecule is needed so as to establish cell entry. The receptor molecules determine the cell-specific HIV-1 tropism. CXCR4 is the major co-receptor for the HIV-1 T-cell tropic strains, and CCR5 the main co-receptor for the HIV-1 macrophage/monocyte-tropic strains (101-103).

The HIV-1 envelope protein is characterized by a high carbohydrate content that masks the critical structures for neutralization and only a quite low number of the infected community named as 'elite controllers' can produce effective neutralizing antibodies (104-105).

- **Expression of accessory genes**

In the family of retroviruses, complex members such as the pandemic HIV-1 and HTLV-1 encode for accessory genes. Accessory genes can regulate gene expression, modulate cell activation and counteract cellular restriction mechanisms (106-109).

1.2.3 The retroviral infectious cell cycle

The retroviral life cycle includes the steps of a) binding to a host cell receptor, b) uncoating, c) reverse transcription, d) integration, e) transcription, processing, and translation of the retroviral genome, and f) assembly and budding of the viral particles (Figure 5).

- **Binding to a receptor**

The retroviral infectious cell cycle is a multi-step process. The first step is characterized by binding of the viral surface envelope glycoproteins through the receptor binding site (RBS) to a host cell receptor, and in many cases also to a co-receptor (110-112). The specificity of the receptor and co-receptor binding defines the viral tropism properties and it is associated with the evolutionary adaptation of retroviruses to overcome various host cell defensive mechanisms. Binding of the RBS to the receptor/co-receptor promotes initiation of both cellular and viral membrane conformational changes and leads to a fusion of the viral envelope lipid bilayer with the host cell membrane with a subsequent internalization of retroviral particle (113-114).

- **Uncoating**

After binding to a receptor, the viral inner core opens and the retroviral genetic material - two copies of a positive stranded RNA molecule- associated with the nucleocapsid (NC), integrase (IN), and reverse transcriptase (RT) is released on the cytoplasmatic compartment. This process is referred as uncoating process. For HIV-1, it is proven that a host protein; cyclophilin A binds to HIV-1 capsid and is required for a proper uncoating of the virus (115).

- **Reverse transcription**

The viral nucleoprotein complex is used as template for the reverse transcription. Reverse transcription is mediated by the reverse transcriptase (RT) enzyme that converts a single-stranded RNA to a double stranded DNA (cDNA). RT can only initiate DNA synthesis when a

host-encoded complementary tRNA anneals to the RNA Primer Binding Site (PBS) of the viral genomic RNA (116). Reverse transcription for retroviruses takes place into the cytoplasm and is dependent on the dNTP pool presence (117). If the host cell is serum starved before infection, reverse transcription is initiated, but is not completed due to a lack of dNTPs (118).

- **Integration**

After the reverse transcription step, the viral DNA molecule is a blunt-ended linear molecule associated with the integrase enzyme. Usually, the viral integrase cleaves two bases from the 3' end DNA. This results in 3'-OH free groups from each 3' DNA end. These preintegration complexes can enter into the nucleus by following two strategies: a) depending on the actively diving cell status when the nuclear membrane is disassembled at mitosis (e.g. Murine leukemia virus (MLV)) (119-120), or b) for HIV-1, the ability of the matrix protein to direct nuclear entry of preintegration complexes (121). The integrated provirus can exist at in either a latent or a productive state. Viral latency is associated with the lack of host cell factors that are required for the activation of the viral gene expression.

- **Transcription, processing, and translation**

The retroviral LTRs contain binding sites for several transcription factors that are induced in infected T lymphocytes or macrophages, such as NF-kB, NF-AT, AP-1. The produced RNA pol II viral pre-mRNA transcripts are subject to a 5' end capping, cleavage and polyadenylation at the 3' end, and splicing processing events. The 5' end capping step is facilitated by RNA pol II pausing that allows RNA guanylytransferase and RNA methyltransferase to synthesize a m7G(5')ppp(5')N cap. In addition, polyadenylation of the 3' end contributes to the RNA stability, and is important for the transport of transcripts out of the nucleus (122). Finally, RNA splicing is the process by which "intronic" RNA sequences are removed and the remaining "exonic" sequences are ligated to provide continuous reading frames for translation. Splicing is mediated through the presence of conserved viral pre-mRNA 5' splice donor (SD), 3' slice acceptor (SA) and branch point (BP) sites. For HIV, the frequency of splicing events is divided into three categories: a)

unspliced mRNA that serves as the viral genomic RNA for encapsidation and as the template for the expression of the Gag-Pol polyproteins, b) single spliced mRNAs lacking *gag-pol* coding region, and c) multiple spiced mRNAs lacking *gag-pol* and *env* coding regions (123).

- **Assembly and budding**

The retroviral assembly is driven by the Gag polyprotein that directs the fusion process and controls the size and shape of the virions. The Gag polyprotein provides all the signals to package all the viral components (Gag-Pro-Pol, Env, RNA, tRNA, etc.). Retroviral RNA is selected out of a large cytoplasmic pool of cellular RNAs through a *cis*-actig signal, the Ψ region. The interaction between the Ψ signal in the retroviral genomic RNA and the *trans*-acting cysteine array (Cys-X2-Cys-X4-His-X4-Cys), which is found within nucleocapsid domain from the Gag precursor, secures packaging of the RNA genome (124-125).
Immature assembled virions are released from the host cell surface taking with them a swatch of cellular membrane containing surface proteins, referred as retroviral budding. The mechanism of budding is supported by the ESCRT cellular protein complexes that involved in the biogenesis of multivesicular bodies (126). After release, retroviruses undergo an obligatory maturation step, where the polyprotein precursors of the immature particle are cleaved by the viral protease. This is a dynamic process that completes viral shaping and final morphogenesis, resulting to infectious viral particles.

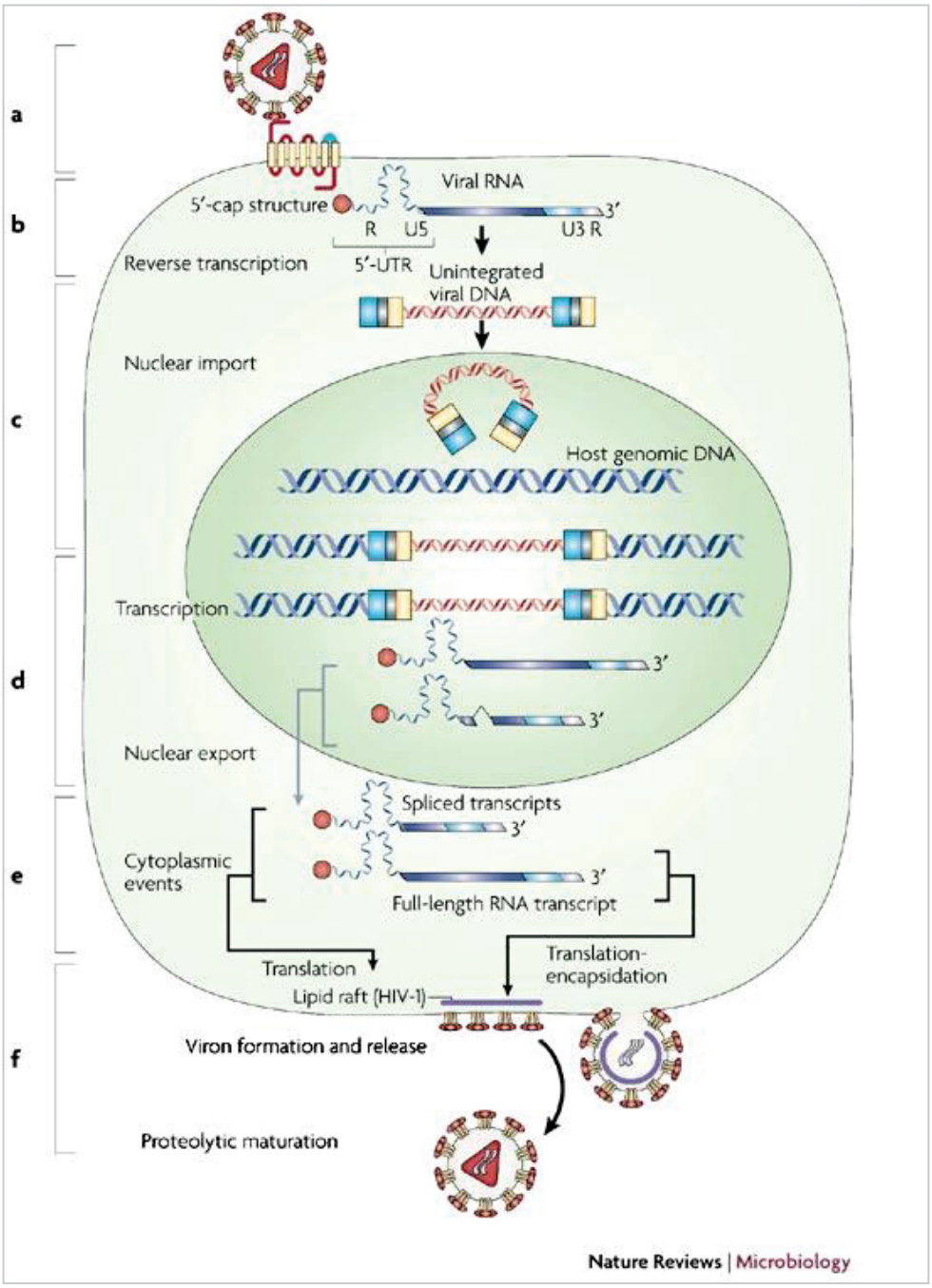

Figure 5 Overview of the retroviral replication cycle. The following seven steps are distinguished: (a) binding to the receptor and uncoating, (b) synthesis of reverse transcribed DNA, (c) integration, (d) transcription, splicing and nuclear export, (e) translation and encapsidation, (f) viral assembly and budding. The figure was adapted from Balvay, L., M. Lopez Lastra, et al., Nature Rev Microbiol, 2007 (127).

1.3 The APOBEC3 family of enzymes

1.3.1 Localization and enzymatic activity of the APOBEC3 family

The APOBEC3 (A3) cytidine deaminases (apolipoprotein B mRNA-editing catalytic polypeptide-like 3) have been shown to be active against a variety of exogenous and endogenous retroviruses, retroelements, and DNA viruses (128-130).

The *A3* genes are located on chromosome 22 in humans and composed of seven related but distinct members (A3-A, -B, -C, -D, -F, -G, and -H) (Figure 6). In high contrast, in mice a single *A3* gene is present on the syntenic chromosome 15. It is believed that the placental ancestor encoded for three *A3* genes that either expanded like in primates, or partially were lost and fused to a single gene like in rodents. (131).

The A3 enzymes catalyze hydrolytic deamination at the C4 position of the cytidine base, converting cytidines to uridines, referred as dC to dU deamination. Their active sites are characterized by the presence of a conserved zinc-binding motif, (Cys/His)-Xaa-Glu-Xaa23∼28-Pro-Cys-Xaa2∼4-Cys. The cytidine deamination reaction is mediated by a key glutamate that is involved in the proton shuttling, and two critical aromatic residues involved in nucleic acid substrate binding (132-134).

The human A3 cytidine deaminases are categorized either as single-domain or as double-domain proteins (Figure 6). Three of the members; A3A, A3C, and A3H display a single deaminase domain (CDA), whereas A3B, A3D, A3F, and A3G consist of a double CDA (131). For A3G, the C-terminal CDA mediates editing activity (135), whereas the N-terminal CDA a) interacts with the viral RNA molecule, b) promotes homo-oligomerization, and c) it is an essential subunit for the A3G packaging into the HIV-1 virions while interacting with the nucleocapsid protein (136-138).

Subcellular localization of A3s is related with the impact of the various A3s on the restriction of retroviruses and retroelements. Immunofluorescence studies in tranfected HeLa cells show that A3A is localized to the nucleus (139). But, significant amounts of A3A are also detectable on the cytoplasmic compartment. However, it was recently reported that endogenous A3A in monocytes and macrophages localizes exclusively within the cytoplasm (140). On the other hand, A3B is found in the nucleus. A3C is equally distributed in both compartments (nuclear and cytoplasmic detection) (139). A3D, A3F, and A3G are localized to the cytoplasm (139). And, A3H is found in the cytoplasm and in the nucleoli (141-142).

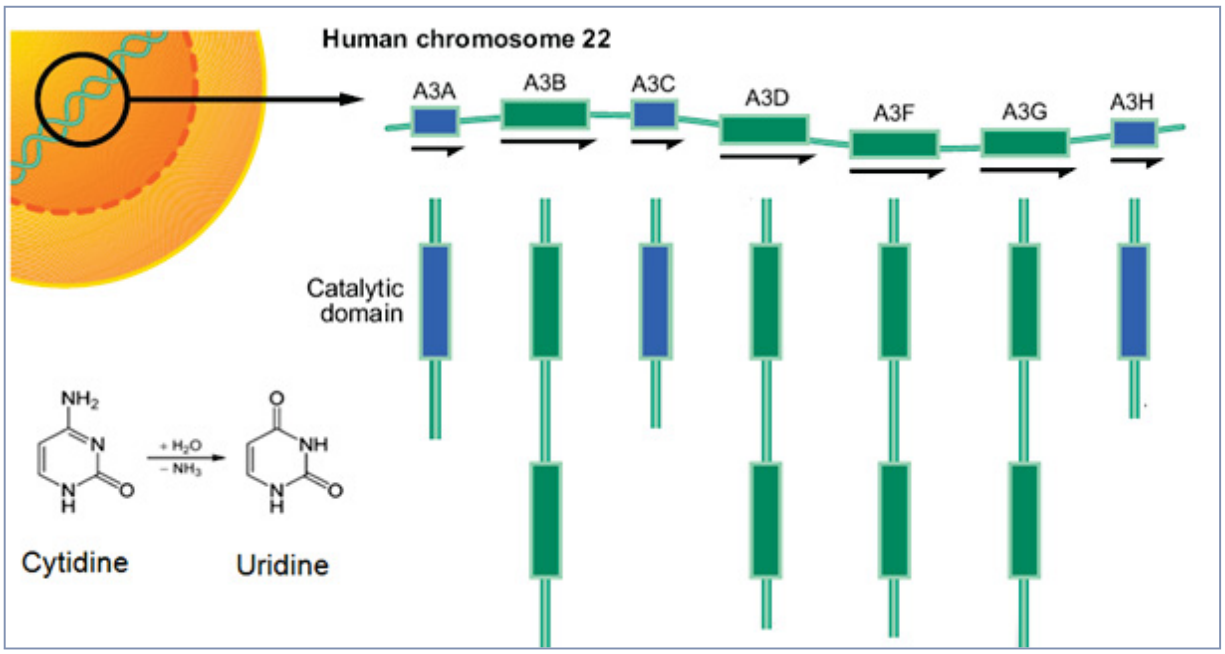

Figure 6 The human APOBEC3 (A3) family of enzymes. The A3 members localize on chromosome 22 in humans and mediate cytidine deamination. Four members (A3B, A3D, A3G, and A3F) carry a double catalytic domain, while A3A, A3C, and A3H a single catalytic domain. The figure was adapted from Chiu, Y. L. and W. C. Greene, Ann. Rev. Immunol., 2008 (143).

1.3.2 Retroviral restriction by APOBEC3

The A3 activity represents a significant mechanism of the host immunity to restrict viral infections. A3s can potently induce C-to-U mutations in the minus strand DNA and therefore inhibit retroviral reverse transcription and integration. In the infected cells (virus producer cells), A3s can be incorporated into the newly synthesized virions by interacting with the retroviral Gag polyprotein and the viral genomic RNA. Then, A3s are carried from producer to target cells in the virus. At the second round of infection, and during reverse transcription, the virion-packaged A3s deaminate cytidines to uridines (C-to-U conversions) in the viral minus-strand DNA (cDNA) in a 5'->3' direction. Subsequent incorporation of adenines instead of guanines in the plus-strand results in extensive G-to-A lethal hypermutation (144-145). However, retroviruses such as the pandemic HIV-1 present an evolutionary advantage against the A3 restriction. In the infected cells, the A3 activity can be neutralized by the HIV-1 viral infectivity factor (Vif) protein. The presence of Vif protein prevents packaging of A3s into the budding viruses (see on page 39).

The A3-induced G->A alterations can lead to a) inactive transcripts that are degraded by the DNA pathways (146-148), b) premature translational termination, or c) contribute to viral variation and evolution (149-151) (Figure 7). For example, a single G->A mutation at TGG generates TAG, TAA, or TGA, the three translational termination codons. Yet, the substrate specificities and nucleotide preferences differ among the A3 members. For example, A3G causes extensive GG->AG hypermutations, while A3A and A3B prefer mainly for GA->AA and GG-AG dinucleotide substitutions (152-155).

On the other side, A3s are also reported to act with a deaminase-independent mechanism of restriction that may involve a) inhibition of the reverse transcription process by interfering with tRNA primer annealing initiation and elongation of DNA synthesis (156), and b) impairment of integration (157).

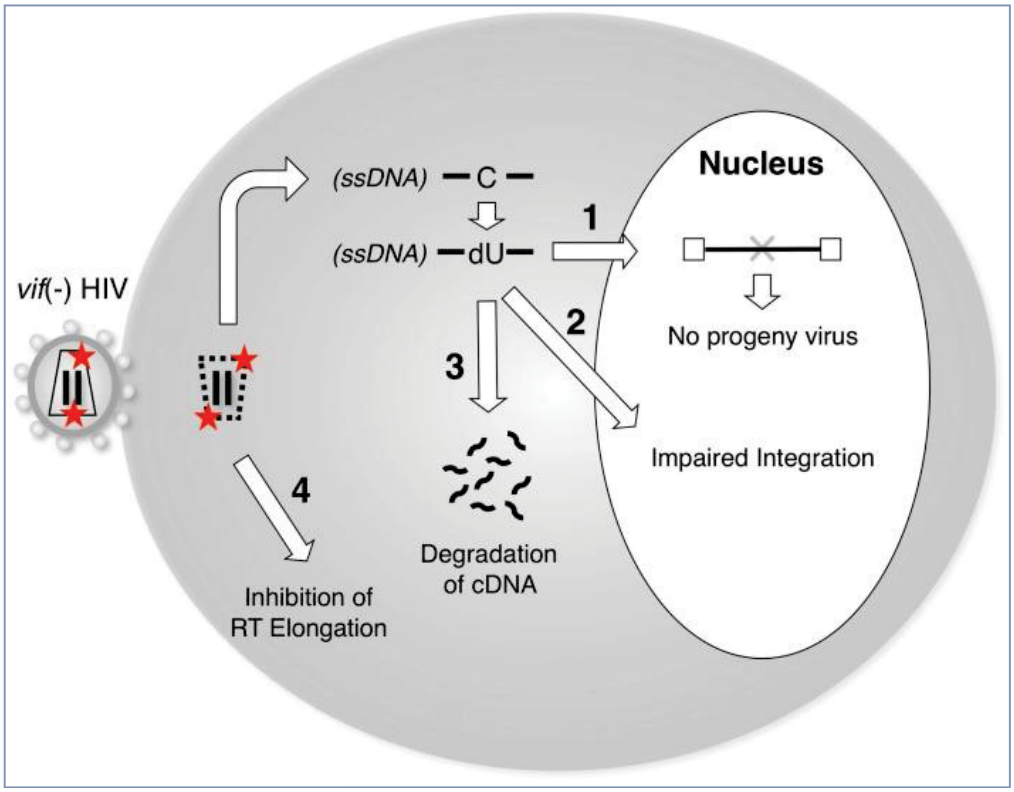

Figure 7 A3-mediated restriction of retroviruses. A3 molecules block post-entry retroviral replication by the following mechanisms: (1) A3s can deaminate the viral cDNA strand during the reverse transcription. (2) A3-mediated editing can create aberrant viral DNA ends that may be unefficient for integration. (3) The A3-induced mutations can be lethal and may induce degradion by cellular DNA repair pathways. (4) Reverse transcription can be blocked by the presence of A3s on the viral templates. (1-3) Exhibit deaminase-dependent restriction and (4) deaminase-independent A3 functions. The figure was originally published by Mayumi Imahashi et al., frontiers in Microbiology, 2012 (158).

1.3.3 Regulation of the APOBEC3 expression

In mammals, the innate immunity network composes the first line of defense against the viral invasions. The most effective mechanisms of the innate immunity defensive line against viral infections are mediated mainly by interferon (INF) production and activation of Natural Killer cells (159-161). INF is a cytokine that is induced at the early stages of viral infections as a response to the sensing of foreign nucleic acid patterns. Production of cytokines is known to regulate induction of expression of various anti-viral factors such as A3s (162).

In myeloid-derived cells (monocytes and macrophages), which serve as reservoirs for HIV spread, type I INF upregulation induces strongly the A3A transcription (163). A3A is described to inhibit the early phases of the R5-tropic HIV-1 infection (164). Nevertheless, a strong induction of A3A transcription by INF is characteristic for macrophages, but not for $CD4^+$ T cells (165).

In $CD4^+$ T cells, cytokines like interleukin-2 and interleukin-15 have been shown to induce A3G and A3F expression (166-167). In addition, a cross-talk between dendritic cells and $CD4^+$ T cells during retroviral infection can also contribute in the induction of A3G and A3F expression via CD40 (dendritic cells)-CD40L ($CD4^+$ T cell) interaction. In the asymptomatic phase of the AIDS disease, dendritic cells exhibit elevated levels of CD40 but, when this signaling pathway attenuates it leads to a lower transcription of A3G and A3F in PBMCs (168).

In B cells, A3B is expressed predominately, but a clearer understanding of the *in vivo* functions and the transcriptional stimuli of this protein need to be further determined (169-170).

Although the immune mediators can induce anti-viral factors such as the A3 family of enzymes, viral infections are usually characterized by an acute and chronic phase of immune-activation. This suggests that disease progression may be associated with various levels of inflammatory factors and A3 expression. Hence, it remains to be explored which concentrations of A3 proteins are required to effectively block viral replication.

1.3.4 Retroviral escape from the APOBEC3 restriction

Retroviruses encode for various accessory genes to counteract the A3 restriction. For instance, the HIV-1 Vif accessory protein helps the virus to escape restriction by A3C, A3F, A3G and A3H. The HIV-2 Vpx accessory protein counteracts A3A, while the Bet protein of foamy viruses inhibits many A3 proteins. On the other hand, HTLV-1 is known to maintain a *cis*-acting mechanism that excludes the A3G incorporation into the viral particles through a C-terminal area of its nucleocapsid protein.

- **HIV-1 *Vif***

Initially, it was observed that productive HIV-1 infections of primary human T lymphocytes and monocytes require the presence of a viral protein (171). Later, the HIV-1 Vif accessory protein was identified to counteract A3G and A3F in primary cells. In $CD4^+$ cells, A3G and A3F are the most potent inhibitors of HIV-1, but only in the absence of Vif (172-173). HIV-1 Vif hijacks an ubiquitin ligase complex, containing CUL5, ELOC, ELOB, a RING-box protein, and the transcription factor CBF-β, to target A3G, A3F, A3C, and A3H by promoting ubiquitination and subsequent proteosomal degradation (174-176). CBF-β is a chaperone protein that was recently reported to stabilize the the Vif–Cul5–EloB/C E3 ligase complex (176). HIV-1 Vif expression is also associated with cell cycle regulation of the infected cells by inducing G2 arrest (177-178). Therefore, structural analysis of the interaction sites between Vif and A3 proteins could lead to the development of novel therapeutic strategies for HIV-1 infection.

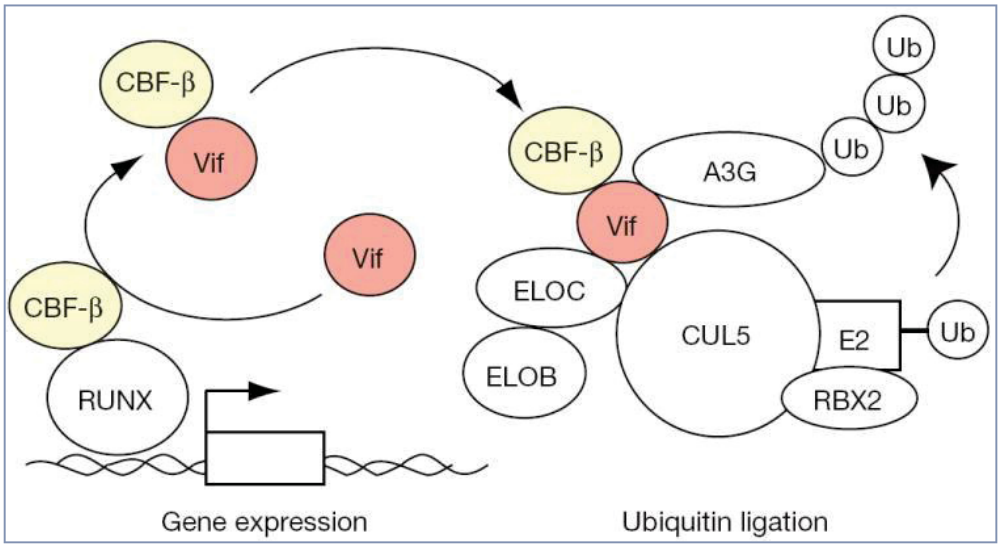

Figure 8 Model for Vif–CBF-β E3 ligase formation and APOBEC3G (A3G) polyubiquitination and degradation. The figure was taken from Jager, S., D. Y. Kim, et al., Nature, 2012 (176).

- **HIV-2 and SIVmac *Vpx***

The accessory protein Vpx is encoded by the Human Immunodeficiency Virus type-2 (HIV-2) and Simian Immunodeficiency SIVmac/SIVsm lineage. The Vpx protein is packaged into the HIV-2 and SIVmac/SIVsm lineage viral particles and facilitates infection of myeloid cells. Vpx is described to counteract and promote degradation of the human A3A protein, a restriction factor that is broadly expressed in myeloid cells (179-180). Vpx recruits A3A to a cullin4A-RING E3 ubiquitin ligase that targets the enzyme for proteasomal degradation.

In addition, Vpx promotes degration of SAMHD1 in myeloid cells. *SAMHD1* is a gamma interferon inducible gene with a triposphohydrolase activity that converts deoxynucleoside triphosphates to deoxynucleoside and inorganic triphosphates. Therefore, SAMHD1 inhibits the retroviral replication in the target cells by regulating the dNTP levels (181-183).

In contrast to HIV-2 and SIVmac, HIV-1 does not encode for Vpx and lacks the ability to establish infection of myeloid cells. Within the myeloid lineage, macrophages are thought to be the most permissive to HIV-1 infections due to a decrease in expression of host restriction factors during differentiation and maturation. However, the pool of A3A and SAMHD1 inhibits the early phases of R5-tropic HIV-1 infection and spread by affecting the amount of viral DNA synthesized over the course of infection (184).

- **HTLV-1 nucleocapsid C-terminal area**

HTLV-1 has evolved a different strategy to counteract the A3G cytidine deaminase activity in T lymphocytes. HTLV-1 does not encode for an accessory protein similar to the HIV-1 Vif, but contains a C-terminal nucleocapsid motif that restricts A3G incorporation into the retroviral particles. HTLV nucleocapsid proteins are different from those expressed by all other retroviruses genera and contain a unique 35-aa nucleocapsid extension on the C-terminal side of the zinc finger domains. Indeed, alignment of the nucleocapsid proteins from HTLV-1 with the HIV-1 and other closely related deltaretrovirus, such as the bovine leukemia virus, shows that these viruses carry only 6-aa C-terminal nucleocapsid extensions. This indicates that retroviruses separated evolutionary and adapted various ways to fight the A3 inhibition: a) by encoding for accessory proteins that sense and degrade A3s, or b) by developing *cis*-acting exclusion mechanisms of the A3 molecules from the viral particles (185).

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Chapter 2
APOBEC3A, APOBEC3B, and APOBEC3H Haplotype 2 Restrict Human T-Lymphotropic Virus Type 1

The following data are published in the Journal of Virology. 2012, 86(11):6097. DOI: 10.1128/JVI.06570-11 by Marcel Ooms*, Aikaterini Krikoni*, Andrea K. Kress, Viviana Simon and Carsten Münk. *Aikaterini Krikoni and Marcel Ooms contributed equally and are the first authors of this article.

Aikaterini Krikoni (A. K.) contributed to this study with the following:

1. A. K. established the HTLV-1 infectivity assay and analysed the effect of the seven human A3s on HTLV-1 replication (Figure 1 A).
2. A. K. performed cloning of the A3A from *Macaca mulatta* and generated the human A3A+SV and A3A+SVR, and the macaque A3A ΔSV and A3A ΔSVR expression plasmids. A.K. compared the human and macaque A3A for HTLV-1 restriction and performed immunoblot analysis from cell and viral lysates (Figure 3 A, B, and C).
3. A.K. analyzed the A3A, A3B, and A3H hap II WT and deaminase mutants for their ability to restrict HTLV-1 (Figure 4 B).
4. A. K. analyzed the A3A, A3B, A3G, and A3H hap II-induced editing in HTLV-1 genomes (Figure 5 A, B, C, and Figure 6 A, B).
5. A. K. analyzed the A3-mediated editing of the HTLV-1 *tax* gene *in vivo* (Figure 7 A, B, and C).
6. A. K. contributed to the manuscript writing

ABSTRACT

The human APOBEC3 family consists of seven cytidine deaminases (A3A to A3H), some of which display potent antiretroviral activity against HIV-1 and other retroviruses. Studies that analyzed the effect of A3G on human T-lymphotropic virus type 1 (HTLV-1) infectivity resulted in conflicting findings, and our knowledge of HTLV-1 restriction by other A3 proteins remains limited. Since HTLV-1, much like HIV, targets CD4 T cells, we hypothesized that A3 proteins other than A3G restrict HTLV-1. All seven human A3 proteins were tested in HTLV-1 reporter and HIV-1 infectivity assays. We show that A3A, A3B, and A3H haplotype 2 (A3H hapII) acted as potent inhibitors of HTLV-1. Wild-type HIV-1, in contrast, was restricted by A3B and A3H hapII, but not by A3A. Catalytic site mutants of A3A, A3B, and A3H hapII showed that A3A and A3B restriction of HTLV-1 required deaminase activity. However, A3H hapII acted in a deaminase-independent manner when restricting HTLV-1, while requiring deaminase activity for HIV-1 restriction. We also analyzed A3 editing of HTLV-1 in five T-cell lines obtained from HTLV-1-infected patients. These cell lines contained extensively edited HTLV-1 sequences with G-to-A mutations in dinucleotide contexts suggestive of APOBEC3 mutagenesis. Comparison of the A3-induced mutations from reporter cells and the patient-derived cell lines indicate that A3G but also other A3 members, possibly A3A and A3B, affect HTLV-1 in vivo. Taken together, our data indicate that HTLV-1 is a likely target for multiple A3 proteins.

BACKGROUND

The deltaretrovirus human T-lymphotropic virus type 1 (HTLV-1) was the very first human retrovirus discovered (67). The results of phylogenetic analyses indicate that HTLV-1 was introduced into human populations thousands of years ago (39,84). This long interaction between HTLV-1 and humans stands in contrast with HIV-1, which is estimated to have crossed the species barrier only a century ago (92). Estimations suggest that approximately 15 to 20 million people are infected with HTLV-1 worldwide (15, 24). HTLV-1 infections are endemic in Southern Japan, sub-Saharan Africa, parts of South America, and the Caribbean islands (reviewed in reference 68). HTLV-1 transmission occurs through mother-to-child transmission (breast milk), sexual transmission, blood transfusion, and intravenous drug use (37, 38, 71). Adult T-cell leukemia/lymphoma (ATLL) develops in up to 5% of HTLV-1-positive individuals (47), and in an additional 1 to 2% of these individuals, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is observed (18, 29). HTLV-1 primarily targets CD4 T lymphocytes, and the spread of infection takes place by cell-to-cell transmission through virological synapses, extracellular viral assemblies, or cellular conduits (27, 42, 53, 63, 85). Upon primary infection, only limited productive replication takes place, but HTLV-1 multiplies by enhancing the proliferation of infected cells (47, 83). As a result, HTLV-1 sequence diversity is extremely low compared to HIV-1. There is currently no cure for HTLV-1 infection, and the lack of efficient antiviral drugs for HTLV-1 offers limited management options. Furthermore, the use of nucleoside analogues in HTLV-1 therapy is subjected to criticism because HTLV-1 infection in vivo is accompanied by limited viremia (8).

The human cytidine deaminase family of apolipoprotein BmRNA editing enzyme catalytic polypeptide 3 (APOBEC3, A3) consists of seven proteins (A3A, A3B, A3C, A3D, A3F, A3G, and A3H) that can inhibit retroviruses, endogenous retroelements, and DNA viruses to different degrees (10, 55, 76, 80, 86). The human A3 locus on chromosome 22 is polymorphic on a genome level with a large structural variation deleting the entire A3B coding region as well as several single-nucleotide polymorphisms in introns and exons of each cytidine deaminase (2, 32, 70). Haplotypes with distinct antiviral phenotypes have

been reported only for A3H: A3H haplotype 2 (A3H hapII) confers strong anti-HIV-1 activity, while most other A3H variants are unstable proteins that lack inhibitory activity (12, 22, 58).The enzymatic activity of virion-packaged A3 molecules results in deamination of cytosine bases in the single-stranded viral DNA during reverse transcription, which leads to guanosine (G)-to-adenosine (A) mutations in the provirus (4, 23, 36, 43, 45). Of note, A3 proteins have also been shown to restrict through deaminase-independent mechanisms by reducing reverse transcription products and integration (3, 25, 28, 49, 50, 57). The accessory HIV-1 protein Vif counteracts the restriction of several A3 proteins (e.g., A3F and A3G) by mediating its proteasomal degradation in the producer cell (11, 46, 51, 77, 93) or by preventing their packaging into virions (21, 31). HTLV-1 encounters A3 proteins in vivo as it replicates in the same CD4 T cells as HIV-1, but it lacks a functional Vif to counteract them (42). Yet, several groups reported that wild-type HTLV-1 is largely resistant to A3G restriction in cell culture assays (41, 56, 59). Only one group reported that HTLV-1 is mildly sensitive to A3G and that its catalytic deaminase activity was dispensable for restriction (74). Moreover, proviruses with multiple G-to-A mutations suggestive of A3 action are frequently detected in HIV-1-infected individuals (30, 33), but HTLV-1 proviruses carrying footprints of past deamination are rarely found in cell culture or HTLV-1-infected patients. In the few cases in which G-to-A mutations have been found, they have been attributed to A3G activity based on the dinucleotide context in which they occur (17, 41, 44). Derse and colleagues reported that HTLV-1 evolved a strategy different from HIV to counteract APOBEC3 proteins (14). In contrast to HIV-1 Vif-mediated proteosomal degradation, a portion of the HTLV-1 nucleocapsid results in A3G exclusion from the virion. Wild-type HTLV-1 particles failed to encapsidate A3G, whereas a deletion of a HTLV-1-specific 20-amino-acid (20-aa) region near the C terminus of NC resulted in enhanced A3G virion encapsidation and restriction, suggesting that this domain excludes A3G from packaging (14).

We hypothesized that one or more A3 cytidine deaminases other than A3G may escape this NC peptide-mediated exclusion from virions and reduce HTLV-1 infectivity. In this study, we tested all seven human A3 proteins for their ability to restrict HTLV-1. We found that A3A, A3B, andA3H hapII potently decrease HTLV-1 infectivity. A3A and A3B required

catalytic deaminase activity for restriction, whereas A3H hapII restricted HTLV-1 in a deaminase-independent manner. Analysis of HTLV-1 sequences in cell lines derived from ATLL and HAM/TSP patients revealed multiple independently mutated proviruses, suggesting that HTLV-1 is targeted by several A3 proteins, such as A3A, A3B, and A3G.

MATERIALS AND METHODS

Cell culture. TZM-bl cells were provided by J. C. Kappes and X. Wu through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, NIH Reagent program (90). The human cell lines HEK-293T and TZM-bl were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's high-glucose modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units/ml penicillin, and 50 g/ml streptomycin. Jurkat cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Roswell Park Memorial Institute medium 1640 (RPMI 1640), supplemented with 10% FBS, 2 mM glutamine, 50 units/ml penicillin, and 50 g/ml streptomycin.

Patient-derived HTLV-1-infected cell lines. Cell lines derived from HTLV-1-infected patients have been established from peripheral blood mononuclear cells (PBMCs) obtained from patients diagnosed with ATLL or HAM/TSP as described previously (65, 72, 88). Cell lines derived from ATLL patients (StEd, Champ, and PaBe cell lines) and from HAM/TSP patients (Eva and Xpos cell lines) were cultured in RPMI 1640 containing 40% Panserin 401 (PAN Biotech), 20% FBS, L-glutamine (0.35 g/liter), and streptomycin-penicillin (0.12 g/liter each). Media for the different cell lines were supplemented with interleukin 2 (IL-2) (Roche Diagnostics) as follows: 40 U of IL-2/ml for StEd cells and 20 U of IL-2/ml for Champ, PaBe, Eva, and Xpos cell lines. Patient-derived cell lines were in continuous culture for at least 4 months.

DNA and RNA isolation from PBMCs. PBMCs were isolated from fresh human or Macaca mulatta blood (Animal Facility Department, Paul-Ehrlich-Institute, Germany) by Histopaque-1077 gradient centrifugation (Sigma-Aldrich). Mononuclear cells at the interface were collected and washed twice with phosphate-buffered saline (PBS), and DNA was isolated by using the DNeasy DNA isolation kit (Qiagen). Macaca mulatta PBMCs were activated with phytohemagglutinin (3 g/ml) for 3 days in RPMI 1640 medium containing 10% FBS, 5 105 mM 2-mercaptoethanol, 2 mM L-glutamine, and 100 units of human recombinant IL-2 per ml at 37°C and 5% CO2. Total RNA was isolated using the RNeasy

RNA kit (Qiagen).

APOBEC3 expression plasmids. All the expression plasmids encoded human A3 proteins (A3s) as triple-carboxy-terminal hemagglutinin (HA)-tagged proteins. A3A, A3B, A3C, A3D, A3F, A3G, A3H hapI, and A3H hapII were PCR amplified using primers carrying 5'- HindIII and 3'-XbaI restriction sites and a 3 HA tag, and inserted in the mammalian expression vector PTR600, as previously described (22). Site-directed mutagenesis was performed to construct the A3A E72A, A3B E68A, A3B E225A, A3B E68A/E255A, and A3H hapII E56A cytidine deaminase mutants, as previously described (22). The PCR products were cloned into the PTR600 vector and confirmed by sequencing.

Cloning of A3A from Macaca mulatta. cDNA was synthesized from 1 g of total Macaca mulatta PBMC RNA using oligo(dT)18 primers and the RevertAid first strand cDNA synthesis kit (Fermentas). A3A cDNA was amplified with forward 5=-ATGGACGGCAGCCCAGCATC-3= and reverse 5'-GTTTCCCTGATTCTGGAGAATGGC-3' primers, using recombinant Pfu polymerase (Fermentas) with the following PCR conditions: (i) 95°C for 3 min; (ii) 32 cycles, with 1 cycle consisting of 95°C for 30 s, 58°C for 30 s; and (iii) 72°C for 4 min. The PCR product was used for a second round PCR with primers 5= HindIII forward (5'-CCAAGCTTATGGACGGCAGCCCAGC-3') and the 3= SmaI HA tag (5'-CCCCCGGGTTAAGCGTAATCTGGAACATCGTATGGGTAGTTTCCCTGATTCTGGAG-3') primer (HA tag underlined) and cloned into the PTR600 vector. The SV and SVR amino acid insertions in human A3A were cloned in the PTR600 vector using site-directed mutagenesis using overlapping primers. Site-directed mutagenesis was used to delete SV at positions 27 and 28 and SVR at positions 27 to 29 from Macaca mulatta A3. The inserts were confirmed by sequencing. Primer sequences are available upon request. Plasmids used for viral infectivity assays. The HTLV-1 packaging plasmid pCMVHT1- Env (CMV stands for cytomegalovirus) (13), which encodes all HTLV-1 genes except env, and the HTLV-1 reporter vector HTLV1-inLuc (Luc stands for luciferase) (48) were kindly provided by Gisela Heidecker and David Derse, NCI-Frederick. HTLV-1 viruses were pseudotyped using the vesicular stomatitis virus G glycoprotein (VSV-G) expression pMD.G plasmid (54). The replication competent molecular clones NL4-3 (1) and NL4-3 Vif (19) were provided

by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

HTLV-1 infectivity assay. Transfections into HEK-293T cells (2*10 cells/well in a 12-well plate) were performed with Lipofectamine LTX (Invitrogen) or Fugene 6 (Roche Diagnostics) according to the manufacturer's instructions. Standard transfection experiments consist of 800 ng of the HTLV-1 packaging construct (pCMVHT1 env), 800 ng of the HTLV-1 reporter vector HTLV1-inLuc, 100 ng of the VSV-G expression plasmid for viral pseudotyping, and 100 ng of the A3 expression plasmids. Green fluorescent protein (GFP)-expressing PTR600 (GFP-PTR600) vectors and LacZ expression plasmids were used as controls. After 24 h, cells were washed with PBS and 105 HEK-293T cells were mixed and cocultured with 105 Jurkat cells. Cells were collected 72 h later, and luciferase activity was measured using SteadyliteHTS luciferase reagent (Perkin Elmer) in black 96-well plates. Infections were done in triplicate, and at least three independent experiments were performed for each A3 protein. HTLV-1 virion release was detected by an anti-HTLV-1 p19 antibody (clone TP-7; Zeptometrix).

HIV-1 infectivity assay. HEK-293T cells (3* 10^5) were cotransfected with 500 ng of wild-type pNL4-3 (pNL4-3 WT) or pNL4-3 Vif and 50 ng of the respective A3 expression plasmids using 4 g/ml polyethylenimine (PEI) (Polysciences, Inc.). Viral supernatants were collected 48 h posttransfection, clarified by centrifugation, and used to infect 1*10^4 TZM-bl cells in black 96-well plates. Infections were done in triplicate with viral supernatants from three independent experiments for each A3 protein tested. Infectivity of the virus particles in the TZM-bl cells was assessed 48 h postinfection by detecting galactosidase activity using the Galacto-Star System (Applied Biosystems).

HTLV-1 virion isolation. HEK-293T cells (6*10^5 cells/well in a 6-well plate) were transfected with 900 ng pCMVHT1 env vector or 900 ng empty pCRV1 control plasmid and 100 ng A3 expression plasmids using Fugene 6 transfection agent (Roche Diagnostics) according to the manufacturer's instructions. Forty-eight hours posttransfection, the supernatants were filtered (0.45- m pore size) and concentrated by centrifugation through

a sucrose cushion (20% sucrose in PBS) at 20,000 g for 3 h at 4°C. The pellets were gently dissolved in PBS and digested with subtilisin A according to the protocol from David Ott (62). Virions were mixed with 1 volume of 2 digestion buffer (40 mM Tris-HCl [pH 8.0], 2 mM CaCl2, 2 mg/ml subtilisin A [Sigma-Aldrich]) and incubated at 37°C for 18 h. Subtilisin was inhibited by the addition of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Boehringer Mannheim Biochemicals) (final concentration of 5 g/ml), and virions were reisolated through a 20% sucrose cushion as described above. Pellets were dissolved in LDS sample buffer (NuPAGE; Invitrogen) and analyzed by immunoblotting.

Immunoblot analysis. Two days posttransfection, HEK-293T cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Protein concentration was measured using Bradford reagent (Bio-Rad). The concentrated virions were also resuspended in radioimmunoprecipitation assay buffer. Lysates containing 20 g of protein were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were probed with mouse anti-HA antibody (1:10,000 dilution) (MMS-101P; Covance), mouse anti-p19 (1:1,000 dilution) (clone TP-7; ZeptoMetrix), or goat antiserum to HTLV-I (1:1,000 dilution), and mouse antitubulin antibody (1:4,000 dilution;clone B5-1-2; Sigma-Aldrich) to ensure equal protein loading. The goat antiserum to HTLV-I was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from P. Szecsi, H. Halgreen, and J. Tang.

Detection of overexpressed A3-mediated HTLV-1 editing. HEK-293T cells (2 *10^5 cells/well in a 12-well plate) were transfected with 800 ng of pCMVHT1 env (a construct which expresses all HTLV-1 proteins except Env), 800 ng of the HTLV1-inLuc plasmid, 200 ng of A3 expression plasmids or GFP-PTR600 vector as a control, and 100 ng of VSV-G expression vector. Twenty-four hours posttransfection, HEK-293T cells were washed with PBS and cocultured with an equal amount of Jurkat cells (1:1 ratio). After 15 h of coculture, DNA was isolated using the DNeasy DNA isolation kit. A 515-bp fragment within the spliced luciferase gene was amplified using the primers 5'-CCGGGAAAACGCTGGGC-3' and 5'-GGCG

ATCTTTCCGCCCTTCTTGG-3'. For selective amplification of the hypermutated products, the PCR denaturation temperatures were lowered stepwise from 88°C to 84°C (84, 85.3, 86.3, 87, 87.5, and 88°C) using a gradient PCR thermocycler. The PCR parameters were as follows: (i) 95°C for 1 min; (ii) 40 cycles, with 1 cycle consisting of 84 to 88°C for 30 s, 58°C for 30 s, and 72°C for 1 min; (iii) 10 min at 72°C. PCRs were performed with recombinant Taq DNA polymerase (Fermentas). PCR products from the lowest denaturation temperatures were cloned into pJet1.2/blunt vector (Fermentas) and sequenced. The nucleotide sequences of at least eight independent clones were analyzed with the Hypermut software
(http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html).

Detection of HIV-1 editing by overexpressed A3G. Viral stocks were generated by transfecting NL4-3 Vif (500 ng) and pTR600-A3G variants (50 ng). Viral supernatants were collected 48 h posttransfection, clarified by centrifugation, and used to infect TZM-bl cells in 24-well tissue culture plates in the presence of DNase I (Invitrogen). After 12 h, cells were washed extensively with PBS, and genomic DNA was extracted using a DNeasy DNA isolation kit (Qiagen). A 1,905-nucleotide-long region of pol (HXB2, nucleotides 2928 to 4833 [22]) was amplified by PCR using Taq DNA polymerase (Qiagen) and gel extracted using the Qiaprep kit (Qiagen). The PCR products were used as a template in the differential DNA denaturation PCR (3DPCR) using different denaturing temperatures (80.5, 80.9, 81.2, 81.5, 81.2, and 83.2°C). PCR products were gel extracted and cloned using a StrataClone kit as previously described (22). Inserts form eight clones were sequenced, manually aligned using Bioedit, and compared using Hypermut software.

Detection of A3-mediated editing of HTLV-1 in patient-derived cell lines. Genomic DNA from the ATLL-derived (StEd, Champ, and PaBe) and HAM/TSP-derived (Xpos and Eva) cell lines were isolated by standard methods. For selective amplification of the HTLV-1 tax gene, two rounds of PCR were performed. The first-round PCR parameters were 95°C for 3 min, followed by 35 cycles (1 cycle consists of 95°C for 1 min, 59.2°C for 30 s, and 72°C for 1 min), and 5 min at 72°C. Second-round nested 3DPCR was performed using 0.5 l of the first-round PCR products as input and a gradient denaturation temperature between

88°C to 84°C (84, 85.3, 86.3, 87, 87.5, and 88°C). The reaction parameters were 95°C for 1 min, followed by 40 cycles (1 cycle consists of 88 to 84°C for 30 s, 54°C for 30 s, and 72°C for 1 min), and 5 min at 72°C. First-round PCR primers tax-fwd (fwd stands for forward) (5'-CAGCCCACTTCCCAGGGTTTGGAC-3') and tax-rev (5'-GTGTGAGAGTAGAAATGAGGGGT-3') (88) amplify an 881-bp fragment. Second-round PCR was performed with primers tax-nested-fwd (5'-TAGGCCTTGGTTTGAAATTTGTG-3') and tax-nested-rev (5'-CCTCCAGGCCATGCGCAAA-3') using recombinant Taq DNA polymerase. The PCR products from the lowest denaturation (88°C) temperature were cloned into pJet1.2/blunt vector and sequenced. The inserts of eight independent clones were analyzed with the Hypermut software (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html). All sequences were compared with the tax template sequence derived after PCR amplification using a recombinant Pfu polymerase (Fermentas) and tax-fwd and tax-rev primers. The PCR conditions were 95°C for 3 min, followed by 30 cycles (1 cycle consists of 95°C for 30 s, 59.2°C for 30 s, and 72°C for 2 min), and 10 min at 72°C.

3DPCR amplification of the human myc exon 2. The myc gene exon 2 was amplified from genomic DNA (500 ng) obtained from HTLV-1-infected T-cell lines (StEd, Champ, PaBe, Xpos, and Eva) and PBMCs from a healthy donor. The first-round reaction parameters were 95°C for 3 min, followed by 35 cycles (95°C for 30 s, 55.3°C for 30 s, and 72°C for 1 min), and 5 min at 72°C. Second-round 3DPCR was performed using 0.5 l of the first-round PCR products as input using a denaturing temperature gradient between 88°C and 84°C. The reaction parameters were 95°C for 1 min, followed by 40 cycles (1 cycle consists of 88 to 84°C for 30 s, 61.5°C for 30 s, and 72°C for 1 min), and 5 min at 72°C. Both rounds were performed using recombinant Taq polymerase. The primers used were described by Suspène et al. (79).

Statistical analysis. Paired t tests were performed using GraphPad Prism 5 software. The values were determined to be statistically significant at 0.05 and 0.01 levels (P values of 0.05 and 0.01).

RESULTS

A3A, A3B, and A3H hapII restrict HTLV-1. To test the hypothesis that several A3 proteins target HTLV-1, we first compared the ability of A3A, A3B, A3C, A3D, A3F, A3G, and two A3H haplotypes (hapI and hapII) to restrict HTLV-1 using a previously established HTLV-1 infectivity assay (13). Briefly, A3 expression plasmids were cotransfected with an HTLV helper construct, which carries genes that encode all HTLV-1 proteins except Env, a packageable luciferase reporter construct, and a VSV-G expression plasmid. Transfected HEK-293T cells were subsequently cocultured with Jurkat T cells, and luciferase activity was measured in the cell lysates. The luciferase reporter contains a reversed CMV promoter-driven luciferase gene with an internal intron preventing expression in transfected cells. The functional luciferase protein is expressed only upon successful splicing and packaging (in the producer cells) as well as completion of reverse transcription and integration in the target cell (the assay is explained in detail in reference 48). A3A, A3B, and A3H hapII inhibited HTLV-1 infectivity up to 10-fold compared to the no-A3 controls (Fig. 1A). The restriction by the deaminases was dose dependent, whereas the other A3 proteins poorly restricted even at higher DNA levels (Fig. 1B). In contrast, HTLV-1 infectivity was largely resistant to human A3C, A3D, A3F, and A3G action (Fig. 1A and B). The observed lack of A3G activity is in agreement with previous reports (14, 41, 56, 59). No luciferase was detected when either HTLV-1 helper construct, luciferase reporter, or the VSV-G plasmid was omitted from the transfection (Fig. 1A). High A3A expression has been reported to degrade plasmid DNA, which could have a negative effect on the HTLV plasmids and, thus, on luciferase expression (78).

To exclude this possibility, we included LacZ expression plasmids in the assay. Equal galactosidase expression was observed, suggesting that, in our experimental system, expression was not affected by A3A or other A3 proteins (data not shown). To provide a framework for the activity spectrum of human A3 proteins against HTLV-1, we additionally assessed the level of restriction of wild-type HIV-1 (WT) and an HIV-1 lacking a functional Vif (Vif) for each of the A3s. The A3 expression plasmids were transfected with HIV molecular clones NL4-3 WT and Vif in HEK-293T cells, and the culture supernatants were used to infect TZM-bl reporter cells. The Vif-sensitive A3D, A3F, and A3G restricted only

HIV-1 Vif, an observation which is in good agreement with numerous other reports (reviewed in reference 2). HIV-1 WT and HIV-1 Vif were both restricted by A3B and A3H hapII to similar levels, indicating their lack of sensitivity to Vif degradation (Fig. 1C). Interestingly, A3A, which potently restricted HTLV-1, did not affect HIV-1 WT and HIV-1 Vif infectivity in our experimental assay system. In summary, both HTLV-1 and HIV-1 WT are restricted by A3B and A3H hapII, whereas A3A exclusively restricts HTLV-1. Of note, this finding makes HTLV-1, next to avian Rous sarcoma virus (91), the only retrovirus that is sensitive to human A3A restriction.

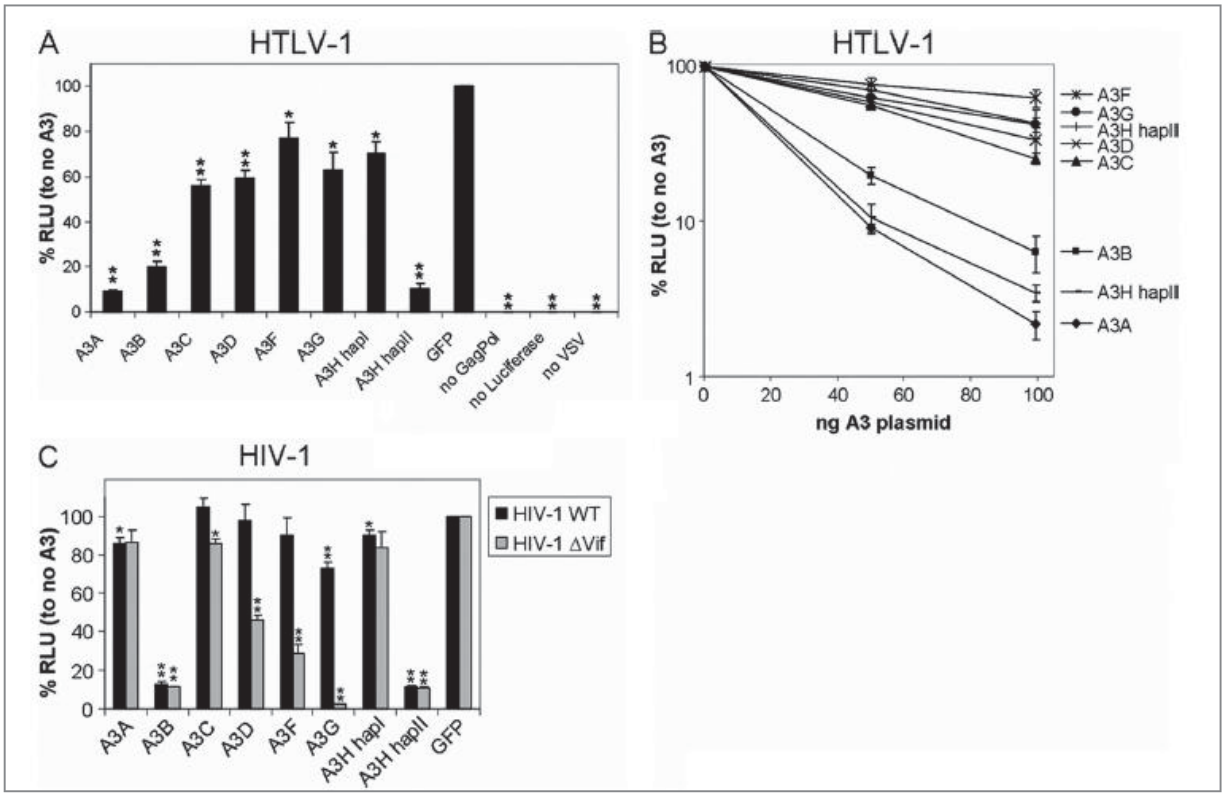

Figure 1 APOBEC3 restriction of HTLV-1 and HIV-1. (A) The indicated A3 expression plasmids were cotransfected with HTLV-1 helper (GagPol and nonstructural proteins), luciferase reporter, and VSV-G plasmids in HEK-293T cells. The cells were overlaid with Jurkat cells, and luciferase (relative light units [RLU]) was measured after 3 days. The GFP control is set at 100% infectivity. Values are means plus standard deviations (error bars) for three independent experiments. (B) Similar to panel A but with increasing amounts of A3 expression plasmids. (C) HIV-1 WT and HIV-1 Vif expression plasmids were cotransfected with theindicated A3 plasmids in HEK-293T cells. Two days posttransfection, supernatants were used to infect TZM-bl reporter cells, and -galactosidase activity (RLU) was measured 2 days postinfection. Infectivity without A3 is set at 100%. Values are means plus standard deviations (error bars) of a representative experiment performed in triplicate. Unpaired t tests were computed to determine whether differences between GFP and each A3 protein reach the level of statistical significance (P 0.05 [*] and P 0.01[**], using GraphPad Prism 5 software).

Packaging of A3 into HTLV-1 virions. A3 proteins need to be packaged into the virion before they can exert their antiviral activity in the target cell during reverse transcription. We therefore analyzed A3 expression and incorporation into HTLV-1 virions by cotransfecting A3 plasmids with HTLV-1 or an irrelevant plasmid in HEK-293T cells. The APOBEC3 expression levels were not affected by HTLV expression, indicating that, unlike WT HIV, HTLV does not encode a protein that degrades A3 proteins in the producer cells (Fig. 2A). Supernatants were concentrated through a 20% sucrose cushion and analyzed by Western blotting (Fig. 2B, left panels). We observed that A3 proteins were present at very low levels in the concentrated pellets compared to HIV-1 (data not shown), but also that A3 levels were similar for the concentrated mock transfected and HTLV-transfected cell culture supernatants (Fig. 2B, left panels). Although some A3 members, like A3A, A3B, A3G, and A3H hapII, pelleted slightly more efficiently in the presence of HTLV-1, the majority of the concentrated A3 proteins were concentrated unspecifically, likely due to association with exosomes and/or microvesicles. To remove these nonviral particles, we incubated the concentrated supernatants with the protease subtilisin A, which digests exosomes and microvesicles, but leaves enveloped virions intact (62). Subtilisin A digestion removed all non-HTLV-1-specific packaging (Fig. 2B, right panels) and showed HTLV-1-specific packaging of A3 proteins. Although all A3 members were specifically packaged into HTLV-1 virions, the active A3A, A3B, A3H hapII, and inactive A3G are packaged more efficiently than the other inactive A3C, A3D, A3F, and A3H hapI proteins. Taken together, HTLV-1 fails to degrade APOBEC3 proteins in the producer cells, but several APOBEC3 members are specifically packaged with low efficiencies into HTLV-1 virions.

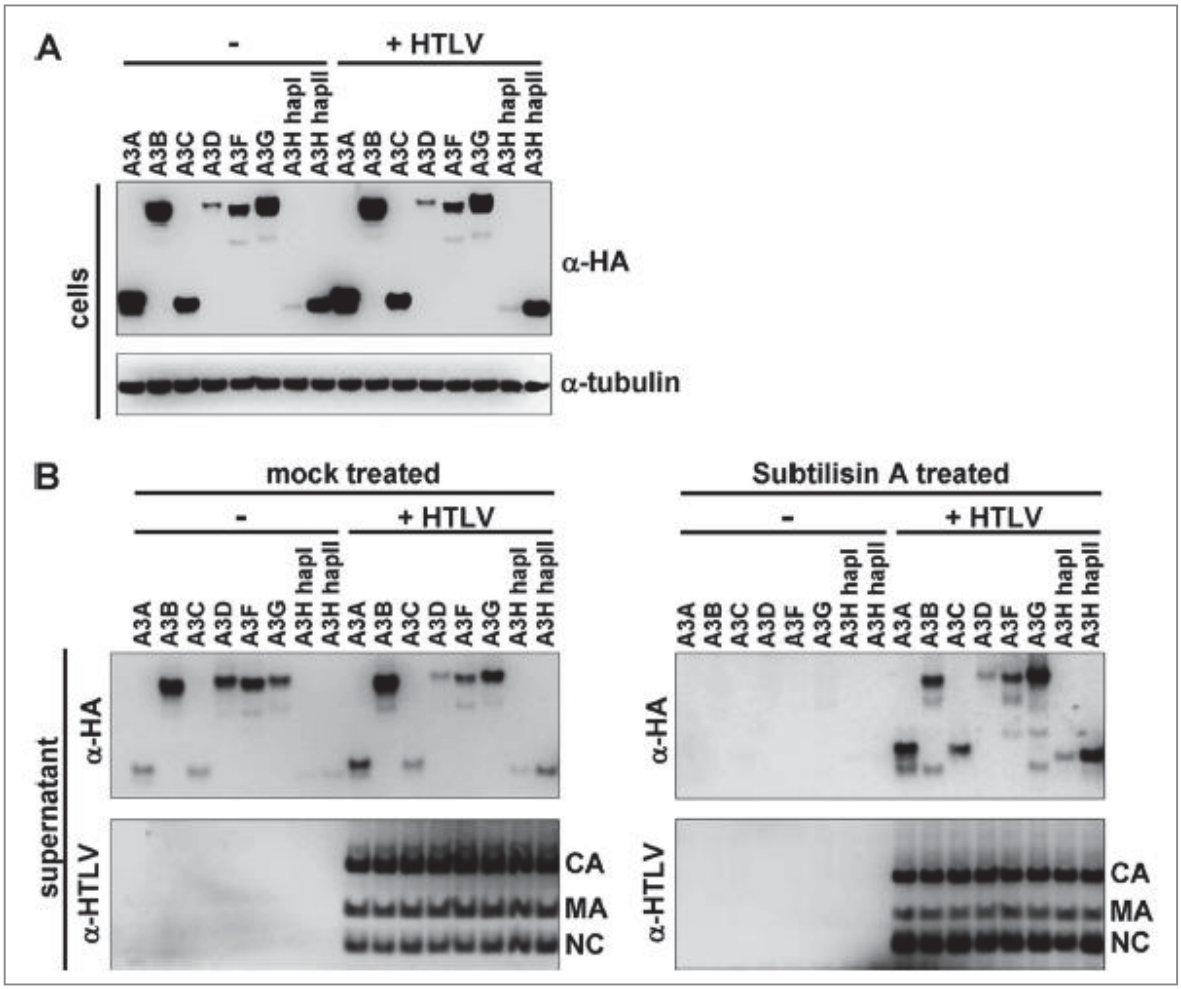

Figure 2 Packaging of APOBEC3 into HTLV-1 virions. (A) The indicated A3 expression plasmids were cotransfected with an irrelevant plasmid (-) or with an HTLV-1 helper plasmid (+ HTLV) (all HTLV proteins except Env) in HEK-293T cells, and the cells were lysed 2 days after transfection. The lysates were analyzed by immunoblotting and probed for A3 expression (HA), and tubulin served as a loading control. -HA, anti-HA antibody. (B) Filtered supernatants from panel A were concentrated through a 20% sucrose cushion and were either mock treated or treated with subtilisin A to remove exosomes/microvesicles and reconcentrated through a 20% sucrose cushion. Virion lysates were analyzed by immunoblotting. HTLV capsid (CA), matrix (MA), and nucleocapsid (NC) were detected by anti-HTLV-1 antibodies (a-HTLV).

Comparison between human and macaque A3A for HTLV-1 restriction. Human A3A is highly expressed in monocytes/macrophages, but its expression is very low or even absent in T cells (69). Thus, the in vivo relevance of A3A for HTLV-1 infection in humans is currently undefined. Rhesus macaques are natural hosts of simian T-lymphotropic virus type 1 (STLV-1), a retrovirus closely related to HTLV-1 (35, 40, 73). Activated T cells from rhesus macaques express high levels of A3A in contrast to humans (75). Moreover, rhesus A3A strongly inhibits HIV-1 Vif but is inactive against adeno-associated virus type 2 (AAV-2) and L1 retrotransposons (75). In contrast, human A3A is inactive against HIV-1 Vif but represses AAV-2 and L1 replication (5, 7, 9, 55). A recent study showed that hominids acquired a three-amino-acid deletion in A3A (ΔSVR at positions 27 to 29 [75] [Fig. 3A, see alignment]). The deletion of these three amino acids in rhesus A3A abrogated its activity against HIV-1 Vif yet failed to bring back the inhibitory capacity against L1, indicating that the difference between human and macaque A3A is not dictated by the indel (75). Because our data identified human A3A as a potent inhibitor of HTLV-1, we analyzed the effect of these three amino acids in rhesus and human A3A proteins on HTLV-1 restriction. Using the described luciferase reporter virus, we show that both human and rhesus A3A displayed a potent inhibition of HTLV-1 (Fig. 3B). Moreover, inserting two or three amino acids in the human A3A (ΔSV and ΔSVR) did not affect its antiviral activity against HTLV-1. Interestingly, deletion of two and three amino acids in rhesus A3A (ΔSV and ΔSVR A3A) reduced its anti-HTLV-1 activity (Fig. 3B). We also observed that WT and mutant A3A proteins were expressed equally in the producer cells, but rhesus A3A was packaged less efficiently than human A3A was (Fig. 3C). We conclude that both rhesus and human A3A proteins share an evolutionarily conserved capacity to inhibit HTLV-1, which indicates that the three-amino-acid deletion in human A3A likely arose because of its beneficial nature in counteracting viruses other than HTLV-1.

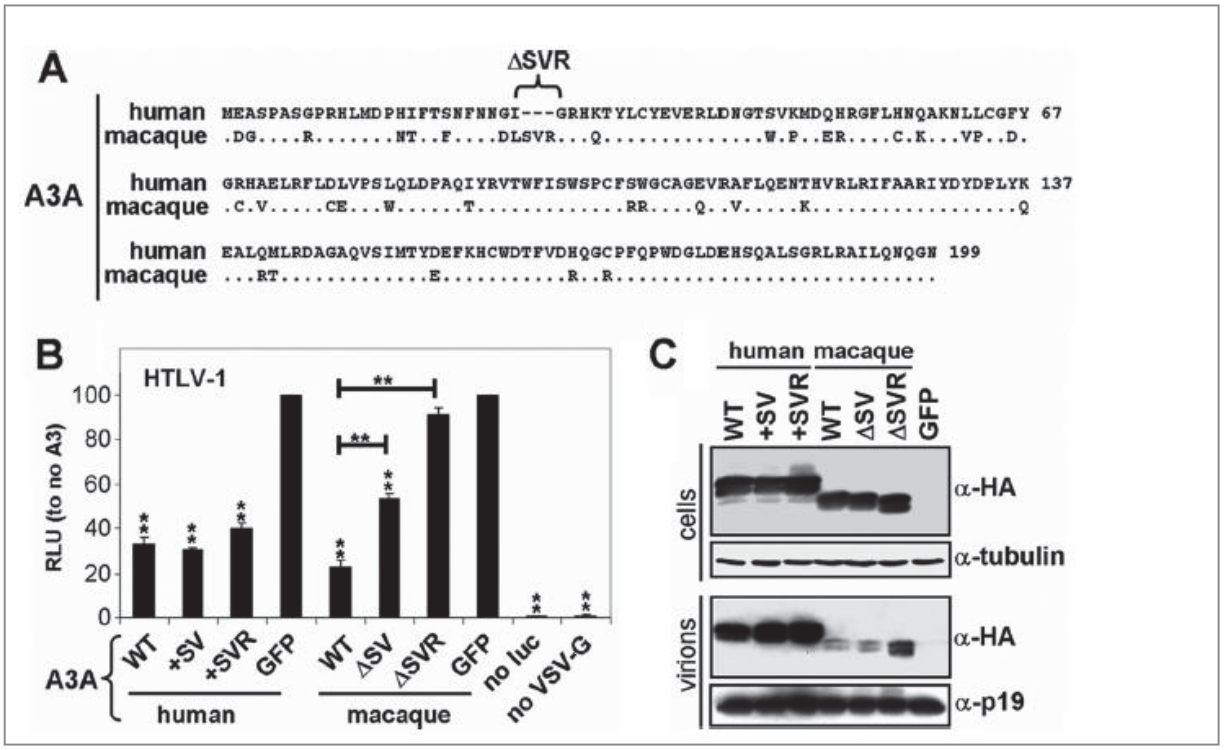

Figure 3 Comparison between human and macaque A3A restriction of HTLV-1. (A) Protein sequence alignment of human A3A and Macaca mulatta A3A. Human A3A contains a specific deletion of 27S, 28V, and 29R. (B) The indicated A3A WT and mutant expression plasmids were cotransfected with HTLV-1 helper (GagPol and nonstructural proteins), luciferase reporter, and VSV-G plasmids in HEK-293T cells. The cells were overlaid with Jurkat cells, and luciferase (RLU) was measured after 3 days. GFP is set at 100%. Values are means plus standard deviations (error bars) for three independent experiments. P values were computed to determine whether differences between GFP and each A3 protein or between A3A mutants reach significance (P 0.05 [*] and P 0.01 [**] by unpaired t test using GraphPad Prism 5 software). no luc, no luciferase. (C) The indicated A3A WT and mutant expression plasmids were cotransfected with HTLV-1 helper (GagPol and nonstructural proteins), luciferase reporter, and VSV-G plasmids in HEK-293T cells, and the cells were lysed 2 days after transfection. Supernatants were cleared and concentrated through a 20% sucrose cushion. Cell and virion lysates were analyzed by Western blotting. Of note, human A3A is 3 HA tagged and is therefore larger than the 1 HA tagged macaque A3A. Tubulin serves as a loading control.

A3A and A3B, but not A3H hapII, require deaminase activity for HTLV-1 restriction. Since A3 proteins can restrict retroviruses by editing and nonediting mechanisms (3, 25, 28, 49, 57), we next determined whether the HTLV-1 restriction exerted by A3A, A3B, and A3H hapII required catalytic deaminase activity. The deaminase activity of each deaminase domain can be abolished by mutating the essential glutamic acid in the catalytic site (22, 52). We introduced these previously described active site mutations into A3A, A3B, and A3H hapII (Fig. 4A) and tested their ability to block HTLV-1 infectivity as described in Materials and Methods. We show that HTLV-1 infectivity was restricted by WT A3A, but not by the corresponding deaminase active site mutant (Fig. 4B). A3B contains two deaminase domains, and both appear to contribute to efficient HTLV-1 restriction, since only virus produced in the presence of the double deaminase mutant A3B (E68A-E255A) showed infectivity levels comparable to the no-A3 control (Fig. 4B). Surprisingly, the A3H hapII deaminase mutant failed to rescue infectivity, suggesting that A3H hapII restricts HTLV-1 in a deaminase-independent manner. We also tested the deaminase mutants for their ability to inhibit HIV-1 to confirm the activity spectra of the deaminase mutants. As expected, rescue was not observed for either WT A3A or A3A E72A, suggesting that both proteins have no activity against HIV-1 (Fig. 4C). The anti-HIV-1 activity of A3B was determined mainly by the C-terminal deaminase motif, and a complete loss of its antiretroviral activity required mutations in both domains (6). Interestingly, the A3H hapII deaminase mutant failed to restrict HIV-1, while A3H hapII deaminase activity was not required for HTLV-1. We conclude that A3A exclusively restricts HTLV-1 in a deaminase-dependent manner, while A3B requires its catalytic sites for restricting HTLV-1 and HIV-1. In contrast, an intact deaminase domain for A3H hapII is dispensable for HTLV-1 restriction but necessary and required for anti-HIV-1 activity.

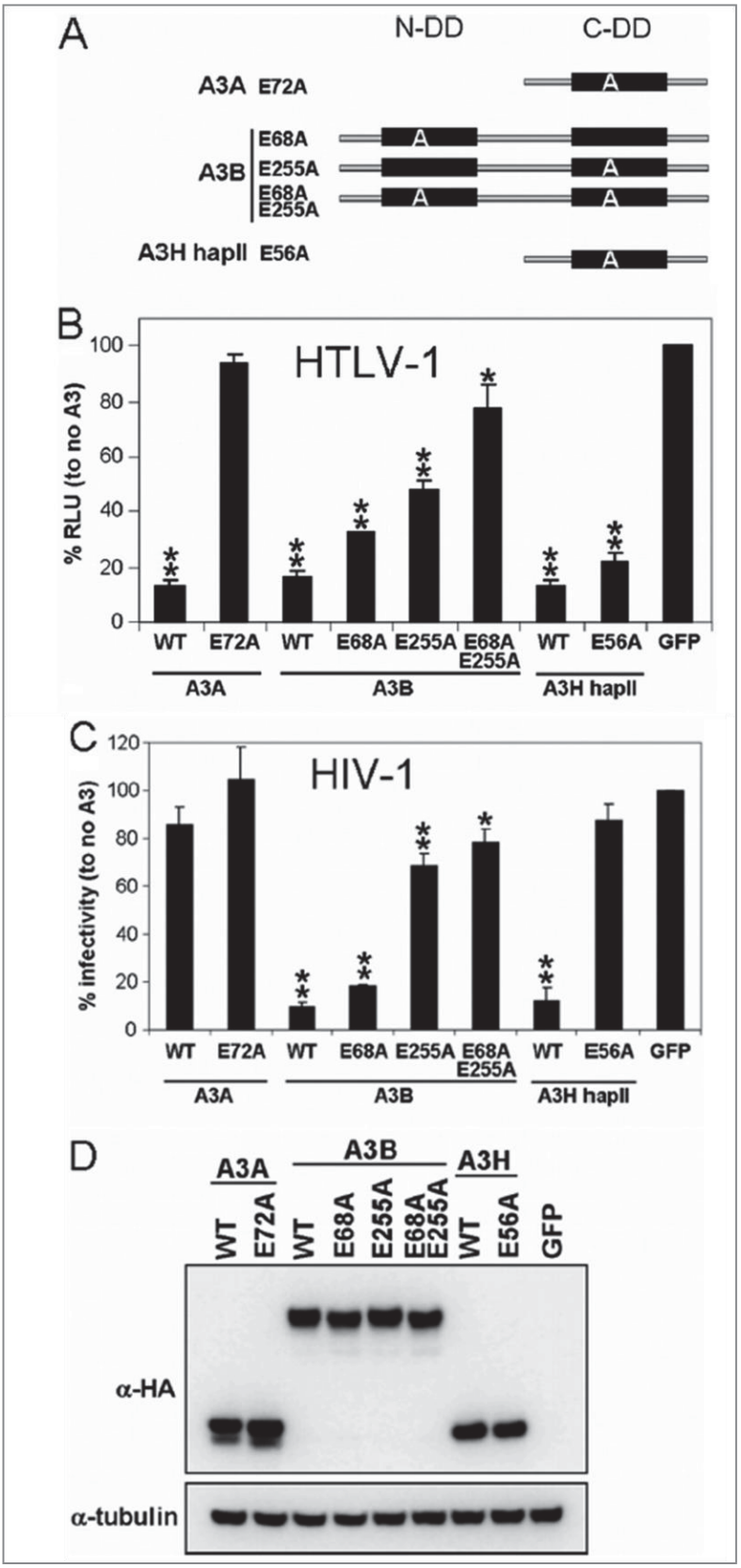
A
N-DD
C-DD
A3A E72A
A3B E68A
E255A
E68A E255A
A3H hapII E56A
B
HTLV-1
% RLU (to no A3)
WT E72A WT E68A E255A E68A E255A WT E56A GFP
A3A
A3B
A3H hapII
C
HIV-1
% infectivity (to no A3)
WT E72A WT E68A E255A E68A E255A WT E56A GFP
A3A
A3B
A3H hapII
D
A3A
A3B
A3H
WT E72A WT E68A E255A E68A E255A WT E56A GFP
α-HA
α-tubulin

Figure 4 Deaminase activity requirements for restriction of HTLV-1 and HIV-1. (A) Schematic representations of the deaminase domains present in A3A, A3B, and A3H. A3A and A3H hapII contain a single deaminase domain, whereas A3B contains two domains, which are indicated by a black box. The letter "A" denotes the alanine mutations of the essential glutamic acid in the catalytic active site. N-DD and C-DD denote the N-terminal and C-terminal deaminase domains, respectively. (B) The indicated A3 WT and deaminase mutants were cotransfected with HTLV-1 helper (GagPol and nonstructural proteins), luciferase reporter, and VSV-G plasmids in HEK-293T cells. The cells were overlaid with Jurkat cells, and luciferase (RLU) was measured after 3 days. GFP is set at 100%. Values are means plus standard deviations (error bars) for three independent experiments. Unpaired t tests were computed to determine whether differences between GFP and each A3 protein are statistically different (P 0.05 [*] and P 0.01[**], using GraphPad Prism 5 software). (C) HIV-1 ΔVif expression plasmids were cotransfected with the indicated A3 WT and deaminase mutants in HEK-293T cells. Two days posttransfection, supernatants were used to infect TZM-bl reporter cells, and -galactosidase (RLU) was measured 2 days postinfection. Infectivity with GFP is set at 100%. Values are means plus standard deviations (error bars) of a representative experiment performed in triplicate. The values were compared for statistical significance (P 0.05 [*] and P 0.01[**] by unpaired t test, using GraphPad Prism 5 software). (D) Immunoblot analysis of A3A, A3B, and A3H hapII and their corresponding deaminase mutants in HEK-293T cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as a loading control.

Mutagenesis of HTLV-1 by A3. We next assessed whether reduction of HTLV-1 infectivity correlated with mutagenesis. We used a previously described 3DPCR approach (82) to amplify mutagenized HTLV-1 sequences. The underlying principle of selective 3DPCR is that edited DNA contains fewer GC base pairs, resulting in a lower melting temperature. Therefore, successful PCR amplification at lower denaturing temperatures (88 to 84°C) is indicative of mutagenized sequences. In order to avoid amplifying transfected plasmids, we selected a region in the spliced luciferase gene for amplification. We performed 3DPCR on DNA extracted from cells obtained from the HTLV-1 infectivity assay (Fig. 1A, GFP, A3A, A3B, and A3H hapII). The GFP control did not yield any PCR products amplified at lower denaturing temperatures, suggesting that no editing took place (Fig. 5A). A3A and A3B resulted in efficient amplification at lower temperatures, which was absent for the A3A E72A and the A3B double deaminase mutant E68A-E255A (Fig. 5A). For A3H hapII, no PCR products could be amplified at lower denaturing temperatures, indicating the absence of editing. Amplified PCR products from the lowest denaturing temperature were cloned, and a minimum of eight clones was sequenced. Figure 5B illustrates the extensive mutagenesis observed in the presence of A3A and A3B, predominantly in an A3-specific G-to-A context (17 to 64 G-to-A mutations per 501 bp for A3A and 4 to 45 G-to-A mutations per 501 bp for A3B). G-to-A mutations were not induced by A3H hapII, which was in stark contrast to its restriction of HIV-1, which required deaminase activity and was previously shown to induce editing (22).

A3 enzymes favor different dinucleotide contexts, and the analysis of the edited sequences reproduced these specific dinucleotide preferences very well (Fig. 5C). In our experiments, A3A preferred GA-to-AA mutations (56%) followed by GG-to-AG (26%), GT-to-AT (13%), and GC-to-AC (4%) mutations. A3B predominantly edited GA-to-AA (73%) and GG-to-AG (25%) mutations, as previously reported (4, 6, 16). In summary, A3A and A3B restricted HTLV-1 in a deaminase-dependent manner, in- ducing G-to-A mutations in a GA-to-AA dinucleotide context, while A3H hapII acted independently of catalytic activity, leaving no specific footprints in HTLV-1 proviruses.

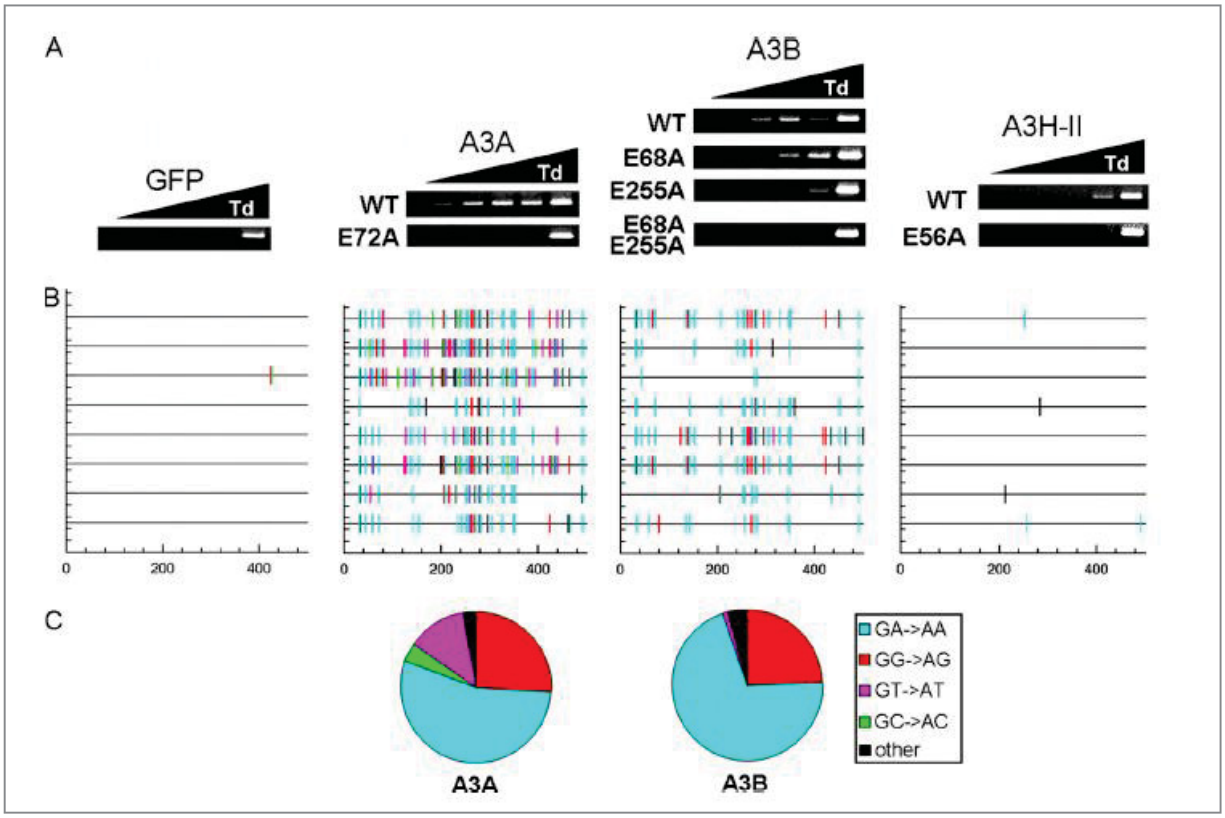

Figure 5 A3-induced editing in HTLV-1 genomes. (A) A region overlapping with the intron in the luciferase gene of the HTLV-1 reporter sequence was PCR amplified from DNA obtained from HTLV-1 infectivity assays (Fig. 1A). PCR was performed using a range of denaturing temperatures (84 to 88°C) and analyzed on agarose gels. The denaturing temperature (Td) (84, 85.3, 86.3, 87, 87.5, and 88°C) is indicated by the height of the black triangle above the gel. (B) PCR products amplified at the lowest denaturing temperature were cloned, and eight individual clones were sequenced (501 bp). The mutations are indicated by color as follows: GA-to-AA mutations (blue lines), GG-to-AG mutations (red lines), GT-to-AT mutations (pink lines), and GC-to-AC mutations (green lines). (C) Pie chart representation of the relative dinucleotide preferences of A3A and A3B.

A3G editing of HIV-1 and HTLV-1. In contrast to HIV-1, HTLV-1 editing by A3 proteins is rarely observed in patients. A recent report analyzed the presence of mutations in HTLV-1 proviral DNA from infected individuals and detected only a relatively low number of mutations (17). Most substitutions were G-to-A mutations in a GG dinucleotide context, which is favored by A3G. However, our data suggest that HTLV-1 is poorly restricted by A3G. To test whether A3G is able to edit HTLV-1 proviral DNA under experimental conditions resulting in minimal restriction, we used 3DPCR to seek evidence for editing. We also performed 3DPCR on HIV-1 proviral DNA by amplifying a 1,905-bp fragment of the HIV-1 pol gene (22). Agarose gel analysis of the 3DPCR products show that selective amplification was possible only with HIV-1 produced in the presence of A3G (Fig. 6A). Cloning and sequencing showed a high number of G-to-A mutations (17 to 45 G-to-A mutations per 800 bp), which were predominantly found in the GG dinucleotide context (Fig. 6B and C). A3G also increased the efficiency of amplification of HTLV-1 proviral DNA at lower temperatures, indicating a moderate level of editing (Fig. 6A). Sequencing showed a low level of mutations (1 to 6 G-to-A mutations per 501 bp), which were mostly in the A3G preferred GG dinucleotide context (Fig. 6B and C). Taken together, A3G is able to introduce high levels of GG-to-AG specific mutations in HIV-1 but also appears to be responsible for low levels of GG-to-AG mutations in HTLV-1 genomic DNA, despite its limited level of restriction in our reporter infectivity assay.

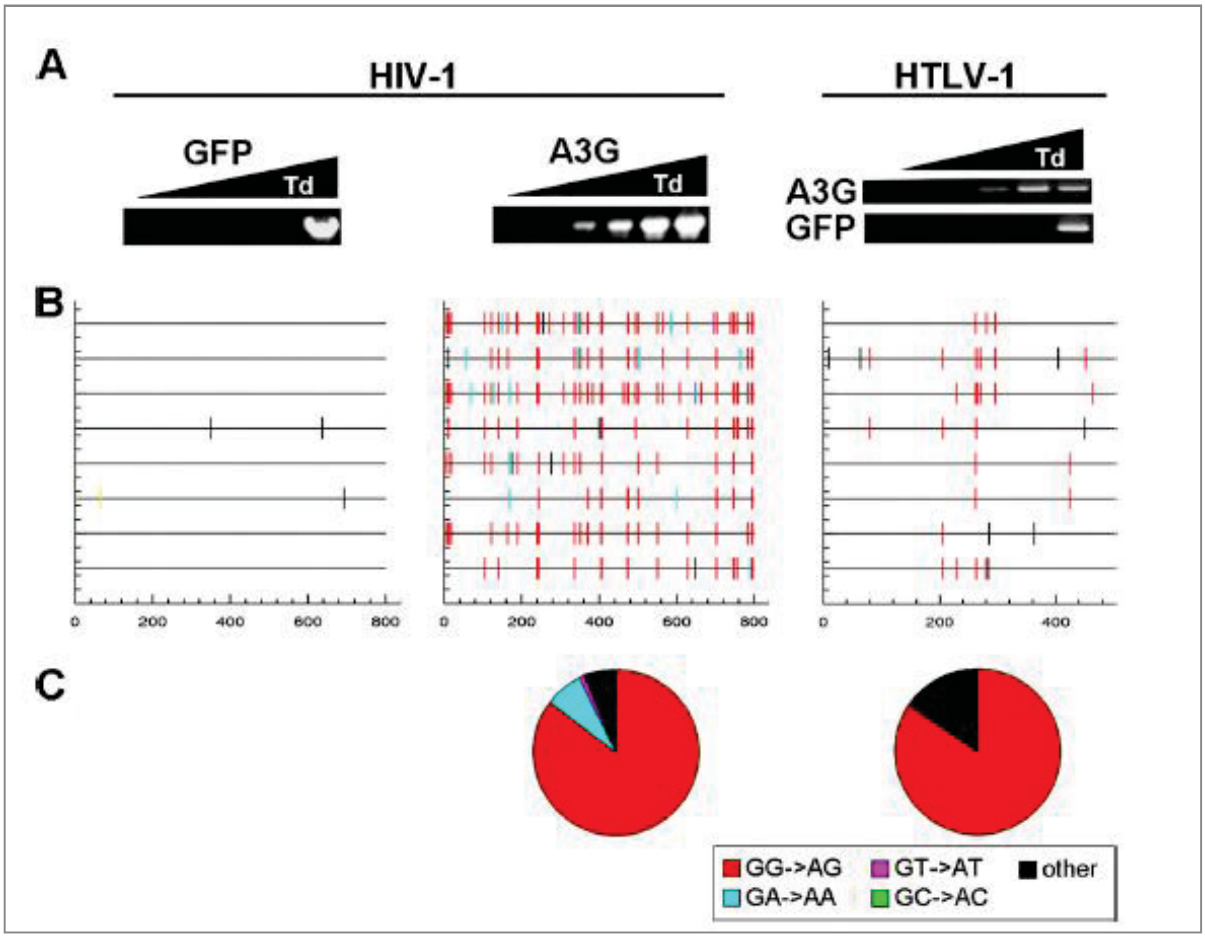

Figure 6 A3G editing of HIV-1 Vif and HTLV-1. (A) Supernatants of cells cotransfected with HIV-1 Vif and GFP or A3G was used to infect TZM-bl cells. Proviral DNA was extracted, and a 1,905-bp fragment in HIV-1 pol was PCR amplified and purified from gel. PCR fragments were subsequently used as a template in the 3DPCR with a range of denaturing temperatures (80.5 to 83.2°C) and analyzed on agarose gels. The denaturing temperature (Td) (80.5, 80.9, 81.2, 81.5, 81.2, and 83.2°C) is indicated by the height of the black triangle above the gel. HTLV-1 editing was performed as described in the legend to Fig. 5. (B) PCR products amplified at the lowest denaturing temperature were cloned, and eight individual clones were sequenced. Red lines indicate GG-to-AG mutations, blue lines indicate GA-to-AA mutations, pink lines indicate GT-to-AT mutations, green lines indicate GC-to-AC mutations, and black lines indicate non-G-to-A mutations. (C) Pie chart representation of the relative dinucleotide preferences of A3G for HIV-1 and HTLV-1.

HTLV-1 editing in patient-derived T-cell lines. We speculated that if A3A and A3B target HTLV-1 in vivo, specific deaminase footprints should be present in HTLV-1 proviral DNA. To assess whether A3A and A3B play a role in HTLV-1 replication in vivo, we analyzed five different T-cell lines obtained from HTLV- 1-infected individuals (65, 72, 88). Three of these donors were diagnosed with ATLL (StEd, Champ, and PaBe cell lines), and two donors were diagnosed with HAM/TSP (Xpos and Eva). To specifically document independently edited HTLV-1 sequences, which likely represent a small fraction of total proviral sequences, we performed 3DPCR using HTLV-1 tax-specific primers. Nested PCR fragments were amplified using lower denaturing temperatures from all but one cell line (Fig. 7A). DNA extracted from 293T cells transfected with HTLV-1 packaging plasmid served as a negative control and did not show amplification at lower temperatures (Fig. 7A, plasmid). To determine whether editing was specific for HTLV-1, a nested 3DPCR was performed on the cellular myc gene (78), which showed no difference compared to DNA extracted from PBMCs from a healthy donor (Fig. 7B). Taken together, HTLV-1 is a specific target for editing in patient-derived cell lines.

Next, we cloned PCR products from the lowest and highest denaturing temperatures. A minimum of 8 clones from each cell line were sequenced and only unique tax sequences (419 nucleotides [nt]) were used for mutational analysis. Comparing the edited sequences to their own reference sequence (high denaturing temperature) showed a large number of mutations in each cell line (48 to 78 mutations for StEd cells, 11 to 15 mutations for Champ cells, 14 mutations for PaBe cells, 5 to 44 mutations for Xpos cells, and 16 mutations for Eva cells [all mutations per 419 bp] [Fig. 7C]). The mutations were mostly G-to-A substitutions, implicating A3 action as the underlying mechanism (85% of all mutations for StEd cells, 96% of all mutations for Champ cells, 50% of all mutations for PaBe cells, 96% of all mutations for Xpos cells, and 100% of all mutations for Eva cells). The dinucleotide contexts observed were GG followed by GA and GC (Fig. 7C), indicating that A3G but also other A3 proteins, like A3A and A3B, could have edited HTLV-1 in these patient-derived cell lines. The low number of unique sequences for Champ, PaBe, and Eva cells may reflect their reported lower HTLV-1 proviral loads (66). Interestingly, one clone from Champ cells (sequence 2) showed C-to-T mutations in the positive strand, and only analysis of the negative strand showed A3-specific G-to-A mutations, indicating that the positive strand

was edited. Evidence of plus-stranded editing has been reported once for HIV-1, but it is more common for hepatitis B virus A3-induced editing (81, 87). In addition, few C-to-T mutations have been found in HTLV-1 proviral DNA, which may have been induced by activation-induced deaminase (17).

In conclusion, multiple independently edited HTLV-1 proviral DNA sequences were detected in HTLV-1-infected cell lines. The pattern of mutagenesis suggests a role for A3G as well as other A3 members in HTLV-1 editing.

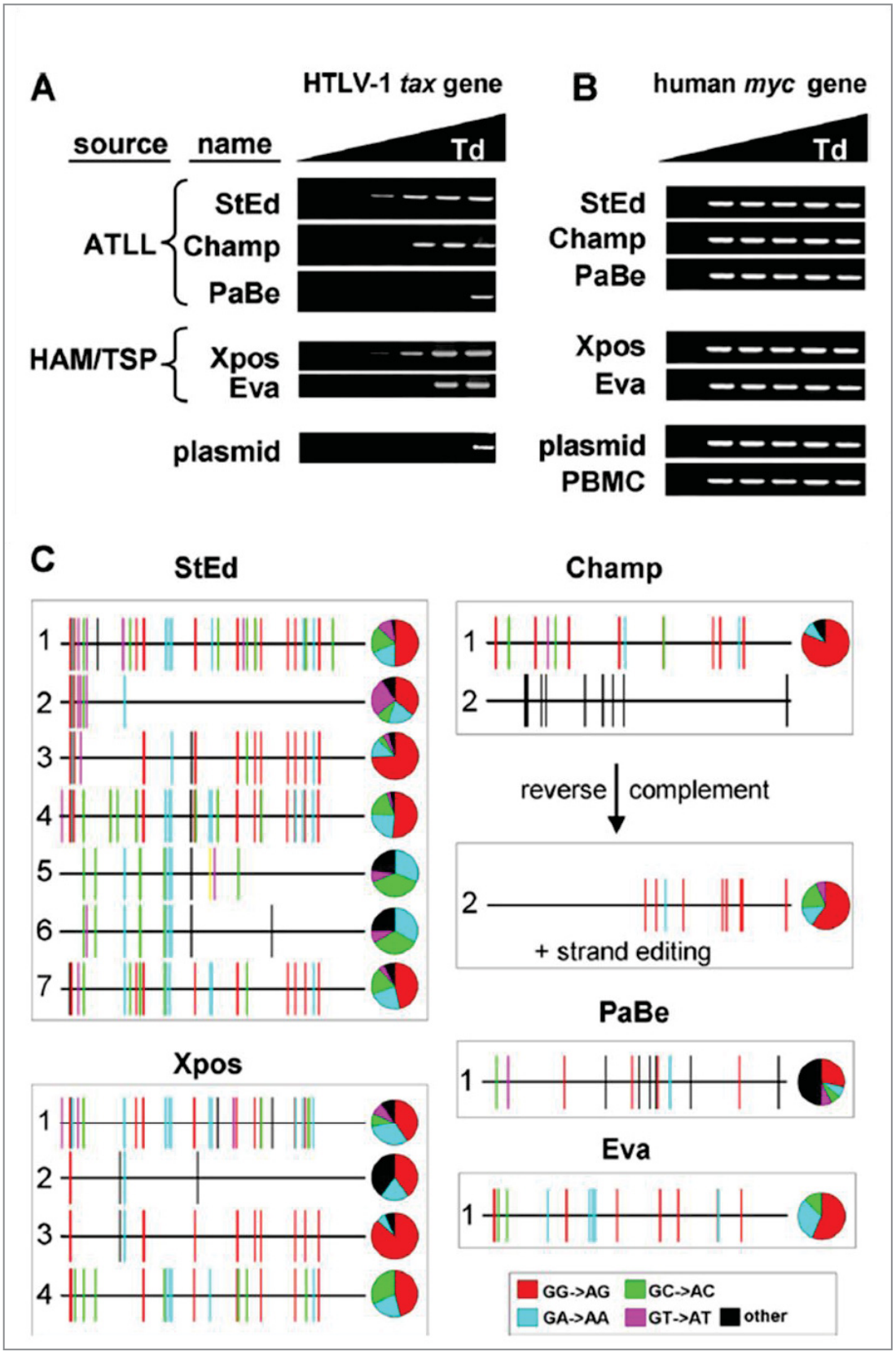
A
HTLV-1 tax gene
source
name
Td
ATLL
StEd
Champ
PaBe
HAM/TSP
Xpos
Eva
plasmid
B
human myc gene
Td
StEd
Champ
PaBe
Xpos
Eva
plasmid
PBMC
C
StEd
1
2
3
4
5
6
7
Champ
1
2
reverse
complement
2
+ strand editing
PaBe
1
Eva
1
Xpos
1
2
3
4
GG->AG
GC->AC
GA->AA
GT->AT
other

Figure 7 A3 editing of HTLV-1 in vivo. (A) Cellular DNA was extracted from several cell lines obtained from HTLV-1-infected individuals diagnosed with ATLL or HAM/TSP. Nested 3DPCR was performed with HTLV-1 tax-specific primers. DNA extracted from HTLV-1 plasmid-transfected HEK-293T cells served as a negative control for editing (plasmid). (B) Nested 3DPCR was performed on DNA extracted from the different HTLV-1 cell lines using specific primers to amplify a fragment of the cellular myc gene. DNA extracted from a healthy donor and from HTLV-transfected HEK-293T cells were used as controls (PBMC and plasmid). (C) PCR fragments from the 3DPCR were cloned and sequenced. Only unique sequences (419 bp) are shown. A3-specific dinucleotide contexts are shown in the indicated colors. All non-G-to-A mutations are represented in black. + strand, plus strand.

DISCUSSION

HTLV-1 is a human retrovirus that infects cell populations that express several A3 proteins, but the full spectrum of their antiviral activity toward HTLV-1 is unknown. We tested all human A3 proteins and show that A3A, A3B, and A3H hapII potently restrict HTLV-1. We confirmed the reported lack of A3G activity and show that A3C, A3D, and A3F also lack anti-HTLV-1 activity. Interestingly, the restriction pattern partially overlaps with the pattern observed for HIV-1 WT, which is also sensitive to A3B and A3H hapII but not to A3A. HTLV-1 does not express a Vif-like protein. The lack of HTLV-1 sensitivity to A3C, A3D, A3F, and A3G does not seem to be mediated by A3 degradation in the producer cells (Fig. 2A), but A3C, A3D, and A3F are packaged with lower efficiency (Fig. 2B) into HTLV-1 particles. It is conceivable that one or more of the many nonstructural HTLV-1 proteins could bind or inactivate packaged A3 proteins. Alternatively, packaged A3 proteins without anti-HTLV-1 activity, like A3G, may not be located within the virion core where reverse transcription takes place. Indeed, intravirion location appeared to be important for A3A and A3H activity against HIV (20, 61).

A3A and A3B both required functional deaminase domains for restriction and introduced extensive G-to-A mutations in HTLV-1, indicating that their restriction is exerted through deamination. However, A3H hapII did not require an intact deaminase domain for restriction nor did it lead to deamination of HTLV-1 proviral DNA. This contrasts with A3H hapII-mediated HIV-1 restriction, which both requires a functional deaminase domain and is accompanied by extensive proviral DNA editing (22). There are numerous reports of APOBEC3 deaminase editing-independent restriction of HIV, including APOBEC3-mediated reduction of reverse transcription activity, strand transfer, or integration (3, 25, 28, 49, 57), and we speculate that one or more of these steps in the HTLV-1 life cycle could be affected by A3H hapII. Elucidating the viral determinants underlying the difference in A3H restriction between HIV-1 and HTLV-1 could lead to an improved understanding of the mechanism of viral A3 restriction.

The entire human A3 locus is highly polymorphic, especially the regions containing A3B and A3H hapII, both of which potently restrict HTLV-1. The A3B coding region is deleted with high frequencies in East Asia, South America, and Oceania (32), while multiple A3H haplotypes have been reported, of which only A3H hapII has strong activity against HIV-1 (12, 22, 58). The frequency of the active A3H hapII is high in African but low in Asian populations (58, 89). If A3B and A3H hapII affect HTLV-1 in vivo, individuals carrying inactive A3H haplotypes (e.g., A3H hapI) combined with a deleted A3B locus would be more susceptible to infection by HTLV-1. Interestingly, HTLV-1 prevalence is extremely high in Japan, which coincides with high A3B deletion and low A3H hapII frequencies at a population level.

We here report that A3A, A3B, and A3H hapII restrict HTLV-1, but the editing profiles observed in the HTLV-1-infected T-cell lines suggest that A3G may also contribute to HTLV-1 proviral DNA editing. The major targets for HTLV-1 are CD4 T cells (42), and depending on their activation state, these cells express only very limited amounts of A3A and A3B transcripts but considerable amounts of A3G and A3H transcripts (26, 69). A3H, however, does not mutate HTLV-1 DNA and does not contribute to the mutations observed in the cell lines. A3A may restrict HTLV-1 replication in cells in monocytes and macrophages, which express high levels of A3A (34, 64, 69). Although A3B expression is low in CD4 T cells, mammary epithelial cells express high A3B levels (60), which may affect mother-to-child transmission of HTLV-1 through breast milk.

In conclusion, HTLV-1 is a target for A3A, A3B, and A3H hapII in cell culture. Further studies will elucidate whether and to what extent they curb HTLV-1 replication in vivo. An individual's genetic A3 profile may have major implications on HTLV-1 transmission and disease progression.

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Chapter 3
APOBEC3A Impairs Plasmid Expression and Blocks Murine Leukemia Virus and Non-Integrating HIV-1

The following data are submitted for publication by Aikaterini Krikoni, Daniela Marino, Wolfgang Goering, Gerald G. Schumann, Renate König, Wolfgang A. Schultz, Dieter Häussinger, and Carsten Münk. Aikaterini Krikoni is the first autor of this study.

Aikaterini Krikoni (A. K.) contributed to this study with the following:

1. A. K. compared the capacity of A3A to restrict HIV-1 and Mo-MLV infection, and performed immunoblot analysis of A3A incorporation in Mo-MLV viral particles (Figure 1 A, and B).
2. A. K. analyzed the ability of A3A to inhibit plasmid expression and induce genomic DNA breaks (Figure 2 A, B, and C).
3. A. K. proved that A3A can inhibit Mo-MLV, but not the AKV-derived MLV strains (Figure 3 A, B, and C).
4. A. K. analyzed the potency of A3A expressed in the target cells to restrict Mo-MLV entry (Figure 4 A, B, and C).
5. A. K. analyzed the ability of A3s expressed in the target cells to restrict Mo-MLV, HIV-1, and HIV-1 IN D64V (Figure 5 A, B, and C).
6. A. K. contributed to the manuscript writing.

ABSTRACT

Background: The APOBEC3A (A3A) polynucleotide cytidine deaminase of the human APOBEC3 (A3) protein family was shown to be antivirally active against HTLV-1 but not HIV-1 when expressed in the virus producer cell. Differing results regarding Murine leukemia virus (MLV) inhibition by A3A have been reported. In viral target cells, high levels of endogenous A3A activity have been associated with restriction of HIV-1 during infection. No cell culture system using ectopic expression of A3A exists to reproduce the antiviral activity of endogenous target cell A3A.

Results: In order to study these issues, we first optimized the experimental system to minimize direct deamination effects of A3A on transfected plasmid DNA. We extend our knowledge of A3A's specific anti-retroviral activity and show that A3A can block the replication of the gammaretrovirus Moloney-MLV (Mo-MLV) but is unable to restrict the related AKV-derived strains of MLV. A3A-mediated inhibition of Mo-MLV can be achieved either by A3A incorporation into nascent virions and subsequent hypermutation of the viral genomes, or by target cell-derived A3A during the early phases of infection. Mo-MLV also was subject to a post-entry restriction by ectopically expressed target cell A3C, A3G and A3H. In contrast, only ectopically expressed target cell A3A moderately inhibited incoming HIV-1. We hypothesized that HIV-1 may evade the post-entry restriction by target cell A3s by a different timing of integration into the host's genomic DNA. Indeed, when expressed in target cells, A3A, A3B, A3C, A3G, and A3H, but not A3D and A3F, significantly restricted non-integrating HIV-1 vectors post-entry.

Conclusion: These data demonstrate that a) AKV MLV but not Mo-MLV has evolved to overcome the A3A restriction by preventing A3A encapsidation through an unknown mechanism and b) that HIV-1's sensitivity to target cell-expressed A3 is modulated by the integration competence of the virus.

BACKGROUND

The human family of APOBEC3 (A3) genes consists of seven members, APOBEC3A (A3A), -B, -C, -D, -F, -G, and –H [1,2]. These genes belong to the group of cytidine deaminases that can deaminate cytidine residues in single-stranded DNA molecules, which are recognized through currently unknown mechanisms [3]. Proteins of the A3 group contain one or two zinc (Z)-coordinating domains and can be classified according to the presence or absence of a Z1, Z2, or Z3 motif [4]. Different lineages of placental mammals show an individual A3 locus with a variable number of A3 genes that appears to result from specific host adaptation to viruses [2,4,5].

Human immunodeficiency virus type 1 (HIV-1) variants lacking expression of the vif gene (HIV-1ΔVif), package A3G and other A3s of their producer cells into viral particles. Incorporated A3G deaminates cytosine residues to uracil in elongating single-stranded DNA during reverse transcription, leading to hypermutation [6-12]. More recent studies indicate that deaminase-independent mechanisms might also contribute to the antiviral activity of A3G [13-23]. In the case of HIV-1, the amount of encapsidated A3G in wild-type HIV-1 virions is dramatically reduced by Vif-dependent degradation of A3G via the ubiquitination-proteasome pathway and by degradation-independent mechanisms [24-32].

Another member of the human A3 family, the A3A protein, was found to have no antiviral activity when tested against HIV-1 or Murine leukemia virus (MLV) by co-expression in the virus producer cells [6,33-41]. However, one study reported that A3A inhibits MLV [42], and A3A can restrict HIV-1 if targeted to the capsid core by fusion to the viral protein Vpr or the N-terminal domain of A3G [36,39].

In addition, A3A expressed in the viral producer cell inhibits other retroviruses, such as the Human T-lymphotropic virus type 1, the Rous sarcoma virus, the Porcine endogenous retrovirus-B and the simian-human immunodeficiency virus ΔVif [34,41-44]. A3A can also restrict hepatitis B virus as well as parvo- and papillomaviruses [35,45-49]. It is a repressor of retrotransposition activity and effectively inhibits long terminal repeat (LTR)-retrotransposons through a novel deamination-independent mechanism [35,38]. A3A is also reported to be a potent inhibitor of human non-LTR retrotransposons, causing a

reduction in LINE-1 (L1) and Alu retrotransposition frequencies [35,50-57]. The mechanism responsible for A3A-mediated L1 and Alu inhibition is still unclear [58]. Other described functions of A3A include deamination of 5-methylcytosine [59,60], induction of DNA breaks, activation of the DNA damage response and induction of cell-cycle arrest [61], hypermutation of nuclear and mitochondrial DNA [62], and reduction of the expression of plasmid DNA by deamination-induced destabilization [59,63].

A3A is preferentially expressed in monocytes, macrophages and dendritic cells, and interferon can further induce its expression [35,63-73]. Monocytes and, to a moderate extent, macrophages are refractory to HIV-1 infection. This restriction is likely caused by two inhibitors, SAMHD1 [74-77] and A3A [64,65,67,68,72]. Silencing of A3A renders myeloid cells vulnerable to HIV-1 infection [64,68], suggesting that A3A expressed in target cells can attack the incoming virion [67,72]. However, ectopic expression of A3A in HeLa cells did not significantly modulate the susceptibility of these cells to HIV-1 infection [64].

Here, we report that in contrast to HIV-1, Moloney-MLV (Mo-MLV) is restricted by producer cell A3A. We also reanalyzed the functional consequences of the presence of A3A on the expression of reporter genes encoded by plasmid DNA and confirm that A3A can inhibit plasmid-derived expression, and that this ability correlated with cytidine deamination on both strands of the plasmid. Ectopically expressed A3A in viral target cells reduced transduction of Mo-MLV and HIV-1. However, using non-integrating HIV-1 vectors, we found that in addition to A3A, other human A3s expressed in the target cell showed a significant post-entry antiviral activity.

METHODS

Cell culture. Cell lines HEK293T, Jurkat and CrFK (feline renal fibroblast cells) were maintained at 37°C in a humidified atmosphere of 5% CO2 in Dulbecco's high-glucose modified Eagle's medium (Biochrom, Berlin, Germany), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Jurkat cells were cultured in Roswell Park Memorial Institute medium 1640 (RPMI 1640, Biochrom), supplemented with 10% FBS, 2 mM glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. PBMCs were isolated from fresh human blood by Histopaque-1077 gradient centrifugation (Sigma-Aldrich, Taufkirchen, Germany). Mononuclear cells at the interface were collected and washed twice with phosphate-buffered saline (PBS).

Plasmids. The MLV packaging plasmids CIG-N, CIG-B, and PHIT60 were kindly provided by Jonathan Stoye, and encode for the gag-pol of AKV N-tropic, AKV B-tropic, and Mo-MLV, respectively [93]. The MLV luciferase reporter plasmid MP71-luc is based on the MP71 plasmid that was previously described by Schambach et al. [94], provided by Harald Wodrich. The MP71-luc plasmid contains the firefly luciferase gene cloned into the EcoRI endonuclease restriction of MP71. The APOBEC3A (A3A)-HA expression construct is coding for the human A3A with three C-terminal hemagglutinin (HA)-tag [33], provided by Bryan Cullen. The E72A mutant of A3A-HA has been described [51]. The pEF-Ugi Hygro plasmid [95] encodes for the UGI (Uracil Glycosylase Inhibitor a gift from Javier Di Noia. The HIV-1 packaging plasmid pMDLg/pRRE encodes gag-pol, and the pRSV-Rev for the HIV-1 rev [96]. The HIV-1 gag-pol packaging plasmid pcDNA3.g/p D64V.4xCTE is an integrase-defective construct that contains a single amino acid mutation from aspartic acid to valine at position 64 (D64V) of the catalytic domain of HIV-1 integrase [84], and was a gift from Christopher Baum [97]. Except D64V, both HIV constructs encode identical Gag and Pol proteins. The HIV-1 vector pSIN.PPT.CMV.Luc.IRES.GFP expresses the firefly luciferase and GFP. The luciferase cDNA (luc 3) was cloned into NheI and BamH1 restriction sites of pSIN.PPT.CMVmcsIRES.GFP [98], a gift of Neeltje Kootsta. MLV and HIV-1 based viral vectors were pseudotyped using the pMD.G plasmid that encodes the glycoprotein of VSV (VSV-G). The PTR600 APOBEC3 (A3)-A, -B, -C, -D, -F, -G, and –H hap II expression plasmids were a gift of Viviana Simon [41].

Production and infectivity of viral vectors. Plasmid transfection into HEK293T cells (2 x 105 cells/well in a 12-well plate) were performed using Lipofectamine LTX (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. MLV vectors: Standard transfection experiments consist of 600 ng of a MLV packaging construct (CIG-N, CIG-B, or PHIT60), 600 ng of the MLV reporter vector plasmid MP71-luc, 100 ng of the VSV-G expression plasmid, and 100 ng to 1 µg of the A3A expression plasmids, or pcDNA3.1 (Invitrogen) to keep total DNA concentrations equivalent for each transfection. Viral vector-containing supernatant was collected and filtered through a 0.45-µm-pore-size syringe tip filter 48 h post-transfection. CrFK cells were harvested 72 h post transduction. MLV virion release was detected and normalized by an anti-MLV p30 capsid antibody (AM26127PU-N, Acris antibodies, Herford, Germany). HIV vectors: Standard transfection experiments consist of 600 ng of the HIV-1 packaging constructs (pMDLg/PRRE or pcDNA3.g/p D64V.4xCTE), 400 ng of the HIV-1 Rev expression plasmid pRSV-Rev, 600 ng of the HIV-1 reporter vector pSIN PPT CMV Luc, and 100 ng of the VSV-G expression plasmid. 48 h post-transfection, viral vector-containing supernatant was collected and filtered through a 0.45-µm-pore-size syringe tip filter. HIV vectors and Mo-MLV-based vectors were used to transduce HEK293T cells. Transduced cells were harvested 72 h later, and luciferase activity was measured. HIV-1 virion release was detected and normalized using the anti-p24 (HIV-1) capsid antibody AG3.0 provided from the NIH AIDS Reagent Program. Luciferase activity was measured using SteadyliteHTS luciferase reagent substrate (Perkin Elmer, Rodgau, Germany) in black 96-well plates on a Berthold MicroLumat Plus luminometer (Berthold Detection Systems, Pforzheim, Germany). Transductions were done in triplicate and at least three independent experiments were performed.

A3A-mediated inhibition of plasmid expression. HEK293T cells (2 x 105 cells/well in a 12-well plate) were co-transfected with the luciferase expression plasmid MP71-luc (200 ng), and increasing amounts of the A3A or A3A E72A expression plasmids (100 ng, 200 ng, 500 ng, and 1 µg). Co-transfections were performed with or without the UGI-encoding plasmid (PEF-Ugi Hygro) in an A3A to UGI plasmid ratio of 1:4 [63]. pcDNA3.1 empty vector was used to keep total DNA concentrations equivalent for each transfection. Two

days post-transfection, luciferase activity was measured as described above. Results are presented as one representative example of three independent experiments. In addition, total DNA was extracted and 100 ng DNA was used as an input for the detection of A3A-mediated MP71-luc plasmid editing. The primers and the 3DPCR parameters are described below. The recovered sequences of clones were analyzed with the Hypermut software (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html).

MLV virion isolation. HEK293T cells (6x105 cells/well in a 6-well plate) were transfected with 900 ng MLV packaging vector (CIG-N, CIG-B, or PHIT60) and 100 ng A3A expression plasmids or empty pcDNA3.1 plasmid, using Lipofectamine LTX transfection agent according to the manufacturer's instructions. 48 h post post-transfection, supernatants were filtered (0.45 µm pore size) and concentrated by centrifugation through a sucrose cushion (20% sucrose in PBS) at 120,000 g for 2 h at 4°C. Pellets were dissolved in radioimmunoprecipitation assay buffer (25 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodiumdeoxycholate, and 0.1% sodium dodecyl sulfate) and submitted to immunoblot analysis.

Immunoblot analysis. Two days post-transfection HEK293T cells were washed with PBS and lysed in RIPA buffer. The concentrated virions were also resuspended in RIPA buffer. Lysates containing 20 µg of protein were separated by sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Schwalbach, Germany). Membranes were probed with mouse anti-HA antibody (1:10,000 dilution; MMS-101P; Covance, Munich, Germany), mouse anti-p30 (1:1,500 dilution; Acris, clone 4B2), mouse anti-p24 (HIV-1) capsid antibody AG3.0 (1:250 Dilution, NIH AIDS Reagents), mouse anti-VSVG (1:20,000 dilution; Sigma-Aldrich, clone P5D4), mouse anti-phospho-Histone H2A.X (Ser139) (1:2,000 dilution; Merck Millipore, clone JBW301), and mouse anti-tubulin antibody (1:4,000 dilution; clone B5-1-2; Sigma-Aldrich) to ensure equal protein loading. As a secondary antibody, we used a horseradish peroxide-conjugated anti-mouse antibody (GE Healthcare, Munich, Germany), and developing was performed with ECL chemiluminescence reagents (GE Healthcare).

Detection of A3A-mediated Mo-MLV editing. HEK293T cells (2 x 105 cells/well in a 12-well plate) were transfected with 600 ng of PHIT60, 600 ng of the MP71-luc plasmid, 100 ng of the A3A expression plasmids or pcDNA3.1 as a control, and 100 ng of VSV-G expression vector. 48h post-transfection, virus supernatants were collected, clarified by centrifugation, filtered (0.45 μm pore size), and treated with DNase I (Thermo Scientific, Schwerte, Germany) at 5 U/ml final concentration for 1 h at 37°C. HEK293T cells (2 x 105 cells/well in a 12-well plate) were transduced with viral supernatants. After 15 h, DNA was isolated using the DNeasy DNA isolation kit (Qiagen, Hilden, Germany). A 714-bp fragment within the luciferase gene was amplified using the primers: 5'-GATATGTGGATTTCGAGTCGTC-3' and 5'-GTCATCGTCTTTCCGTGCTC-3'. For selective amplification of the hypermutated products, the PCR denaturation temperatures were lowered stepwise from 88°C to 81°C (81, 83, 84, 85.3, 86.3, 87, 87.5, and 88°C) using a gradient PCR thermocycler. The PCR parameters were as follows: (i) 95°C for 1 min; (ii) 40 cycles, with 1 cycle consisting of 81 to 88°C for 30 s, 54°C for 30 s, and 72°C for 1 min; (iii) 10 min at 72°C. PCRs were performed with recombinant Taq DNA polymerase (Thermo Scientific). PCR products visualized on agarose gels, and the products from the lowest denaturation temperatures were cloned into pJet1.2/blunt vector (Thermo Scientific) and sequenced. The nucleotide sequences of independent clones were analyzed with the Hypermut software.

Detection of Mo-MLV and HIV-1 vector inhibition of A3s expressed in the target cells. HEK293T cells (2 x 105 cells/well in a 12-well plate) were transfected with 0.5 μg of A3 expression plasmids or pcDNA3.1(+), and UGI expression plasmid in a ratio 1:4 respectively. One day post-transfection, HEK293T cells were transduced with viral vector containing supernatant. Transduced cells were collected 72 h later, and luciferase activity was measured. In addition, 18 h after transduction, HEK293T cells were washed with PBS, and genomic DNA was obtained (DNeasy Kit, Qiagen) and analyzed for A3-derived vector genome editing. The first round PCR reaction parameters were 95°C for 3 min, followed by 28 cycles (95°C for 30 s, 54°C for 30 s, and 72°C for 1 min), and 5 min at 72°C. Second-round 3DPCR was performed using 0.3 μl of the first-round PCR products as input using a denaturing temperature gradient between 88°C and 81°C (81, 83, 84, 85.3, 86.3, 87, 87.5,

and 88°C). Reaction parameters were 95°C for 1 min, followed by 40 cycles (1 cycle consists of 88 to 81°C for 30 s, 46°C for 30 s, and 72°C for 1 min), and 5 min at 72°C. Both PCR rounds were performed using recombinant Taq polymerase (Thermo Scientific). The 714-bp first round luciferase gene product was amplified with the primers: 5'-GATATGTGGATTTCGAGTCGTC-3' and 5'-GTCATCGTCTTTCCGTGCTC-3', and the second round 512 bp product with 5'-CCAACCCTATTTTCATTCTT-3' and 5'-AACAATATCGATTCCAATTCA-3'. PCR products from the second PCR round were visualized on agarose gels and the products from the lowest denaturing temperatures were cloned into pJet1.2/blunt (Thermo Scientific) and sequenced. The nucleotide sequences of independent clones were analyzed with Hypermut software (see above).

RNA extraction and quantitative reverse transcription–polymerase chain reaction. Total RNA was isolated from naïve human PBMCs, Jurkat T cells and HEK293T cells using the RNeasy kit (Qiagen), according to the manufacturer's instructions. Synthesis of complementary DNA including a DNA removal step by DNase using the QuantiTect Reverse Transcription Kit (Qiagen) was conducted according to the manufacturer's protocol. Quantitative reverse transcription (qRT)–PCR was performed as described previously [99] on a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the QuantiTect SYBR Green PCR Kit (Qiagen) with specific primers for human A3s listed in Table 1. All qRT–PCR data were adjusted to TATA-box-binding protein (TBP) mRNAs measured by a specific QuantiTect assay (Qiagen). All measurements were performed in at least duplicates; assay variance was <10%. Relative expression was calculated by the ΔΔCt method. Data are presented as normalized to the expression in naïve PBMCs.

Table 1: Oligonucleotides

	Sequence fwd primer 5' to 3'	Sequence rev primer 5' to 3'
A3A	AACTTTAACAATGGCATTGG	CAGAGAAGATTCTTAGCCTGGT
A3B	AATAAAGAGGGGCCGCTCAAA	TGCGTGGTACTGAGGCTTGAAAT
A3C	TTCACCGTGGAAGGTATAAAG	TATGTCGTCGCAGAACCA
A3D	AGAGGGGCCGCTCAAATCT	TGCCTGTGATTCGACTGACGT
A3F	TCCTGAAACCTGGAGCCT	GAAAGATCTTTGCGTCCAAAC
A3G	AGCGCAGGGGCTTTCTAT	AGGTCCAGCTTCCAAAAGG
A3H	GGGGCAAGTCTGCTAAGGAA	CGGCGCTTGTTGTTAAACTG

Statistical Analysis. Statistical analysis was performed using the statistics software GraphPad Prism version 5. The results were analyzed for statistical significance by Student's t test and P values of less than or equal to 0.05 ($p \leq 0.05$) are defined as statistically significant difference.

RESULTS

Producer-cell-derived A3A restricts Mo-MLV but not HIV-1. First, we wanted to compare the so far described activities of A3A expressed in the viral producer cell against HIV-1 and MLV. Single-round luciferase reporter vectors based on HIV-1 or Mo-MLV were generated by transfection of HEK293T cells together with increasing amounts of expression plasmids for A3A and its cytidine deaminase mutant E72A. Equal volumes of vector particle containing cell culture supernatant were used to transduce CrFK cells to study effects of A3A on virus release and infectivity in one step. The vector-derived luciferase activity was determined from lysates two days post transduction (Figure 1A). Our data show that A3A did not much affect HIV-1, as expected [6,33,35,36,38-41], but significantly reduced Mo-MLV-mediated luciferase expression in the transduced cells by up to 80%, in a dose-dependent manner. We did not observe any inhibition of viral vectors by the A3A E72A mutant. There was no detectable luciferase activity when the viral helper constructs, luciferase reporter, or the VSV-G plasmid were omitted from the transfection reaction (data not shown).

A3 proteins must be packaged into the virion before they can exert their antiviral activity in the newly infected cell. We therefore analyzed A3A expression in the transfected cells and A3A incorporation into the Mo-MLV virus-like particles (VLPs) by immunoblotting (Figure 1B). We found a dose-dependent correlation of increasing A3A expression with reduced signals of the Mo-MLV packaging construct and of the VSV-G plasmid (Figure 1B). Normalized viral supernatants of the transfected cells were concentrated through a 20% sucrose cushion and analyzed. A3A protein was detectable in the Mo-MLV particles, and A3A packaging was correlated with the A3A expression level of the producer cells (Figure 1B).

Taken together, the results suggest that (1) Mo-MLV fails to block encapsidation of A3A into nascent viral particles, and (2) the A3A protein can repress the expression of co-transfected plasmids [63]. Thus, the A3A-dependent loss of MLV reporter transduction might be caused by an antiviral activity of A3A on MLV cDNA and by it targeting the expression of the viral plasmids.

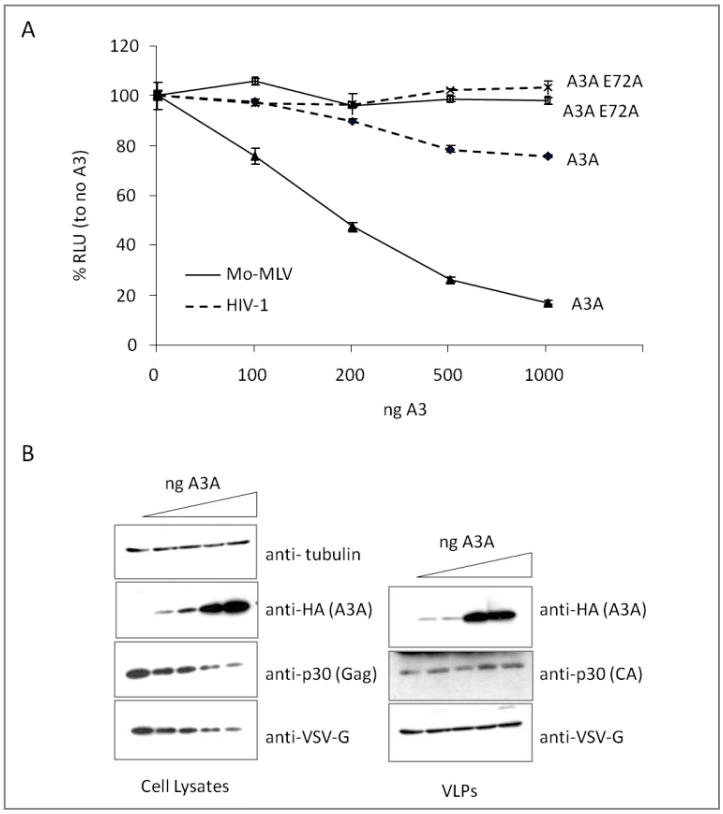


Figure 1 A3A restricts Mo-MLV, but not HIV-1. (A) Increasing amounts of the A3A and A3A E72A cytidine deaminase mutant were co-transfected with plasmids for HIV-1 or Mo-MLV luciferase reporter vectors in HEK293T cells. The viral supernatants were used to infect CrFK cells, and luciferase relative light units (RLU) counts per sec were measured 3 days later. Values are means plus standard deviations (error bars) for three independent experiments. (B) A3A was detected in lysates of transfected Mo-MLV vector producer cells and in viral like particles (VLPs) using immunoblots probed for A3A (HA), VSV-G, p30 (MLV Capsid), and tubulin.

A3A mediates inhibition of plasmid expression and induces DNA breaks. Next, we wanted to use our experimental system to provide a more quantitative assessment of whether A3A impairs the expression and integrity of co-transfected plasmids, as initially described by Stenglein et al. [63]. Other recent studies using co-transfection systems with A3A plasmids observed either a slight (<20%) [34,41,51] or a strong (2-to-4-fold) diminution of reporter gene expression [48], possibly indicating a dose- and reagent-specific effect of A3A. To delineate any side effects of A3A during viral vector production, we co-transfected cells with the MLV-based luciferase transfer vector plasmid and increasing amounts (0 to 1000 ng) of A3A or A3A E72A expression plasmids and analyzed the luciferase activity in the transfected cells two days post transfection. The results demonstrate an A3A dose-dependent inhibition of luciferase expression (Figure 2A, left panels). Considering that A3A is a cytidine deaminase, we tested whether uracil N-glycosylase (UNG) could partially eliminate the negative effect of A3A on the plasmid luciferase expression by coexpressing UGI, an inhibitor of the major cellular uracil DNA glycosylase UNG2 from the bacteriophage PBS2 [78]. UNG detects uracil molecules in DNA, initiates cleavage of the N-glycosylic bond, and activates the base-excision repair pathway (see [79] for review). The action of UNG can therefore result in repair or induction of DNA degradation [63]. UGI expression did however not change the detected luciferase activity, indicating either that UGI was inefficient or A3A-mediated damage of the luciferase plasmid cannot be efficiently repaired (Figure 2A, left panel). Next, we used the well-established 3DPCR method [80] to amplify a segment of the luciferase plasmid from the co-transfected cells. In this technique, the detection of PCR products at lower denaturing temperatures is indicative of a higher A-T content of the amplicons and thus a sign of A3-induced cytidine deaminations [80]. DNA amplified from transfected cells without UGI expression did not show differences in 3DPCR. In contrast, UGI expression in A3A transfected cells allowed us to detect PCR products of the luciferase plasmid amplified at lower denaturing temperatures (Figure 2A, right panel). No such products were obtained from cells that were transfected with A3A E72A or from cells where the A3A expression plasmid was omitted. Notably, the results of the 3DPCR experiments (Figure 2A) were generated using DNA from cells transfected with only 100 ng of A3A, which barely reduced the luciferase activity, indicating that A3A-induced editing of plasmid DNA also occurs in the presence of

small amounts of A3A. Our results support the model that A3A-generated uracils in plasmid DNA are removed by cellular UNG2 [63].

PCR products from cells expressing UGI and generated with the lowest denaturing temperatures were cloned and sequenced to verify the presence of A3A-induced cytidine deamination in the luciferase gene. Results shown in Figures 2B demonstrate the presence of hypermutations on both strands of the luciferase plasmid. Most of the cytidine deaminations, here described for the coding strand, occurred in GA dinucleotides generating AA, typical for A3A [35,41,63,81], followed by mutations in the GG motif resulting in AG dinucleotides. Of note, a smaller proportion of mutations also occurred in GC or GT dinucleotides, leaving AC or AT.

Formation of DNA double-strand breaks induces phosphorylation of the histone variant H2AX on Ser139. The phosphorylated form of H2AX is referred to as γH2AX and triggers recruitment of DNA-binding proteins to the site containing damaged chromatin and activates cell cycle arrest (see [82] for review). Using our co-transfection system of A3A and the luciferase vector plasmid (without UGI expression), we asked whether H2AX phosphorylation indicative of DNA double-strand breaks was detectable, as reported by Landry et al. [61]. Cells treated with the DNA damage mimetic antibiotic Zeocin, which causes DNA damage by cleaving both strands of the DNA molecule [83], served as a positive control. Immunoblots of cell lysates of HEK293T cells co-transfected with increasing amounts of A3A expression plasmid and of lysates of cells treated with Zeocin demonstrated a dose-dependently intensifying signal of H2AX phosphorylation. No phosphorylated H2AX was detectable in samples transfected with the empty control plasmid (pcDNA3.1), with low amounts (100 ng) of the A3A expression plasmid, or with the A3A E72A expression plasmid (Figure 2C). Our data support the hypothesis that A3A is associated with DNA breaks in transfected HEK293T cells, whereas the A3A E72A mutant showed no capacity to induce DNA breakage.

The results demonstrate that A3A can both induce cytidine deamination in plasmid DNA and cause double-strand breaks, likely in the chromosomal DNA. We conclude that the use

of A3A in studies of viral replication requires a careful investigation of unwanted and possibly misleading side effects.

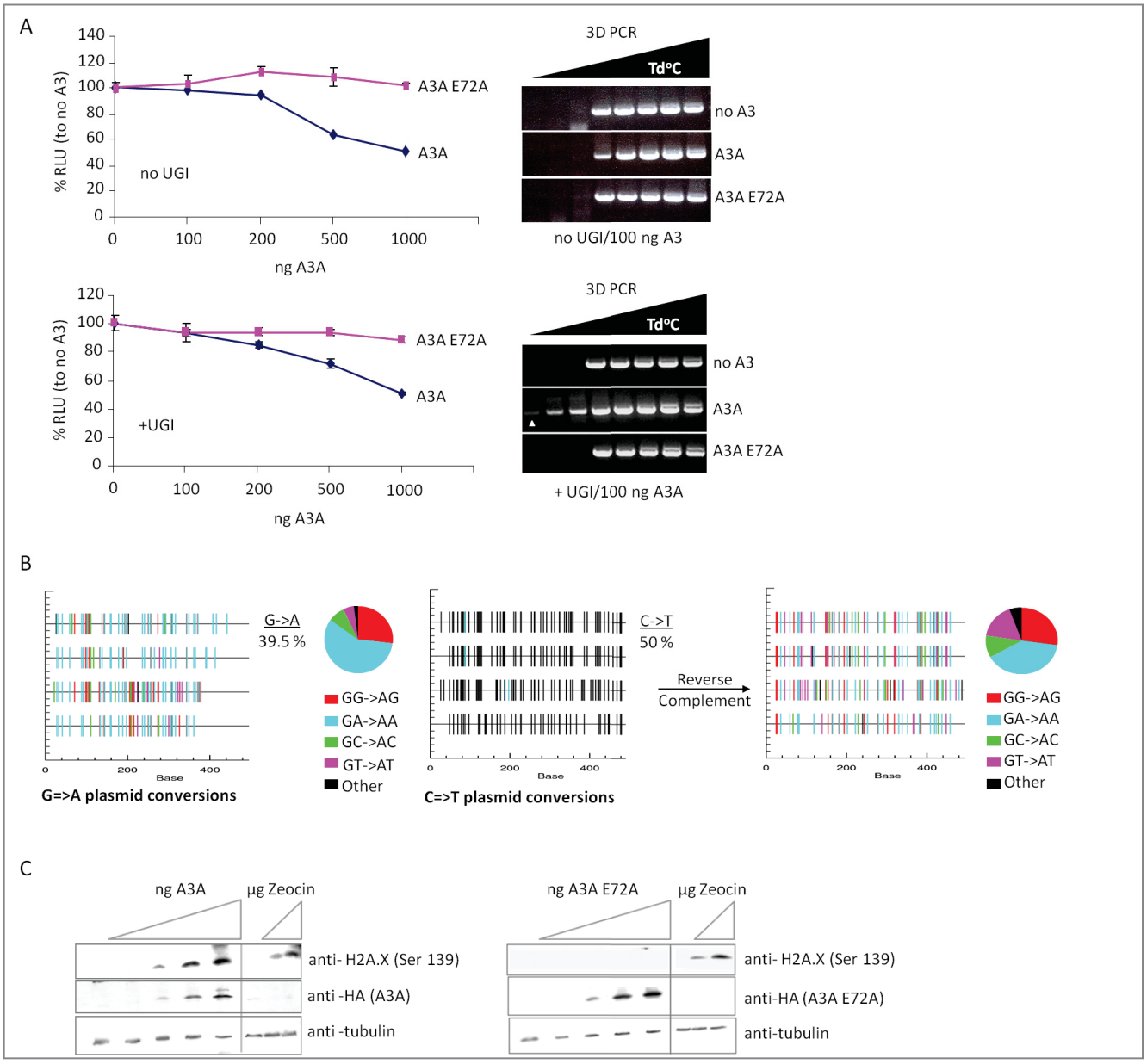


Figure 2 A3A inhibits luciferase plasmid expression and induces DNA breaks. (A) Increasing amounts of A3A or A3A E72A expressing plasmids were co-transfected without or with an Uracil Glycosylase Inhibitor (UGI) expression plasmid. Two days post-transfection, luciferase activity was measured in relative light units (RLU). The transfection with no A3 (pcDNA3.1) is set at 100% activity. Values are means plus standard deviations (error bars) for three independent experiments. Total DNA was isolated from the transfected cells and a portion of the luciferase gene was amplified using a gradient of PCR denaturation temperatures (Td°C). Comparison of the 3DPCR products demonstrates that UGI facilitates detection of PCR products at lower denaturation temperatures for A3A. (B) Sequencing analysis of 714-bp region of the luciferase encoding plasmid DNA recovered from A3A and UGI expressing HEK293T cells (cloned fragment indicated by a triangle). Eight different PCR products were recovered and sequenced. A3A mediates editing of both plasmid strands. Both G-to-A and C-to-T substitutions were identified in the plasmid. The conversions (vertical lines) are shown for each clone of a PCR product (horizontal line). Mutations are indicated by color: GA-to-AA (blue), GG-to-AG (red), GT-to-AT (pink), GC-to-AC (green), and other (black). The pie charts represent the relative dinucleotide preferences detected for A3A. (C) HEK293T cells were transfected with increasing amounts of A3A and A3A E72A expression plasmids (0 - 1000 ng). Two days post-transfection, cells were lysed and extracts were analyzed for A3A expression and H2A.X Ser 139 phosphorylation by immunoblot analysis using anti-HA and anti-H2A.X (Ser 139) antibodies. Tubulin expression served as loading control. The effect of increasing concentrations of Zeocin (0 - 1000 μg/ml) on double-strand breaks, served as a positive control for the H2A.X Ser 139 phosphorylation.

Mo-MLV but not AKV is restricted by A3A. To understand whether A3A can restrict MLV, we repeated the analysis using packaging constructs derived from Mo-MLV and two variants of the related AKV MLV (N-tropic or B-tropic). The resulting vector particles differed only in the Gag-Pol protein. The amount of A3A plasmid was limited to 100 ng per transfection for all experiments to reduce the likelihood of mutations in plasmids and of DNA double-strand breaks. Luciferase activity measured in the transfected HEK293T producer cells confirmed this assumption by showing equal luciferase activity with pcDNA3.1 (no A3), A3A or A3A E72A expression plasmid (Figure 3A, upper panel). Normalized viral supernatants were subsequently used to infect CrFK cells, and luciferase activity was measured in CrFK lysates (Figure 3A, lower panel). Using these experimental conditions, the infectivity of the Mo-MLV vector was significantly inhibited and transduced cells yielded only 60% luciferase activity compared to cells that lacked the A3A expression. MLV vectors based on the AKV MLV were resistant to A3A, and the A3A E72A mutant did not inhibit any vector (Figure 3A, lower panel). Immunoblot analysis of cell lysates of vector producer cells further demonstrated that A3A did not diminish expression of the viral vector proteins (Figure 3B). Western blot analysis of VLPs detected A3A packaged into Mo-MLV particles and, at a much lower amount, into AKV particles. This finding is consistent with the particle infectivity data (Figure 3A, lower panel). While Mo-MLV encapsidated A3A, the E72A mutant was barely detectable in the viral particles. Similarly, A3A E72A was not found in the AKV particles. Why the Mo-MLV particles failed to encapsidate A3A E72A is not clear, but it is independent of its protein expression.

To understand the effect of A3A on Mo-MLV infectivity, we used 3DPCR to characterize the integrity of viral cDNA produced in the presence of A3A during transduction of target cells (Figure 3C). The cDNA samples of Mo-MLV particles generated in the absence of A3A or A3A E72A did not result PCR products amplified at lower denaturing temperatures (Figure 3C). In contrast, Mo-MLV particles assembled in the presence of A3A yielded PCR amplification products at the lower denaturing temperatures indicative of A3-mutagenized sequences. PCR products from the lowest denaturing temperature each were cloned and sequenced. Sequencing confirmed G-to-A hypermutations caused by A3A (G-to-A changes 16%), and that none were elicited by the corresponding A3A E72A in which the deaminase

active site is mutated (G-to-A changes 0.2%) (Figure 3D). Virions made in the absence of any A3A plasmids displayed 0.14% G-to-A changes in their cDNA. The dinucleotide substitutions occurred in an A3A-specific pattern of GA > GG > GT > GC (Figure 3D).

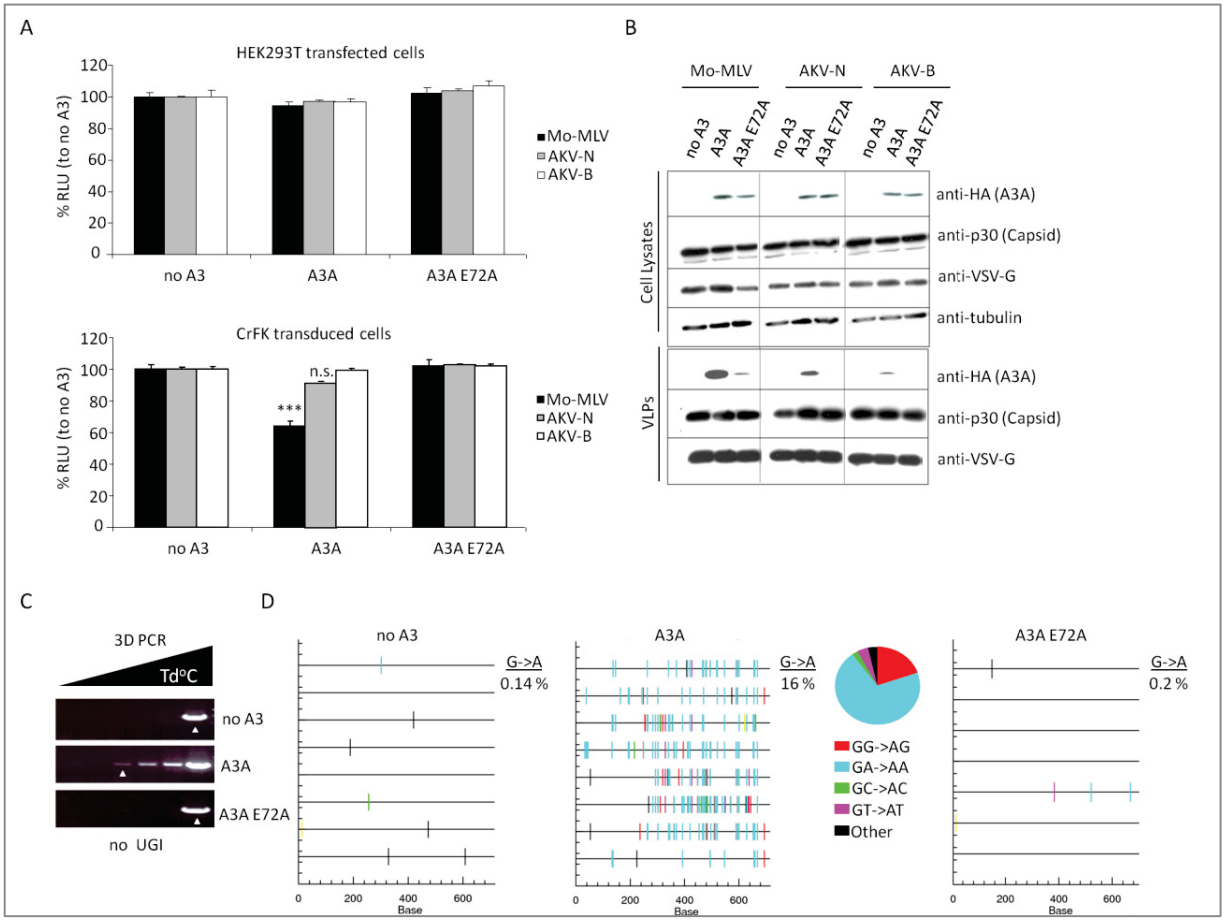


Figure 3 A3A restricts Mo-MLV, but not AKV. (A) 100 ng expression plasmid coding for A3A or A3A E72A mutant, were co-transfected into HEK293T cells together with plasmids for luciferase reporter vectors based on the Mo-MLV or the AKV-derived N- and B-tropic MLV. The viral supernatants were used to infect CrFK cells. Luciferase relative light units (RLU) were measured from the transfected HEK293T cells, and from vector particle-transduced CrFK cells. Luciferase activity of cells transfected with the empty pcDNA3.1 plasmid was set as 100%. Values are means ± standard deviations (error bars) for three independent experiments ($P < 0.001$ [***], relative to no A3 sample). (B) A3A and A3A E72A expression plasmids were co-transfected with the MLV helper plasmids for Mo-MLV, AKV N or AKV B in HEK293T cells. Supernatants were collected and the cells were lysed two days after transfection. The viral supernatants were filtered and concentrated through a 20% sucrose cushion. Viral and cell lysates were analyzed by immunoblotting and probed for A3A (anti-HA), VSV-G, p30 (MLV Capsid), and tubulin. (C) A 714-bp region of the luciferase gene of

the MLV reporter vector was PCR amplified from DNA obtained from Mo-MLV vector transduced cells. PCR (3DPCR) was performed using a range of denaturing temperatures (84°C to 88°C) and PCR products were analyzed on agarose gels. (D) PCR products amplified at the lowest denaturing temperature were cloned (cloned fragments indicated by triangles), and independent nucleotide sequences were determined. The conversions (vertical lines) are shown for each clone (horizontal line). Mutations are color-coded: GA-to-AA (blue) mutations, GG-to-AG (red) mutations, GT-to-AT (pink), GC-to-AC (green), and others (black). The pie chart represents the relative dinucleotide preferences detected for A3A.

A3A in target cells mediates a post-entry restriction for Mo-MLV. In the next experiment, we wanted to test whether ectopically expressed A3A in target cells can inhibit MLV infection. Mo-MLV luciferase vectors were used to transduce HEK293T cells that were transfected with pcDNA3.1 (no A3), A3A, and A3A E72A expression plasmids, with or without the UGI expression plasmid. A3A produced in target cells reduced the vector-transferred luciferase counts to 60-70% of the control (Figure 4A). Expression of UGI did not modulate the inhibitory effect of A3A against the incoming viral vector (Figure 4A). 3DPCR of transduced cells was combined with a nested PCR to detect A3A-induced hypermutations (Figure 4B). Cloned and sequenced nested PCR products with the lowest denaturing temperatures of the A3A-expressing cells confirmed a high frequency of A3A-typical G-to-A mutations (Figure 4C).

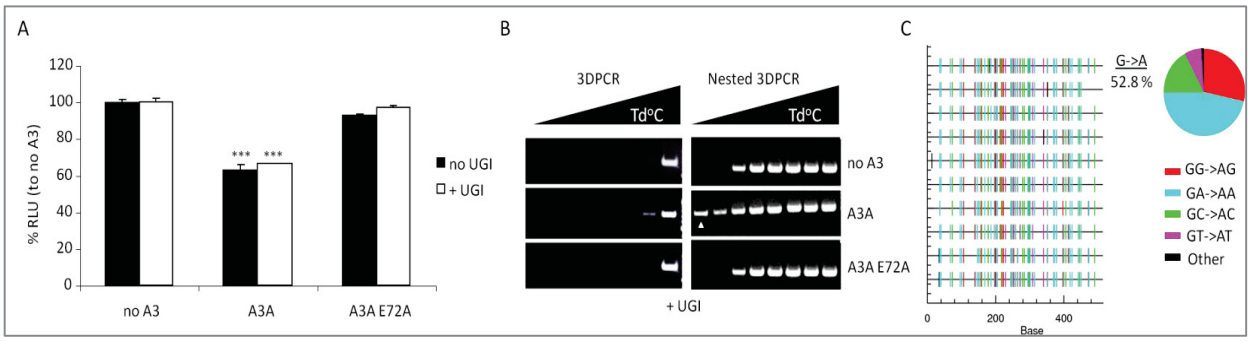


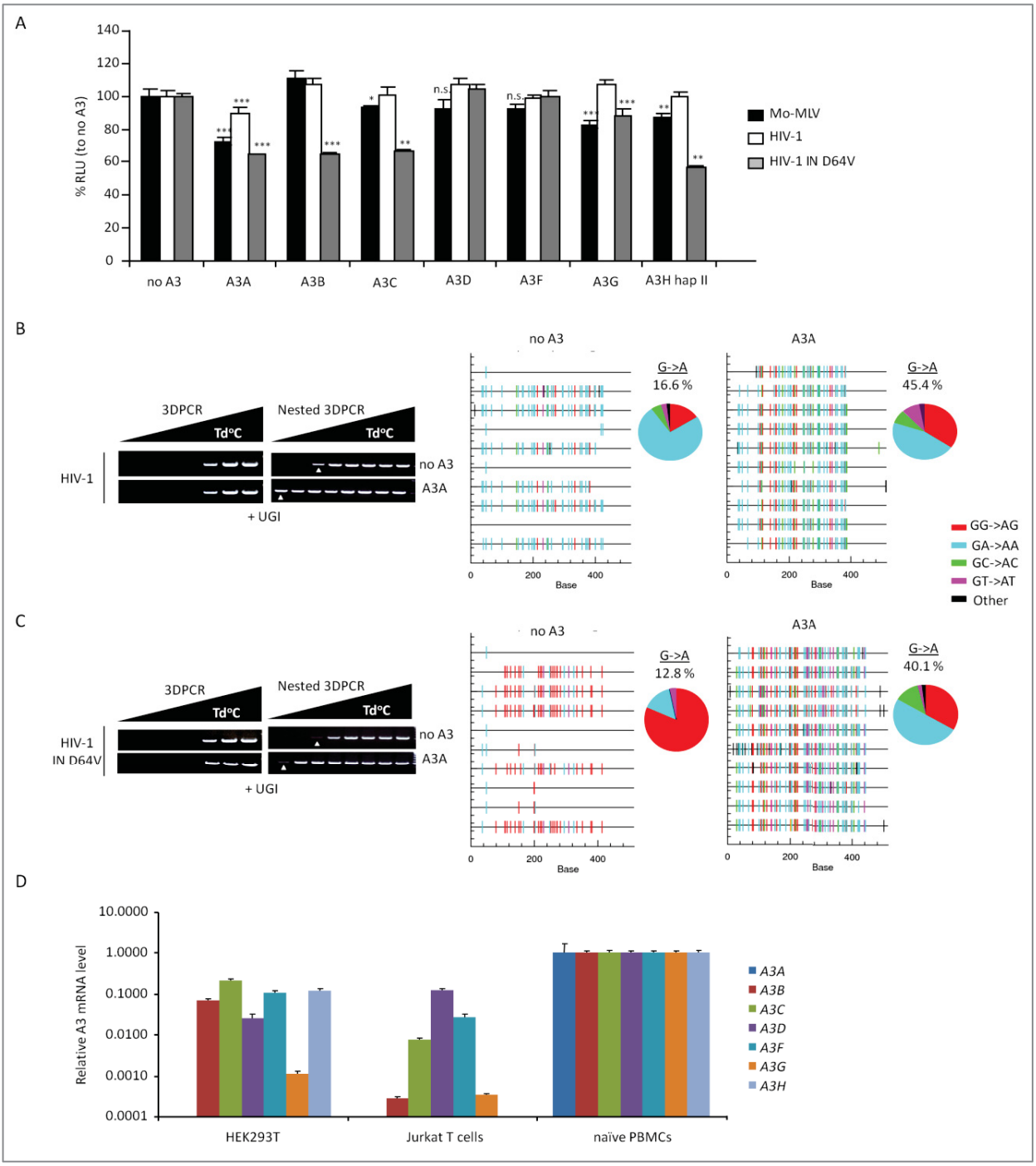
Figure 4 A3A expressed in the target cell inhibits Mo-MLV. (A) HEK293T cells were transfected with expression plasmids for A3A, A3A E72A or with pcDNA3.1 (no A3) without or with an UGI expression plasmid. Two days post-transfection, cells were transduced with Mo-MLV luciferase reporter vectors. Luciferase data of three independent experiments are shown. The control (no A3, pcDNA3.1) is set at 100% infectivity. Asterisks indicate statistically significant differences after a Student t test ($p<0,001$ [***]), relative to no A3 sample. (B) DNA was isolated from Mo-MLV vector transduced cells that expressed UGI. A region of the viral luciferase gene of the Mo-MLV reporter vector was amplified with 3DPCR (714 bps) and nested 3DPCR (512 bps) (81°C to 88°C) and PCR products were analyzed on agarose gels. (C) PCR products amplified at the lowest denaturing temperature of the nested 3DPCR were cloned (cloned fragment indicated by a triangle), and individual clones were sequenced. The conversions (vertical lines) are shown for each clone (horizontal line). The mutations are indicated by color as follows: GA-to-AA (blue), GG-to-AG (red), GT-to-AT (pink), GC-to-AC (green), and others (black). The pie chart represents the relative dinucleotide preferences detected for A3A.

A3A, A3B, A3C, A3G and A3H in target cells form a post-entry restriction for non-integrating HIV-1. The results in Figure 4 indicate that target cell A3 can inhibit retroviruses post-entry. Next, the other human A3 proteins were tested for their ability to establish a post-entry restriction of Mo-MLV in HEK293T cells (Figure 5A). We found that A3C, A3G, and A3H haplotype (hap) II in target cells can also inhibit MLV transduction, like A3A. A3C, A3G and A3H inhibited the MLV-mediated luciferase expression to ~ 80 – 90% of the control (cells transfected with empty expression plasmid) in comparison to the 70% achieved by A3A. Mo-MLV-mediated luciferase gene transduction was not impaired in cells expressing A3B, A3D or A3F.

Next, we asked whether HIV-1-based vectors also exhibit a post-entry inhibition in A3-expressing cells. In contrast to the results obtained with Mo-MLV, target cell A3A induced a less efficient post-entry restriction to HIV vectors to ~90% of the control (Figure 5A). Cellular infection by gammaretroviruses, but not by lentiviruses, depends on the break-down of the nuclear membrane. Since MLV cDNA may therefore stay longer in an unintegrated state than HIV cDNA and thus be more vulnerable for target cell restriction, we hypothesized that integration-deficient HIV-1 vectors might be more susceptible to the A3 activity in target cells than integration-competent vectors. To address this question, we analyzed the sensitivity of HIV-1 vectors with the integrase mutant D64V (HIV-1 IN D64V) to all human A3s, ectopically expressed in HEK293T target cells. Viruses with the well-characterized integrase mutation D64V show a specific block during the integration reaction [84,85]. Quite different from wild type HIV vectors, HIV-1 IN D64V vectors were not only susceptible to a post-entry restriction by A3A, but also were inhibited by A3B, A3C, A3G, and A3H hap II (Figure 5A). A3A, A3B, A3C, and A3H reduced the HIV-mediated gene expression to ~ 60 to 70% of the control, while A3G showed lower activity. In these experiments, the presence or absence of UGI had no impact on the luciferase activity (data not shown). Most of the A3-plasmid-transfected cells showed full permissiveness to at least one of the three viral vectors (HIV-1, HIV-1 IN D64V, Mo-MLV), supporting the existence of specific antiviral activities among the tested A3 proteins (Figure 5A).

In addition, we analyzed whether the observed A3A-dependent restriction of HIV-1 and HIV-1 IN D64V resulted in deamination of the viral genomes. Briefly, HEK293T cells were co-transfected with A3A and UGI expression plasmids, and the cells were transduced with luciferase vectors based on HIV-1 or HIV-1 IN D64V at day one post-transfection. Total DNA was isolated from the transduced cells at 18 h post transduction and a region of the luciferase reporter vector was amplified with nested 3DPCR (Figure 5B and 5C, left panels). Amplified products from the lowest denaturation temperatures were cloned and analyzed. The sequences revealed typically A3A-induced G-to-A hypermutations of the HIV-1 (Figure 5B, right panel) and HIV-1 IN D64V (Figure 5C, right panel) viral genomes. In the presence of A3A, we detected ~40 – 45% G-to-A changes in the amplified sequences of both vector systems. Unexpectedly, we also found G-to-A mutations in the control reactions in viral cDNAs isolated of HEK293T that did not ectopically express A3A (Figure 5B and 5C, middle panels). These viral cDNAs showed a lower number of G-to-A changes of ~ 13 – 17%. The dinucleotide context of the mutations analyzed was GA > GG for for both HIV vectors (wild type and IN D64V) testing A3A and HIV-1 wild type vectors omitting A3A. In the control reaction applying the HIV IN D64V vector without A3A overexpression in the target cells, the detected mutated dinucleotide was mostly GG resulting in AG motifs. These findings suggest active A3 proteins present in untransfected HEK293T cells. Using quantitative PCR with A3 isoform specific primers, we found that HEK293T express low amounts of mRNAs for several A3s (Figure 5D). Specifically, A3B, A3C, A3D, A3F, and A3H were expressed at levels of 7%, 22%, 3%, 11%, and 12%, respectively, relative to naïve human PBMCs that were used as a reference in this experiment. A3A mRNA was not detectable and A3G mRNA was found only in negligible amounts of 0.1% relative to A3G mRNA in PBMCs.

To sum up, the above data provide evidence that Mo-MLV and non-integrating HIV-1 vectors are sensitive to A3A and several other target-cell-expressed A3s, while, whereas, wild type HIV-1 vectors show a moderate post-entry restriction only in A3A-expressing cells.


A
% RLU (to no A3)
Mo-MLV
HIV-1
HIV-1 IN D64V
no A3
A3A
A3B
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A3H hap II
B
3DPCR
Nested 3DPCR
Td°C
HIV-1
no A3
A3A
+ UGI
G->A
16.6 %
45.4 %
Base
GG->AG
GA->AA
GC->AC
GT->AT
Other
C
HIV-1
IN D64V
12.8 %
40.1 %
D
Relative A3 mRNA level
HEK293T
Jurkat T cells
naïve PBMCs
A3A
A3B
A3C
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A3F
A3G
A3H

Figure 5 Target cell expressed A3A, A3B, A3C, and A3H hap II restrict non-integrating HIV-1. (A) The indicated A3 expression plasmids (A3A, -B, -C, -D, -F,-G, and H hap II) were transfected into HEK293T cells. At the second day of post-transfection, HEK293T were transduced with luciferase reporter vectors based on Mo-MLV, HIV-1, or HIV-1 IN D64V. The graph presents luciferase activity relative light units (RLU) at day 5 post-transduction from three independent experiments. Luciferase activity in pcDNA3.1 transfected control cells lacking an A3 expression plasmid (no A3) was set at 100% infectivity. Asterisks indicate statistically significant differences towards cells lacking an A3 expression plasmid by a Student t test ($p<0.05$ [*], $p<0.01$ [**], and $p<0.001$ [***]). Genomic DNA was isolated from HIV-1 vector (B) or HIV-1 IN D64V vector (C) transduced cells expressing no A3 and UGI or A3A and UGI and a region of the viral luciferase gene (512 bps) was amplified with nested 3DPCR. PCR products amplified at the lowest denaturing temperature of the nested 3DPCR were cloned and sequenced (cloned fragments indicated by triangles). The conversions (vertical lines) are shown for each clone (horizontal line). The mutations are indicated by color as follows: GA-to-AA (blue), GG-to-AG (red), GT-to-AT (pink), GC-to-AC (green), and other (black). The pie charts represent the relative dinucleotide preferences of the detected G-to-A changes. (D) RNA levels of A3A, A3B, A3C, A3D, A3F, A3G, and A3H were measured by qRT PCR in HEK293T, Jurkat T cells and naïve PBMCs. RNA levels were each normalized to TBP and standardized to the respective RNA level in PBMCs that was set as 1.

DISCUSSION

This study was designed to expand our knowledge of the anti-retroviral restriction of A3A. We recently reported that producer cell A3A could restrict HTLV-1, but not HIV-1 [41]. Here we show that HIV-1 vectors are sensitive to A3A expressed in target cells. Mo-MLV, in contrast, can be inhibited by target cell as well as by producer cell A3A. Mo-MLV was also sensitive to target cell A3C, A3G, and A3H hap II, while HIV-1 vectors were not inhibited by any target cell A3 other than A3A. By testing non-integrating HIV-1 vectors, we found that A3A, A3B, A3C, A3G, and A3H hap II can inhibit the incoming virus under these circumstances, indicating that HIV-1 has a previously unknown resistance to target cell A3s.

A3A appears to have broader and stronger activities than the other human A3 proteins [41,51,59-64]. It's extremely high interferon inducibility in myeloid cells [59,63,66,69-73] makes it likely that A3A plays an important role in the mammalian innate immunity against pathogens. As yet, however, gammaretroviruses and HIV-1 have not been clearly identified as targets of A3A [6,33-37,39,41,42,64,72]. Experimental research with A3A is challenging, because A3A may induce double strand breaks in the chromosomal DNA [61] and repress the expression of co-transfected plasmids by deaminating cytidines [59,63]. The biological consequences of A3A-induced DNA breaks for production and use of viral vectors could not be addressed in this study. However, we found that HEK293T may tolerate a low amount of DNA breaks for the short time of the assays we performed. Our data confirm the seminal findings by Stenglein et al. [63] and we show that increasing amounts of A3A inhibit plasmid expression in target cells by hypermutating both plasmid strands. As suggested by these authors [63], the use of UGI to inhibit the cellular UNG2-driven DNA repair pathway was instrumental in demonstrating deamination of plasmid DNA by A3A. This finding supports the conclusion that A3A can sense and edit plasmid DNA and indicates that UNG2 mediates clearance of the severe mutated episomal DNA.

The ability of A3A to inhibit plasmid expression needs to be considered in experiments working with A3A, in order to reduce A3A-mediated side effects. For this reason, we used only 100 ng of A3A expression plasmid to transfect the producer cells to generate MLV

vector particles. The experiments conducted under this condition confirmed and extend our previous observation that Mo-MLV can be inhibited by producer cell A3A [42] and that A3A induces G-to-A mutations in the viral cDNA. Our data clearly show that A3A is packaged into Mo-MLV particles but is poorly encapsidated into B- or N-tropic AKV particles. In agreement with our findings, Aguiar et al. described that B-tropic MLV is resistant to A3A expressed in producer cells [36]. In contrast to our results, Doehle et al. found no inhibition of Mo-MLV by A3A [37]. Mo-MLV and AKV Gag have differences beyond residue 110 that mediates the N-, B-, or NB-tropism of the Capsid, and only 87% of the amino acids constituting the Gag proteins are identical. Hence, the determinant of the MLV resistance to A3A can likely not be identified without extensive mutagenesis and testing of chimeric virions. In addition to its efficiency in the viral producer cells, A3A also showed anti-Mo-MLV activity when expressed in the target cells. The ectopic expression of A3C, A3G, and A3H hap II likewise caused a post-entry restriction of Mo-MLV. Pertinently, in murine cells, an A3-mediated post-entry restriction infection was recently identified, in which Mo-MLV counteracts the antiviral activity of target-cell-expressed murine A3 by its glycosylated Gag [86].

These observations motivated us to further study the role of A3 expressed in target cells. HIV-1 wild type vectors showed resistance to all target-cell-expressed human A3s except A3A in transfected HEK293T cells. We postulate that when expressed in target cells, among A3s, A3A has some unidentified superior activity against HIV-1. The exceptional function of A3A is supported by four reports describing that endogenously expressed A3A in myeloid cells forms a post-entry restriction for HIV-1 [64,67,68,72]. Moreover, the HIV-1-related Simian immunodeficiency virus accessory protein Vpx can counteract target cell A3A by triggering its degradation [64,65], and together with Vpx induced degradation of SAMHD1 [74-77]; this correlates with infection of monocytes and macrophages by SIV.

We speculate that integration of the viral cDNA or the timing of integration protects HIV-1 from the A3s present in the target cell. Integration, the insertion of proviral DNA into the host chromosome, is an intermediate step in the HIV or MLV life cycle. The two viruses might not only differ in the capacity to form proviruses in resting cells, but also in other subtle kinetic aspects related to integration. If integration does not occur after HIV-1 cDNA

enters the nucleus, it circularizes upon itself and forms a 2-LTR circle. Recent measurements of HIV-1 infection kinetics in a T cell line demonstrated that 2-LTR circles begin accumulating as early as 7 h post-infection, with integration beginning 1.5 h later; all viral transcripts emerged by 15 h and the release of viral particles started 18 h after infection [87]. To address the role of integration, we used an HIV-1 gag-pol construct that encodes the catalytically inactivated D64V integrase. D64V is a well-characterized specific mutation that prevents integration and does not affect reverse transcription or other viral functions [84,85,88]. In this case, HIV-1 can form a pre-integration complex, but cannot integrate its cDNA, which remains episomal and accessible to DNA sensors and antiviral proteins. Indeed, HIV-1 IN D64V vectors showed an increased sensitivity to target-cell-expressed A3A compared to wild type HIV vectors. The HIV-1 IN D64V vectors also lost the resistance to target-cell-expressed A3B, A3C, A3G, and A3H hap II and were inhibited by up to 40%. For unknown reasons, neither A3D nor A3F expressed in target cells showed antiviral activity.

MLV and HIV-1 vectors are profoundly sensitive to producer cell-expressed A3s, but are, in comparison, rather resistant to target cell-expressed A3s. Even the more sensitive D64V integrase mutant of HIV-1 retained significant resistance to target cell A3s. The reason ectopically expressed A3s in target cells show a rather weak inhibition of incoming viruses is unclear. The editing of viral DNA by target cell A3s is likely less efficient than cytidine deaminations by virion-encapsidated A3s. In the viral core/reverse transcription-/pre-integration-complex, viral cDNA intermediates are temporarily single-stranded and encapsidated A3s are directly associated with the viral polynucleotides. Of note, although, the diminished transduction of target cells expressing A3A correlated with detectable A3A-induced cytidine deaminations, we cannot rule out additionally non-enzymatic activities of A3A and other A3s that inhibit incoming virions.

3DPCR is a sensitive tool to identify even extremely rare A3-induced mutations; unfortunately, it will not reveal the proportion of molecules that contains these mutations. Using a nested 3DPCR, we also detected A3-induced mutations in viral cDNAs of HIV vectors that were generated by transfection and transduction of HEK293T cells without overexpression of A3s. These results challenge the generally assumed lack of significant

endogenous expression of most A3 proteins in this cell line. In full agreement, by comparing to naïve PBMCs, we detected in the HEK293T cells low expression of several A3s (A3B, A3C, A3D, A3F, A3H and extremely low amounts of A3G). Two previous studies also reported the detection of A3 mRNAs in HEK293T cells; Gärtner et al. found A3F and A3G by a quantitative RT PCR [89] and Bourara et al. used a semi-quantitative RT PCR to identify transcripts for A3B and A3C [90]. It is not established whether these mRNA levels correlate with protein levels, since the available anti-A3 antibodies fail to identify endogenous A3 proteins in HEK293T cells (data not shown). In agreement with expression level of endogenous A3s in the HEK293T, the detected G-to-A mutations in the viral cDNAs were lower compared to experiments overexpressing A3A. Interestingly, but mechanistically unclear, HIV-1 IN D64V vectors showed preferentially G-to-A mutations in the GG dinucleotide recognition motif, while wild type HIV vectors were preferentially edited in the GA dinucleotide context. The dinucleotide GG is the favored recognition sequence for A3G, while most other human A3s show a higher preference for the GA dinucleotide [41]. Our findings cannot explain whether the endogenous A3s of HEK293T cells interacted with the viral vector in the producer or in the target cell. But in experiments using HEK293T cells for HIV-1 infection, Bourara et al. [90] identified endogenous A3C of the target cells as causing G-to-A mutations in the HIV cDNA. These data suggest that endogenous A3C of the HEK293T target cells partakes in the editing we found here.

In addition to the antiviral role of A3C in target cells, A3F and A3G are also reported to restrict incoming HIV-1 [90-92]. The resistance of immature dendritic cells and the relative resistance of $CD4^+$ Th1 lymphocytes to HIV-1 infection compared with Th2 lymphocytes have both been ascribed to target cell A3G [91,92]. Together these and our present data support that the target cell A3s are relevant for the biology of HIV-1.

CONCLUSIONS

Mo-MLV, but not its relative AKV MLV, is sensitive to co-expressed A3A. Target cell A3 proteins can further interfere with Mo-MLV replication. Whether differences between replication cycles of gammaretroviruses and lentiviruses can explain why HIV-1 is only moderately inhibited by target cell A3A and no other human A3s is unknown. The sensitivity of HIV-1 to an A3-mediated post-entry restriction is significantly influenced by its integration competence, and A3 proteins in the target cell might cause further genetic variability in HIV-1. The exact mechanism involved presents an open question for future research, but in a first model we speculate that the timing of integration might influence the susceptibility of HIV-1 to A3 proteins. Lentiviral gene therapy using non-integrating vectors [88] might thus face a previously unrecognized problem by cytidine deamination of the vector genome in target cells expressing A3 proteins.

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Chapter 4
Final Discussion

Chapter 2

- **Summary**

HTLV-1 infects $CD4^+$ and $CD8^+$ T lymphocytes, and $CD14^+$ macrophages that express for various A3s. But, the complete spectrum of the A3 inhibition against HTLV-1 is unknown. This study aimed to associate the HTLV-1 tropism with the viral ability to overcome the A3 restriction pattern. The results demonstrated that HTLV-1 can be extensively inhibited by A3A, A3B, and A3H haplotype 2, while A3C, A3D, A3F, and A3G presented a low inhibition capacity. On the other hand, a direct comparison of HTLV-1 and HIV-1 showed that both viruses share a common susceptibility to A3B and A3H haplotype 2, while HIV-1 could overcome the A3A inhibition. A3A and A3B mediated HTLV-1 inhibition with a deaminase-dependent manner by inducing fatal G-to-A hypermutations on the viral genome, whereas A3H haplotype 2 was found to act with a deaminase-independent mechanism of restriction. Indeed, catalytic inactivation of the A3H haplotype 2 did not modulate the molecular restriction capacity. Finally, recovery of A3-deaminated sequences from T cell lines, derived from patients with HTLV-1 adult T-cell leukemia/lymphoma or HTLV-1 associated Myelopathy/Tropical Spastic Paraparesis, demonstrated a high correlation with the A3A, A3B, and the less active A3G dinucleotide-specific G-to-A substitutions.

- **Discussion**

HTLV-1 nucleocapsid C-terminus region modulates A3 restriction

A3s need to establish a proper interaction with the viral Gag polyprotein and the viral RNA so as to be effectively incorporated into the produced viral particles. On the other side, retroviruses antagonize the A3 sensing and encapsidation by encoding for accessory proteins, such as the HIV-1 Vif, and/or by hiding viral motifs from the A3 recognition. For HTLV-1, a C-terminus 20 amino acid region of the nucleocapsid (NC) (referred here as *Derse-domain*) was initially reported by Derse et al. as a novel *cis*-acting mechanism that modulates A3G incorporation into the HTLV-1 viral particles (1). NC is highly conserved among retroviruses, but HTLV-1 is characterized by a C-terminus extension that is absent from all the other retroviral NC proteins. Deletion of this area results in increasing levels of A3G incorporation into virions and subsequent increase of HTLV-1 susceptibility to A3G. Critical residues of the NC C-terminal region that regulate the A3G resistance are a small cluster of acidic amino acids and an adjacent leucine-rich motif (LLLDL).

Here, a global analysis of the human A3 encapsidation profile into the released HTLV-1 virions revealed that the active A3A, A3B, and A3H haplotype 2, but also the less active A3C, A3G, and A3F, are packed sufficiently into the released HTLV-1 viral particles. To eliminate an unspecific A3 association with exosomes and/or microvesicles the viral pellets were digested with the protease subtilisin A, which removes exosomal and microvesicle formations but leaves the enveloped virions intact. It is likely that the non-active A3s may be excluded from the HTLV-1 viral core formation. After release, retroviruses undergo an obligatory maturation for the construction of infectious viral particles. In mature HIV-1 ΔVif viral particles, A3G localizes in the viral core in association with the NC protein (2). Nevertheless, the specific A3 localization profile in mature HTLV-1 virions needs to be further explored with electron microscopy and core isolation studies. Possibly, HTLV-1 NC and viral RNA are closely related during maturation by abolishing A3C, A3G, and A3F incorporation into the viral core.

Next, HTLV-1 uses ribosomal frameshifting to regulate the relative expression of the Gag proteins and of the viral enzymes (3-4). In HTLV-1 two ribosomal frameshifts occur: one where the *gag* and *pro* gene overlap, and a second one within the *pro/pol* overlap. The HTLV-1 *gag/pro* frameshift region is located only few amino acids downstream from the NC C-terminus 20 amino acid region. In the released viruses, protease is directly involved to the formation of the viral core by processing the Gag, Gag-Pro, and Gag-Pro-Pol polyproteins. Deletion of the HTLV-1 NC C-terminal 20-aa region may affect protease production and cleavage of the encapsidated A3s. Interestingly, the retroviral protease from Moloney murine leukemia virus was found to be an important determinant of viral resistance against murine A3 (5). To test whether the HTLV-1 protease contributes to the viral resistance against the human A3s, the viral protease levels could be quantified by applying immunoblot analysis from the producer cells and viral particle analysis with wt and delta 'Derse-domain' viral constructs. In addition, I propose the generation of a frameshifting assay, where in the HTLV-1 *gag-pro* sequence the protease gene could be replaced by a luciferase reporter gene. Luciferase activity could be quantified in transient transfections of constructs with wt NC and delta 'Derse-domain' NC to identify whether the 'Derse-domain' deletion affects the protease expression.

Analysis of the A3H haplotype 2 mechanism of HTLV-1 restriction

A3s are cytidine deaminases and therefore known as DNA dC->dU-editing enzymes. However, a flurry of studies show that deamination activity is not always required for viral inhibition. For instance, A3A deaminase-deficient mutants are reported to effectively inhibit parvoviruses (6). A3G and A3F are also able to function as anti-viral factors and reduce HIV-1 replication in the absence of cytidine deamination (7-11). In addition, several other publications report that A3s are able to inhibit retrotransposition of long terminal repeat (LTR) and non-LTR retrotransposons by deaminase-independent mechanisms (12-15).

In this study, A3H haplotype 2 was found to inhibit HTLV-1 replication without the requirement of deaminase activity. In contrast, an intact deaminase domain for A3H haplotype 2 is dispensable for the HIV-1 restriction. Here, further analysis of the A3H haplotype 2-mediated HTLV-1 inhibition with real-time PCR experiments can reveal the quantity of reverse transcripts in target cells. The A3H haplotype 2 protein may act by abolishing the HTLV-1 reverse transcriptase activity. Consequently, the amount of the quantified cDNA transcripts should correlate to the degree of viral infectivity. An A3H-mediated suppression of the HTLV-1 cDNA accumulation in the target cells would be important evidence that A3s harbor on the viral genome and disrupt the reverse transcription step and/or formation of the pre-intregration complex. Thus, sequencing and southern blot analysis of the viral genome may show whether a subsequent defect on the integration ability is associated with aberrant DNA structures of the 3' and 5' LTRs or with the incapacity of the reverse transcriptase to complete synthesis of the viral plus DNA strand. Nevertheless, it remains a mystery whether the A3 deaminase-independent restriction of viruses is a supplementary mechanism to the dC->dU substitutions or applies to the variability of the viral substrates.

Impact of the individual's A3 genetic profile on disease progression

The individual's genetic profile in combination with the environmental conditions has an enormous impact on disease phenotype and progression. It is calculated that over 90% of the HTLV-1 infected individuals remain lifelong asymptomatic (16-17), up to 5% develop adult T-cell leukemia/lymphoma disease (ATLL), and about 2% HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). On the other hand, the incubation period from HIV-1 infection until the development of AIDS disease is estimated approximately 10 years for young adults (18). However, a small percentage (less than 1%) of HIV-1 infected individuals -known as elite controllers- can suppress the viral replication below the limit of detection in the absence of antiretroviral therapy (ART) (19-22).

A growing number of genomic studies describe that the *A3* gene variation may be associated with the host's response to the early viral post-entry events. Indeed, the seven members of the human A3 family of enzymes are characterized by a large number of polymorphisms, splice variations, and copy number variations. Xuan D. et al. reported that a common deletion located in the *A3A* and *A3B* genes is significantly associated with breast cancer among women of European ancestry (23). Besides, a 29.5-kb deletion on the *A3* locus that removes the *A3B* gene is fixed in Ocean populations (92.2%), is common in East Asians and Amerindians (36.9% and 57.7%), but presents a low frequency in Africans and Europeans (frequency of 0.9% and 6%) (24). In addition, the *A3H* gene locus contains several single nucleotide polymorphisms and up to seven haplotypes have been detected in humans with various anti-viral functions and subcellular localizations (25-27). For HIV-1, it is reported that A3H haplotype 2 confers a strong anti-viral activity, while most of the other A3H haplotypes appear to lack an inhibitory capacity (27). Interestingly, A3H haplotype 2 is mainly detected in individuals of African descent. Finally, A3G and A3F protein variants appear to differ sharply in their HIV-1 Vif sensitivity (28).

This study presents that HTLV-1 is susceptible to A3A, A3B, and A3H haplotype 2 restriction, while HIV-1 WT only to A3B and A3H haplotype 2. A3A is known to be expressed in myeloid derived cells and its expression is regulated by INF-a. A3B is widely expressed in B cells, and A3H in T cells. Though, further analysis of the contribution of the individual's A3 genetic variance on the HTLV-1 and HIV-1 restriction with relevance to the clinical phenotype is critical to gain new insights into promising treatment and prevention strategies.

Chapter 3

- **Summary**

A3A is highly expressed in myeloid-derived cells and up-regulated by INF-a. In macrophages, the endogenously expressed A3A inhibits the early phases of infection and spread of HIV-1. However, A3A can not undergo incorporation in HIV-1 viral particles and inhibit the viral replication in next round of infection. Here, I aimed to compare the A3A activity against HIV-1 with a simple gamma-retrovirus, the Mo-MLV. The results demonstrated that A3A was able to block Mo-MLV infectivity by achieving encapsidation in the released viruses. I could also confirm that A3A causes *in vitro* genomic DNA breaks and mediates repression of plamid DNA co-transfected with A3A expression plasmids. Next, I compared the ability of A3s expressed in the target cells to inhibit infection of HIV-1, HIV-1 integrase-deficient (D64V), and Mo-MLV viral vectors. The results demonstrated that the HIV-1 integrase deficient viruses were sensitive to A3A, A3B, A3C, A3G, and A3H haplotype 2 indicating that a rapid HIV-1 integration into the target cell genome may protect the virus from the A3 entry barrier.

- **Discussion**

Integration timing and cell tropism might protect viruses from the A3 clearance in the target cell

Integration of the reverse transcribed retroviral genome into the host cell chromosomal DNA is the hallmark for the establishment of a retroviral infection. However, HIV-1 is detectable either in the form of integrated proviruses or as unintegrated DNA (29). Indeed, in cell culture experiments the unintegrated proviral forms are more abundant than the integrated forms (30-31).

HIV-1 unintegrated DNA appears to be transcriptionally active during the early phases of infection and before the viral integration. Probably, HIV-1 needs to express for accessory gene products (Nef, Vpr, and Tat proteins) to initiate integration of non-dividing cell populations (32-34). Some other studies support also the idea that episomal HIV-1 DNA plays the role of a viral reservoir in resting T cells and macrophages (35-39).

The existing model for the A3-mediated antiviral activity proposes that A3s interact with the Gag polyprotein motifs and viral RNA to achieve incorporation into the assembled viruses. The structural morphology of the released viruses is unstable and the Gag polyprotein is processed to smaller proteins (matrix, capsid, and nucleocapsid) resulting in the core formation. A3s need to localize in the viral core in close contact with the viral genome and the nucleocapsid protein to edit the viral genome in the next round of infection. Interestingly, the data of this thesis demonstrate that when expressed in target cells, A3A, A3B, A3C, A3G, and A3H haplotype 2, but not A3D and A3F, could significantly restrict non-integrating HIV-1 viruses. HIV-1 presents a tropism for $CD4^+$ T lymphocytes and $CD14^+$ macrophages that express A3G, A3C, A3H, and A3A respectively. Thus, it is possible that the timing of integration (integration kinetics) may influence an A3-mediated clearance of the virus in the target cell.

Another virus which is reported to be vulnerable to genetic editing by the host cell A3 cytidine deaminases is HBV (Hepatitis B Virus). HBV infection of hepatocytes includes the steps of viral entry, uncoating, and transfer of the viral DNA genome to the nuclear compartment. In the host nucleus, a covalently closed circular DNA (cccDNA) is formed by covalent ligation of both viral DNA strands (40-41). In contrast to retroviridae, HBV integration into the host nuclear is not required for viral replication. In fact, a rare accidental HBV integration event disrupts the virus ORFs (Open Reading Frames) and prevents transcription of functional RNAs.

Quantification of A3 mRNAs levels in human primary hepatocytes shows low or even absent hepatic transcription of these enzymes (42). But, in chronic inflammatory responses to HBV and upon stimulation of primary hepatocytes with INF-a a remarkable mRNA up-regulation of A3B, A3G, and A3F is observed (43-44). HBV tropism for the human hepatocytes may indicate a viral strategy to escape from the A3 restriction. However, INF-a production in the early stages of infection could contribute to the nonlytic A3-mediated clearance of HBV.

Episomal viral DNA may be the Achilles heel of viruses to A3 restriction. Insights on the A3 promotor transcriptional regulation could contribute to the development of strategies to induce selective up-regulation of the A3 expression in the infected cells.

A3 therapeutic approaches

Retroviruses are highly adaptable to evolution. Their genomic plasticity determines their ability to adapt in new environments by overcoming the host restriction barriers. For example, the pandemic HIV-1 counteracts the A3 restriction by expressing for the Vif accessory protein. Yet, isolation of HIV-1 viral sequences harboring various Vif mutants challenges the development of effective anti-retroviral therapies (45-47).

Novel targets for the HIV-1 spread inhibition are the Vif-A3 (Vif-A3G, and Vif-A3F) and/or Vif-ElonginB/C-Cullin5 E3 ubiquitin ligase interactions (48-50). Antagonists that may disrupt the Vif-mediated A3 binding and subsequent proteasomal degradation are considered as an attractive therapeutic approach. Inhibition of the Vif-A3 binding would facilitate the A3 molecules to escape from the Vif restriction, undergo encapsidation into the synthesised viral particles in the producer cells, and hypermutate effectively the viral DNA at the second-round of infection. Interestingly, several studies of Vif-defective HIV-1 (HIV-1 ΔVif) viruses report that their replication is significantly reduced in nonpermissive cells (51-54). A structural determination of the A3-Vif interactions would indicate potential targets for the design of effective Vif inhibitors.

On the other side, integrase inhibitors are also regarded nowadays as a common therapeutic strategy to control HIV-1 disease progression. The observed sensitivity of the unintegrated HIV-1 proviruses to the A3 restriction (chapter 3) may be associated with the positive clinical impact of HIV-1 inhibitors on patient treatment. Further analysis of the A3 activity on episomal DNA clearance is critical to understand the fate of the unintegrated viral sequences in the infected cells.

Relevance of A3s on the virus evolution and host cell transformation

The A3 enzymes are cytidine deaminases that restrict a variety of retroviruses and retrotransposons, but may also cause pro-mutagenic uracil lesions and DNA breaks disrupting the cellular genomic integrity (55). A3B was recently associated with the breast and other cancers (23, 56-57), while other studies report that A3A, A3B, and A3D can impede the cell cycle during mitosis and cause chromosomal DNA damage (58).

The data of this thesis confirm that A3A can cause DNA breaks and deamination of episomal DNA in a dose-dependent manner. *In vivo*, A3A is known to be exclusively expressed in macrophages and keratinocytes, and its expression is up-regulated by INF-a. Possibly, *in vivo*, the A3A-depended genomic mutagenic effects are limited through a controlled A3A expression in the terminally differentiated myeloid cells and keratinocytes. However, the A3A implications for the skin physiology are unknown and the role of A3A on the microbe skin invasion need to be characterised.

In addition to the fatal A3 effects on the genomic integrity, many studies associate the A3 activity with the HIV-1 viral evolution. The A3-hypermutated viral sequences are eventually degraded by the uracil DNA glycosylase UNG2 triggered pathways (59), but HIV-1 can tolerate a low mutation rate and escape degradation. HIV-1 viruses that survive the A3 restriction may develop drug resistance to therapy and new strategies to escape from the immune control (60-63). On the other side, Ebrahimi et al. report that A3G and A3F have not left a significant footprint on HIV-1 evolution (63). Here, it should be highlighted that most of the established knowledge on the A3-mediated HIV-1 restriction is being developed using the HIV-1 pNL4-3 viral isolate (J. Virol. 1986) (64). Thus, the possible impact of A3s on HIV-1 evolution can be only analyzed with a global population study.

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Publications and submitted manuscripts

- APOBEC3A Impairs Plasmid Expression, Blocks Murine Leukemia Virus Replication, and Non-Integrating HIV-1. **Aikaterini Krikoni**, Daniela Marino, Wolfgang Goering, Gerald G. Schumann, Renate König, Wolfgang A. Schultz, Dieter Häussinger, and Carsten Münk**,** submitted for publication.
- An intronic G-run within HIV-1 intron 2 is critical for splicing regulation of vif-mRNA. Marek Widera, Steffen Erkelenz, Frank Hillebrand, **Aikaterini Krikoni**, Darius Widera, Wolfgang Kaisers, René Deenen, Michael Gombert, Rafael Dellen, Tanya Pfeiffer, Barbara Kaltschmidt, Carsten Münk, Valerie Bosch, Karl Köhrer, and Heiner Schaal, J Virol 87(5): 2707-2720.
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