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NOTE

Species-specific differences in the ability of feline lentiviral Vif to degrade feline APOBEC3 proteins

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ABSTRACT

How host–virus co-evolutionary relationships manifest is one of the most intriguing issues in virology. To address this topic, the mammal–lentivirus relationship can be considered as an interplay of cellular and viral proteins, particularly apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 (APOBEC3) and viral infectivity factor (Vif). APOBEC3s enzymatically restrict lentivirus replication, whereas Vif antagonizes the host anti-viral action mediated by APOBEC3. In this study, the focus was on the interplay between feline APOBEC3 proteins and two feline immunodeficiency viruses in cats and pumas. To our knowledge, this study provides the first evidence of non-primate lentiviral Vif being incapable of counteracting a natural host's anti-viral activity mediated via APOBEC3 protein.

Key words apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3, feline immunodeficiency virus, lentivirus, viral infectivity factor.

Shedding light on the co-evolutionary history of viruses and hosts is one of the most intriguing topics in the field of virology. However, because viruses have usually become highly diversified through their high evolutionary rates and cross-species swapping, accessing the co-evolutionary history of viruses and hosts has been challenging. Focusing on interactions between viral and cellular proteins is a potential strategy for investigating the evolutionary arms race between viruses and hosts, particularly lentiviruses and mammals (1–3). In particular, the interaction between Vif and APOBEC3 represents a microcosm of the co-evolutionary relationship (1).

HIV-1, which causes AIDS in humans, is the most studied lentivirus (4). HIV-1 replication is robustly restricted by an intrinsic anti-viral APOBEC3 family protein, APOBEC3G (5). APOBEC3 proteins, including APOBEC3G, are incorporated into nascent viral particles and enzymatically introduce G-to-A substitutions in the newly synthesized viral DNA, which results in the abrogation of viral replication (6, 7). To counteract APOBEC3-mediated anti-viral action, Vif, an HIV-1-encoding protein, induces degradation of APOBEC3 proteins via a ubiquitin/proteasome-dependent pathway (8, 9).

In contrast to the effects of HIV-1 infection in humans, it is known that several lineages of OWMs residing in Africa do not develop any AIDS-like disorders when naturally infected with SIVs, another lineage of lentiviruses (10, 11). Interestingly, the functional interaction between a various lineage of SIV Vif proteins and OWM APOBEC3G proteins has been elucidated (1, 12, 13), possibly providing a clue to understanding the history of co-evolution and

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List of Abbreviations: APOBEC3, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3; FIV, feline immunodeficiency virus; HIV-1, human immunodeficiency virus type 1; OWM, Old World monkey; PLV, puma lentivirus; PBMC, peripheral blood mononuclear cells; SIV, simian immunodeficiency virus; Vif, viral infectivity factor.

co-divergence of SIVs and monkeys in the Old World. Furthermore, it has been demonstrated that OWM APOBEC3G can be the barrier that restricts lentiviral cross-species infection (14, 15).

Another lentivirus, FIV, was first isolated in 1987 from domestic cats (*Felis catus*) with chronic AIDS-like disorders (16). Subsequent studies revealed that several lineages of felids are infected with FIV (17–19).

The puma (*Puma concolor*; also commonly known as the cougar, panther, or mountain lion) is a felid that resides in the New World (20). An FIV that has been designated FIVpco (also known as PLV), circulates among pumas in the wild (21). The prevalence of FIVpco in wild pumas differs between the geographical areas investigated and/or the epidemiological studies reported (17, 21–23). Interestingly, because of its lower genetic diversity (22), it has been assumed that FIVpco has not undergone any selective pressures from the host and that wild pumas are naturally infected (i.e., with little or no pathogenicity), as is true for SIV infection in OWMs in Africa (24). However, prominent G-to-A substitutions, the mutation signature mediated by APOBEC3, have reportedly been detected in proviral DNA isolated from wild pumas naturally infected with FIVpco (25). Therefore, it is assumed that endogenous puma APOBEC3 protein(s) are resistant to FIVpco Vif-mediated degradation, which would affect the replication kinetics of FIVpco in pumas.

To elucidate the mechanism of inter-species viral transmission, domestic cats have been experimentally infected with FIVpco (26–29). However, PLV1695, a prototypic infectious molecular clone of FIVpco (30), replicates poorly in domestic cats, usually being eradicated around 100 days post-infection without any treatment (30, 31). FIVpco expands in cultures of PBMCs from pumas *in vitro*, whereas it only replicates very little in *in vitro* cultures of domestic cat PBMCs (26). These observations suggest that a barrier inhibits cross-species transmission of FIVpco into domestic cats. Particularly noteworthy, G-to-A hypermutation in proviral DNA, which is the signature of APOBEC3-mediated mutation, has been observed in cultures of domestic cat PBMCs infected with FIVpco (30).

The fact that human APOBEC3G robustly limits HIV-1 replication in the absence of Vif both *in vitro* (8, 5) and *in vivo* (32–34), is convincing evidence that human APOBEC3G confers intrinsic immunity restricting HIV-1 replication. Similar to what occurs in HIV-1 infection in humans, certain APOBEC3 proteins from domestic cats exhibit strong anti-FIV activity, whereas this antiviral action is counteracted by the Vif protein of FIV in domestic cats (FIVfca) (35–37). The presence of the *vif* gene in FIV strongly suggests that there was co-evolutionary interplay between feline APOBEC3 and FIV Vif proteins. In fact, as described above, one study has suggested that feline APOBEC3 is the barrier that inhibits cross-species transmission of FIVpco (strain PLV1695) into domestic cats (30). However, the counteracting activity of FIVpco Vif against APOBEC3 proteins from both domestic cats and pumas has not been elucidated. To address these issues, we herein focused on the role of FIVpco Vif in antagonizing APOBEC3 proteins from domestic cats and pumas.

We set out to compare the amino acid sequences of APOBEC3s from domestic cats and pumas and those of Vifs from FIVfca and FIVpco. Human APOBEC3s and HIV-1 Vif, respectively, were used as the putative outgroups. It should be noted that domestic cats and pumas express five different APOBEC3 proteins; namely APOBEC3Z2a, APOBEC3Z2b, APOBEC3Z2c, APOBEC3Z3 and APOBEC3Z2Z3 (35–38), whereas humans encode seven APOBEC3 proteins (APOBEC3A, B, C, D, F, G and H) (7, 8). As shown in Figure 1, we found that puma APOBEC3Z2 (Fig. 1a) and APOBEC3Z3 (Fig. 1b) are similar to cat orthologs rather than human orthologs. On the other hand, although FIVpco Vif are more closely related to FIVfca Vif than to HIV-1 Vif (Fig. 1c), the values for identity and similarity of FIVpco Vif to FIVfca Vif are relatively low compared to those for puma APOBEC3s to cat APOBEC3s (Fig. 1a,b). These results indicate that lentiviral *vif* is relatively diversified in comparison with host APOBEC3 proteins. Moreover, we compared the FIV Vif sequences from various felids; namely FIVfca in cats, FIVpco in pumas, FIVple in lions (*Panthera leo*) and FIVoma in Pallas' cats (*Otocolobus manul*). As shown in Figure 1d, FIV *vif* is highly diversified but forms a cluster in the same lineage. This figure also shows that the sequence of PLV1695 *vif* is close to that of the *vif* of the other FIVpco from wild pumas (Fig. 1d), suggesting that the use of PLV1695 Vif in our subsequent experiments is biologically relevant.

Next, we used an *in vitro* cell culture system to evaluate whether FIVpco Vif can degrade feline APOBEC3 proteins. For this assay, we obtained the FLAG-tagged codon-optimized open reading frames of FIVfca Vif strain Petaluma (39), FIVfca strain C36 (40) and FIVpco strain PLV1695 (26) from GeneArt Gene Synthesis service (Life Technologies, Carlsbad, CA, USA) (the GenBank accession numbers are presented in the legend of Figure 1, and the codon-optimized sequences are available on request). The obtained DNA fragments were inserted the *Bam*HI-*Sal*I site of pDON-AI plasmid (Takara, Kyoto, Japan). The expression plasmids for HA-tagged APOBEC3Z3 and APOBEC3Z2Z3 from domestic cats (35) and pumas (37) were kindly provided by Dr. Carsten Münk (Heinrich

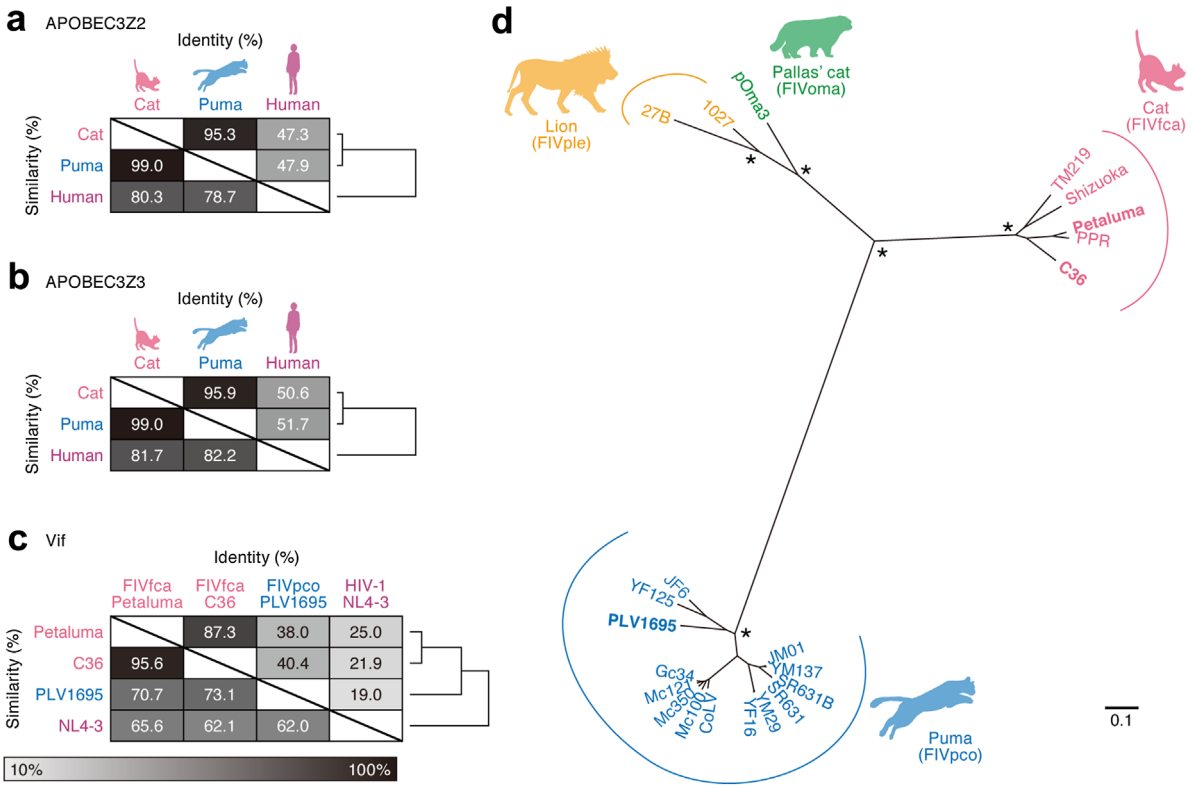

Fig. 1. Comparison of APOBEC3 and Vif sequences. The identity and similarity of amino acid sequences of (a) APOBEC3Z2, (b) APOBEC3Z3 and (c) Vif. Unrooted phylogenetic trees are shown on the right of the respective matrix. The sequences of human APOBEC3 (a, b) and HIV-1 Vif (c) were used as the outgroups. The identity and similarity of amino acid sequences were analyzed using GENETYX v 9.0. The following sequences were used in this analysis; GenBank accession numbers are indicated in parentheses: domestic cat, APOBEC3Z2a (EU109281), APOBEC3Z3 (EU011792); puma, APOBEC3Z2 (GU097659), APOBEC3Z3 (GU097659); human, APOBEC3Z2 (NM_014508), APOBEC3Z3 (NM_001166002); FIVfca Vif strain Petaluma (M25381), strain C36 (AY600517); FIVpco strain PLV1695 (DQ192583) and HIV-1 strain NL4-3 (M19921). Note that human APOBEC3Z2 and APOBEC3Z3 are identical to APOBEC3C and APOBEC3H, respectively (55). A3Z2, APOBEC3Z2; A3Z3, APOBEC3Z3. (d) Unrooted phylogenetic tree of FIV *vif* reconstructed by the maximum likelihood method. The common names of the hosts of each FIV are provided below each illustration. The names of the cognitive FIV lineage of each host are shown in parentheses. The FIV strains used in the experiments, the results of which are shown in Figures 2 and 3, are shown in bold. Note that the bootstrap values on the nodes of respective FIV lineages (FIVfca, FIVpco, FIVoma, and FIVple) are more than 80 (indicated with asterisks). The scale bar indicates an evolutionary distance of 0.1 nucleotide substitutions per site. We used the following 23 FIV strains to construct this phylogenetic tree (GenBank accession numbers in parentheses): FIVfca, Petaluma (M25381), TM219 (M59418), C36 (AY600517), Shizuoka (LC079040), PPR (M36968), BM3070 (AF474246); FIVoma, pOma3 (AY713445); FIVpco, PLV1695 (DQ192583), Gc34 (EF455603), Mc350 (EF455604), Mc100 (EF455605), Mc121 (EF455606), YM29 (EF455607), YF16 (EF455608), JM01 (EF455609), JF6 (EF455610), YM137 (EF455611), YF125 (EF455612), SR631 (EF455613), SR631B (EF455614), CoLV (EF455615); FIVple, 27B (EU117991) and 1027 (EU117992).

Heine University, Düsseldorf, Germany). To produce FIV-based lentivirus vector, pFP93 (pFIV*gagpolΔvif*; we used a replication-incompetent *vif*-deficient FIV packaging construct derived from clone FIV 34TF10 (GenBank accession no. M25381; 430 ng) (41), pTiger-luc (pFIVΨ-luc) (430 ng; Addgene, Cambridge, MA, USA), and pMD.G (pVSVg, a VSVg expression plasmid; 150 ng). We co-transfected these plasmids into HEK293T cells by using PEI Max (Polysciences, Warrington, PA, USA) and harvested the cells and viral particles from culture supernatants at 48 hr post-transfection. The harvested samples were used for SDS-PAGE/western blotting or lentiviral reporter assays as previously described (32, 39, 42, 43). Briefly, we used the following antibodies for western blotting: an anti-FLAG polyclonal antibody (Sigma, St Louis, MO, USA), an anti-HA antibody (3F10; Roche, Indianapolis, IN, USA), an anti-FIV $p27^{Capsid}$ antibody (PAK3-2C1; Santa Cruz Biotechnology, Santa Cruz, CA, USA); an anti-α-tubulin antibody (DM1A; Sigma) and an anti-VSVg antibody (P5DA; Roche). For FIV reporter assays, we used HEK293T cells for the target cells (44).

It is known that feline (i.e., both cats and pumas) APOBEC3Z3 and APOBEC3Z2Z3 can impair FIV

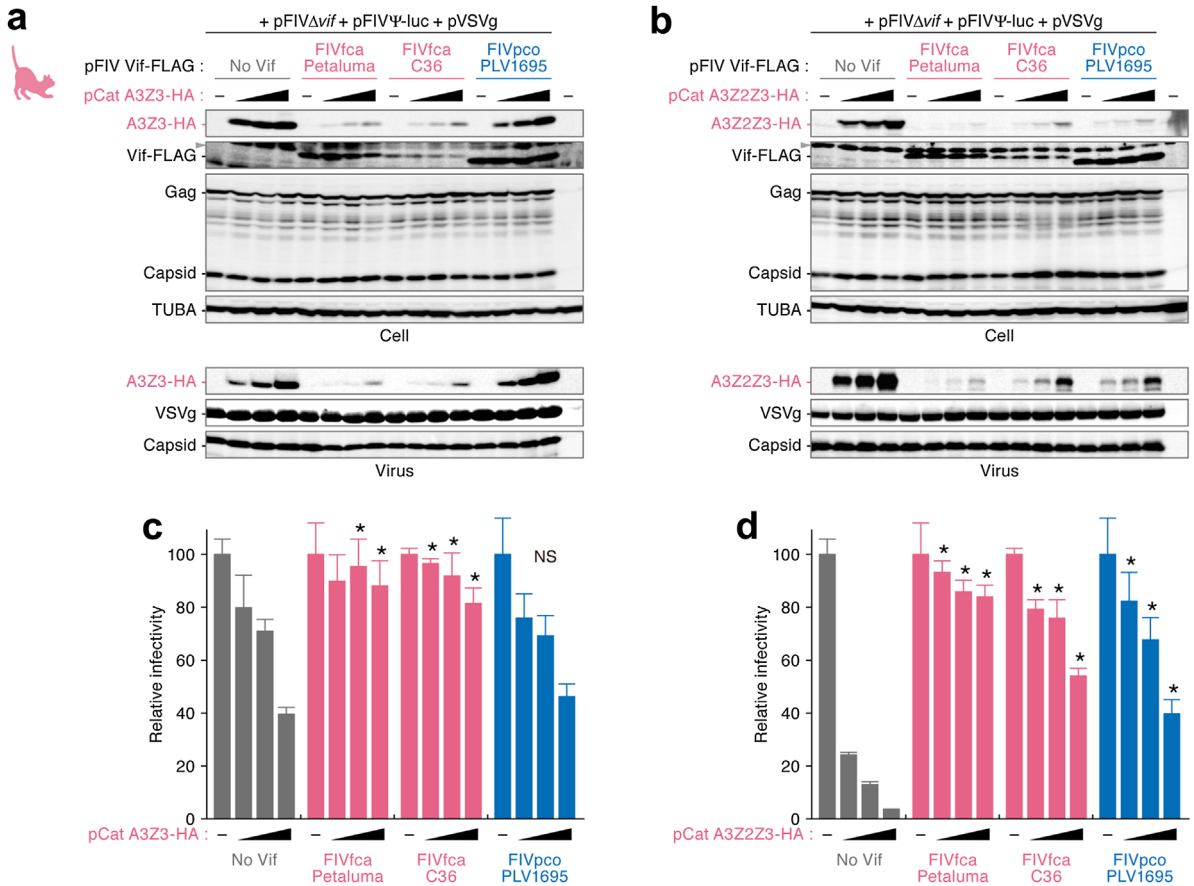

Fig. 2. Resistance of domestic cat APOBEC3Z3 to FIVpco Vif. The plasmids expressing FLAG-tagged FIV Vif (800 ng) and HA-tagged domestic cat APOBEC3Z3 or APOBEC3Z2Z3 (0, 50, 100 and 290 ng) were co-transfected with the plasmids for producing FIV-based lentiviral vector (see the main text for detail) into HEK293T cells. The cell extracts and viral particles from culture supernatants were analyzed by (a, b) western blotting and (c, d) FIV-based reporter assay. The assays were performed in triplicate. Representative results of western blotting are shown in (a) and (b). In panels (a) and (b); gray arrowheads indicate non-specific bands. (c) and (d) show the percentages of viral infectivity compared to the value without APOBEC3 expression. The error bars indicate SDs. *, $P < 0.05$ versus "No Vif" by Student's *t*-test. A3Z3, APOBEC3Z3; A3Z2Z3, APOBEC3Z2Z3; NS, no statistically significant difference compared to the values for "No Vif".

infection (36, 37, 39). Consistent with these previous observations, we incorporated domestic cat APOBEC3Z3 (Fig. 2a) and APOBEC3Z2Z3 (Fig. 2b) into the released virions and found that the packaged APOBEC3 proteins decreased FIV infectivity in a dose-dependent manner (Fig. 2c,d). Also, FIVfca Vif (strains Petaluma and C36) significantly augmented viral infectivity by impairing the incorporation of these proteins into released virions (Fig. 2).

We then assessed the ability of FIVpco Vif to degrade feline APOBEC3 proteins. Although FIVpco Vif degraded domestic cat APOBEC3Z2Z3, as in the case of FIVfca Vif (Fig. 2b), it was of interest that FIVpco Vif was unable to degrade domestic cat APOBEC3Z3 (Fig. 2a). The undegraded cat APOBEC3Z3 proteins were efficiently incorporated into the released viral particles (Fig. 2a,b, bottom panels) and suppressed viral infectivity (Fig. 2c,d). These findings suggest that domestic cat APOBEC3Z3 is resistant to FIVpco Vif-mediated degradation.

Next, we analyzed the functional relationship between puma APOBEC3 and FIV Vif proteins. As in the case of domestic cat APOBEC3 proteins, puma APOBEC3Z3 and APOBEC3Z2Z3 proteins suppressed viral infectivity in a dose-dependent manner (Fig. 3). We also found that the anti-viral action of puma APOBEC3 proteins is significantly antagonized by FIVfca Vif (Fig. 3). However, although puma APOBEC3Z2Z3 is counteracted by FIVpco Vif (Fig. 3b,d), we found that FIVpco Vif is unable to degrade the APOBEC3Z3 protein of the puma, its natural host (Fig. 3a), and that viral infectivity is suppressed by the packaged APOBEC3Z3 protein as in the case of an absence of Vif expression (Fig. 3c).

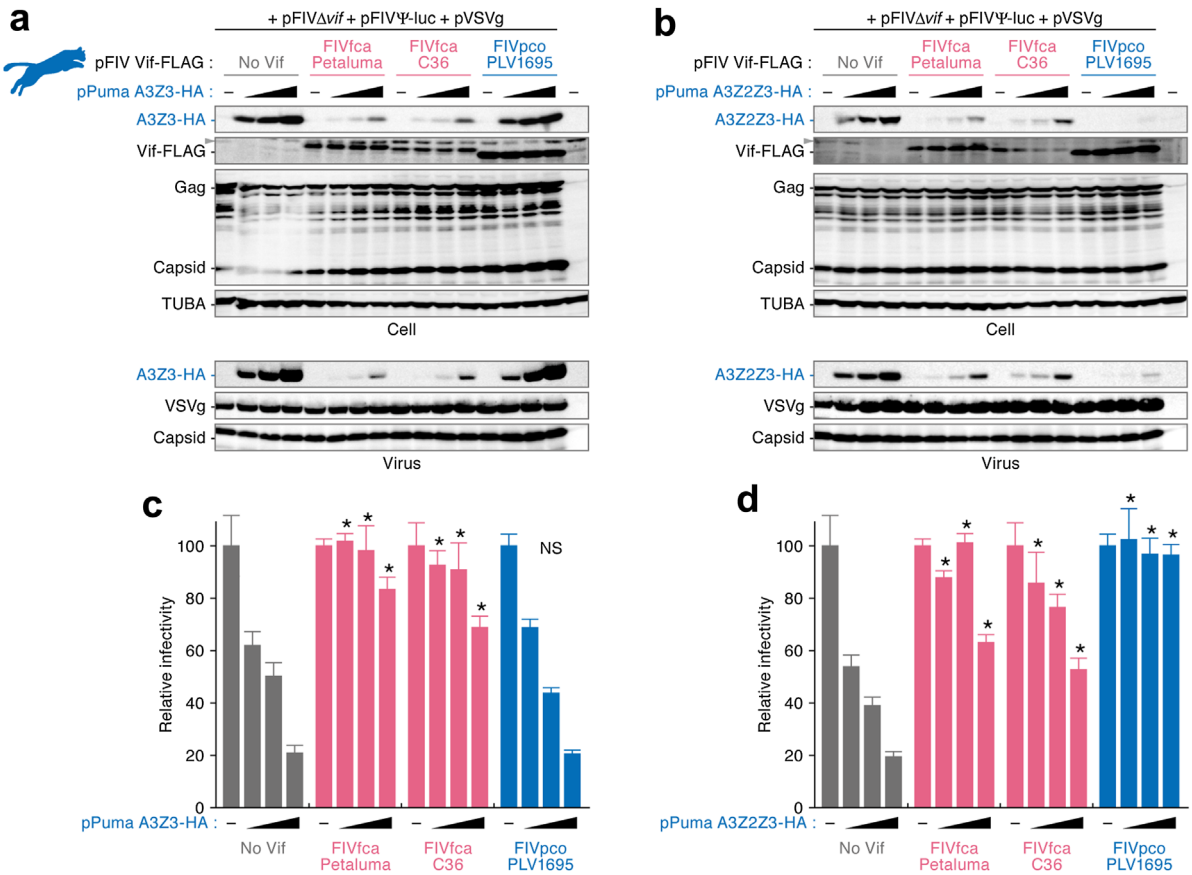

Fig. 3. Resistance of puma APOBEC3Z3 to FIVpco Vif. The plasmids expressing FLAG-tagged FIV Vif (800 ng) and HA-tagged puma APOBEC3Z3 or APOBEC3Z2Z3 (0, 50, 100 and 290 ng) were co-transfected with the plasmids for producing FIV-based lentiviral vector (see the main text for detail) into HEK293T cells. The cell extracts and viral particles from culture supernatants were analyzed by (a, b) western blotting and (c, d) FIV-based reporter assay. The assays were performed in triplicate. Representative results of western blotting are shown in (a) and (b); gray arrowheads indicate non-specific bands. (c) and (d) show the percentages of viral infectivity compared to the value without APOBEC3 expression. The error bars indicate SDs. *, $P < 0.05$ versus "No Vif" by Student's *t*-test. A3Z3, APOBEC3Z3; A3Z2Z3, APOBEC3Z2Z3; NS, no statistically significant difference compared to the values of "No Vif".

In this study, we found that FIVpco Vif is unable to degrade domestic cat APOBEC3Z3. This finding raises the possibility that anti-FIV activity of feline APOBEC3Z3 is not crucial for FIV replication and that the antagonizing ability of FIV Vif against feline APOBEC3Z3 is dispensable for FIV. However, here we also demonstrated that feline APOBEC3Z3 proteins significantly attenuate FIV infectivity (Fig. 2). Moreover, it has been reported that Vif is a prerequisite for FIV replication in both *in vitro* cell culture systems (45, 46) and *in vivo* (47). Taken together, these observations argue against the possibility described in the second sentence of this paragraph.

Rather, we found that FIVpco Vif is incapable of counteracting the APOBEC3Z3 protein of pumas, its natural host (Fig. 3a,c). Even in the case of SIVs naturally infected with OWMs in Africa, SIV Vif proteins counteract the APOBEC3G proteins of their natural hosts (13). Therefore, to the best of our knowledge, this is the first report demonstrating that a non-primate lentiviral Vif protein is unable to degrade an anti-viral APOBEC3 protein of its natural host (Fig. 3a,c). FIVpco strain PLV1695, which we used this study and which is the only infectious clone of FIVpco reported so far, is able to replicate in puma PBMCs (26), and pumas in the wild are naturally infected with FIVpco (17, 21–23). However, as described above, a previous study has demonstrated that G-to-A mutations, which are presumably mediated by endogenous APOBEC3, are detectable in the proviral DNA of wild pumas naturally infected with FIVpco (25). Therefore, taken together with our findings, puma APOBEC3Z3 may partially suppress, but not completely prevent, FIVpco replication.

As described above, several lentiviruses have been identified in mammals and most of them encode Vif (48, 49). This fact strongly suggests that there are various patterns of cross-species transmissions and evolutionary histories between lentiviruses and their hosts. For instance, Etienne and colleagues have recently reported that chimpanzee APOBEC3 proteins play a pivotal role as the barrier that protects interspecies transmission of OWM lentiviruses (50). In addition, D'arc and colleagues have reported that gorilla APOBEC3G is resistant to the degradation mediated by the Vif protein of SIV in chimpanzee (51). In contrast, here we found that FIVfca Vif degrades anti-viral APOBEC3 proteins (i.e., APOBEC3Z3 and APOBEC3Z2Z3) of both cat and puma (Figs 2,3). These results suggest that FIVfca Vif has a potent and broader ability to counteract the anti-viral actions of various feline APOBEC3 proteins. In this regard, it is known that a predator–prey system is one of the triggers leading to the interspecies transmission of viruses (52). In fact, previous studies have reported that pumas and domestic cats are sympatric in North America and share certain pathogens such as feline leukemia virus (53, 54), strongly supporting the possibility that FIVfca-infected domestic cats can be puma's prey and that FIVfca can be transmitted to puma.

In summary, our observations suggest that the evolutionary interplay between viruses and hosts, particularly Vif and APOBEC3, is more complicated than expected. Clarifying the interplay between lentiviral Vif and mammalian APOBEC3 may a provide clues to elucidating the evolutionary bottleneck of emerging/re-emerging viruses, including lentiviruses.

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DISCLOSURE

All authors have no interests to declare.

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