

APOBEC1-mediated editing and attenuation of HSV-1 DNA implicates an antiviral role in neurons during encephalitis

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Abstract

APOBEC1 (A1) is a cytidine deaminase involved in the regulation of lipids in the small intestine. HSV-1 is a ubiquitous pathogen that is capable of infecting neurons in the brain, causing encephalitis. Here, we show that A1 is induced during encephalitis in neurons of rats infected with HSV-1. In A1-stably expressing cells, HSV-1 infection resulted in significantly reduced virus replication when compared with infection in control cells. Infectivity could be restored to levels comparable to those observed in control cells if A1 expression was silenced by specific A1 shRNA. Moreover, cytidine deaminase activity appeared to be essential for this inhibition and lead to an impaired accumulation of viral mRNA transcripts and DNA copy number. The sequencing of the viral gene UL54 DNA, extracted from infected A1-expressing cells, revealed G to A and C to T transitions, indicating that A1 associates with HSV-1 DNA. Taken together, our results demonstrate a model in which A1 induction during encephalitis in neurons may aid to thwart HSV-1 infection.

Running Title: APOBEC1 inhibits HSV-1 infection

INTRODUCTION:

The human apolipoprotein B editing catalytic polypeptide (APOBEC) family is a group of zinc-dependent DNA and RNA cytidine deaminases and consists of AID, APOBEC1 (A1), APOBEC2 (A2), seven APOBEC3s (A3A-H), and APOBEC4 (A4). A1, the first APOBEC to be discovered, is known to introduce a premature stop codon into host apolipoprotein B mRNA in the gastrointestinal tract, an event critical for lipid metabolism (17, 39, 60). The editing by A1 is highly precise and specifically converts C to U at position 6666 of the apolipoprotein B mRNA substrate (45). Along with apobec 1 complementation factor ACF, these two proteins constitute the minimal required components necessary for the editing of apolipoprotein B mRNA *in vitro* (36).

Cytidine deaminases as antiviral factors first came into the limelight after A3G was identified as a cellular restriction factor capable of inhibiting HIV-1 dissemination in the absence of HIV-1 virus infectivity factor (vif) (55). This molecule was later shown to inhibit retrovirus infection by inducing massive hypermutation of the murine leukemia virus (MLV) genome (22). Further detailed studies revealed that APOBEC molecules are packaged into HIV-1 virions in virus producer cells via a specific interaction with gag and viral RNA and then exert their deaminase activity in subsequent target cells on a single ssDNA intermediate synthesized by the reverse transcriptase (3, 27, 54). Editing can lead to non-synonymous mutations, such as premature stop codons, in critical proteins (e.g. reverse transcriptase) necessary for virus replication and infectivity, severely impairing the next round of infection (53, 63). Extensive studies to assess the antiviral nature of these APOBEC enzymes have been performed across a broad range of retroviruses and the hepatitis B virus (HBV) (7, 21, 34, 35, 41, 42, 49, 55, 57).

56 Herpes simplex virus (HSV) is an enveloped, dsDNA virus and a member of the genus
57 alphaherpesviridae. One in every 250,000 to 500,000 individuals infected with HSV type
58 1 (HSV-1) or type 2 (HSV-2) experiences a devastating disease known as HSV
59 encephalitis (HSE), characterized by acute inflammation and/or hemorrhaging in the
60 central nervous system (CNS) (61). HSV-1 is the predominant causative agent of
61 sporadic encephalitis in western countries and it is estimated to be responsible for over
62 90 % of HSE cases (61).

63 In this study, we show a novel finding that A1 expression is induced during HSV-1
64 infection in neurons of infant rat brains, more than 30 % of which were able to recover
65 from HSE. Moreover, an investigation into the potential antiviral role of A1 *in vitro*
66 revealed that A1 inhibited virus replication directly by mainly targeting viral DNA in a
67 deaminase dependent manner, resulting in a stall of virus replication. To the best of our
68 knowledge, this is the first report implicating the potential antiviral function of A1 against
69 herpes viruses in the context of HSE.

70

Materials and methods

Cells, plasmids, viruses and infection. Vero and RS cells were maintained in DMEM and supplemented with FCS and antibiotics as previously described (4). Rat A1 cDNA was obtained by RT PCR of mRNA derived from HSV-1-infected rat brain tissue and inserted into pcDNA3.1/Zeo(+) (Invitrogen). An A1 mutant, E63A, was generated by site-directed mutagenesis using a QuickChange® II Site-Directed Mutagenesis Kit (Stratagene). HA-tagged empty vector, A1-WT, or E63-mutant DNA were transfected into RSC and then cultured for 10 days in the presence of Zeocin. Colonies were then screened and confirmed for protein expression by western blotting with an anti-HA Mab. GFP-expressing replication-competent HSV-1, YK333 (59), was used as previously described (4). YK333 HSV-1 was created by the intergenic insertion of a GFP expressing cassette between UL3 and UL4 of the virus genome. Green fluorescence can be detected after HSV infects host cells and starts viral gene expression (59); however, since this event occurs before viral DNA replication, the GFP signal detected in these cells is not indicative of HSV-1 progeny production. For inhibition of HSV-1, we added 25 µg/ml of acyclovir (Sigma-Aldrich, St. Louis, MO, USA) in culture medium.

Generation of shRNA and shRNA-expressing cells. shRNA plasmids were generated by cloning A1 specific or luciferase control ORF targeted sense and antisense sequences into a pBAsi-hU6 Puromycin vector (TaKaRa), via *Bam*H1 and *Xba*I restriction sites. The targeted A1 and luciferase sequences are as follows: A1-736, 5'-GATCCCCGACGCTCCGTTACCCGGTTACGTGTGCTG
TCCGTAACCAGGTAATGGAGCATCTTTTGGAAAT-3'; A1-1119, 5'-GATCCCCGTTC
TTCAAGGCTGCCGTTACGTGTGCTGTCCGTAATGGCAGCTTTGAAGAGCTTTTGG

AAAT-3'; Luciferase, 5'-

GATCCCCGTGCGTTGTTGGTGTTAATACGTGTGCTGTCCGTATTGGCA

CCAGCAGCGCACTTTTTGGAAAT-3'. A1-expressing RSCs were transfected with the shRNA plasmids, respectively, using lipofectamine 2000, selected by puromycin for individual clones and finally analyzed by western blotting for the suppression of A1.

Animal models and tissue collection. Fourteen-day-old Wistar Hannover GALAS rats (CLEA Japan, Inc.) were anaesthetized and then intracranially inoculated with 1.0×10^6 TCID₅₀ HSV-1 (YK333), or phosphate-buffered saline (PBS) as a negative control. Rats exhibiting a range of symptoms were classified into four groups as described in Table 1. For histological examinations, tissue samples were fixed by immersion in 4% PFA and processed as described before (4). All animal experiments were carried out according to the guidelines for animal experimentation at Kyoto University.

Transcriptome analysis. RNA was prepared from rat brains using an RNeasy extraction kit (QIAGEN) and transcriptome analysis was performed using a GeneChip Rat Genome 230 2.0 Array (Affymetrix) according to the manufacturer's instructions. Microarray data was analyzed using Gene Spring software (Agilent).

Real-time PCR. Nucleic acids were extracted as previously described (4). For the analyses of HSV-1 mRNA products, 1 µg of RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (QIAGEN), according to the manufacturer's instructions. The cDNA (50 ng) was used as a template to amplify UL54 (immediate early), UL30 (early), and UL27 (late) genes by real-time PCR using a Power SYBRGreen PCR Master Mix (Applied Biosystems). The threshold was set to a value

of 1.00, and the number of cycles required to reach the threshold cycle, Ct, was determined and then normalized with the Ct of the housekeeping gene, GAPDH. The HSV-1 DNA copy number was measured as previously described (4). Thermocycler conditions for the real-time PCR reaction are as follows: 95°C for 10 min, (95°C for 15s, 60°C for 1 min) x 40 cycles. The primers used for real-time PCR are as follows: A1 Forward, 5'-ACCACGCAGATCCTCGAAAT-3'; A1 Reverse, 5'-TCTTGCTCCGTCATGATCTGG-3'; HSV-1 UL54 Forward, 5'-CCGCGACGACCTGGAATCGG-3'; HSV-1 UL54 Reverse: 5'-GGCGAGCGGCGTCGAGTATC-3'; HSV-1 UL30 Forward, 5'-AGAGGGACATCCAGGACTTTGT-3'; HSV-1 UL30 Reverse, 5'-CAGGCGCTTGTTGGTGTAC-3'; HSV-1 UL27 Forward, 5'-TCGCCTTTCGCTACGTCAT-3'; HSV-1 UL27 Reverse, 5'-GGTTCTTGAGCTCCTTGGTGG-3'; GAPDH Forward, 5' - ACTAAAGGGCATCCTGGGCTA-3'; GAPDH Reverse: 5' - TGGAAGAATGGGAGTTGCTGT- 3'.

Immunohistochemistry and antibodies. Brain sections and culture cells were treated as described before (4, 30). The following primary and secondary antibodies were used: goat anti-apobec1 polyclonal antibody (Pab) (Santa Cruz Biotechnology), rabbit anti-HSV-1 Pab (DakoCytomation), mouse anti-MAP2 monoclonal antibody (Mab) (Upstate), anti-HA Mab (Roche), Alexa Fluor 594-conjugated anti-goat IgG (Invitrogen), Cy5-conjugated anti-mouse IgG or Alexa Fluor 647-conjugated streptavidin (Invitrogen). Nuclei were stained using Hoechst33342 (Invitrogen). Each sample was examined

under a confocal laser microscope (TCS SP2 AOBS, Leica Microsystems) using 405, 543, 633 nm excitations with 10x, 20x and 40x objectives.

Sequencing analysis of UL54. Nested PCR of UL54 was carried out with Pfu Ultra II Fusion DNA polymerase (Stratagene). In the first round of PCR, 100 ng of template cDNA or DNA, derived from either HSV-1 infected A1-expressing or control cells, was used with the appropriate primers in a total volume of 25 µl under the following thermocycler conditions: 1 min at 98°C, (20 s at 98°C, 20 s at 57°C, and 30 s at 72°C) x 35, and 3 min at 72°C. For the second round of PCR, 2.5 µl of the first PCR reaction was used as a template in a total volume of 25 ul under the following thermocycler conditions: 1 min at 93.4°C, (20 s 93.4°C, 20 s at 57°C, and 30 s at 72°C) x 35, and 3 min at 72°C. The UL54 amplicon was cloned into a pUC19 vector and the DNA was extracted from transformed bacteria. A BigDye® Terminator Cycle Sequencing Kit (v3.1) was next used and samples were read on an ABI® Prism 3130 sequencer. Sequences were analyzed using Sequencher 4.9 software. The primers used for the nested PCR are listed as follows: First PCR UL54 forward, 5'-AGCTTTGGCCGCAGCGCACA-3'; First PCR UL54Reverse, 5'-GAGTTGCAATAAAAATATTTGCCGTGCAC-3'; Second PCR UL54 Forward, 5'-GGTCTAGAAGCTTTGGCCGCAGCGCACA-5'; Second PCR UL54 Reverse, 5'-ATCAAGCTTCCTCGCGCCTTCAGGTAGCA-3'.

Statistical analysis. Statistical significance was determined using the student's t test and χ^2 analysis. A p value of less than 0.05 was considered statistically significant.

Results

Survival rat model from encephalitis with intracranial HSV-1 inoculation

In the past, rats have been reported to be useful models for examining HSE induced by both HSV-1 and HSV-2 and, depending on the age of the animal, there appears to be an age-dependent resistance to infection for survival (5, 15). In line with these reports, our preliminary studies revealed that infant GALAS rats before the age of 14 days old suffered from 100% mortality when infected by an intracranial injection of GFP-expressing HSV-1 (YK333) (Supplementary Data 1). On the other hand, rats 18 days or older displayed 100% survival after HSV-1 inoculation. Interestingly, the infection of 14 day-old-rats resulted in half of the animals surviving (Supplementary Data 1). These results indicated that possible intrinsic factors expressed in rats from this age may help to protect against HSV.

In order to elucidate factors contributing to the survival of the HSV-1-infected 14-day-old rats, we focused on the observed phenotype in this age group. We proceeded to inoculate 63 animals with HSV-1 for a more in-depth assessment of HSE. We found that 17 HSV-1-injected rats (27%, indicated as severe in Table 1 and Figure 1A) died after showing severe signs of encephalitis such as weight loss and quadriplegia within 5 days after inoculation. Fourteen injected rats (22%, indicated as mild in Table 1 and Figure 1A) died after showing a milder degree of weight loss and quadriplegia between 5 and 7 days. Interestingly, 24 injected rats (38.1%, indicated as survived in Table 1 and Figure 1A) recovered from HSE after showing temporary paralysis; HSV-1 infection in these mice was confirmed by GFP⁺ and HSV-1⁺ regions. The final group of 8 injected rats did not exhibit symptoms of encephalitis and did not possess GFP⁺ and HSV-1⁺

regions (indicated as healthy in Table 1). The range of symptoms displayed by the rats indicated that some were able to cope with HSV-1 infection more than others, which we speculated to be due to the containment of virus infection.

In the brain tissue of the severe and mild rats, taken at the time of death from HSE, we found vast hemorrhagic and necrotic damage (Figure 1A). Furthermore, disseminated GFP⁺ regions, which were confirmed as HSV-1⁺ cells by immunostaining with an anti-HSV antibody, indicated massive spreading and a multifocal distributed infection in the brain, typical of encephalitis in human infants (Table 1 and Figure 1A) (61). Severe and mild rats were also associated with extensive leukocyte infiltration by CD3⁺ T cells and CD68⁺ macrophages (Figure 1B, Red), however, brain samples from survived rats taken 36 h after the disappearance of paralysis showed less infiltration in parenchyma and fewer GFP⁺ cells (Table 1 and Figure 1B, 3rd row of panels). Meanwhile we could not detect pathological changes, GFP⁺ or HSV⁺ cells in the brains of mock-infected rats (in the bottom panels Figure 1A), confirming that the observed results were indeed dependent on HSV-1 infection. These data also indicated that limited but clear HSV-1 infection occurred in the brain of the survived rats.

To investigate whether our initial observation of reduced HSV-1 infection in survived rats was due to the control of HSV-1 dissemination over the course of infection or simply due to an inadequate initial infection, brain samples of survived rats were taken 12, 24, and 36 h after the disappearance of paralysis, and the extent of GFP⁺ regions was determined. Results showed that the HSV-1-infected regions diminished over a span of 36 h, suggesting that the virus was being contained and cleared (Figure 1C). These data also suggest the existence of an anti-HSV factor(s) induced upon HSV-1

infection, which appears to be expressed in CNS parenchymal cells, including neurons or glia, since the number of invading lymphocytes and macrophages did not seem to correlate with clearance of the virus.

Apolipoprotein B editing catalytic subunit 1 is induced during HSV-1 infection in brain tissue

In order to identify potential candidate molecules which are induced in the brain upon HSV-1 infection, we isolated RNA from the GFP⁺ and neighboring regions of two survived rat brains at 4 and 6 days post infection (corresponding to 12 and 36 h after the disappearance of paralysis, respectively) and performed microarray analysis of mRNA from both survived or mock-infected rat brains. In comparing the level of mRNA from these rats, 47 genes showed augmented expression (Supplementary Data 2). Among them, the level of A1 mRNA was clearly increased by 4.59 and 41.6 fold at 4 and 6 days post infection, respectively.

Since A1 and related A3 family proteins are known to be strong inhibitors of HIV-1 infection, we examined the possibility of an association between A1 and HSV-1 infection. From rat brain tissue, we were able to detect significantly elevated levels of A1 cDNA exclusively in HSV-1-infected rats by real-time PCR (Figure 2A). As expected, we confirmed high levels of A1 cDNA expression in the small intestine of mock and HSV-1-infected rats, where A1 is constitutively expressed (Figure 2E). Immunofluorescence staining of brain tissue with an A1-specific antibody demonstrated A1⁺ staining only after HSV-1 infection, but not after mock infection (Figure 2B), corroborating PCR findings.

To further investigate the cell type specific expression of A1 after HSV-1 infection, we performed co-immunostaining with an anti-MAP2 (neuron-specific) and an anti-A1 antibody. A1⁺ staining was predominantly observed in MAP2⁺ cells which were HSV-1 infected but not in MAP2⁻ cells, indicating that the A1 expression was induced in neurons within the HSV-1 infected brain (Figure 2B-D). On the other hand, the expression levels of A1 in the small intestine were high regardless of HSV-1 infection (Figure 2F). These data suggested that A1 may serve as an intrinsic neuronal tissue factor for the control of HSV-1 infection in the CNS.

A1 inhibits HSV-1 infection in a deaminase-dependent manner

To examine the potential anti-HSV-1 activity of A1, we generated N-terminal HA-tagged wild type rat A1 (A1_{WT}) stably-expressing rabbit skin cells (RSC). A1 is a zinc-dependent deaminase with 3 residues, (His⁶¹, Cys⁹³, Cys⁹⁶) needed for coordinating zinc binding and a glutamate residue at position 63 in the catalytic domain essential for cytidine deaminase activity (38). Hence, we also generated N-terminal HA-tagged A1 deaminase-deficient mutant (A1_{E63A}) stably-expressing RSC. Control A1 (A1_{Ctrl}) RSC were generated using an empty HA vector. Western blotting analysis using an anti-HA antibody confirmed that the expression of A1_{WT} and A1_{E63A} was equivalent in these cell lines (Figure 3A). Immunofluorescence staining of A1_{WT} and A1_{E63A} showed similar localization which was comparable to that in A1⁺ neuronal cells of HSV-1-infected brain tissue (Figure 2B and 3B, respectively).

We next proceeded to assess the potential HSV-1 antiviral activity of A1 by infecting A1_{WT} or A1_{E63A} RSC with GFP-expressing HSV-1 at a MOI of 0.01. As shown in Figure

3C, the level of GFP expression was significantly attenuated in A1_{WT} RSC in comparison to A1_{E63A} and A1_{Ctrl} RSC 48 h post infection. Interestingly, A1_{E63A} cells also had a modest inhibitory effect on HSV-1, although this activity did not appear to be as potent as in wild type expressing RSC, suggesting a somewhat deaminase-independent inhibition mechanism (Figure 3C). When two different shRNA were used to specifically target A1_{WT}, the infectivity of HSV-1 was restored and comparable to that observed in A1_{Ctrl} (Figure 3D). Overall, the low and high level of A1 protein expression resulting from specific shRNA (A1/736 and A1/1119) against A1 and control luciferase, respectively, correlated inversely with HSV-1 infectivity (Figure 3D) and suggested that A1 expression is essential for the inhibition of HSV-1 infection.

In order to analyze the level of attenuation in HSV-1 replication, we looked at the virus titer of supernatants taken from A1_{ctrl}, A1_{WT} and A_{E63A} cells infected at higher MOI. The virus titer of supernatant from HSV-1-infected A1_{WT} cells was significantly attenuated when compared with that from empty vector or A1_{E63A} stably expressing cells in a dose dependent manner (Figure 3E). Additionally, the A1_{E63A} mutant also modestly attenuated virus titers, although not to the same extent as wild type A1. These data indicated again that cytidine deaminase activity was also crucial for potent inhibition to take place. Taken together, even at a high MOI, HSV-1 replication was being significantly inhibited in A1_{WT} expressing cells when compared with A1_{Ctrl} RSC in a deaminase-dependent manner.

A1 inhibits HSV-1 gene transcription and DNA replication

271 HSV-1 gene expression is highly ordered and occurs in a cascade-dependent manner
272 which is divided into three stages with the transcription products subdivided into 3 broad
273 groups termed α , β , and γ , also known as immediate early (IE), early (E), and late (L),
274 respectively (26). The α genes, produced soon after infection, promote β gene
275 transcription and increase viral protein synthesis. Thereafter, β genes are transcribed
276 and enhance DNA replication which, in turn, signals γ gene transcription for the
277 production of viral proteins necessary for virion assembly (12).

278 To better understand the mechanism by which A1 inhibits HSV-1 replication, we first
279 examined the expression level of representative genes from α , β , and γ groups. UL54,
280 an α gene that plays a role in the shut off of host protein synthesis and also enhances
281 viral gene expression, UL30, a β gene and the DNA polymerase responsible for DNA
282 replication, and UL27, a γ gene and envelope glycoprotein, were analyzed by real-time
283 PCR and normalized to GAPDH. As can be seen from the expression level of HSV-1
284 cDNA extracted from A1_{Ctrl} and A1_{WT} stably expressing cells at 8 h post infection at a
285 MOI of 0.01, all three genes analyzed were significantly lower in A1_{WT} cells compared to
286 A1_{Ctrl} cells (Figure 4A).

287 Given that α gene synthesis precedes DNA replication, we expected that the observed
288 low level of the immediate early genes would affect HSV-1 DNA replication and, thus,
289 the viral DNA copy number. Indeed, at 8 h post infection, the HSV-1 DNA copy number
290 was nearly 4 times lower in the A1_{WT} cells when compared with A1_{Ctrl} cells (Figure 4B).
291 As an additional control, infection of control RSC with a UV-inactivated virus did not
292 result in viral DNA replication (Figure 4B). Altogether, our results indicated that A1
293 affected viral gene expression and also DNA replication.

A1 induces mutations in HSV-1 DNA

A1 homologues are putative inducers of mutations in retrovirus DNA and also in DNA of A1-expressing bacteria (8, 23, 28, 43, 48). Thus, to investigate the mechanism of A1-dependent inhibition of HSV-1 gene expression, we looked at the potential editing of HSV-1 viral DNA. The UL54 DNA extracted from HSV-1-infected A1_{WT} RSC showed evidence of both G→A and C→T transitions, indicating that plus and minus strands were being edited (Figure 5B). The majority of the 51 clones analyzed from A1_{WT} RSC contained 1 mutation within the sequenced region; however, a few clones harbored 2 or 3 mutations (Figure 5E). On the other hand, A1_{Ctrl} derived clones showed no signs of mutations (Figure 5A and 5D), suggesting that the observed nucleotide changes were induced by A1.

Given that rat A1 is also known to edit retroviral RNA, we looked at cDNA extracted from A1_{WT} RSC 8 hpi at a MOI of 0.01 for evidence of additional deaminase activity (8). Similar to DNA sequencing results, G→A and C→T transitions in viral cDNA were observed, with the majority of clones containing 1 mutation, while clones with 2 or 3 mutations were also observed (Figure. 5C and 5F). Interestingly, 1 clone contained 10 G→A mutations, suggesting that a second round of editing may be occurring on mRNA transcribed from the mutated DNA (Figure 5F); yet, the main editing appeared to be occurring on the viral DNA which was mirrored by the mutations observed in viral cDNA (Figure 5B and 5C). In looking at the type of mutations arising from 39 commonly mutated positions in UL54 cDNA and 30 commonly mutated positions in UL54 DNA, approximately 70% of mutations were found to lead to non-synonymous substitutions in the analyzed gene (Figure 5G).

317 APOBECs, such as A3G, are known to have an editing preference for 5'CpC and 5'TpC
318 and we attempted to assess the dinucleotide preference of rat A1 (58). As can be seen
319 in Figure 5H, we found the preferred context to be 5'GpC on both viral DNA and RNA,
320 although previous studies have indicated a tendency of rodent A1 to prefer 5'TpC
321 (Figure 5H) (11, 28, 48). Altogether, these results indicate that rat A1 directly acts on
322 DNA for editing.

323 ***A1 inhibits HSV-1 transcription independently of viral mRNA editing***

324 To further elucidate the mechanism by which A1 inhibits HSV-1 infection, we focused on
325 the initial transcription of viral genes in the presence of the nucleoside analogue
326 inhibitor, acyclovir, which effectively inhibits nascent DNA synthesis. If A1 mainly acts
327 on ssDNA substrates for mutagenesis then we expected to see reduced deamination of
328 HSV-1 cDNA during transcription and few clones harboring signature A1-mediated
329 mutations. At a MOI of 1, the sequencing of UL54 mRNA extracted 2 hpi revealed a low
330 frequency of C → U and G → A transitions in samples taken from A1_{WT} expressing cells
331 similar to nucleic acids obtained from A1_{Ctrl}, further indicating that A1 is acting on DNA
332 and not mRNA (Figure 6A and 6B). In addition, only 1 clone containing these mutations
333 was observed from each A1_{Ctrl} and A1_{WT} RSC (Figure 6C and 6D). However, when we
334 looked at the levels of viral gene transcripts at this time point, α, β, and γ gene
335 expression was still significantly lower in A1_{WT} than A1_{Ctrl} RSC (Figure 6E). Altogether,
336 these data suggest that although A1 did not induce mutations in viral RNA, its
337 association with ssDNA may serve to block and inhibit transcription.

Discussion:

From our findings, we propose a novel role for A1 as an inhibitor of HSV-1 infection in the context of HSE in rats. Although the induction mechanism remains to be elucidated, we posit that HSV-1 infection triggers the expression of A1 in neuronal cells which inhibits the early stages of the virus life cycle. The molecule may edit viral DNA in the nucleus, and/or serve as a physical blockade by binding to DNA during transcription, resulting in reduced expression and dysfunction of the edited viral genes. Stalling of the HSV-1 gene cascade may prevent the virus from ramping up to full scale production and allow for a controlled immune response.

We found that both viral DNA and RNA harbored G → A transitions, however, DNA was the main target of A1 during HSV-1 infection (Figure 5B and 5C). Furthermore, when DNA synthesis was blocked, cDNA isolated from virus-infected cells displayed few mutations, further reinforcing the assumption that A1 is acting on a DNA substrate (Figure 6A and 6B). HSV-1 is known to replicate within the nucleus and while A1 has been reported to shuttle between the cytosol and nucleus in the presence of apobec 1 complementation factor (ACF) for a nuclear distribution, if ACF is mutated or absent, then A1 will primarily have a cytoplasmic localization (9, 24). Here, we show similar distribution of A1 in the cytosol *in vivo* in HSV-1-infected neurons and *in vitro* in A1 expressing RSC (Figure 2B and 3B). Yet, because we observed mutations in HSV-1 DNA *in vitro* (Figure 5B), this suggests that a small pool of A1 also exists in the nucleus and is involved in the direct interaction with and mutation of genomic viral DNA.

Although we obtained evidence of cytidine deaminase dependent mutations in HSV-1 nucleic acids, it is possible that our observed results could be an underestimate of the

actual degree of mutagenesis which is taking place inside of A1-expressing cells. HSV encodes a uracil DNA glycosylase which is able to remove uracil bases from DNA allowing for subsequent repair of the damaged DNA (31, 37, 62). This activity may be masking the true extent of deamination taking place by A1.

It is worth noting that the editing of HSV-1 DNA alone may not completely account for the inhibition of HSV-1. We found 33% of mutated positions in the sequenced region of UL54 DNA and 28% of UL54 RNA to be synonymous mutations, meaning that their function would remain intact (Figure 5G). Furthermore, mutations alone do not entirely account for the observed inhibition of α gene transcription (Figure 4A) since transcription of these genes are highly dependent on incoming tegument protein VP16 which acts as a transactivator (1, 2). Therefore, it is possible that A1 may also inhibit gene transcription through an undefined mechanism similar to the A3 deaminase-independent inhibition of HIV-1 reverse transcription products, perhaps through a physical block of transcription or DNA replication (6, 18, 19, 40, 56). Supporting this assumption is data from our deaminase-deficient mutant A_{E63A} that showed partial inhibition of HSV-1 infection, although not to the same extent as the A1_{WT} protein (Figure 3C and 3E). Moreover, diminished HSV-1 transcript levels were observed in A1_{WT} RSC when DNA synthesis was inhibited, even in the absence of RNA cytidine deamination (Figure 6E). Yet, because of the presence of repair mechanisms encoded by HSV-1, such as the uracil DNA glycosylase described above, we were not able to rule out the possibility that viral ssDNA is mutated during transcription.

If mutated viral transcripts are synthesized and make their way to the cytoplasm for translation, we suspect the functionality of the synthesized viral protein may be affected.

384 As mentioned above, the gene expression of HSV-1 is tightly regulated and defects in
385 certain genes can have detrimental effects on the virus life cycle. For instance, studies
386 on HSV-1 temperature mutants have revealed that defects in the IE genes ICP4, and
387 UL54 render the virus replication deficient (16, 46, 50). Furthermore, IE genes have
388 pleiotropic functions as transcriptional activators of viral genes and suppressors of host
389 protein synthesis. In the case of UL54, other functions include the inhibition of mRNA
390 splicing, an increase in viral mRNA levels, and the export of viral mRNA from the
391 nucleus (10, 20, 29, 51, 52). Given that HSV-1 proteins have essential regulatory roles,
392 it is conceivable that disruptions of these genes can cause a stall in the virus replication
393 cycle. Our results also support this possibility as evidenced by the reduced HSV-1
394 mRNA and DNA levels, GFP expression, and virus titer in infected A1 expressing RSC
395 (Figure 3C, 3E, 4A, and 4B, respectively). This effect was dependent on A1 expression
396 as silencing of A1 by specific shRNA restored HSV-1 infection (Figure 3D).

397 Our *in vitro* results raise the issue of whether the expression of A1 in rat brains induced
398 during HSE aids to inhibit virus infection or not. Rat A1 is ubiquitously expressed in
399 virtually all tissues such as the spleen and liver and while A1 functions within an editing
400 complex in the small intestine for the deamination of apolipoprotein B mRNA, it is also
401 expressed in tissues where apolipoprotein B mRNA is not present, bringing into
402 question whether there is an additional role for this enzyme (25). Indications that A1
403 also has antiviral activity on top of its already known physiological function have been
404 shown using mouse models infected with either MLV or HBV. Splenocytes taken from
405 MLV infected mice or hepatocytes taken from HBV infected transgenic mice both
406 displayed signs of A1 specific editing of viral genomes, implicating a direct role in virus

407 inhibition (44, 48). From our *in vivo* findings, survived and mild rats showed an increase
408 in A1 mRNA expression which was not observed in mock infected rats (Figure 2A and
409 2B). Moreover, rat brain tissue infected by HSV-1 revealed that the induction of A1
410 occurred predominantly in HSV-1 infected neuronal cells and suggests a possible
411 antiviral function for this molecule in the brain.

412 The consequence of increased cytidine deamination of the HSV-1 genome on virus
413 infectivity has been exemplified by a previous report showing that HSV-1 mutants
414 containing a defective uracil DNA glycosylase were 100,000 times less neuroinvasive in
415 mice intracranially inoculated with HSV-1 than their wild type counterparts (47). On the
416 other hand, uracil DNA glycosylase deficient-HSV-1 did not have issues replicating *in*
417 *vitro*, suggesting that this enzyme is necessary for virus infectivity in rodent neurons
418 because HSV-1 is more prone to cytidine deamination in these cells (47). It is tempting
419 to speculate that A1 in rats may also be playing a role in mutating the HSV-1 DNA *in*
420 *vivo* to inhibit HSV-1 infection in the brain in our model.

421 A1 may also help to reduce HSE *in vivo* by slowing down virus dissemination long
422 enough to allow for an appropriate immune response to be mounted, thus eliminating
423 the virus without causing excessive damage from infiltrating macrophages during
424 encephalitis or lytic damage resulting from massive viral infection. In fact, it has been
425 speculated that an overzealous immune response may be causing more harm than
426 good in the CNS (13). For instance, the depletion of macrophages during HSV-1
427 infection in mice has been demonstrated to lead to a higher survival rate (33). On the
428 other hand, uncontrolled virus dissemination can also lead to a fatal outcome in mice
429 missing type I interferon receptor (14, 32). Needless to say, a balance must be struck

430 between the host immune response and the extent of virus infection. It is plausible that
431 A1 helps to achieve both of these feats by inhibiting virus infection, resulting in a toned
432 down immune response, as was shown *in vitro* (Figure 3C and 3E). Here, we observed
433 diminished leukocyte infiltration in survived rats compared to mild and severe rats which
434 died within 5-6 days after infection in our *in vivo* model (Figure 1A). Moreover, HSV-1
435 infected areas, diminished over time in survived rats, suggesting the containment of
436 virus dissemination (Figure 1C).

437 In conclusion, we identified rat A1 as a novel anti-HSV-1 molecule induced during virus
438 infection in rat brains. The mechanism by which A1 inhibits virus replication appears to
439 be through the interference of the HSV-1 gene cascade by a predominantly deaminase
440 dependent manner involving the editing of viral DNA. In order to assess the extent of A1
441 inhibition on HSV-1 infection during encephalitis, future studies using knockout animals
442 may provide insights into the contribution of A1 in controlling HSE.

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Footnotes

Author contributions: Y.K., Y.A., H.K., and P.G. designed research; Y.A., H.K., S.P.Y., Y.K., H.E., and P.G. performed research; Y.K. contributed new reagents/analytic tools; Y.K., Y.A., H.K., and P.G. analyzed data; and Y.K. and P.G. wrote the paper.

The authors declare no conflict of interest.

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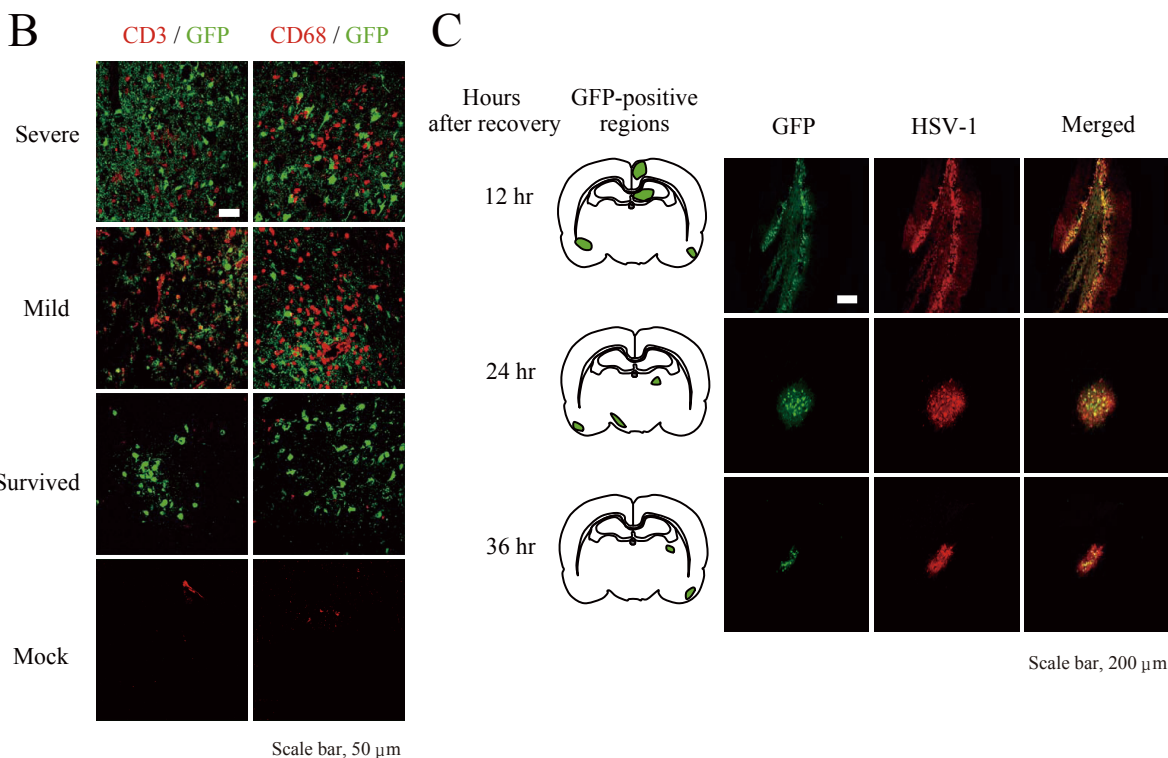
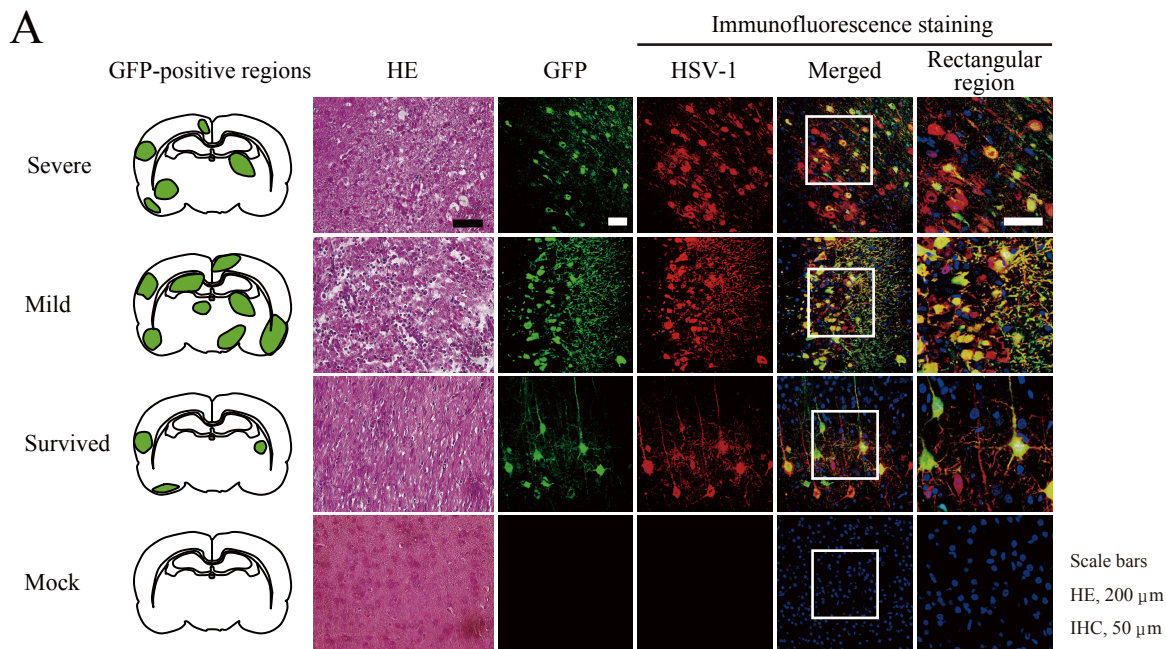


Figure 1. HSE model induced by GFP-HSV-1 inoculation of infant rats. Fourteen day-old infant rats were intracranially injected with GFP-expressing HSV-1 (YK333), and brain tissues were collected from rats exhibiting severe or mild encephalitis as described in Table 1 or from survived rats 36 h after the disappearance of paralysis. PBS (Mock)-inoculated rats were used as negative controls and brain tissues were collected at the end of the experiment. (A) GFP+ (green) regions in the coronal plane, HE, GFP+ (green) and HSV antigen+ (red) brain cells in the tissue section. Nuclei were also stained with Hoechst33342 (blue) and shown in the merged image. A zoom in view of the rectangular region in the merged image is shown to the right. (B) GFP+ (green), CD3+ (red) or CD68+ (red) cells in the tissue section. (C) GFP+ regions in the coronal plane (right panels) and GFP+ and HSV antigen+ cells in the tissue section (left panels) are shown in survived rats at indicated h after the disappearance of paralysis. Representative results are shown. Scale bars are 200 μ m in HE section and 50 μ m in the other sections.

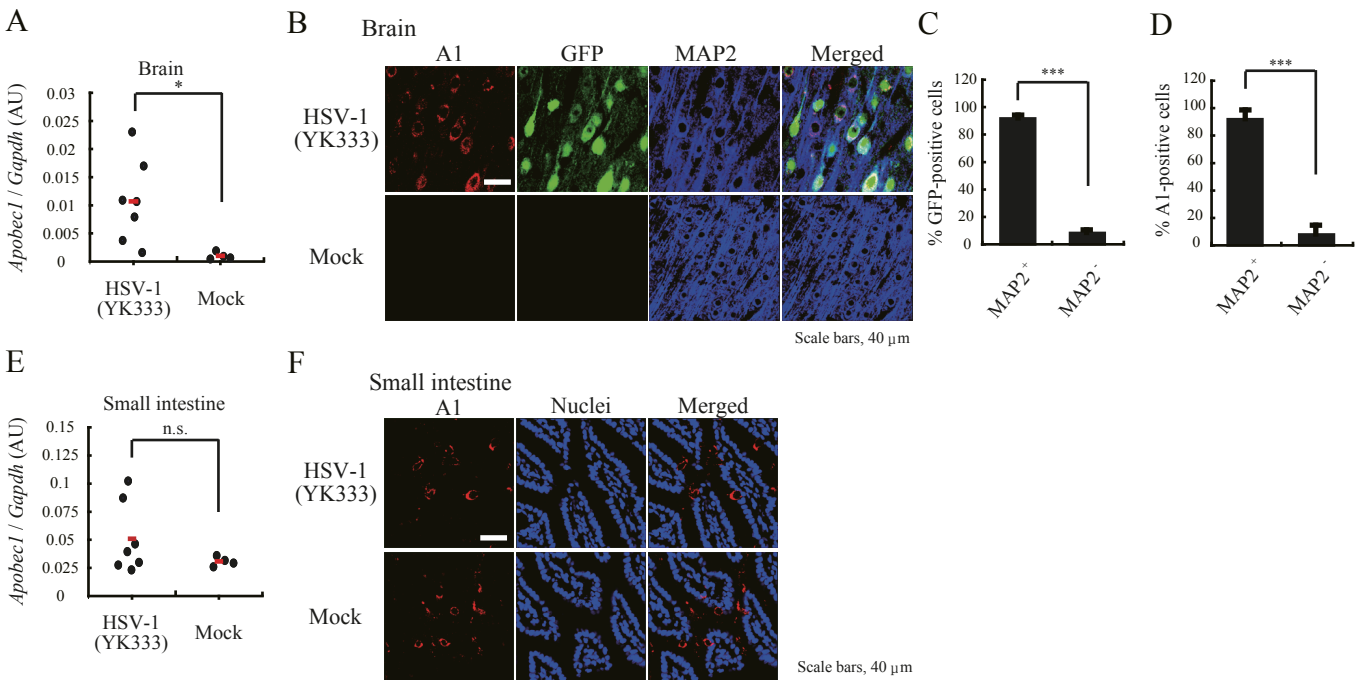
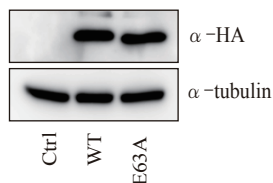
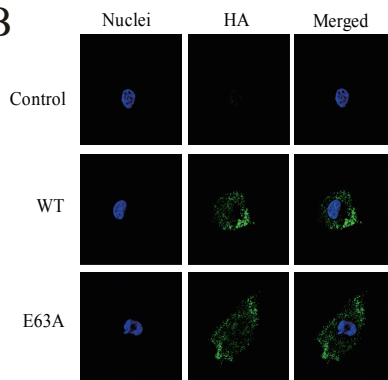


Figure 2. Expression of A1 in HSV-1-infected rat brains. (A) The level of A1 cDNA expression quantified by real-time PCR in the brain and small intestine of HSV-1-inoculated rats, which consisted of 4 mild rats and 2 recovery rats, and of 4 PBS-inoculated rats was assessed. (B) A1+ (red), GFP+ (green), and MAP2+ (blue) neuronal cells of an HSV-1 and mock-infected rat brain (upper panels) and A1+ cells in the small intestine of HSV-1 and mock-infected rats (bottom panels). Representative results are shown. Scale bars are 200 μ m. Quantification data were presented as a percentage of MAP2 antigen-positive cells in GFP+ (C) or A1+ (D). *, $P < 0.05$ compared to mock, *** $P < 0.005$ compared to MAP2 antigen-negative cells.

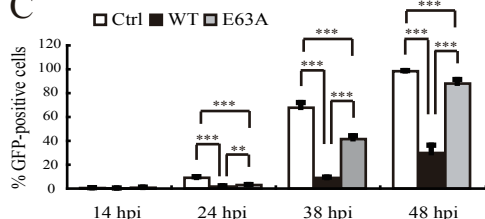
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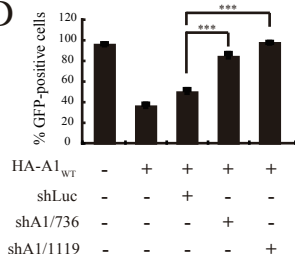
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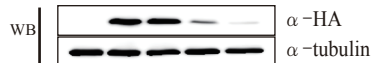
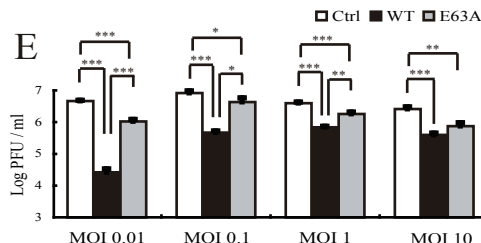


Figure 3. A1 inhibits HSV-1 infection. (A) Western blotting of HA-tagged WT, E63A, and Ctrl stably transfected cells probed with an anti-HA for A1 expression or an anti-tubulin antibody as an internal standard. (B) Immunofluorescence staining of HA-tagged A1WT, A1E63A or A1Ctrl RSC with an anti-HA antibody (green). The nucleus was stained with Hoechst33342 (blue). (C) Longitudinal analysis of GFP-expressing HSV-1 infection in A1WT, A1E63A mutant, or Control stably transfected cells. **, $P < 0.05$ and *** $P < 0.005$. (D) Level of GFP in HA-tagged A1WT RSC stably expressing A1 targeted shRNAs or luciferase (Luc) 48 h after YK333 infection. *** $P < 0.005$ compared to shLuc-treated cells. Western blot probed with an anti-HA antibody for the expression of A1 and anti-tubulin as an internal standard. (E) Level of PFU in the supernatant of Ctrl, WT, and E63A RSC 48 h after the infection by YK333 at varying MOI. *, $P < 0.05$, **, $P < 0.005$, and *** $P < 0.0005$.

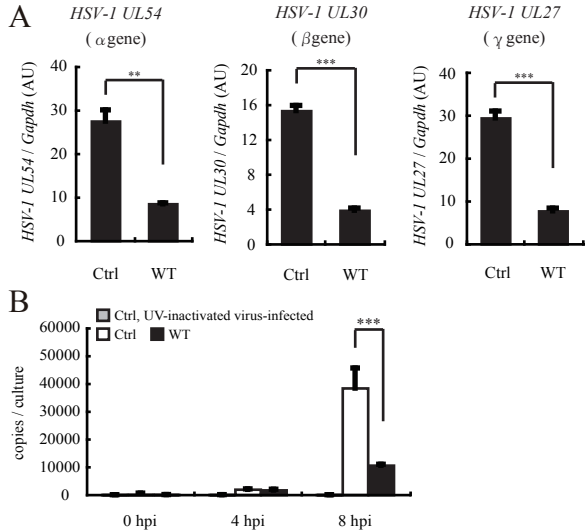


Figure 4. Inhibition of HSV-1 mRNA and DNA. (A) HSV-1 gene expression for UL54, UL30, and UL27 were measured by real-time PCR and normalized to GAPDH. Nucleic acids were extracted from HSV-1 infected WT and Ctrl RSC 8 hpi. (B) DNA copy number was measured by real-time PCR in A1 and control RSC 4 and 8 h after infection. As a control, UV-inactivated virus was also used to infect control RSC. **, $P < 0.05$, and *** $P < 0.005$

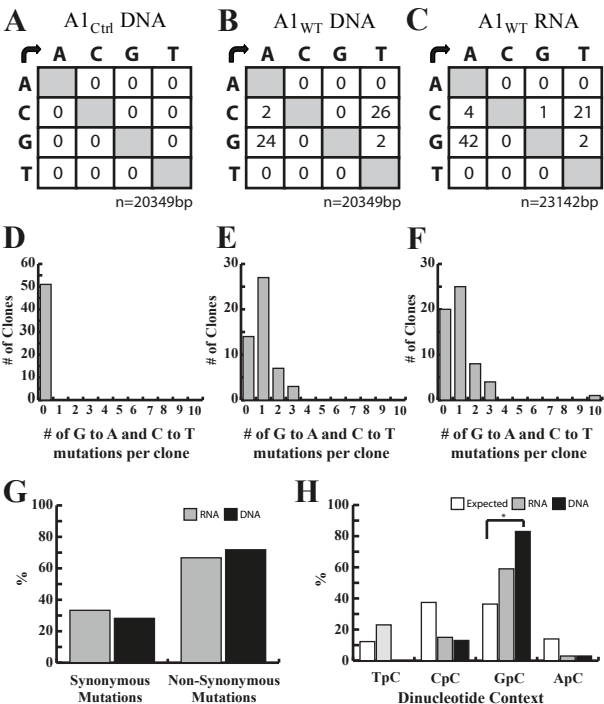


Figure 5. Sequencing analysis of UL54 DNA and cDNA from A1WT and A1 Ctrl HSV-1 infected RSC. A 399 bp region of UL54 DNA or cDNA, extracted 8 hpi, was amplified by nested PCR and cloned for sequencing. Mutation matrices are depicted for (A) DNA extracted from A1Ctrl RSC, 51 clones; (B) DNA extracted from A1WT RSC, 51 clones; and (C) cDNA extracted from A1WT RSC, 58 clones. The number of clones harboring G to A and C to T transitions was assessed and represented in (D) DNA extracted from A1 Ctrl RSC; (E) DNA extracted from A1WT RSC; and (F) cDNA extracted from A1WT RSC. (G) Mutations from UL54 cDNA and DNA were narrowed down to 39 and 30 commonly mutated positions, respectively. The % of synonymous or non-synonymous mutations is depicted in the bar graph. (H) Rat A1 had a tendency in dinucleotide context for 5'GpC in viral UL54 DNA and cDNA and was statistically significant in the DNA. A χ^2 analysis showed that when compared to expected values, 5'GpC was significantly favored on UL54 DNA as indicated by the asterisk ($p < 0.001$).

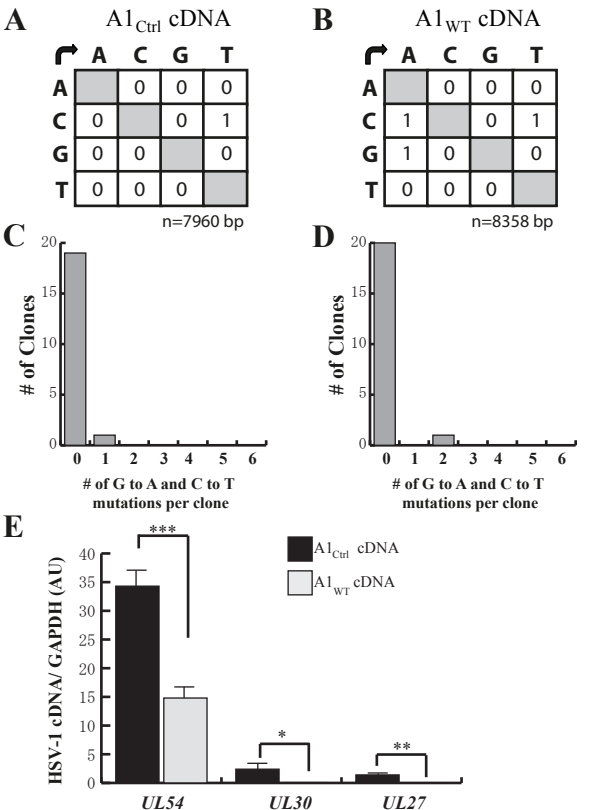


Figure 6. $A1$ inhibition of HSV-1 RNA transcripts is independent of RNA mutagenesis. $A1_{WT}$ and $A1_{Ctrl}$ RSC were infected with YK333 at a MOI of 1 for 2 h in the presence of 25 $\mu\text{g}/\text{mL}$ of acyclovir. Mutation matrices for UL54 cDNA extracted from (A) $A1_{Ctrl}$ RSC and (B) $A1_{WT}$ RSC are shown. The number of clones harboring G to A and C to T transitions from (C) cDNA extracted from $A1_{Ctrl}$ RSC, 20 colonies and (D) cDNA extracted from $A1_{WT}$ RSC, 21 colonies, was assessed and represented in the bar graphs. (E) The level of HSV-1 mRNA for UL54, UL30 and UL27 in $A1_{Ctrl}$ and $A1_{WT}$ RSC were measured by real time PCR and normalized to GAPDH levels. *, $p < 0.05$; **, $p < 0.0005$; ***, $p < 0.00005$.

Table I Classification of rats exhibiting varying degrees of HSE

Classification ^a	Number of rats (%)	Number of rats showing the following neurological symptoms ^b			Pathological Change			GFP-positive regions ^f	HSV-1-positive regions ^f
		Monoplegia	Quadriplegia	Seizure	Tissue damage ^c	CD3-positive cells ^d	CD68-positive cells ^e		
Severe	17 (27)	1	15	11	+	++	++	++	++
Mild	14 (22.2)	7	7	4	++	+++	+++	+++	+++
Survived	24 (38.1)	22	0	0	-	+	+	+	+
Healthy	8 (12.7)	0	0	0	-	-	-	-	-
Total	63								

a HSV-1-infected rats were classified as follows: Severe, which died within 5 days after showing symptoms of severe encephalitis; Mild, which died between 5-7 days after showing symptoms of mild encephalitis; Survived, which recovered after transient encephalitis; Healthy, which did not display HSE symptoms.

b The number of rats exhibiting neurological symptoms such as monoplegia, quadriplegia, and seizures.

c The percentage of damaged areas including cell loss, degeneration of cells, and/or hemorrhaging in the coronal section was presented as follows: -, <5%; +, 5-10%; ++, > 15%

d The number of CD3-positive cells in one infected area of the coronal section was quantified and presented as follows: -, not detected; +, <5 cells; ++, 5-20 cells; +++, >20 cells.

e The number of CD68-positive cells in one infected area of the coronal section was quantified and presented as follows: -, not detected; +, <5 cells; ++, 5-50 cells; +++, >50 cells.

f The percentage of GFP- or HSV-1-positive areas in the coronal section was presented as follows: -, not detected; +, <5%; ++, 5-20%; +++, >20%.