

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

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16 **Abstract**

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

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31

32 Running Title: APOBEC1 inhibits HSV-1 infection

33 **INTRODUCTION:**

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

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

71 **Materials and methods**

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

87 **Generation of shRNA and shRNA-expressing cells.** shRNA plasmids were
88 generated by cloning A1 specific or luciferase control ORF targeted sense and
89 antisense sequences into a pBAsi-hU6 Puromycin vector (TaKaRa), via *Bam*H1 and
90 *Xba*I restriction sites. The targeted A1 and luciferase sequences are as follows: A1-
91 736, 5'-GATCCCCGACGCTCCGTTACCCGGTTACGTGTGCTG
92 TCCGTAACCAGGTAATGGAGCATCTTTTTGGAAAT-3'; A1-1119, 5'-GATCCCCGTTC
93 TTCAAGGCTGCCGTTACGTGTGCTGTCCGTAATGGCAGCTTTGAAGAGCTTTTTGG

94 AAAT-3'; Luciferase, 5'-
95 GATCCCCGTGCGTTGTTGGTGTAAACGTGTGCTGTCCGTATTGGCA
96 CCAGCAGCGCACTTTTTGGAAAT-3'. A1-expressing RSCs were transfected with the
97 shRNA plasmids, respectively, using lipofectamine 2000, selected by puromycin for
98 individual clones and finally analyzed by western blotting for the suppression of A1.

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

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

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

116 of 1.00, and the number of cycles required to reach the threshold cycle, Ct, was
117 determined and then normalized with the Ct of the housekeeping gene, GAPDH. The
118 HSV-1 DNA copy number was measured as previously described (4). Thermocycler
119 conditions for the real-time PCR reaction are as follows: 95°C for 10 min, (95°C for 15s,
120 60°C for 1 min) x 40 cycles. The primers used for real-time PCR are as follows: A1
121 Forward, 5'-ACCACGCAGATCCTCGAAAT-3'; A1 Reverse, 5'-
122 TCTTGCTCCGTCATGATCTGG-3'; HSV-1 UL54 Forward, 5'-
123 CCGCGACGACCTGGAATCGG-3'; HSV-1 UL54 Reverse: 5'-
124 GGCGAGCGGCGTCGAGTATC-3'; HSV-1 UL30 Forward, 5'-
125 AGAGGGACATCCAGGACTTTGT-3'; HSV-1 UL30 Reverse, 5'-
126 CAGGCGCTTGTTGGTGTAC-3'; HSV-1 UL27 Forward, 5'-
127 TCGCCTTTCGCTACGTCAT-3'; HSV-1 UL27 Reverse, 5'-
128 GGTTCTTGAGCTCCTTGGTGG-3'; GAPDH Forward, 5' -
129 ACTAAAGGGCATCCTGGGCTA-3'; GAPDH Reverse: 5' -
130 TGGAAGAATGGGAGTTGCTGT- 3'.

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

138 under a confocal laser microscope (TCS SP2 AOBS, Leica Microsystems) using 405,
139 543, 633 nm excitations with 10x, 20x and 40x objectives.

140 **Sequencing analysis of UL54.** Nested PCR of UL54 was carried out with Pfu Ultra II
141 Fusion DNA polymerase (Stratagene). In the first round of PCR, 100 ng of template
142 cDNA or DNA, derived from either HSV-1 infected A1-expressing or control cells, was
143 used with the appropriate primers in a total volume of 25 μ l under the following
144 thermocycler conditions: 1 min at 98°C, (20 s at 98°C, 20 s at 57°C, and 30 s at 72°C) x
145 35, and 3 min at 72°C. For the second round of PCR, 2.5 μ l of the first PCR reaction
146 was used as a template in a total volume of 25 μ l under the following thermocycler
147 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
148 min at 72°C. The UL54 amplicon was cloned into a pUC19 vector and the DNA was
149 extracted from transformed bacteria. A BigDye® Terminator Cycle Sequencing Kit
150 (v3.1) was next used and samples were read on an ABI® Prism 3130 sequencer.
151 Sequences were analyzed using Sequencher 4.9 software. The primers used for the
152 nested PCR are listed as follows: First PCR UL54 forward, 5'-
153 AGCTTTGGCCGCAGCGCACA-3'; First PCR UL54Reverse, 5'-
154 GAGTTGCAATAAAAATATTTGCCGTGCAC-3'; Second PCR UL54 Forward, 5'-
155 GGTCTAGAAGCTTTGGCCGCAGCGCACA-5'; Second PCR UL54 Reverse, 5'-
156 ATCAAGCTTCCTCGCGCCTTCAGGTAGCA-3'.

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

159

160 **Results**

161 ***Survival rat model from encephalitis with intracranial HSV-1 inoculation***

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

172 In order to the elucidate factors contributing to the survival of the HSV-1-infected 14-
173 day-old rats, we focused on the observed phenotype in this age group. We proceeded
174 to inoculate 63 animals with HSV-1 for a more in-depth assessment of HSE. We found
175 that 17 HSV-1-injected rats (27%, indicated as severe in Table 1 and Figure 1A) died
176 after showing severe signs of encephalitis such as weight loss and quadriplegia within 5
177 days after inoculation. Fourteen injected rats (22%, indicated as mild in Table 1 and
178 Figure 1A) died after showing a milder degree of weight loss and quadriplegia between
179 5 and 7 days. Interestingly, 24 injected rats (38.1%, indicated as survived in Table 1
180 and Figure 1A) recovered from HSE after showing temporary paralysis; HSV-1 infection
181 in these mice was confirmed by GFP⁺ and HSV-1⁺ regions. The final group of 8 injected
182 rats did not exhibit symptoms of encephalitis and did not possess GFP⁺ and HSV-1⁺

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

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

199 To investigate whether our initial observation of reduced HSV-1 infection in survived
200 rats was due to the control of HSV-1 dissemination over the course of infection or
201 simply due to an inadequate initial infection, brain samples of survived rats were taken
202 12, 24, and 36 h after the disappearance of paralysis, and the extent of GFP⁺ regions
203 was determined. Results showed that the HSV-1-infected regions diminished over a
204 span of 36 h, suggesting that the virus was being contained and cleared (Figure 1C).
205 These data also suggest the existence of an anti-HSV factor(s) induced upon HSV-1

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

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

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

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

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

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

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

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

250 3C, the level of GFP expression was significantly attenuated in A1_{WT} RSC in
251 comparison to A1_{E63A} and A1_{Ctrl} RSC 48 h post infection. Interestingly, A1_{E63A} cells also
252 had a modest inhibitory effect on HSV-1, although this activity did not appear to be as
253 potent as in wild type expressing RSC, suggesting a somewhat deaminase-independent
254 inhibition mechanism (Figure 3C). When two different shRNA were used to specifically
255 target A1_{WT}, the infectivity of HSV-1 was restored and comparable to that observed in
256 A1_{Ctrl} (Figure 3D). Overall, the low and high level of A1 protein expression resulting
257 from specific shRNA (A1/736 and A1/1119) against A1 and control luciferase,
258 respectively, correlated inversely with HSV-1 infectivity (Figure 3D) and suggested that
259 A1 expression is essential for the inhibition of HSV-1 infection.

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

270 **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.

294 ***A1 induces mutations in HSV-1 DNA***

295 A1 homologues are putative inducers of mutations in retrovirus DNA and also in DNA of
296 A1-expressing bacteria (8, 23, 28, 43, 48). Thus, to investigate the mechanism of A1-
297 dependent inhibition of HSV-1 gene expression, we looked at the potential editing of
298 HSV-1 viral DNA. The UL54 DNA extracted from HSV-1-infected A1_{WT} RSC showed
299 evidence of both G→A and C→T transitions, indicating that plus and minus strands
300 were being edited (Figure 5B). The majority of the 51 clones analyzed from A1_{WT} RSC
301 contained 1 mutation within the sequenced region; however, a few clones harbored 2 or
302 3 mutations (Figure 5E). On the other hand, A1_{Ctrl} derived clones showed no signs of
303 mutations (Figure 5A and 5D), suggesting that the observed nucleotide changes were
304 induced by A1.

305 Given that rat A1 is also known to edit retroviral RNA, we looked at cDNA extracted
306 from A1_{WT} RSC 8 hpi at a MOI of 0.01 for evidence of additional deaminase activity (8).
307 Similar to DNA sequencing results, G→A and C→T transitions in viral cDNA were
308 observed, with the majority of clones containing 1 mutation, while clones with 2 or 3
309 mutations were also observed (Figure. 5C and 5F). Interestingly, 1 clone contained 10
310 G→A mutations, suggesting that a second round of editing may be occurring on mRNA
311 transcribed from the mutated DNA (Figure 5F); yet, the main editing appeared to be
312 occurring on the viral DNA which was mirrored by the mutations observed in viral cDNA
313 (Figure 5B and 5C). In looking at the type of mutations arising from 39 commonly
314 mutated positions in UL54 cDNA and 30 commonly mutated positions in UL54 DNA,
315 approximately 70% of mutations were found to lead to non-synonymous substitutions in
316 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.

338 **Discussion:**

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

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

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

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

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

382 If mutated viral transcripts are synthesized and make their way to the cytoplasm for
383 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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450 **Footnotes**

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

454 The authors declare no conflict of interest.

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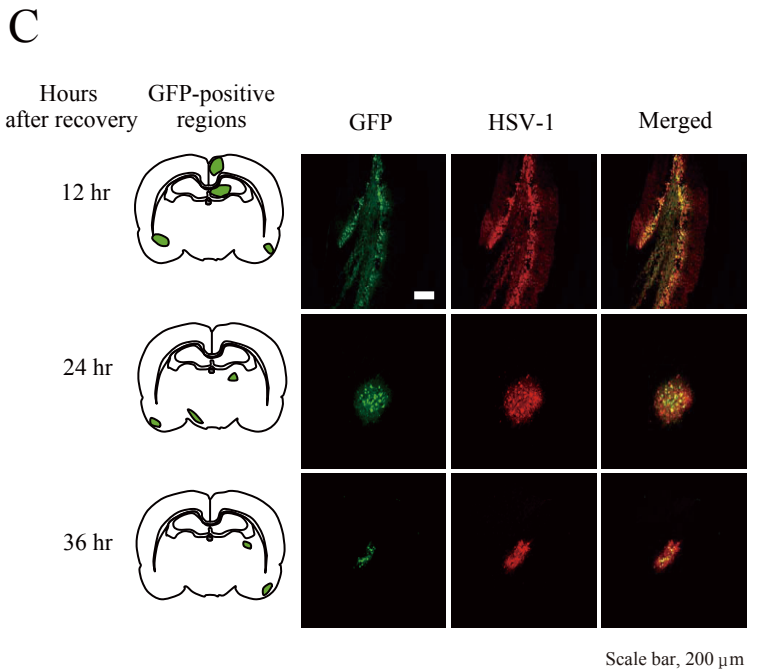
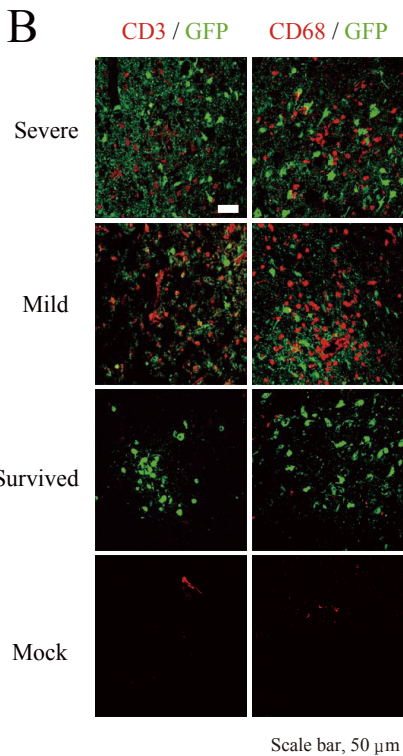
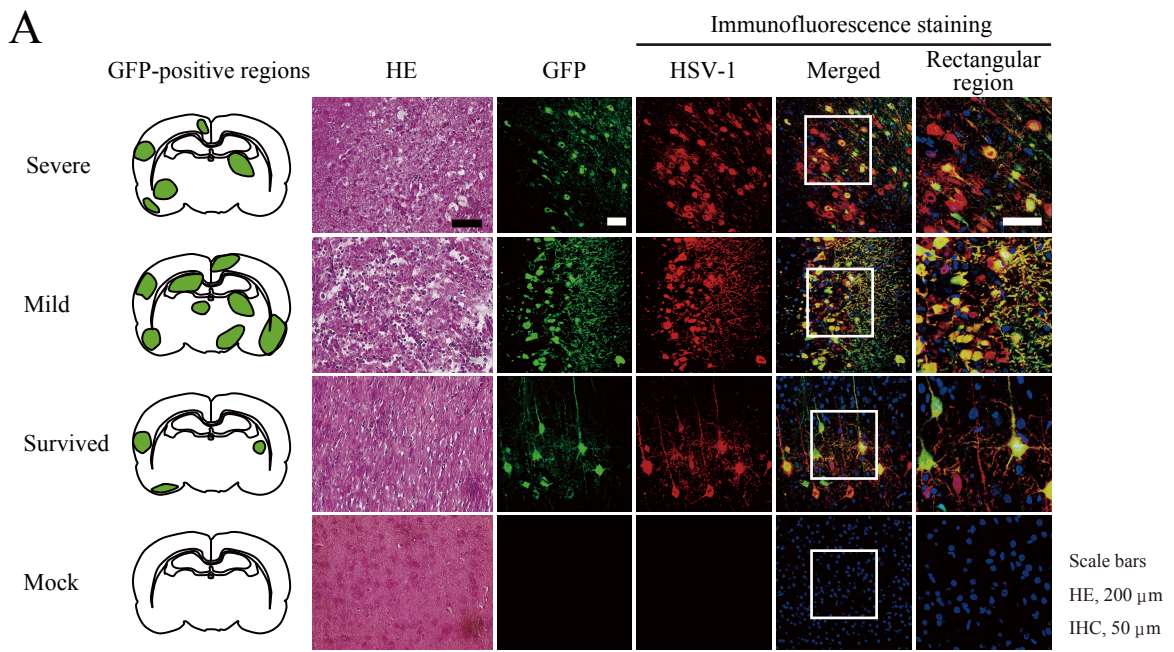


Figure 1. HSE model induced by GFP-HSV-1 inoculation of infant rats. Fourteen day-old infant rats were intracranially injected with GFP-expressing HSV-1 (YK333), and brain tissues were collected from rats exhibiting severe or mild encephalitis as described in Table 1 or from survived rats 36 h after 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.

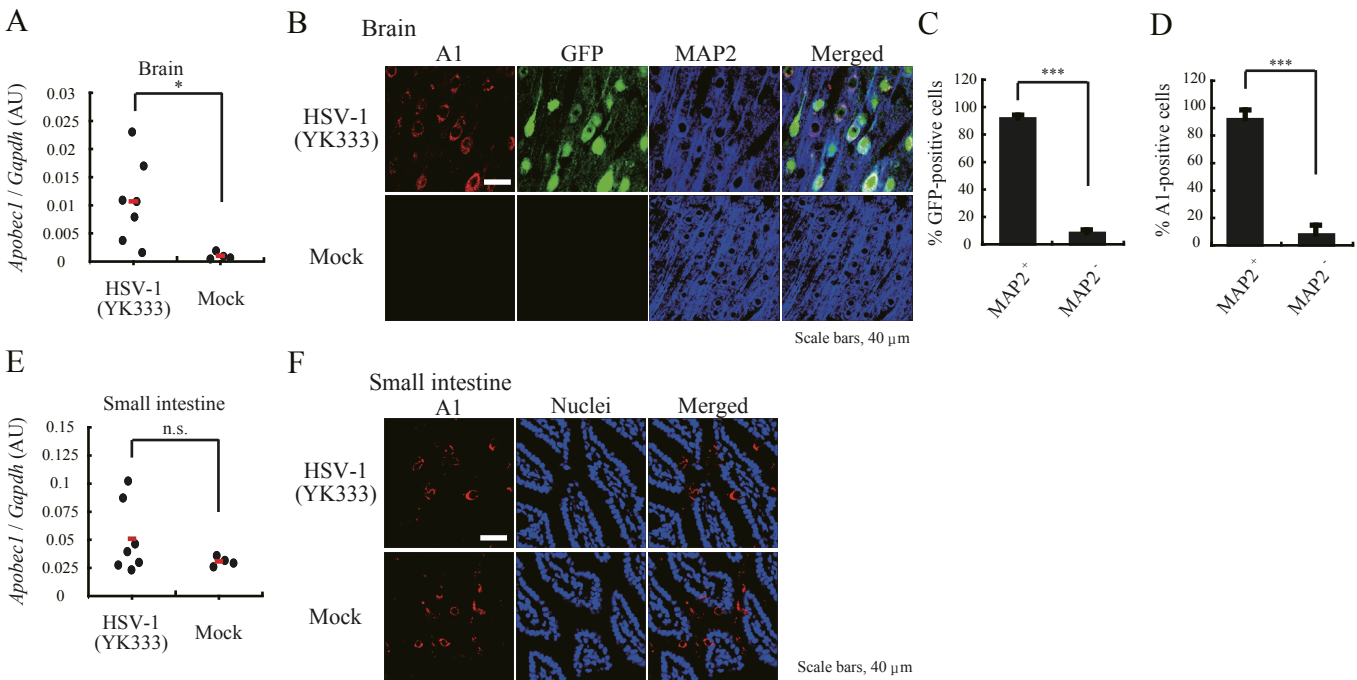


Figure 2. Expression of A1 in HSV-1-infected rat brains. (A) The level of A1 cDNA expression quantified by real-time PCR in the brain and small intestine of HSV-1-inoculated rats, which consisted of 4 mild rats and 2 recovery rats, and of 4 PBS-inoculated rats was assessed. (B) A1+ (red), GFP+ (green), 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.

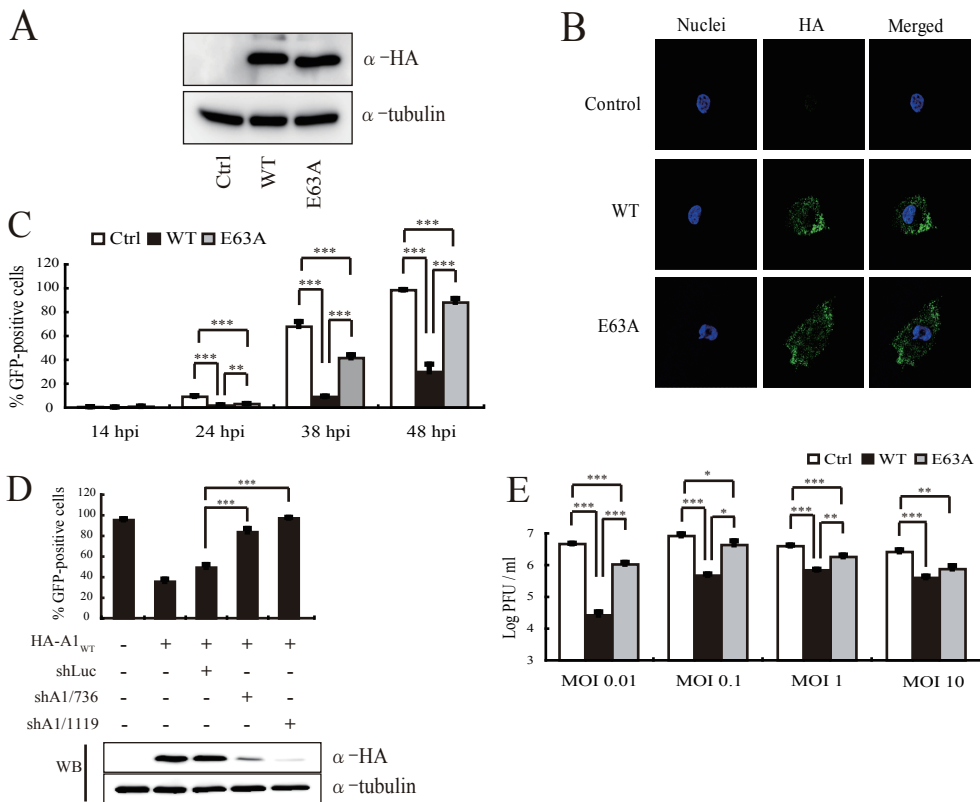


Figure 3. A1 inhibits HSV-1 infection. (A) Western blotting of HA-tagged WT, E63A, and Ctrl stably transfected cells probed with an anti-HA for A1 expression or an anti-tubulin antibody as an internal standard. (B) Immunofluorescence staining of HA-tagged A1WT, A1E63A or A1Ctrl RSC with an anti-HA antibody (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 *** $P < 0.0005$.

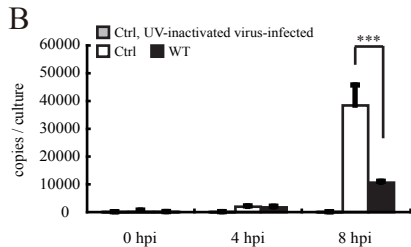
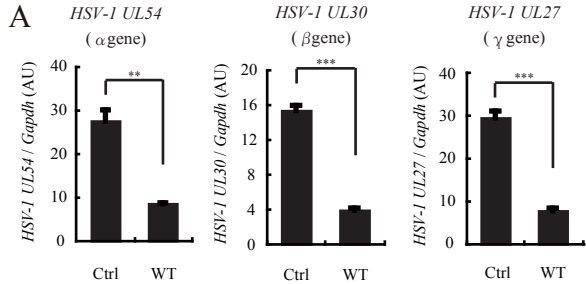


Figure 4. Inhibition of HSV-1 mRNA and DNA. (A) HSV-1 gene expression for UL54, UL30, and UL27 were measured by real-time PCR and normalized to GAPDH. Nucleic acids were extracted from HSV-1 infected WT and Ctrl RSC 8 hpi. (B) DNA copy number was measured by real-time PCR in A1 and control RSC 4 and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

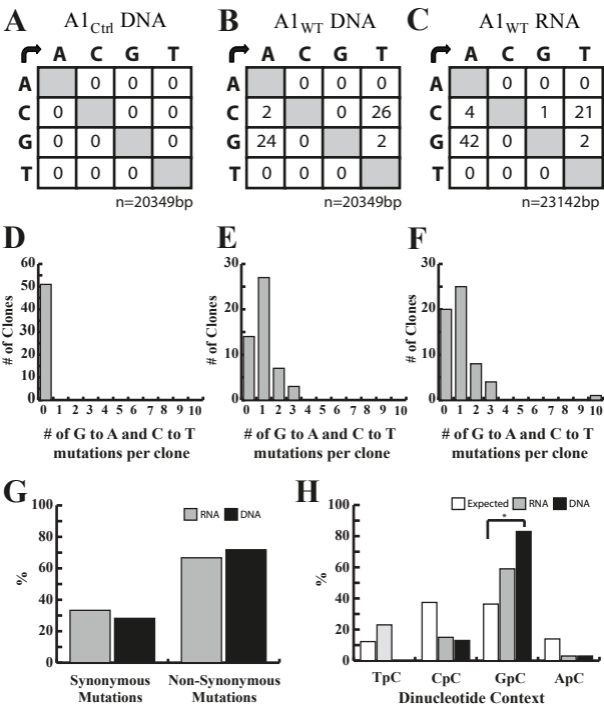


Figure 5. Sequencing analysis of UL54 DNA and cDNA from A1_{WT} and A1_{Ctrl} HSV-1 infected RSC. A 399 bp region of UL54 DNA or cDNA, extracted 8 hpi, was amplified by nested PCR and cloned for sequencing. Mutation matrices are depicted for (A) DNA extracted from A1_{Ctrl} RSC, 51 clones; (B) DNA extracted from A1_{WT} RSC, 51 clones; and (C) cDNA extracted from A1_{WT} 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 A1_{WT} RSC; and (F) cDNA extracted from A1_{WT} 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 ($p < 0.001$).

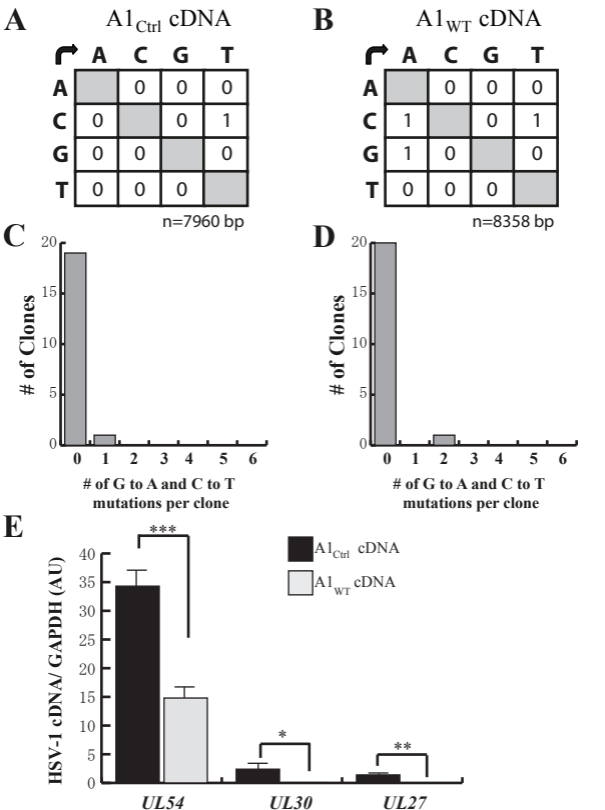


Figure 6. A1 inhibition of HSV-1 RNA transcripts is independent of RNA mutagenesis. $A1_{WT}$ and $A1_{Ctrl}$ RSC were infected with YK333 at a MOI of 1 for 2 h in the presence of 25 $\mu\text{g}/\text{mL}$ of acyclovir. Mutation matrices for UL54 cDNA extracted from (A) $A1_{Ctrl}$ RSC and (B) $A1_{WT}$ 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%.