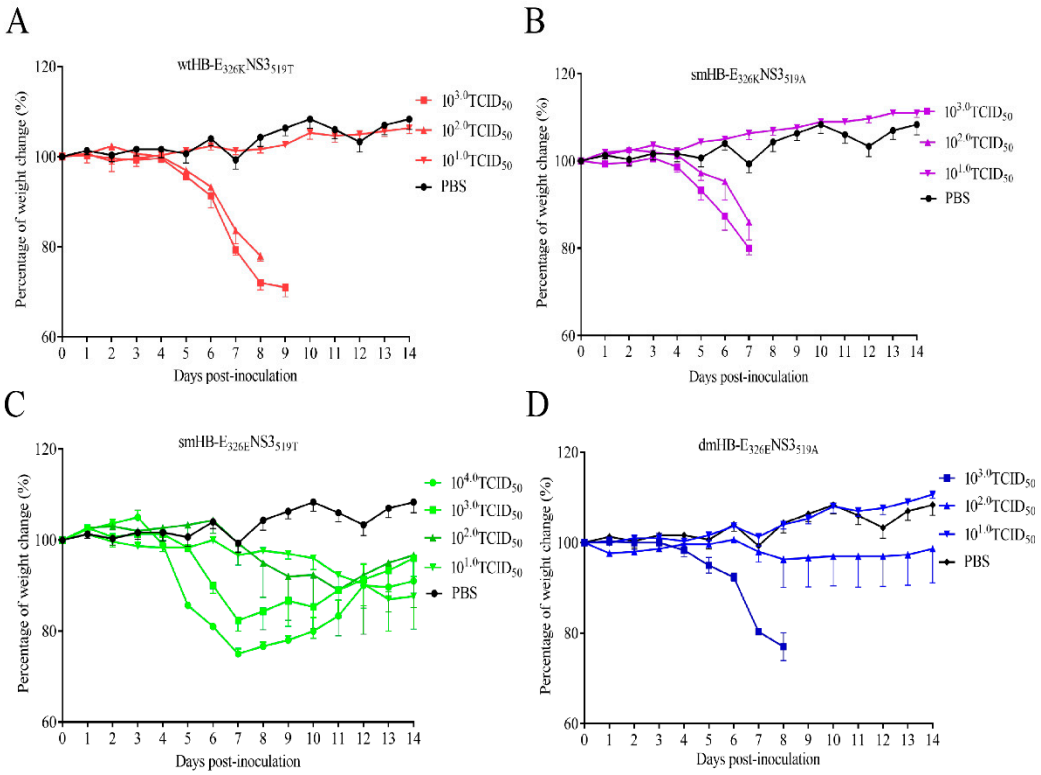

Figure S1. Weight change curves of mice. (A to D) Five-week-old BALB/c mice were infected by the i.c. route with four viruses at different doses (10^1.0, 10^2.0, 10^3.0 or 10^4.0 $TCID_{50}$), respectively or mock infected. Animals were monitored daily for the appearance of symptoms and weight changes were recorded during the two weeks period of observation.

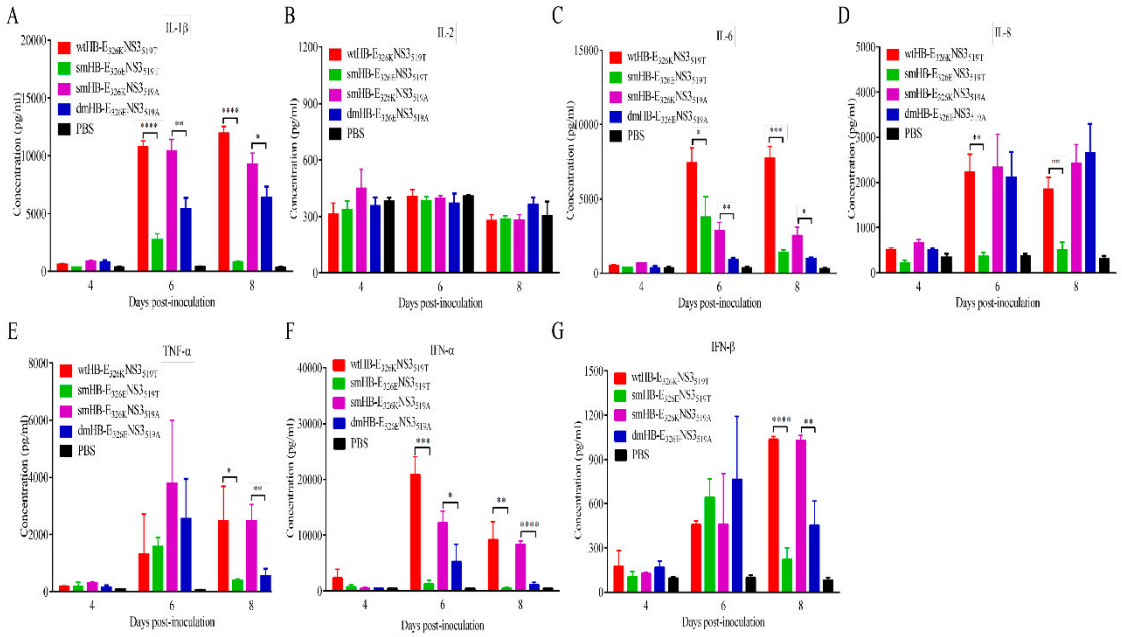

Figure S2. The wtHB-$E_{326K}NS3_{519T}$ and mutant viruses induce different protein levels inflammatory cytokines in the mouse brain. Five-week-old BALB/c mice were infected by the i.c. route with virus at a dose 10^3.0 $TCID_{50}$. Supernatants of infected mouse brain homogenates

were collected for measurement of pro-inflammatory cytokines(A-E) and IFN-α/β (F and G) at protein levels by ELISA. (****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

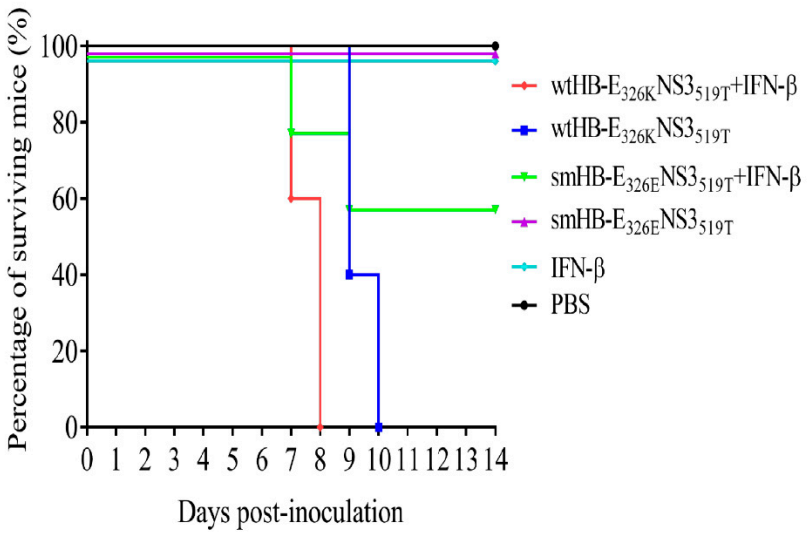

Figure S3. The death of infected mice was promoted by i.c. injection of IFN-β. Five-week-old BALB/c mice were infected by the i.c. route with virus at a dose 10^3.0 $TCID_{50}$. Human recombinant IFN-β was injected into the brain of mice at a dose of 3000 U at 3 dpi and 5 dpi to observe the survival of the mice.

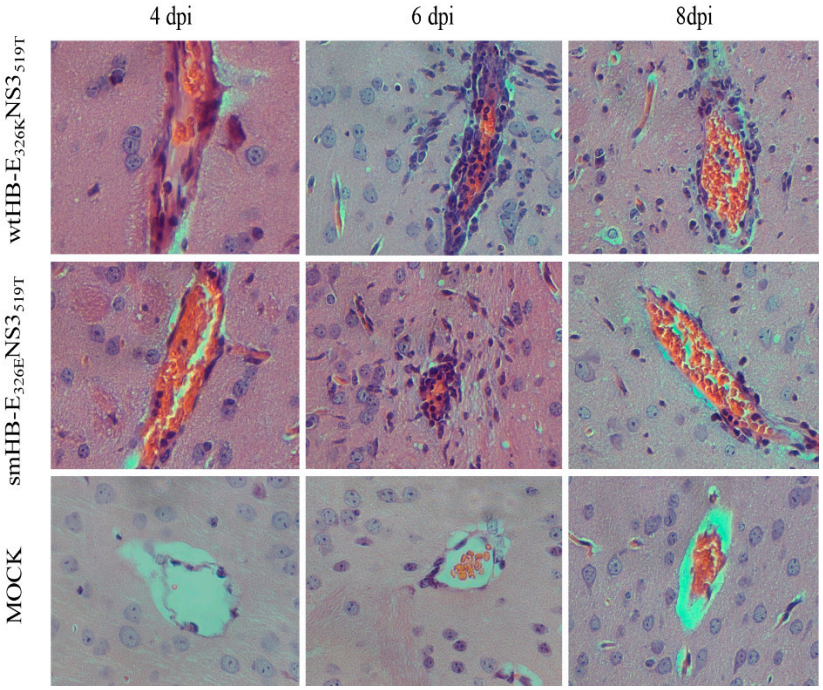

Figure S4. Histopathologic analysis of the hematoxylin and eosin (HE)-stained brain tissue of mice infected. Five-week-old female BALB/c mice were infected by the i.c. route with wtHB-$E_{326K}NS3_{519T}$ and smHB-$E_{326E}NS3_{519T}$ at a dose 10^3.0 $TCID_{50}$. Brain tissues of infected mice was collected at 4, 6, and 8 dpi for histopathological analysis.

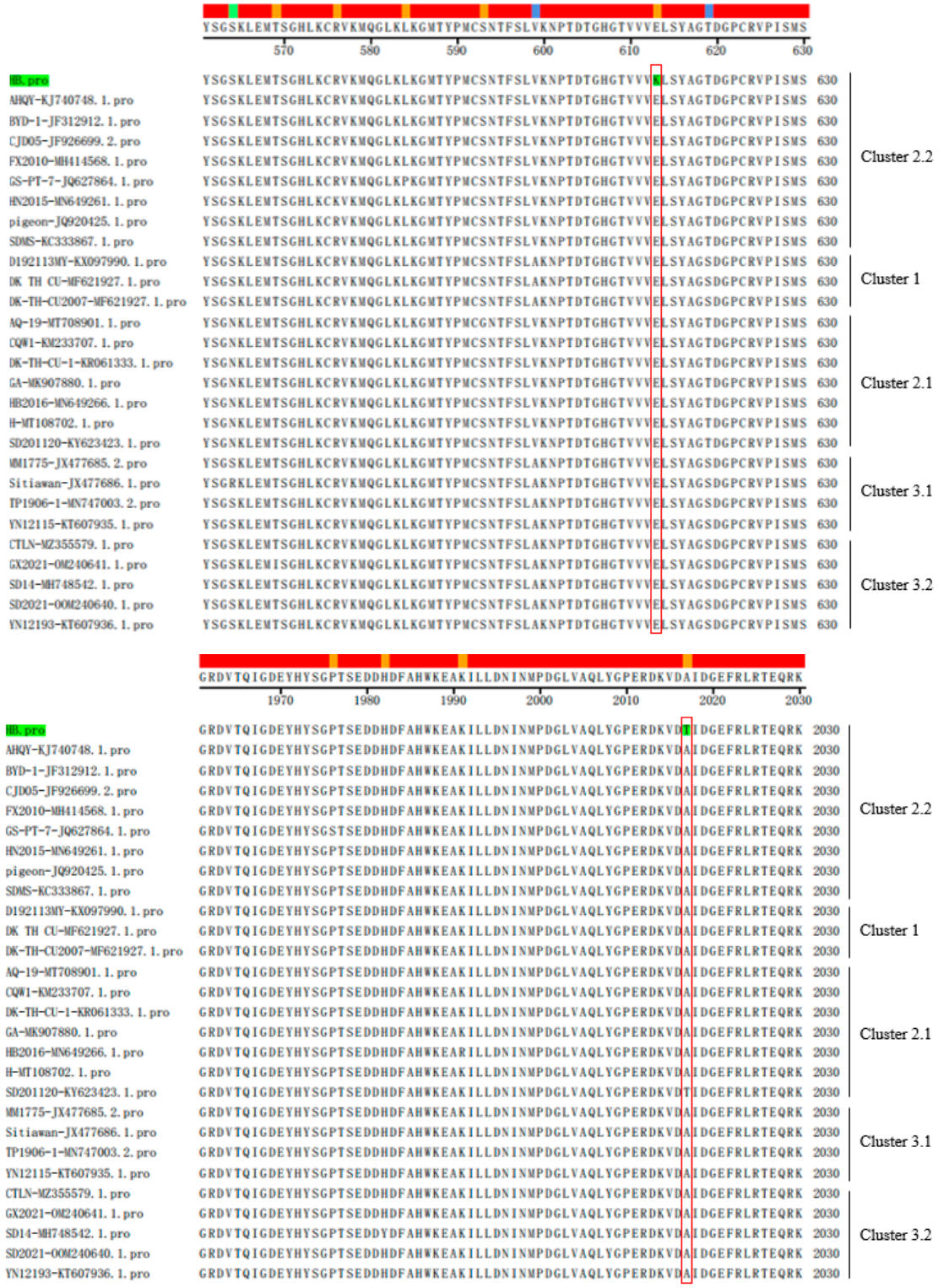

Figure S5. Sequence analysis results. The sequencing results were analyzed using DNASTAR software. The reference virus strain information have been supplemented in Table S2.

Table S1. Primers used in the study

Primer name	Sequence (5′to 3')	Positions	Application
HBRT1	ACACATCGGGTAGGTC	1856-1871	Specific reverse transcription primer
HBRT2	CCTTCTCAAAATCTCC	3578-3593	Specific reverse transcription primer
HBRT3	ATCTCTGTGATGCTCC	4404-4419	Specific reverse transcription primer
HBRT4	GTCAACCTTGTCCCGC	2433-2457	Specific reverse transcription primer
HBRT5	TGCCGTCGCTGGTCTCAA	3806-3831	Specific reverse transcription primer
HBRT6	TAACCTCTCACTTCCT	3656-3680	Specific reverse transcription primer
HBRT7	AGACTCTGTGTTCTAC	10957-10991	Specific reverse transcription primer
HBT7-1F	CCCGGGTAATACGACTCACTATAGGGA GAAGTTCATCTGTGTGAACTTATTCC	1-27	Amplifying HB
HB-956R	GCTGTACGCTGGGGC	942-956	Amplifying HB
HB-942F	GCCCCAGCGTACAGCTT	942-958	Amplifying HB
HB-2459R	GGCATTGACATTTACTGCC	2441-2459	Amplifying HB
HB-2433F	GTCTTCCTGGCAGTAAATGTCAATG	2433-2457	Amplifying HB
HB-3831R	ATCTGCTTTCCTGATTGCTCCACTTC	3806-3831	Amplifying HB
HB-3656F	AGGAATCACGTACAGTGATCTGGTC	3656-3680	Amplifying HB
HB-10991R	AGACTCTGTGTTCTACCACCACCAGCC ACACTTTC	10957-10991	Amplifying HB
E-K326E-F	GGCACTGTCGTGGTG**GAA**TTGT	1917-1938	E K326E mutation
E-K326E-R	ACAA**TTC**CACCACGACAGTGCC	1917-1938	E K326E mutation
NS3-T519A-F	GACAAGGTTGAC**GCA**ATTGATG	6132-6153	NS3 T519A mutation
NS3-T519A-R	CATCAAT**TGC**GTCAACCTTGTC	6132-6153	NS3 T519A mutation
HB-E-1936-F	TGTCTTATGCAGGTACCGATG	1936-2040	qRT-PCR primer
HB-E-2040-R	CGTATGGGTTGACTGTTATCA	1936-2040	qRT-PCR primer
HB-E-probe	FAM-AGTTCCCATATCCATGTC-TAMRA	1967-1984	Probe primer
qMouse-IL-1β-F	GGTGTGTGACGTTCCCATTA	_	qRT-PCR primer
qMouse-IL-1β-R	ATTGAGGTGGAGAGCTTTCAG	_	qRT-PCR primer
qMouse-IL-2-F	TGAGCAGGATGGAGAATTACAG	_	qRT-PCR primer
qMouse-IL-2-R	GAGGTCCAAGTTCATCTTCTAGG	_	qRT-PCR primer
qMouse-IL-6-F	CTTCCATCCAGTTGCCTTCT	_	qRT-PCR primer

qMouse-IL-6-R	CTCCGACTTGTGAAGTGGTATAG	_	qRT-PCR primer
qMouse-IL-8-F	TGGGAGAATTCAAGGTGGATAAG	_	qRT-PCR primer
qMouse-IL-8-R	GACAGCATCTGGCAGAATAGAG	_	qRT-PCR primer
qMouse-TNF-α-F	GTTCTATGGCCCAGACCCTCA	_	qRT-PCR primer
qMouse-TNF-α-R	CAGCAAGCATCTATGCACTTAG	_	qRT-PCR primer
qMouse-IFN-α-F	CCCGCAGGAGAAGGTGGAT	_	qRT-PCR primer
qMouse-IFN-α-R	GAGCTGCTGGTGGAGGTCA	_	qRT-PCR primer
qMouse-IFN-β-F	GCTGCGTTCCTGCTGTGCT	_	qRT-PCR primer
qMouse-IFN-β-R	CATCTTCTCCGTCATCTCCA	_	qRT-PCR primer
qMouse-IFN-γ-F	ACTGGCAAAAGGATGGTGAC	_	qRT-PCR primer
qMouse-IFN-γ-R	TGAGCTCATTGAATGCTTGG	_	qRT-PCR primer
qMouseGAPDH-F	GAGGCCGGTGCTGAGTATGT	_	qRT-PCR primer
qMouseGAPDH-R	CGGCAGAAGGGGCGGAGATG	_	qRT-PCR primer
qMouse-RIG-I-F	GAGCCAGCGGAGATAACAATA	_	qRT-PCR primer
qMouse-RIG-I-R	CCCACGTACTCATAGAGAATGAC	_	qRT-PCR primer
qMouse-MDA5-F	GCTAAAGACGGAAATCGCAAAG	_	qRT-PCR primer
qMouse-MDA5-R	GAATGTCTCCAGGTGGCTATATG	_	qRT-PCR primer
qMouse-TLR3-F	ACCTCCAGAAGAACCTCATAAC	_	qRT-PCR primer
qMouse-TLR3-R	GAACGGATTGAAGCGCATATC	_	qRT-PCR primer
qMouse-TLR7-F	AACCTTTCCCAGAGCATACAG	_	qRT-PCR primer
qMouse-TLR7-R	GGAGCCTCTGATGAGACAAATAA	_	qRT-PCR primer
qMouse-IRF3-F	GTCTTAAGGAGCTGTTAGAGATGG	_	qRT-PCR primer
qMouse-IRF3-R	TGGTCAGAGGTAAGGGAGATAG	_	qRT-PCR primer
qMouse-IRF7-F	TTCTGCAGTACAGCCACATAC	_	qRT-PCR primer
qMouse-IRF7-R	GCATAGGGTTCCTCGTAAACA	_	qRT-PCR primer

Table S2. The reference virus strain information

Strains	GenBank No.	Source	Date
FX2010	MH414568.1	Duck	2010
BYD-1	JF312912.1	Duck	2010
pigeon	JQ920425.1	pigeon	2012
CJD05	JF926699.2	Chicken	2010
AHQY	KJ740748.1	Duck	2013
HN2015	MN649261.1	Duck	2015
SDMS	KC333867.1	Mosquito	2012
GS-PT-7	JQ627864.1	Goose	2010
CQW1	KM233707.1	Duck	2013
SD201120	KY623423.1	Duck	2011
GA	MK907880.1	Duck	2018
AQ-19	MT708901.1	Goose	2019
HB2016	MN649266.1	Duck	2016
H	MT108702.1	Duck	2019
DK/TH/CU-1	KR061333.1	Duck	2013
D1921/1/3MY	KX097990.1	Duck	2012
D1977/1/MY	KX097989.1	Duck	2012
DK-TH-CU2007	MF621927.1	Duck	2007
Sitiawan	JX477686.1	Chicken	2002
MM1775	JX477685.2	Mosquito	1995
TP1906	MN747003.2	Mosquito	2019
YN12115	KT607935.1	Mosquito	2012
GX2021	OM240641.1	Chicken	2021
SD2021	OM240640.1	Chicken	2021
YN12193	KT607936.1	Mosquito	2012
SD14	MH748542.1	Duck	2014
CTLN	MZ355579.1	Chicken	2020