An RNA interference therapeutic potentially achieves functional cure of chronic hepatitis B virus infection

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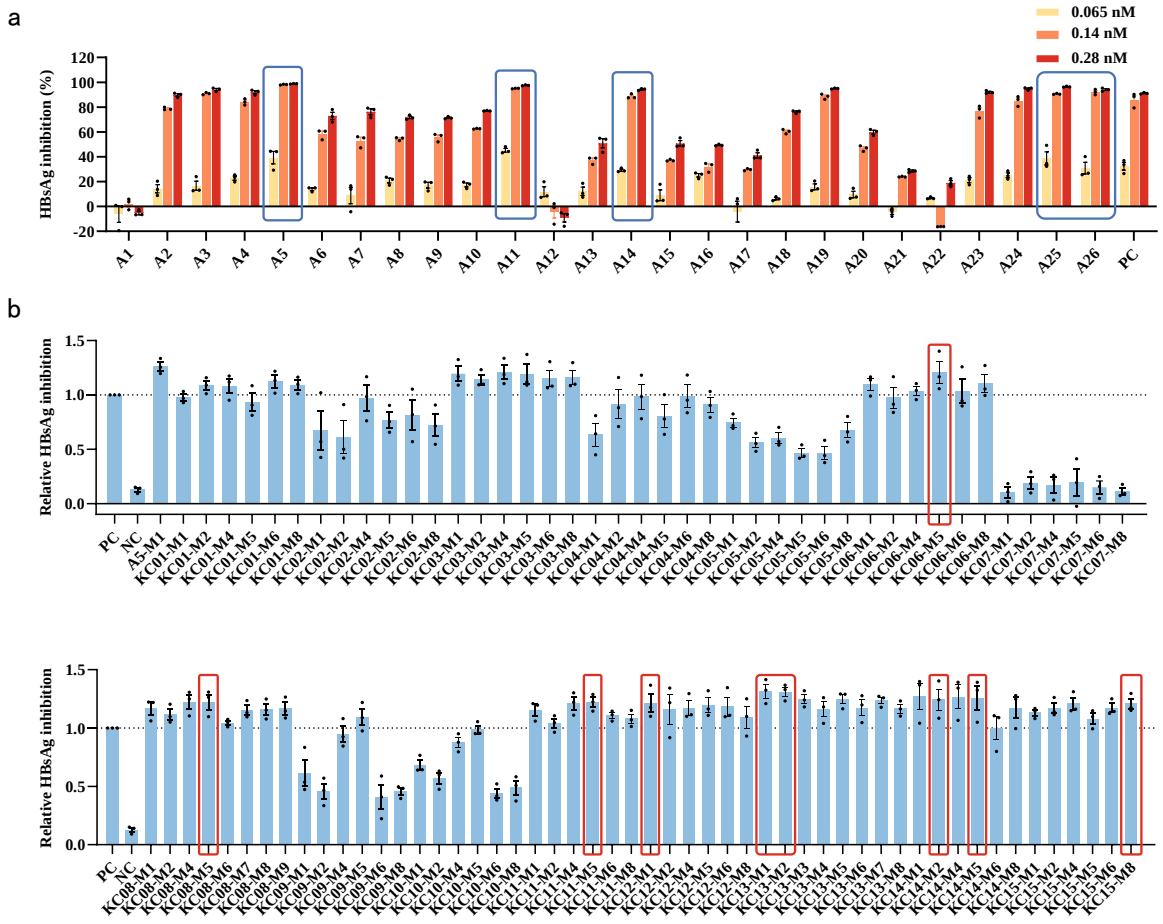
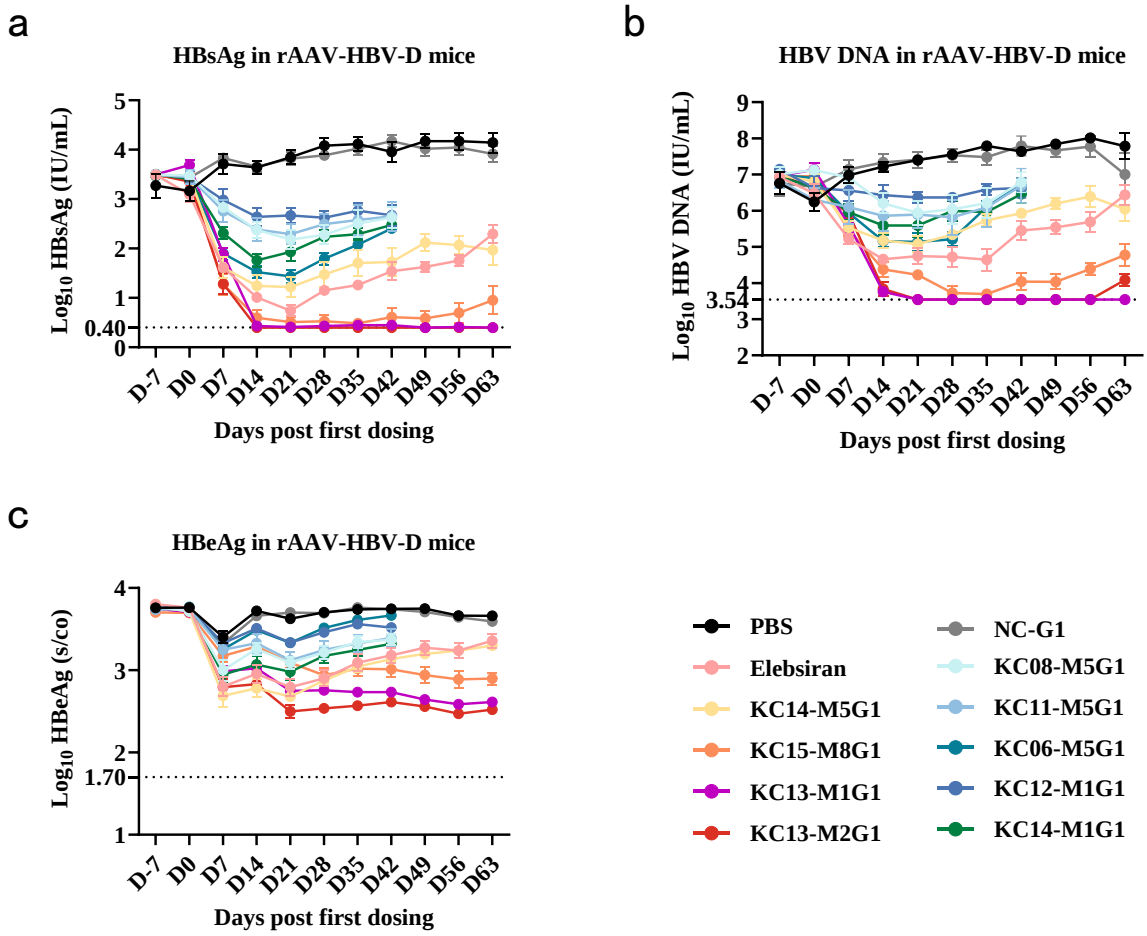
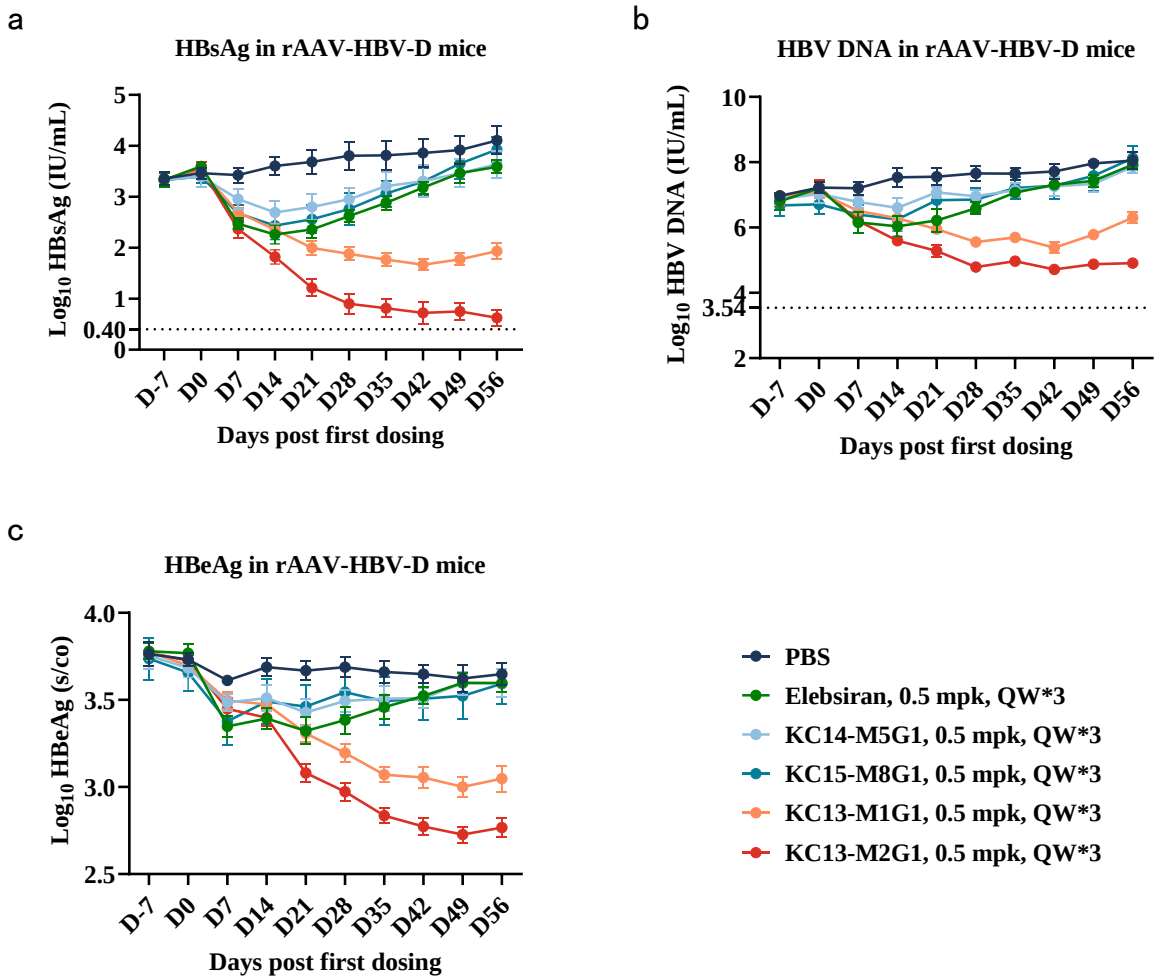
Supplementary Figures



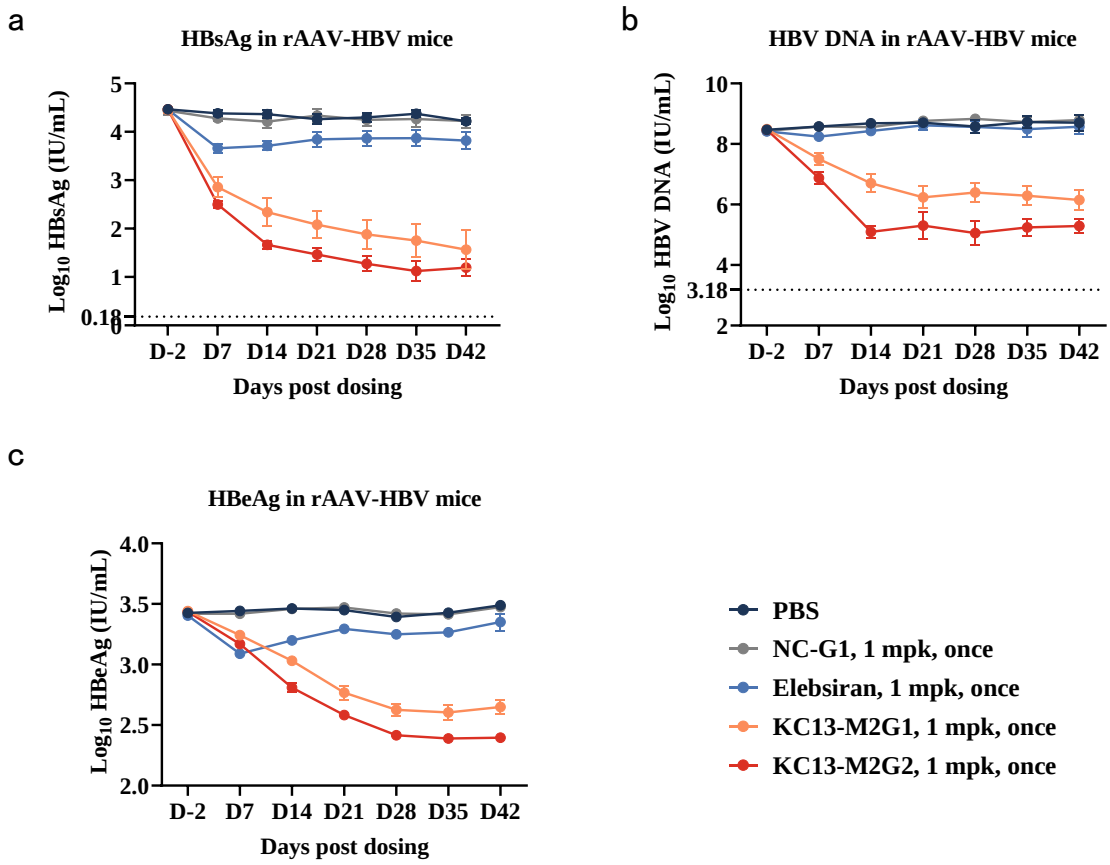
Supplementary Fig. 1 *In vitro* screening of anti-HBV siRNA agents in HepG2.2.15 cells. a The first screening round identified sensitive target sites, from which five candidates were selected for the second screening round. **b** The second screening round identified optimal modification patterns, from which nine candidates were selected for further evaluation in rAAV-HBV mice. The siRNAs were transfected into cells at 0.065, 0.14, and 0.28 nM (**a**) or 0.3 nM (**b**). On day 6 post-transfection, HBsAg in supernatants were quantified using chemiluminescence immunoassay (CLIA) commercial Kits. Data are shown as mean ± SEM (n=3 biologically independent replicates). Source data are provided as a Source Data file.



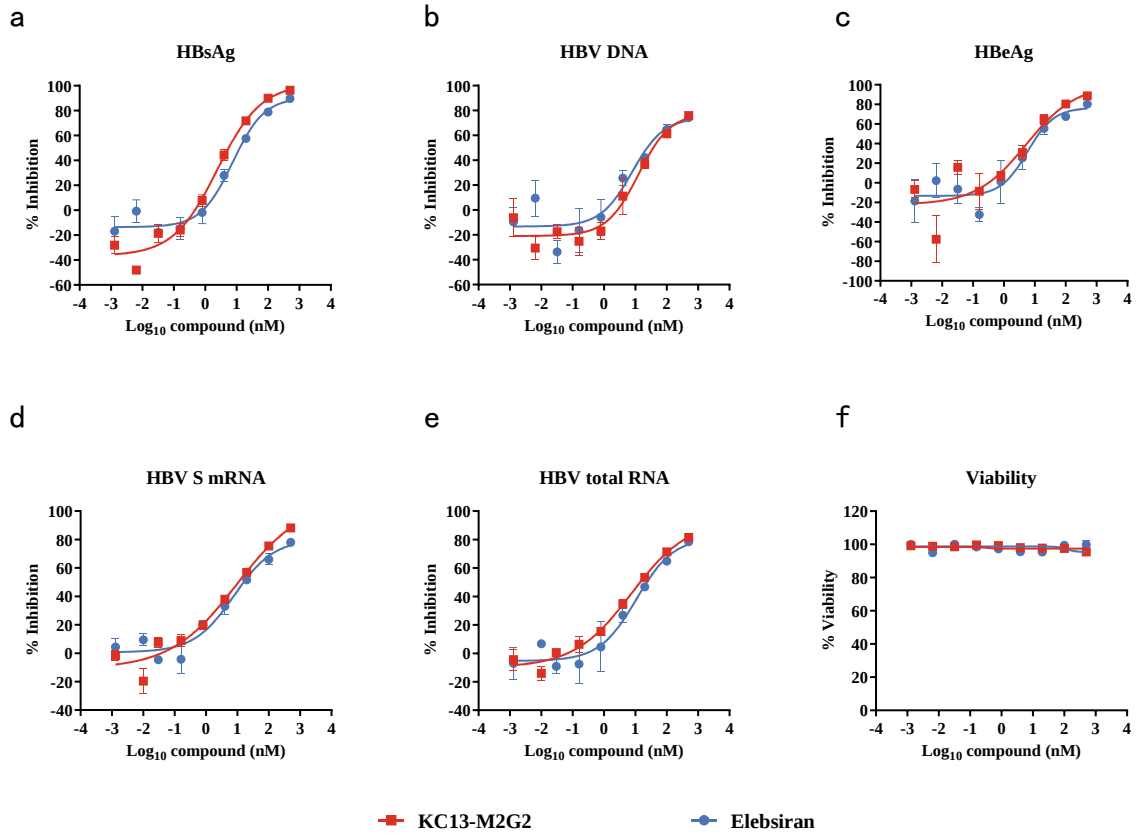
Supplementary Fig. 2 *In vivo* screening of GalNAc-conjugated siRNA candidates in rAAV-HBV-D mice. Nine selected siRNAs conjugated with the proprietary GalNAc ligand G1 were administered subcutaneously at a dose of 3 mg/kg once weekly for three weeks. Plasma levels of HBsAg (**a**) and HBeAg (**c**) were quantified by chemiluminescence immunoassay (CLIA) assays on commercial analytical systems, HBV DNA (**b**) by PCR-fluorescence probing assay at certain timepoints, with the first dosing day defined as D0. Data are showed as mean ± SEM (n=6 mice). Dashed lines indicate LLOQs. Source data are provided as a Source Data file.



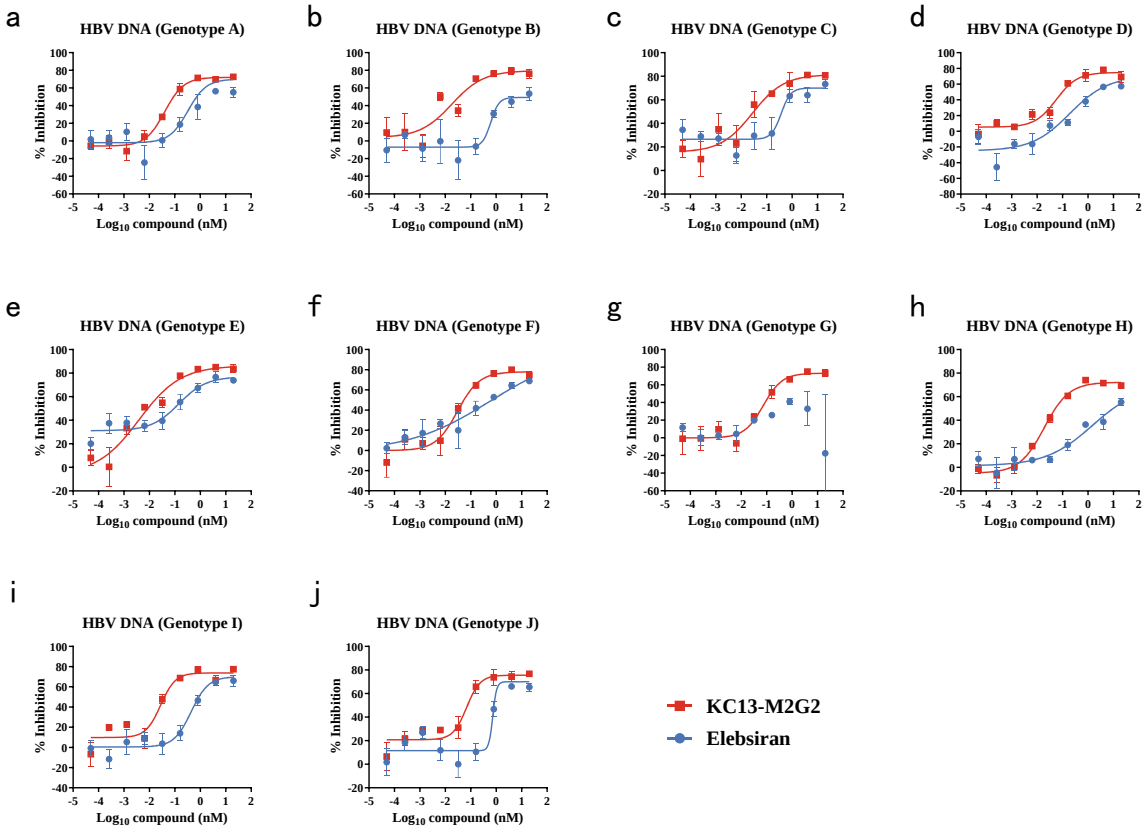
Supplementary Fig. 3 Lower-dose study (0.5 mg/kg, once weekly for three weeks) for siRNA screening in rAAV-HBV-D mice. Four selected siRNAs, conjugated with the proprietary GalNAc ligand (G1), were evaluated. Plasma levels of HBsAg (**a**) and HBeAg (**c**) were quantified by chemiluminescence immunoassay (CLIA) assays on commercial analytical systems, HBV DNA (**b**) by PCR-fluorescence probing assay at certain timepoints. The first dosing day was defined as D0. Data are shown as mean ± SEM (n=5 mice for D56 in PBS group and n=6 for the other groups and timepoints). Dashed lines indicate LLOQs. Source data are provided as a Source Data file.



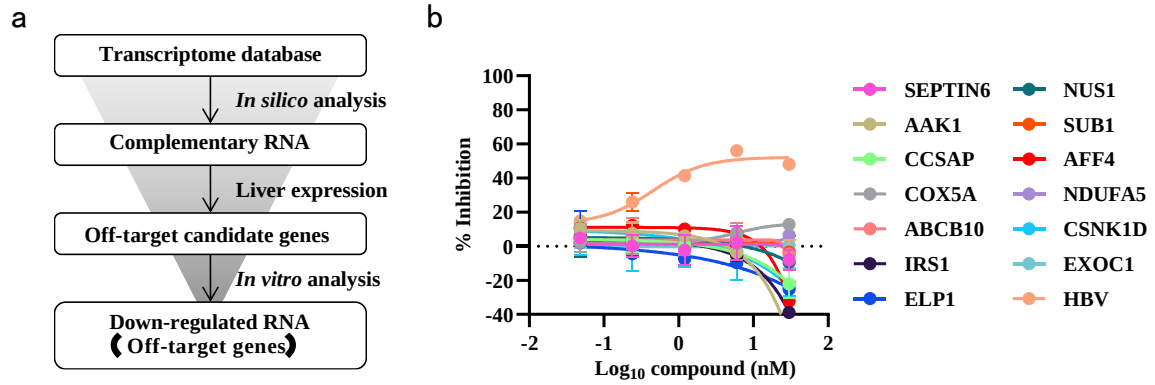
Supplementary Fig. 4 GalNAc screening for KC13-M2 in rAAV-HBV mice. KC13-M2G2 conjugated with either proprietary GalNAc ligand G1 or G2 was administered subcutaneously at a single dose of 1 mg/kg. Plasma levels of HBsAg (**a**) and HBeAg (**c**) were quantified by chemiluminescence immunoassay (CLIA) assays on commercial analytical systems, HBV DNA (**b**) by PCR-fluorescence probing assay at certain timepoints. The dosing day was defined as D0. Data are shown as mean ± SEM (n=6 mice). Dashed lines indicate LLOQs. Source data are provided as a Source Data file.



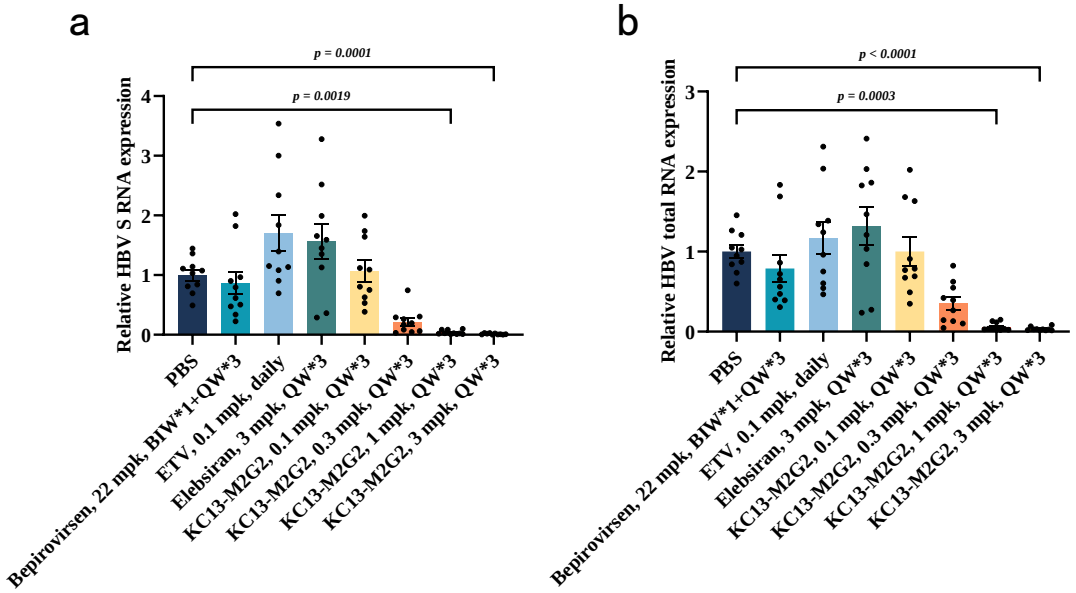
Supplementary Fig. 5 *In vitro* performances of KC13-M2G2 in HBV-infected PHHs. KC13-M2G2 and elebsiran were freely uptaken into cells at concentrations of 500 to 0.0013 nM. On day 8 post-transfection, HBsAg (**a**), and HBeAg (**c**) in supernatants were quantified using chemiluminescence immunoassay (CLIA) commercial Kits, and HBV DNA (**b**) was quantified using HBV primers and qPCR. HBV S mRNA (**d**) and total RNA (**e**) in cells were detected by RT-qPCR. The primers used were listed in Supplementary Table 5. Cell viabilities (**f**) were determined using CTG according to the manuals. Data are shown as mean ± SEM (n=3 biologically independent replicates). Source data are provided as a Source Data file.



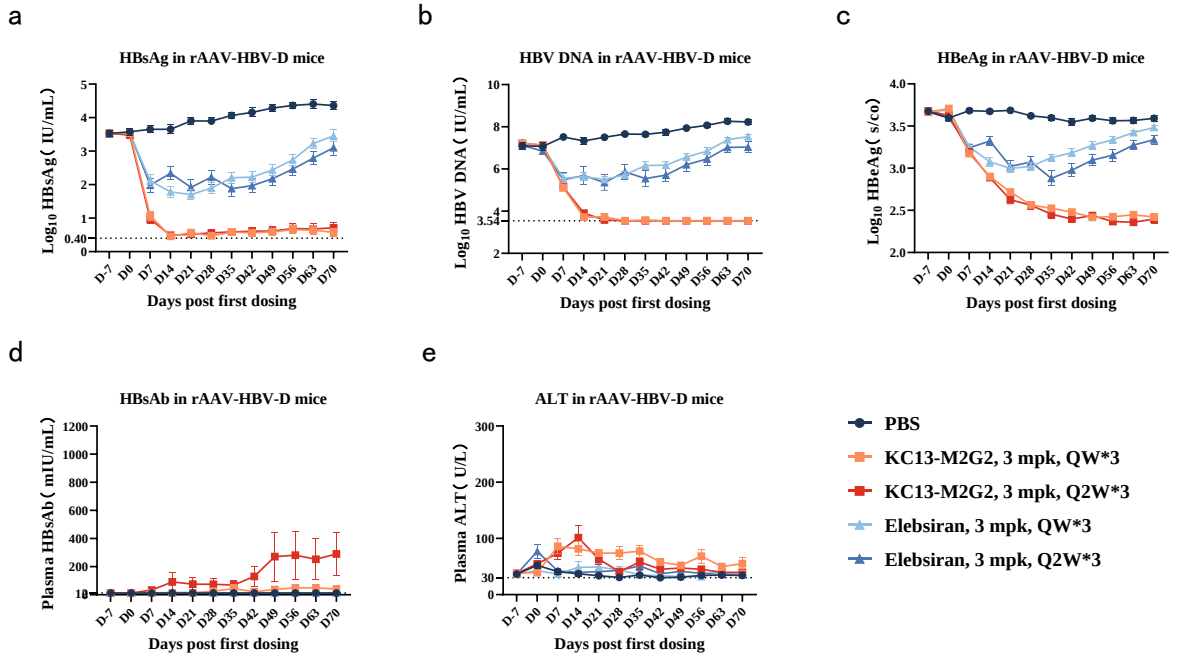
Supplementary Fig. 6 HBV DNA inhibitions of KC13-M2G2 in HBV-transfected HepG2 cells related to Fig. 2. HepG2 cells were transfected with 10 kinds of HBV genotype plasmids (**a-j**) on day 0 and siRNA agents on day 1. HBV DNA content in supernatants were detected using HBV primers and qPCR on the eighth day. The primers used were listed in Supplementary Table 5. Data are shown as mean ± SEM (n=3 biologically independent replicates). Source data are provided as a Source Data file.



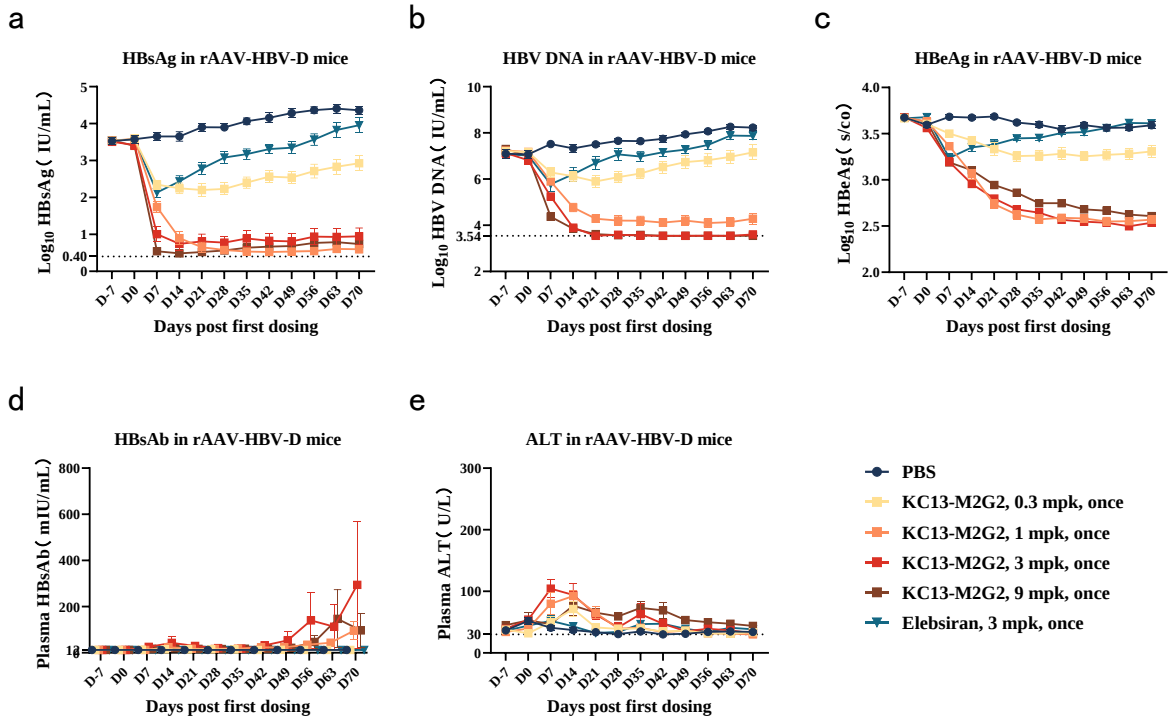
Supplementary Fig. 7 *In silico* off-target analysis and validation of KC13-M2G2. a Scheme for miRNA-like off-target effect assessment. **b** Off-target gene inhibitions of KC13-M2G2 evaluated in hepG2.2.15 cells. KC13-M2G2 was transfected into cells at concentrations of 30 to 0.048 nM. At 48 h post-transfection, off-target gene mRNAs in cells were detected by RT-qPCR. The primers used were listed in Supplementary Table 5. Data are shown as mean ± SEM (n=3 biologically independent replications). Source data are provided as a Source Data file.



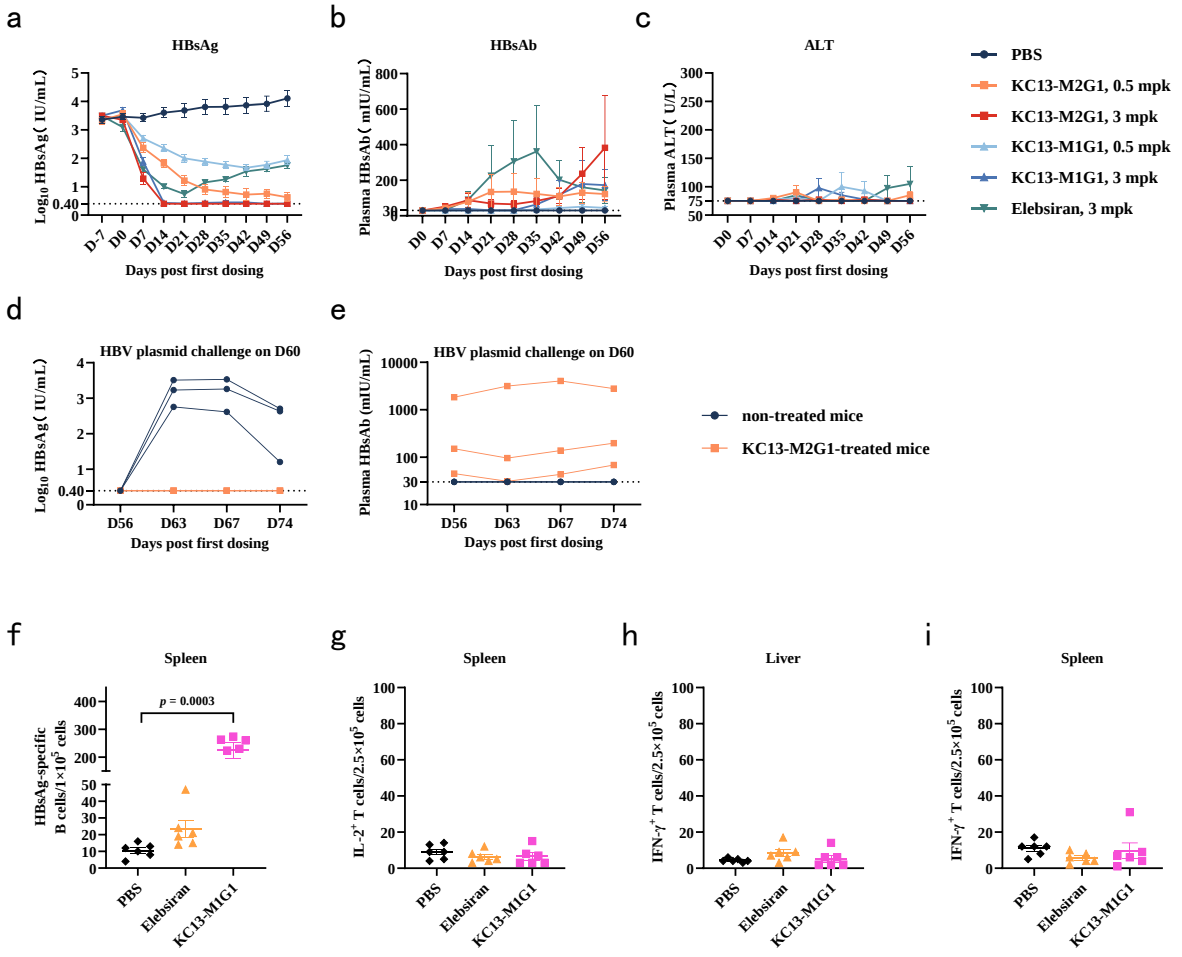
Supplementary Fig. 8: Intrahepatic HBV RNA levels corresponding to Fig. 4. The intrahepatic HBV S RNA **(a)** and total RNA **(b)** levels were measured at the terminal timepoint (Day70) by RT-qPCR. Data are shown as mean ± SEM (n=10 mice). Only statistically significant differences are indicated (Kruskal-Wallis test with Dunn's post-hoc test for multiple groups). Source data are provided as a Source Data file.



Supplementary Fig. 9 Efficacy of KC13-M2G2 in rAAV-HBV-D mice at three weekly or every-two-week doses of 3 mg/kg. The first dosing day was defined as D0. Plasma levels of HBsAg (**a**), HBV DNA (**b**), HBeAg (**c**), HBsAb (**d**) and ALT (**e**) were detected at certain timepoints. The levels of HBsAg, HBeAg, and HBsAb were quantified by chemiluminescence immunoassay (CLIA) assays on commercial analytical systems, HBV DNA by PCR-fluorescence probing assay, and ALT by enzymatic method. Data are shown as mean ± SEM (n=10 mice). Dashed lines indicate LLOQs. Source data are provided as a Source Data file.

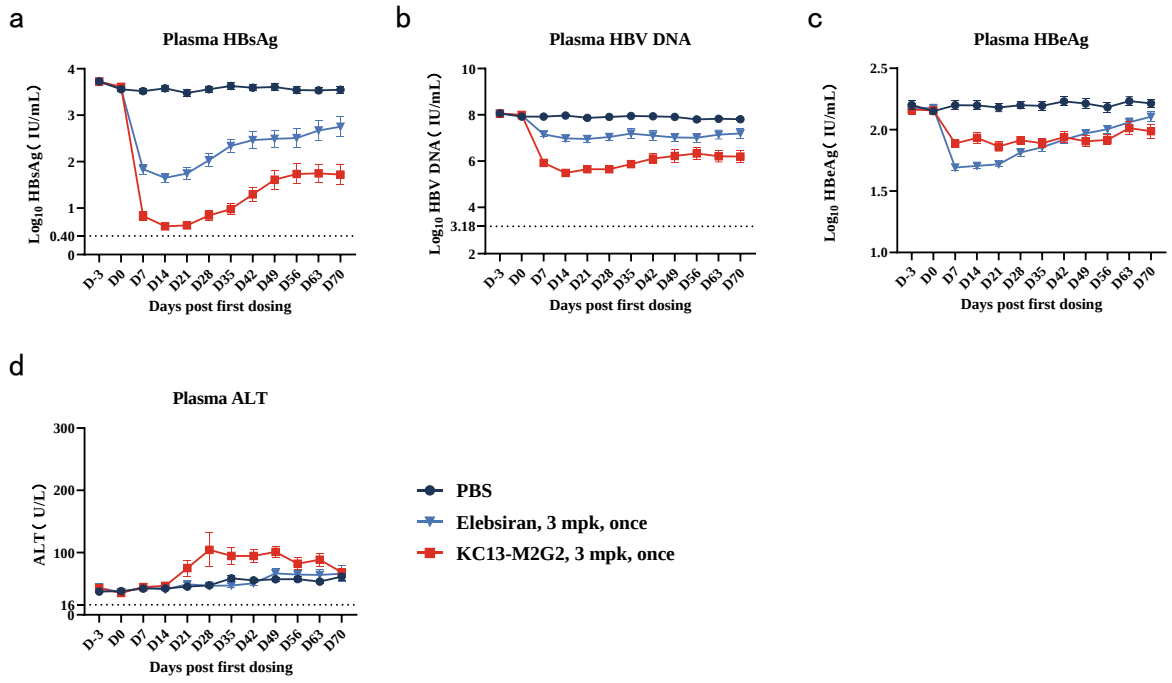


Supplementary Fig. 10 Efficacy of KC13-M2G2 in rAAV-HBV-D mice at single dose of 0.3, 1, 3, and 9 mg/kg. The first dosing day was defined as D0. Plasma levels of HBsAg (**a**), HBV DNA (**b**), HBeAg (**c**), HBsAb (**d**) and ALT (**e**) were detected at certain timepoints. The levels of HBsAg, HBeAg, and HBsAb were quantified by chemiluminescence immunoassay (CLIA) assays on commercial analytical systems, HBV DNA by PCR-fluorescence probing assay, and ALT by enzymatic method. Data are shown as mean ± SEM (n=10 mice). Dashed lines indicate LLOQs. Source data are provided as a Source Data file.



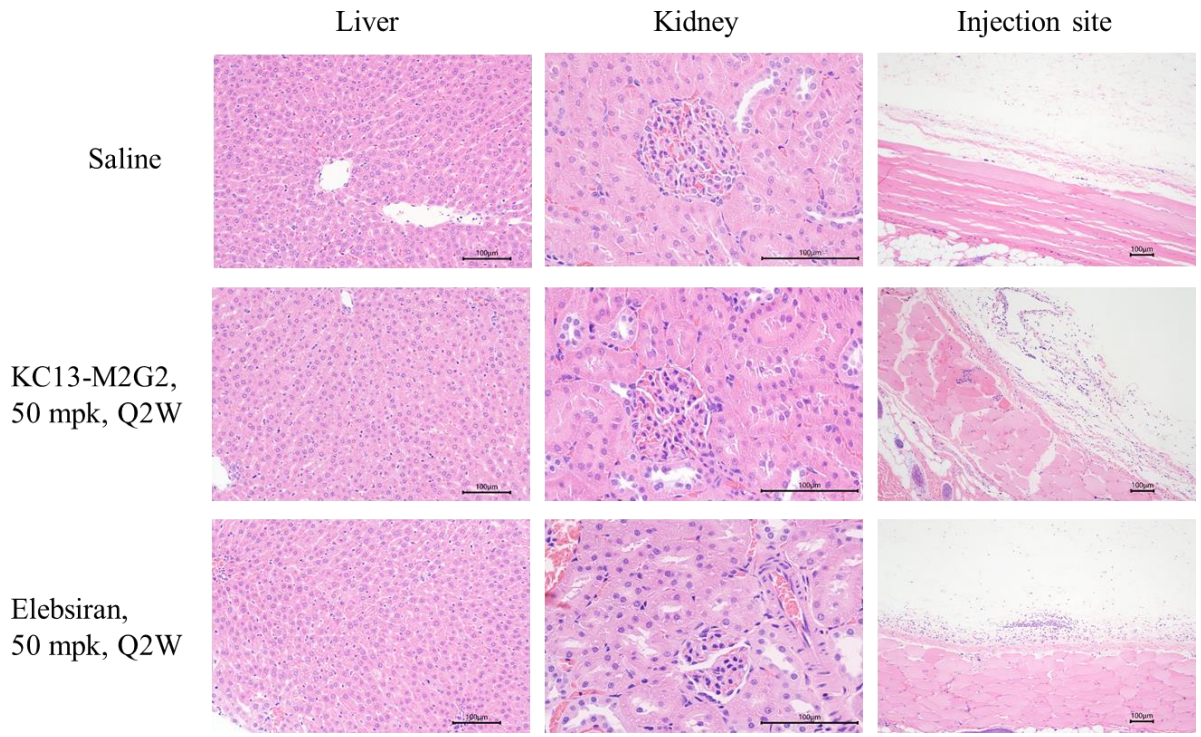
Supplementary Fig. 11 HBV plasmid challenge and immune responses evaluation in rAAV-HBV-D mice. Mice received three weekly doses of 0.5 or 3 mg/kg indicated siRNA agents. The first dosing day was defined as D0. Plasma levels of HBsAg (**a**), HBsAb (**b**) and ALT (**c**) were detected at certain timepoints. The levels of HBsAg and HBsAb were quantified by chemiluminescence immunoassay (CLIA) assays on commercial analytical systems, and ALT by enzymatic method. Data are shown as mean ± SEM (n=6 mice). Dashed lines indicate LLOQs. **d-e** Mice administered 3 mg/kg KC13-M2G1 were challenged intravenously with pAAV-HBV1.2 plasmids on day 60. Plasma levels of HBsAg (**d**) and HBsAb (**e**) were subsequently measured at the indicated timepoints (n=3 mice). **f-i** Humoral and cellular immune response in mice treated with 3 mg/kg KC13-M1G1. Splenocytes were isolated on

day 56 and evaluated using ELISpot assays to detect antibody-secreting cells (ASCs) and antigen-specific T cell responses via IL-2 and IFN-γ secretion. Liver-associated lymphocytes were isolated on day 56 and evaluated using ELISpot assays to detect antigen-specific T cell responses via IFN-γ secretion. Data are shown as mean ± SEM (n=6 mice). Only statistically significant differences are indicated (one-way ANOVA test or Kruskal-Wallis test with Dunn's post-hoc test for multiple groups). Source data are provided as a Source Data file.

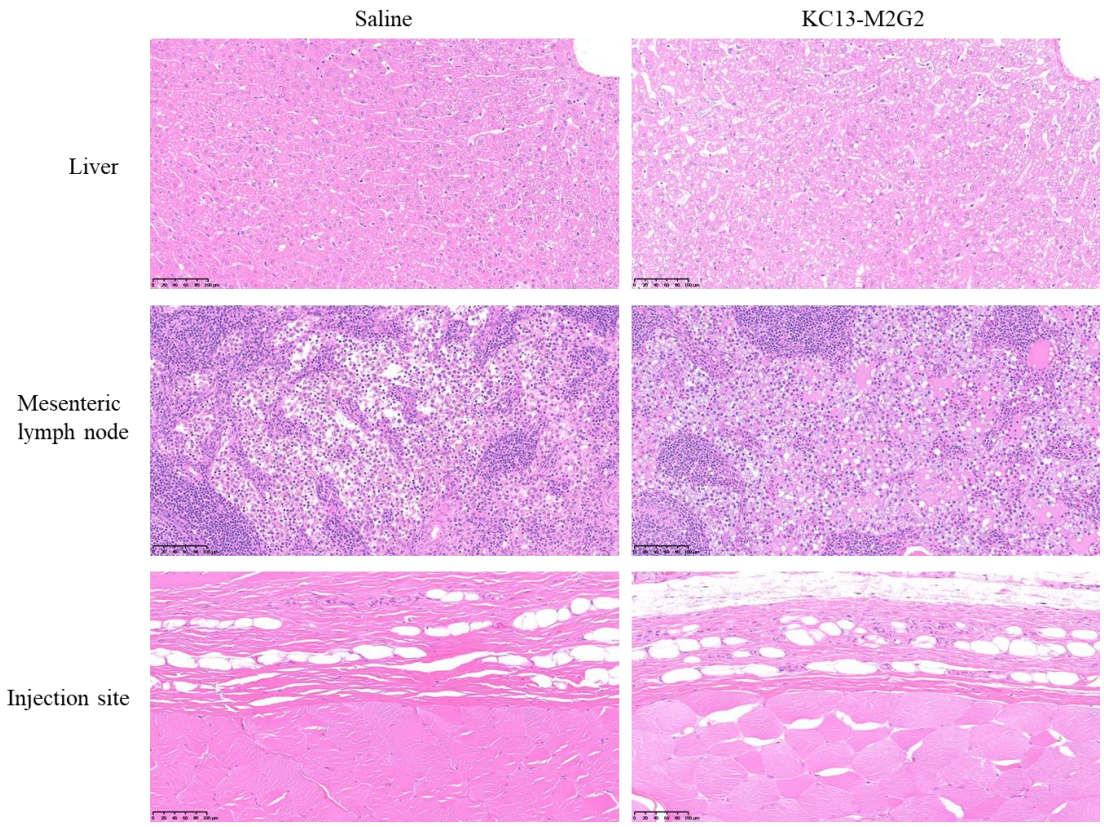


Supplementary Fig. 12 Efficacy of KC13-M2G2 in HBV-transgenic mice at single dose of 3 mg/kg. The first dosing day was defined as D0. Plasma levels of HBsAg (**a**), HBV DNA (**b**), HBeAg (**c**), and ALT (**d**) were detected at certain timepoints. The levels of HBsAg, and HBeAg, were quantified by chemiluminescence immunoassay (CLIA) assays on commercial analytical systems, HBV DNA by PCR-fluorescence probing assay, and ALT by enzymatic method. Data are shown as mean ± SEM (n=12 mice). Dashed lines indicate LLOQs. Source data are

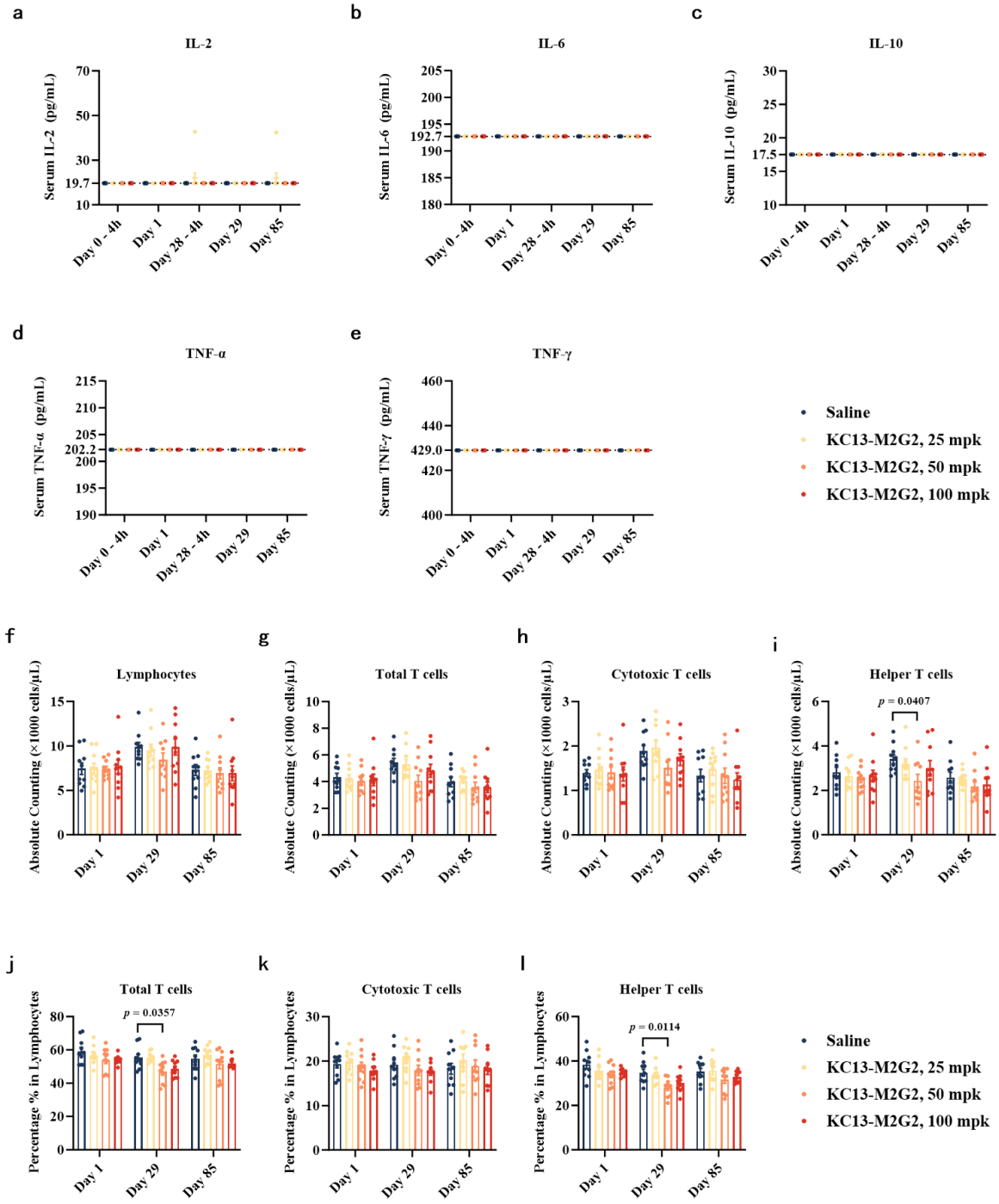
provided as a Source Data file.



Supplementary Fig. 13 Representative hematoxylin and eosin (H&E)-stained liver, kidney, and injection site tissue sections from rats treated with KC13-M2G2 or Elebsiran. Rats received three repeat doses of KC13-M2G2 on day 0, 14, and 28. Tissues were harvested on day 29, and stained with H&E (n=6 rats).

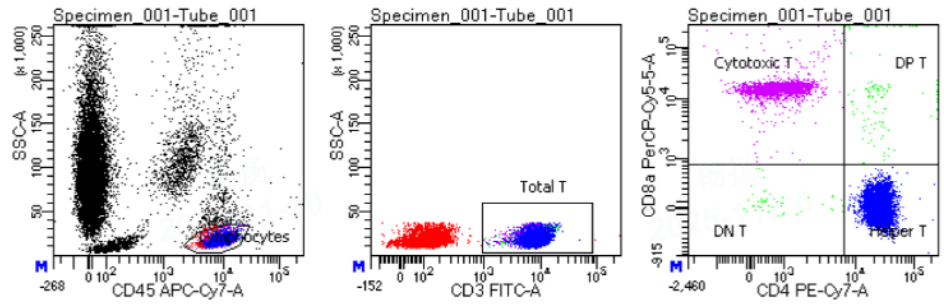


Supplementary Fig. 14 Representative H&E-stained liver, mesenteric lymph node, and injection site tissue sections from cynomolgus monkeys treated with KC13-M2G2 or saline. Monkeys received three repeat doses of KC13-M2G2 on day 0, 14, and 28. Tissues were harvested on day 30, and stained with H&E (n=6 monkeys).



Supplementary Fig. 15 Immunogenicity of KC13-M2G2 in SD rats. KC13-M2G2 was administered repeatedly via subcutaneous injection to SD rats at 25, 50 or 100 mg/kg on day 0, 14, and 28 with an 8-week recovery period. **a-e** Serum cytokine levels (including IL-2, IL-6, IL-10, TNF-α and IFN-γ) were assayed 4 h after the first dose and the final dose (Day 0-4 h and Day 28-4 h), 24 h after the first dose and the final dose (Day 1 and Day 29) and at the

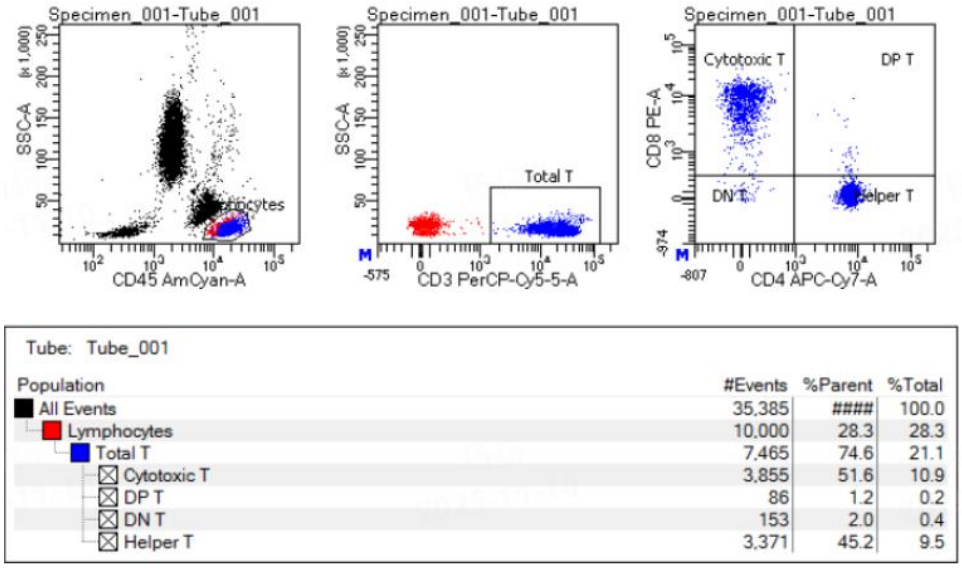
recovery period terminus (Day 85), using Rat Luminex® Discovery Assay. **f-l** Serum absolute numbers and percentages of lymphocyte subpopulations of total T cells ($CD45^+$ $CD3^+$), cytotoxic T cells ($CD45^+$ $CD3^+$ $CD4^-$ $CD8a^+$), and helper T cells ($CD45^+$ $CD3^+$ $CD4^+$ $CD8a^-$) were detected on day 1, day 29 and day 85 by flow cytometry, employing an immunophenotyping panel. The first dosing day was defined as Day 0. Data are shown as mean ± SEM (n=10 rats). Dashed lines indicate LLOQs. Only statistically significant differences are indicated (one-way ANOVA test or Kruskal-Wallis test with Dunn's post-hoc test for multiple groups). Source data are provided as a Source Data file.



Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	66,738	####	100.0
Lymphocytes	11,000	16.5	16.5
Total T	7,055	64.1	10.6
Cytotoxic T	2,312	32.8	3.5
DP T	118	1.7	0.2
DN T	53	0.8	0.1
Helper T	4,572	64.8	6.9

Supplementary Fig. 16 The gating strategy of flow cytometry analysis for Supplementary Fig. 15f-l.



Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	35,385	####	100.0
Lymphocytes	10,000	28.3	28.3
Total T	7,465	74.6	21.1
Cytotoxic T	3,855	51.6	10.9
DP T	86	1.2	0.2
DN T	153	2.0	0.4
Helper T	3,371	45.2	9.5

Supplementary Fig. 17 The gating strategy of flow cytometry analysis for Fig. 9f-l.

Supplementary Tables

Supplementary Table 1: Calculated IC_{50}s of HBsAg and HBV DNA against ten HBV genotypes A throughout J.

Construct	Genotype	HBsAg IC_{50}/nM		HBV DNA IC_{50}/nM	
		KC13-M2G2	Elebsiran	KC13-M2G2	Elebsiran
HE974371	A	0.0024	0.0896	0.1484	2.3052
JN406371	B	0.0032	0.2355	0.8592	>20
AB246345	C	0.0064	0.0311	0.1566	0.4180
U95551	D	0.0015	0.0986	0.0973	2.8852
HE974380	E	0.0059	0.0079	0.1827	0.0935
AB036920	F	0.0209	0.0539	0.1895	0.4171
AB625342	G	0.0004	0.0566	0.0041	1.0782
AP007261	H	0.0085	0.0524	0.4464	9.7865
FJ023673	I	0.0026	0.0391	0.0984	1.0365
AB486012	J	0.0018	0.0747	0.0833	0.8677

Supplementary Table 2: Proportion of mice with HBsAg loss, HBV DNA loss and HBsAb positivity in rAAV-HBV-B mice corresponding to Figure 4.

HBsAg loss											
Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
KC13-M2G2	0/10	3/10	5/10	7/10	7/10	8/10	9/10	10/10	10/10	10/10	10/10
HBV DNA loss											
Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Elebsiran	0/10	1/10	2/10	2/10	1/10	1/10	2/10	3/10	1/10	1/10	0/10
KC13-M2G2	0/10	10/10	9/10	10/10	10/10	10/10	10/10	10/10	8/10	10/10	10/10
HBsAb positivity											

Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Elebsiran	0/10	0/10	0/10	0/10	0/10	1/10	1/10	1/10	1/10	1/10	1/10
KC13-M2G2	0/10	0/10	0/10	1/10	2/10	2/10	2/10	4/10	6/10	6/10	8/10

Supplementary Table 3: Proportion of mice with HBsAg loss, HBV DNA loss and HBsAb positivity in rAAV-HBV-C mice corresponding to Figure 4.

HBsAg loss											
Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Elebsiran	0/10	0/10	0/10	1/10	1/10	1/10	1/10	1/10	1/10	0/10	1/10
KC13-M2G2	0/10	5/10	7/10	8/10	8/10	8/10	9/10	9/10	9/10	9/10	9/10
HBV DNA loss											
Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
KC13-M2G2	0/10	1/10	5/10	9/10	8/10	8/10	10/10	10/10	10/10	10/10	10/10
HBsAb positivity											
Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10	0/10	1/10
KC13-M2G2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	2/10	3/10

Supplementary Table 4: Proportion of mice with HBsAg loss, HBV DNA loss and HBsAb positivity in rAAV-HBV-D mice with high HBsAg baseline corresponding to Figure 6.

HBsAg loss

Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
ETV	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Bepirovirsen	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
KC13-M2G2	0/10	0/10	0/10	1/10	2/10	2/10	3/10	3/10	3/10	4/10	5/10
HBV DNA loss											
Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
ETV	0/10	0/10	4/10	7/10	8/10	9/10	9/10	7/10	7/10	6/10	9/10
Bepirovirsen	0/10	0/10	8/10	10/10	10/10	9/10	6/10	1/10	0/10	0/10	0/10
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
KC13-M2G2	0/10	0/10	0/10	0/10	6/10	7/10	9/10	10/10	9/10	10/10	10/10
HBsAb positivity											
Days	0	7	14	21	28	35	42	49	56	63	70
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
ETV	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Bepirovirsen	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10	1/10	1/10	1/10
KC13-M2G2	0/10	0/10	0/10	1/10	1/10	1/10	2/10	3/10	3/10	4/10	4/10

Supplementary Table 4 (continued): Proportion of mice with HBsAg loss, HBV DNA loss and HBsAb positivity in rAAV-HBV-D mice with high HBsAg baseline corresponding to Figure 6.

HBsAg loss										
Days	77	84	91	105	119	133	147	161	175	189
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
ETV	ND									
Bepirovirsen	ND									
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
KC13-M2G2	4/10	5/10	6/10	6/10	6/10	6/10	6/10	6/10	7/10	6/10
HBV DNA loss										
Days	77	84	91	105	119	133	147	161	175	189
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
ETV	ND									
Bepirovirsen	ND									
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
KC13-M2G2	9/10	10/10	10/10	8/10	8/10	8/10	8/10	6/10	7/10	7/10
HBsAb positivity										
Days	77	84	91	105	119	133	147	161	175	189
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
ETV	ND									
Bepirovirsen	ND									
Elebsiran	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10	1/10	1/10
KC13-M2G2	5/10	4/10	3/10	5/10	5/10	5/10	6/10	7/10	7/10	7/10

ND: not determined.

Supplementary Table 5: Primer and probe list.

Primers	Forward primer	Reverse primer

SEPTIN6	TTTGACAGCTTGCCTGACCA	CCCAAACCTGTCTCTCCCAC
AAK1	TGTTGGCGGAAGGTGGATTT	TGCACACCTGGAGATCATGC
CCSAP	GCTCCTGTGCACGAGATTCA	TCCACATCCACAGAGTGAGC
COX5A	TCATTGATGCTGCTTTGCGG	TGAGGTCCTGCTTTGTCCTTA
ABCB10	GTGGTGCTGCCGCCAATG	CCTCCTGCCTCAGAATGGAGGA
IRS1	TCAGTTTCCAGAAGCAGCCA	GGTCATTTAGGTCTTCATTCTGCT
ELP1	GAGAAGTCACAGAAGGATCCCA	ATGGCTTTTTCATATCGTTTCAAGT
NUS1	GGTCCTGTGGACAGCACATT	GGTGGGAAGGCAAAGAGACAA
SUB1	TTCCGGTTCTCTGTCAGTCG	TGATTTAGGCATCGCTTCGC
AFF4	CGGGACTGGAGCAACATGA	TGATTCCGCCTTTCCCGTTC
NDUFA5	TACTCCTCACGAGAGGCTAAG	TAGCCAGCTTCTCATTTGTAATCT
CSNK1D	TCGGAGACATCTATCTCGGTACG	GGAGCTGAGGGTGTTTGGTT
EXOC1	CAAACAGCAACAGCAGCGAT	ACTCTGATCATGACCCTGTTGAA
HBV total RNA	CCGACCTTGAGGCATACTTCA	AATTTATGCCTACAGCCTCCTAGTACA
HBV S mRNA	CCAAACCTTCGGACGGAAA	TGAGGCCCACTCCCATAGG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
Probes		
HBV total RNA	TTAAAGACTGGGAGGAGTTG	
HBV S mRNA	CCCATCATCCTGGGCTTTCGGAAAAT	

Supplementary Table 6: Antibody list.

Antibody	Manufacturer	Cat No.	Clone name	Lot No.	Dilution
APC-Cy™7 Mouse anti-rat CD45	BD	561586	OX-1	3258955	1:50
FITC Mouse Anti-Rat CD3	BD	554832	G4.18	3114240	1:50
PE-Cy™7 Mouse Anti-Rat CD4	BD	561578	OX-35	4024523	1:20
PerCP Mouse Anti-Rat CD8a	BD	558824	OX-8	4015835	1:50
NHP CD45 Horizon V500	BD	561489	D058-1283	3160468	1:50
Hu/NHP CD3 PerCP-Cy5.5	BD	552852	SP34-2	4003828	2:50
Hu/NHP CD4 APC-H7	BD	560837	L200	2059633	1:50
Hu CD8 PE	BD	555367	RPA-T8	1116651	2:50