

Retreatment with HBV siRNA Results in Additional Reduction in HBV Antigenemia and Immune Stimulation in the AAV-HBV Mouse Model

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Supplementary Tables

Supplementary Table S1: antibodies used for immunohistochemistry

| Marker | Catalog Number | Supplier | clone |
|--------|----------------|----------|------------|
| HBcAg | B0586 | Dako | polyclonal |
| HBsAg | Ab859 | Abcam | 3E7 |

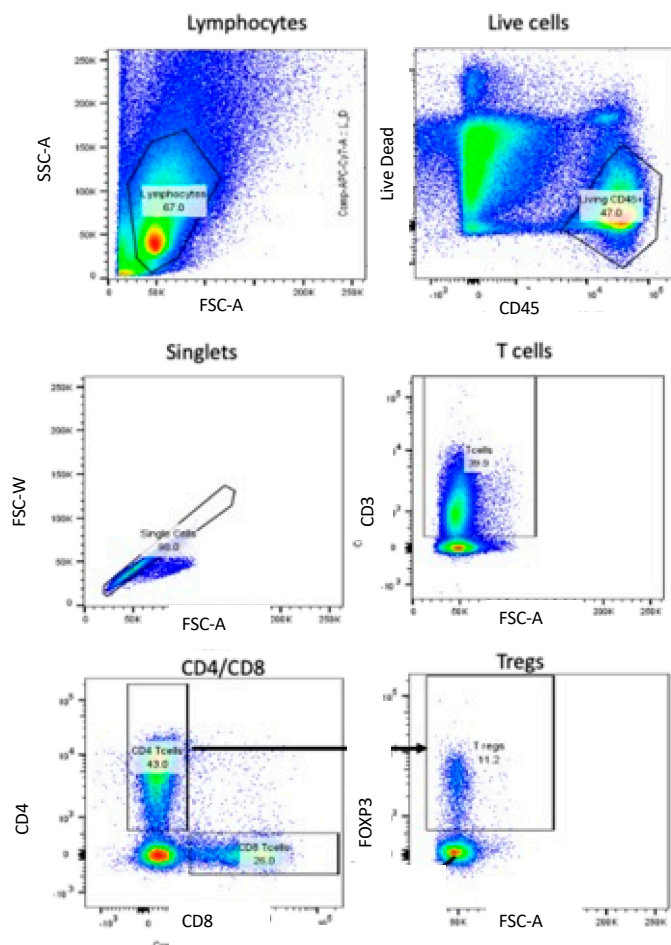
Supplementary Table S2: Flow cytometry antibody panel.

| Marker | Fluorochrome | Catalog Number | Supplier | clone |
|-----------|--------------|----------------|-------------|---------|
| CD16/CD32 | Purified | 553142 | BD | 2.4G2 |
| CD45 | BUV-395 | 564279 | BD | 30-F11 |
| CD8a | BUV496 | 750024 | BD | 53-6.7 |
| CD4 | BV-786 | 563331 | BD | GK1.5 |
| CD3 | Percpcy5.5 | 560527 | BD | 17A2 |
| PD-1 | BV-605 | 563059 | BD | J43 |
| LAG-3 | BUV737 | 741820 | BD | C9B7W |
| TIM-3 | BB515 | 567810 | BD | 5D12 |
| TIGIT | PE | 622206 | biolegend | A17200C |
| CD154 | PE-Cy7 | 106512 | biolegend | MR1 |
| TOX | A647 | 568356 | BD | NAN448B |
| FOXP3 | PE-CF594 | 567456 | BD | 3G3 |
| TCF1 | A405 | FAB8224V | Rnd systems | #812145 |

Supplementary figures

Supplementary Figure S1: T-cell gating.

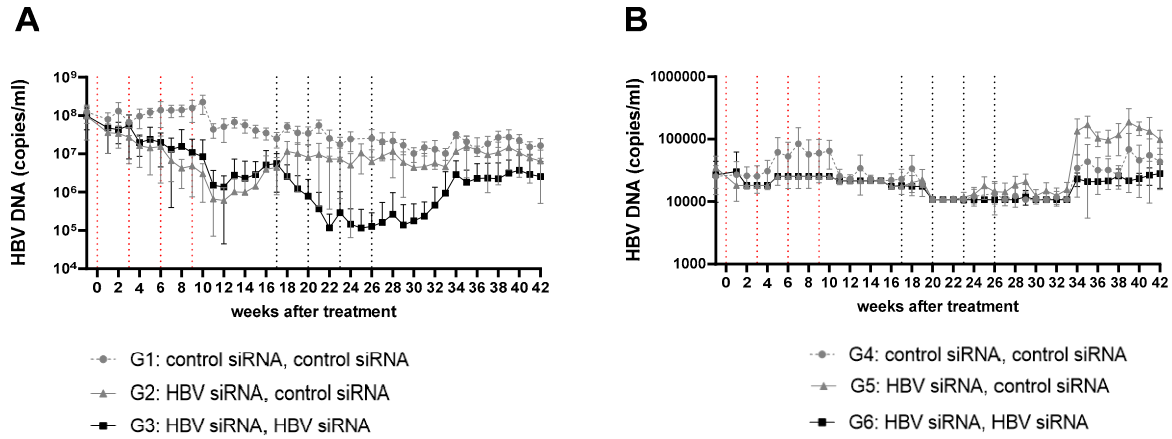
The following gating strategy is followed to determine pre-exhausted, exhausted and terminally exhausted T-cells. Lymphocyte gate was made based on forward scatter (FSC)-side scatter (SSC). Next single cells are selected based on FSC-Area/SSC-Area. Live dead stain was plotted against CD45 to select the living CD45⁺ cells. Next SSC was plotted against CD3 to select T-cells. CD4 was plotted against CD8 within the CD3 + T cell gate to distinguish between CD8 and CD4 T-cells. In both these gates the expression of PD-1, TOX, TIM-3, LAG-3 and TIGIT was evaluated. Tregs were characterized as FOXP3⁺ within the CD4 T-cells.



Supplementary Figure S2: HBV DNA in serum of mice transduced with AAV-HBV and treated with GalNac-HBV siRNA

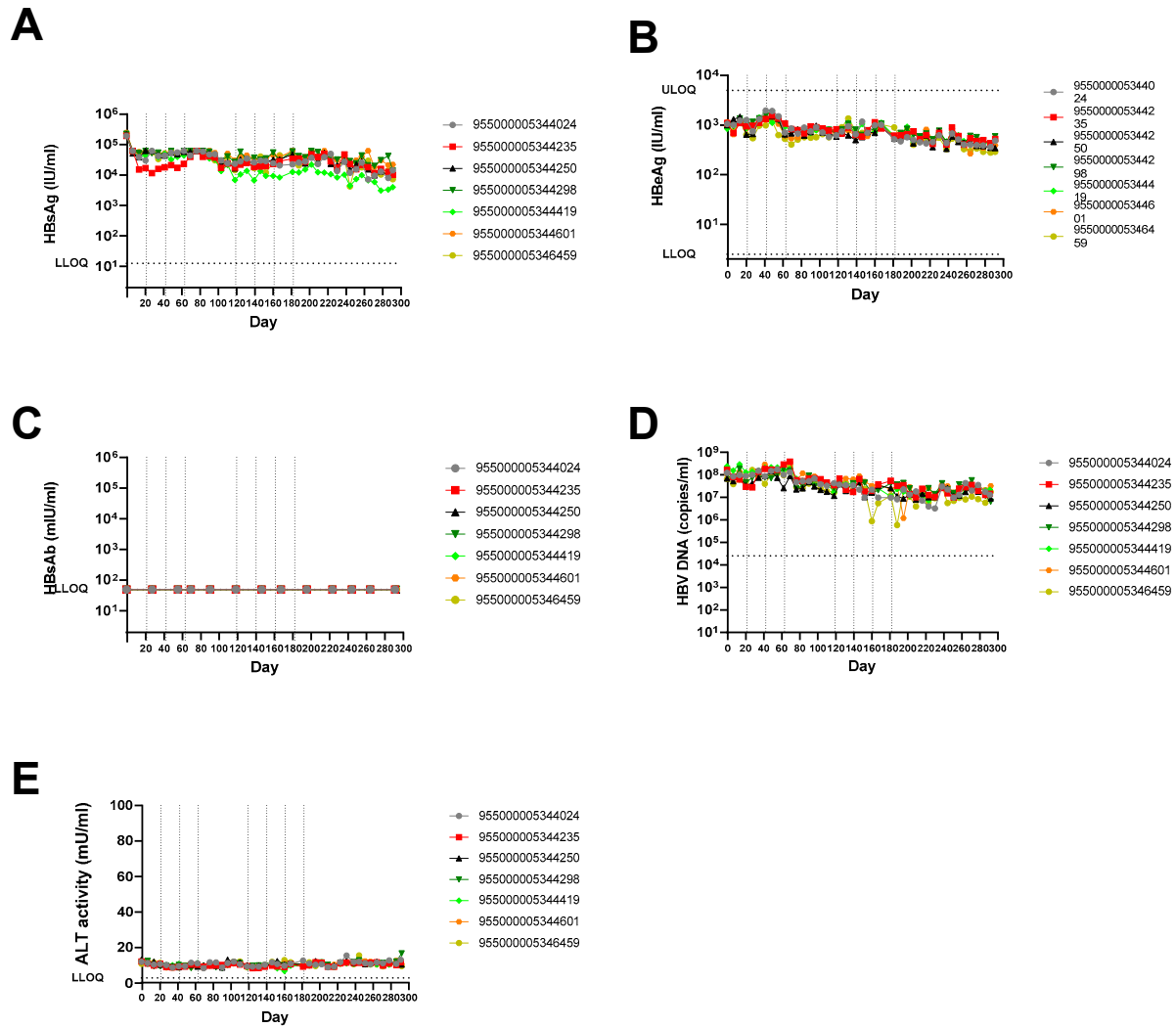
High titer mice, transduced with 3×10^{10} vge/mice of AAV-HBV (A) or Mid titer mice:transduced with 2.5×10^9 vge/mice of AAV-HBV (B), start treatment twenty-eight days after transduction. The first treatment cycle of 4 doses of 3mg/kg siRNA every 3 weeks is indicated with red dotted lines. The second treatment cycle is indicated with black dotted lines. G1 and G4 received twice a treatment with GalNac-control-siRNA (grey diamonds). G2 and G5 received first GalNac-HBV-siRNA and then GalNac-control-siRNA (grey

triangle). G3 and G6 received twice GalNac-HBV-siRNA (black squares). Blood was taken on weekly basis to evaluate viral parameters.



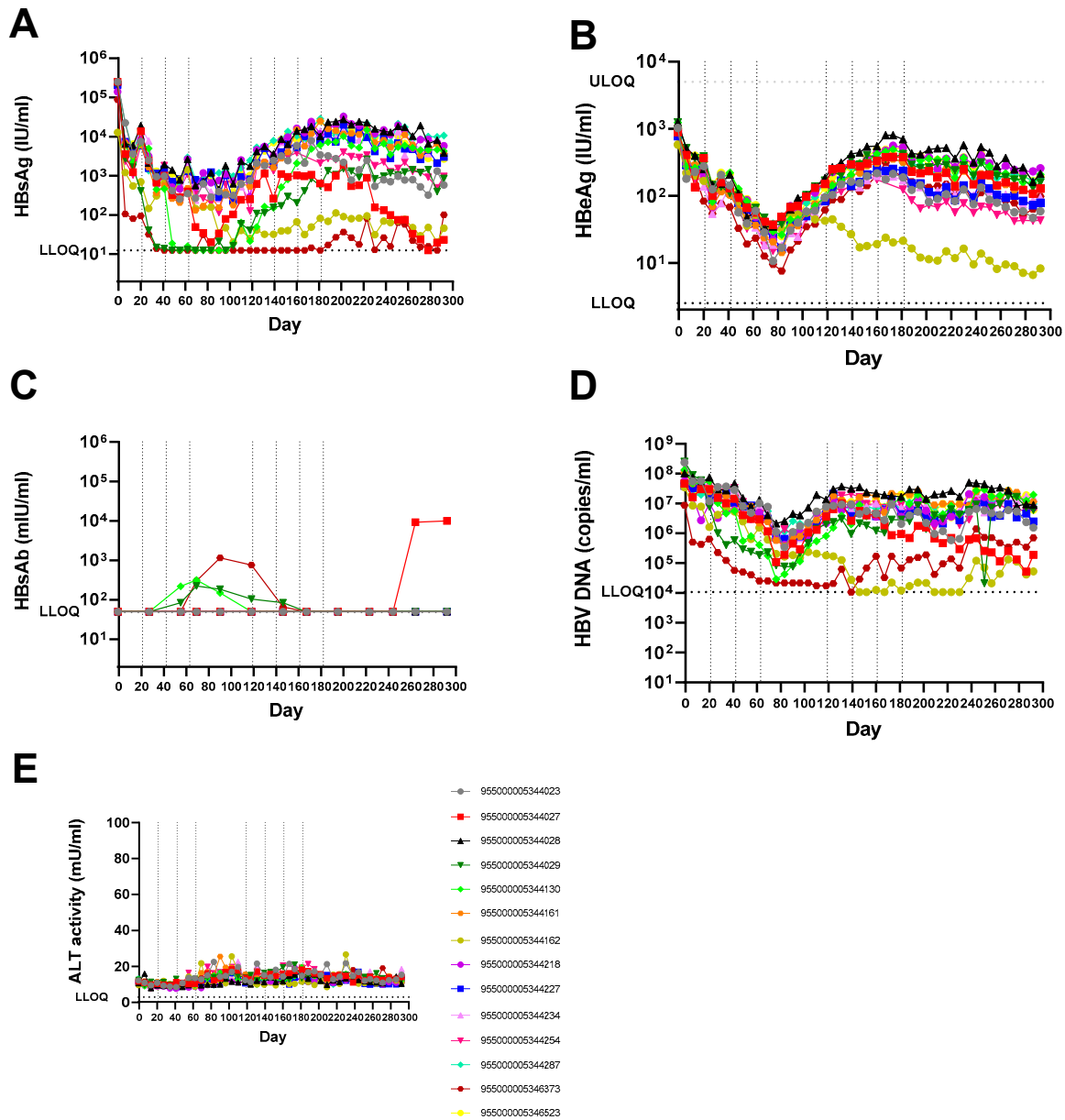
Supplementary Figure S3: Viral parameters in serum and liver of mice transduced with high titer virus and treated twice with control GalNac siRNA

High titer mice are mice transduced with 3×10^{10} vge/mice of AAV-HBV. Twenty-eight days after transduction treatment started. The two-treatment cycle of 4 doses of 3mg/kg siRNA every 3 weeks are indicated with dotted lines. Blood was taken weekly to evaluate viral parameters. (A) HBsAg levels, (B) HBeAg levels, (C) HBs antibodies, (D) viral load, (E) ALT.



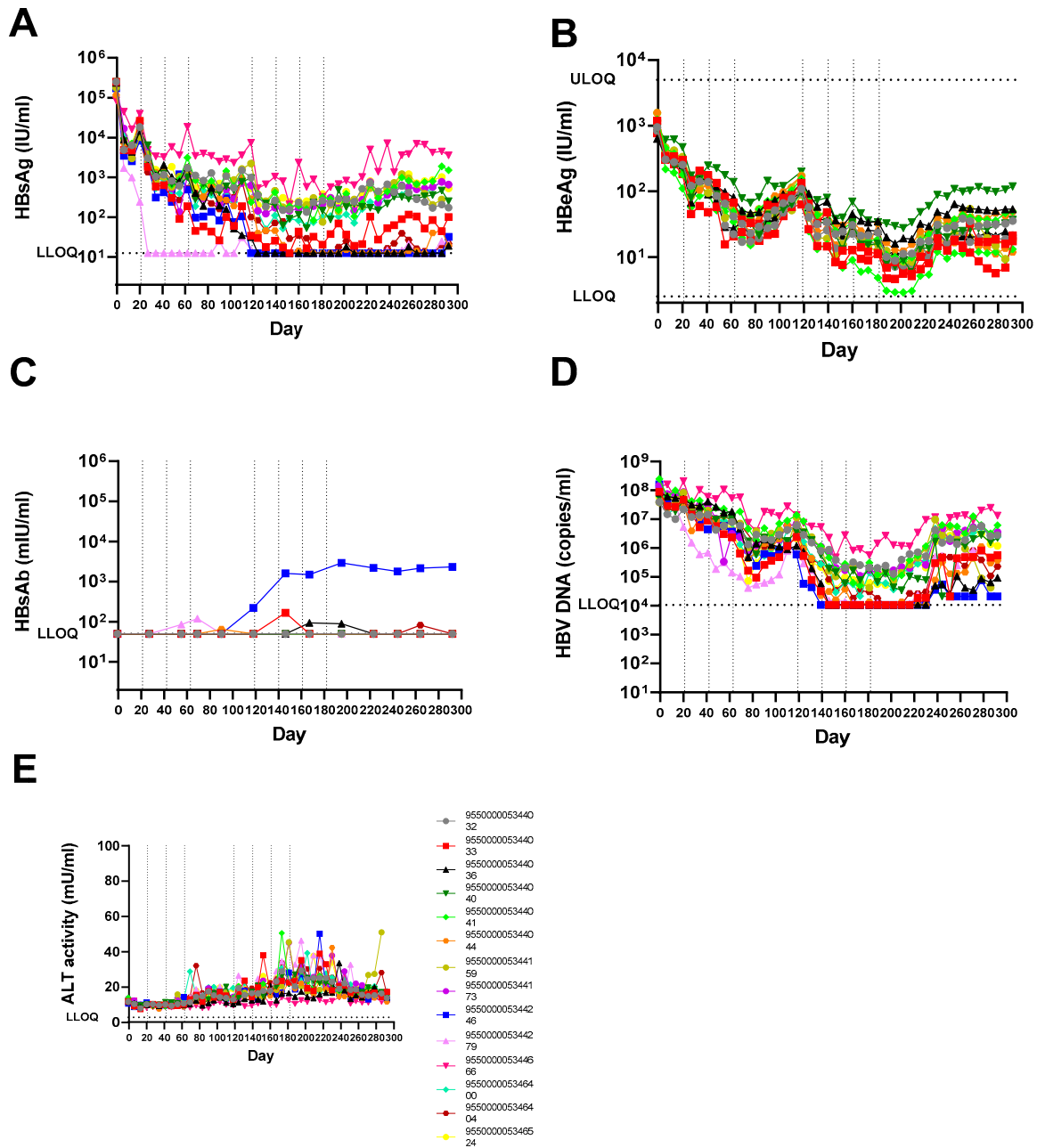
Supplementary Figure S4: Viral parameters in serum and liver of mice transduced with high titer virus and treated with GalNac-HBV-siRNA and with GalNac-control-siRNA

High titer mice are mice transduced with 3×10^{10} vge/mice of AAV-HBV. Twenty-eight days after transduction treatment started. The treatment cycles of 4 doses of 3mg/kg HBV-siRNA every 3 weeks are indicated with dotted lines. Blood was taken weekly to evaluate viral parameters. **(A)** HBsAg levels, **(B)** HBeAg levels, **(C)** HBs antibodies, **(D)** viral load, **(E)** ALT.



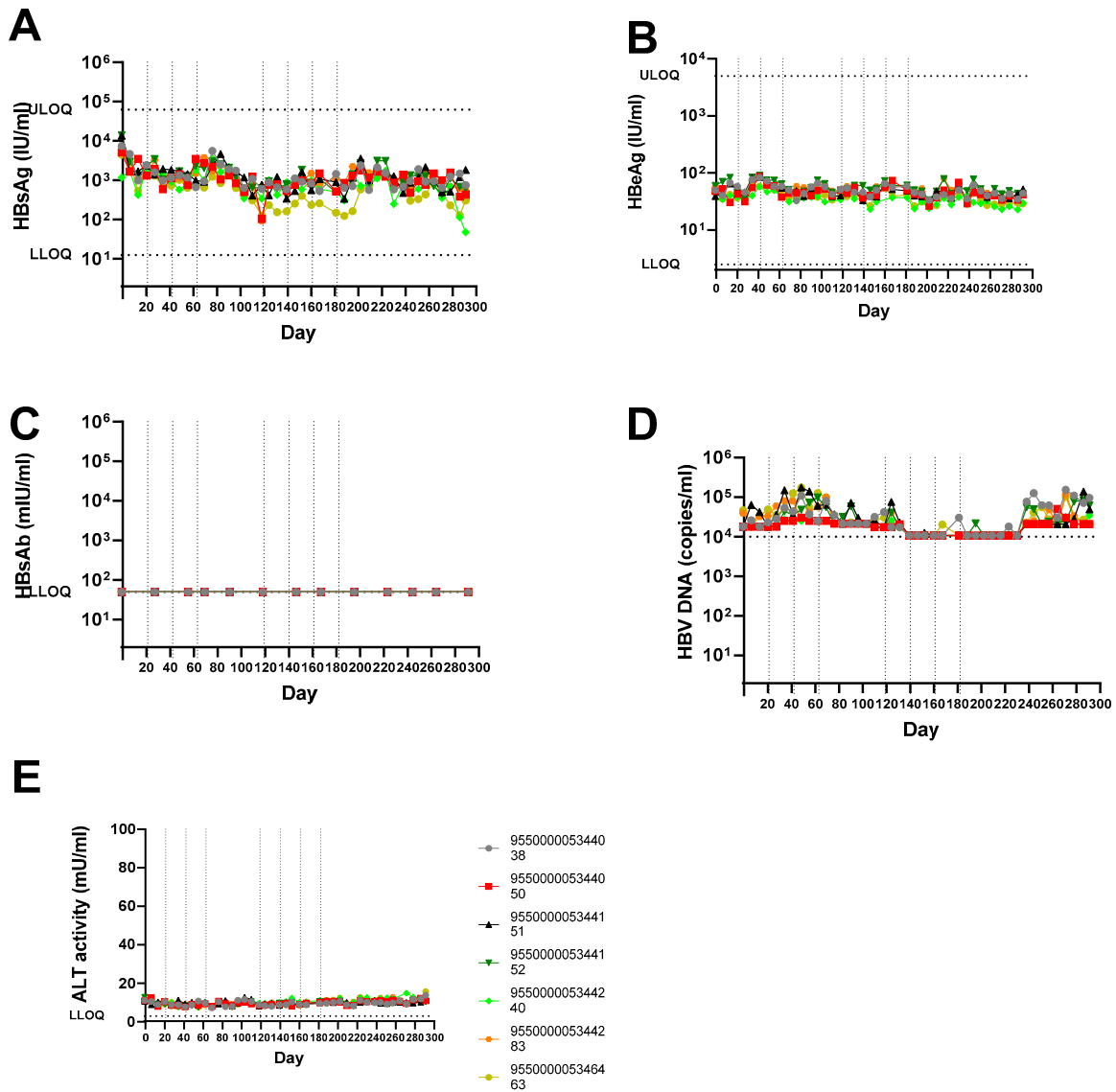
Supplementary Figure S5: Viral parameters in serum and liver of mice transduced with high titer virus and treated twice with GalNac-HBV-siRNA.

High titer mice are mice transduced with 3×10^{10} vge/mice of AAV-HBV. Twenty-eight days after transduction treatment started. The treatment cycles of 4 doses of 3mg/kg HBV-siRNA every 3 weeks are indicated with dotted lines. Blood was taken weekly to evaluate viral parameters. **(A)** HBsAg levels, **(B)** HBeAg levels, **(C)** HBs antibodies, **(D)** viral load, **(E)** ALT.



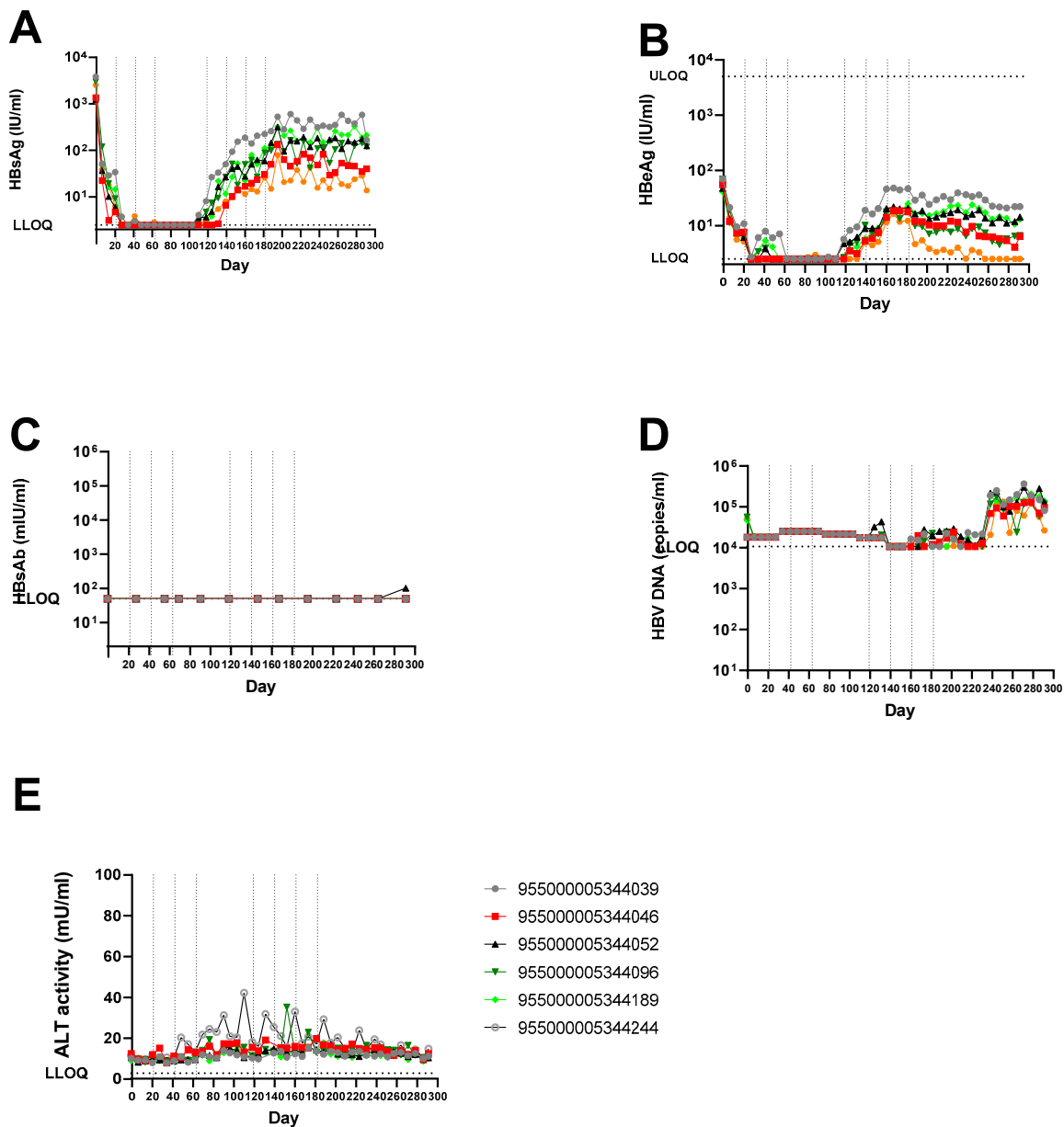
Supplementary Figure S6: Viral parameters in serum and liver of mice transduced with mid titer virus and treated twice with GalNAc-control-siRNA.

Mid titer mice are mice transduced with 2.5×10^9 vge/mice of AAV-HBV. Twenty-eight days after transduction treatment started. The treatment cycles of 4 doses of 3mg/kg HBV-siRNA every 3 weeks are indicated with dotted lines. Blood was taken weekly to evaluate viral parameters. **(A)** HBsAg levels, **(B)** HBeAg levels, **(C)** HBs antibodies, **(D)** viral load, **(E)** ALT.



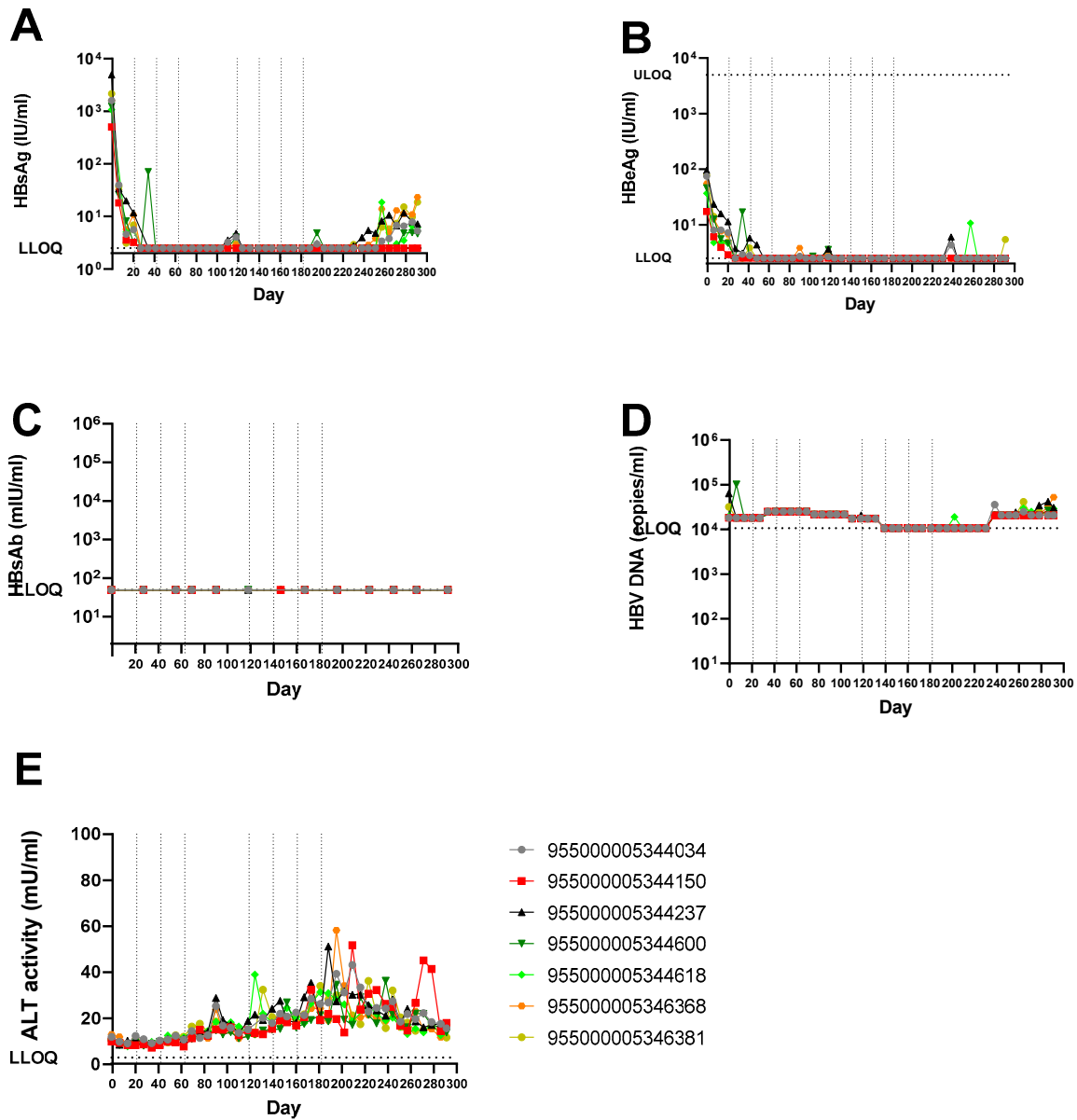
Supplementary Figure S7: Viral parameters in serum and liver of mice transduced with mid titer virus and treated with GalNAc-HBV-siRNA and retreated with GalNAc-control-siRNA

Mid titer mice are mice transduced with 2.5×10^9 vge/mice of AAV-HBV. Twenty-eight days after transduction treatment started. The treatment cycles of 4 doses of 3mg/kg HBV-siRNA every 3 weeks are indicated with dotted lines. Blood was taken weekly to evaluate viral parameters. **(A)** HBsAg levels, **(B)** HBeAg levels, **(C)** HBs antibodies, **(D)** viral load, **(E)** ALT.



Supplementary Figure S8: Viral parameters in serum and liver of mice transduced with mid titer virus and treated twice with GalNac-HBV-siRNA.

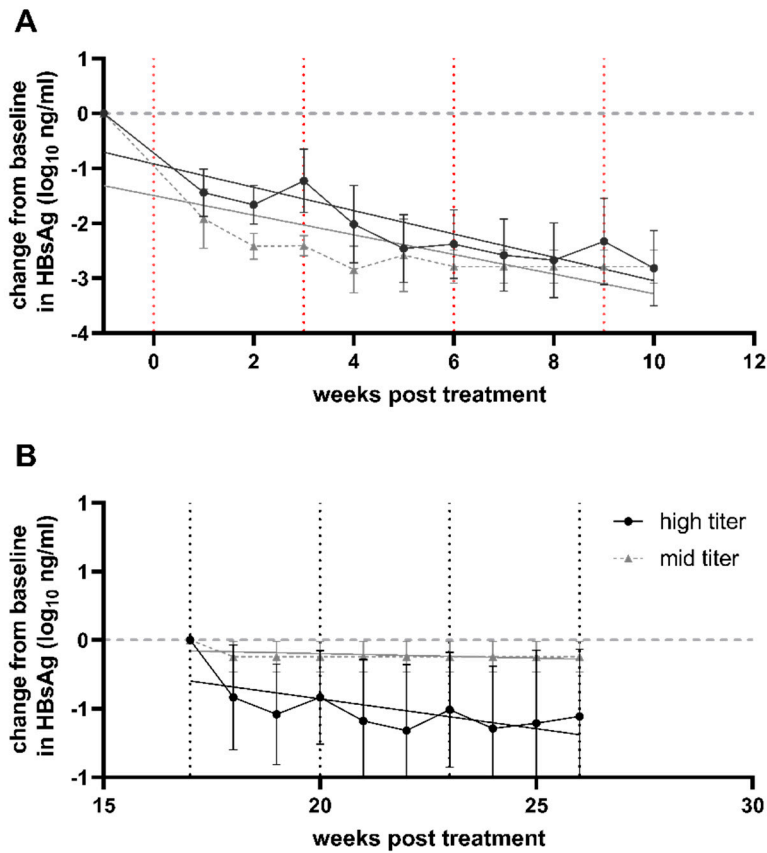
Mid titer mice are mice transduced with 2.5×10^9 vge/mice of AAV-HBV. Twenty-eight days after transduction treatment started. The treatment cycles of 4 doses of 3mg/kg HBV-siRNA every 3 weeks are indicated with dotted lines. Blood was taken weekly to evaluate viral parameters. **(A)** HBsAg levels, **(B)** HBeAg levels, **(C)** HBs antibodies, **(D)** viral load, **(E)** ALT.



Supplementary Figure S9: Changes from baseline in HBsAg levels during first and second treatment cycle with HBV-GalNAc-siRNA

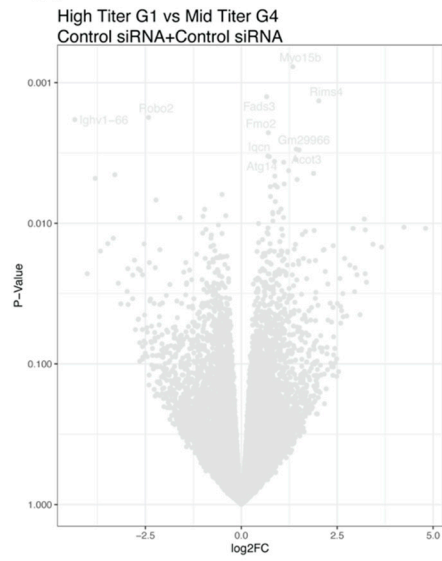
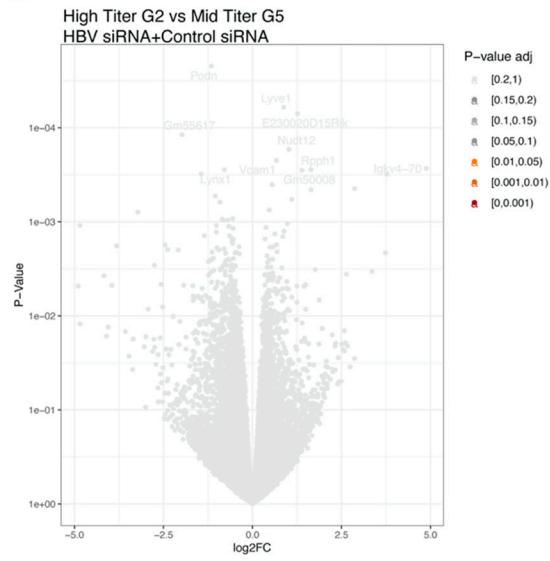
Changes from baseline were calculated for mice treated with HBV-GalNAc-siRNA during the first treatment cycle (A). Slope of decline in HBsAg was calculated using simple linear regression method. The slope of decline was comparable in mid titer (grey triangle, dotted line) and high titer mice (black circle and line). Changes from baseline (defined as last value of HBsAg levels before new cycle of treatment

started) were calculated for mice treated with HBV-GalNac-siRNA during second treatment cycle (B). The slope of decline was significantly different between mid-titer and high titer arm. But this is due to concentrations of HBsAg levels at start of second treatment cycle. Which was just above detection limit in mid titer mice.



Supplementary Figure S10: Differential expression analysis between high and mid titer

Mid titer mice are mice transduced with 2.5×10^9 vge/mice of AAV-HBV. High titer mice are mice transduced with 3×10^{10} vge/mice of AAV-HBV. (A) Volcano plot comparing controls from high vs mid titer (B) single treated mice with HBV-GalNac-siRNA from high and mid titer mice and (C) retreated mice with two rounds of HBV-GalNac-siRNA from high and mid titer mice. Colors indicate p-value fdr correction.

A**B****C**