Supplementary Figures and Tables to:

IL-21-based therapies induce clearance of hepatitis B virus persistence in mouse models

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LIST

Supplementary Figure 1-19

Supplementary Table 1 (see separate xls file)

Supplementary Table 2
Figure S1. Validation of IL-21 expression by AAV-IL-21. Cultured Huh-7 cells were infected with AAV-IL-21 or AAL-Ctrl at 10:1 multiplicity of infection and IL-21 in supernatants was measured using ELISA. Dotted line represents cut-off threshold. Infections were performed in triplicates, and means and SEM for each treatment are presented.
Figure S2. Serum total bilirubin and creatine kinase levels in BPS persistence mice treated with recombinant AAV. Male BALB/c mice aged 6-8 weeks were injected through hydrodynamic injection via tail vein with 10 µg of BPS replicon plasmid, and mice remaining positive for serum HBV antigens for 4 weeks were injected (solid arrows) with $2 \times 10^{11}$ geq of AAV-IL-21 or AAV-Ctrl, or left untreated. Sera were collected at indicated time points and analyzed for total bilirubin (A) and creatine kinase (B) using commercial quantitative assay. Dotted lines represent pre-treatment baselines. Group means and SEM within group are presented with the group size (n) indicated. Data were obtained from the same mice represented in Figure 1. w.p.i., weeks post injection of AAV.
Figure S3. AAV-IL-21 treatment cleared intrahepatic BPS DNA and viral replication intermediates from BPS persistence mice. BPS HDI mice remaining positive for serum HBV antigens for 4 weeks were injected with $2 \times 10^{11}$ geq of AAV-IL-21 ($n = 2$), AAV-Ctrl ($n = 2$), or left untreated ($n = 2$). Serum HBsAg was tested every two weeks. At 10 weeks post injection (w.p.i.) of AAV, mice were sacrificed and liver samples taken (A). AAV-IL-21 treated mice had displayed HBsAg negativity for over 4 weeks by the time of sacrifice. BPS DNA and viral replication intermediates in total cellular DNA extracted from liver tissue samples were measured using Southern blot (B) with serum HBsAg status indicated.
Figure S4. Extended follow-up confirmed that AAV-IL-21 treatment cleared intrahepatic BPS DNA from BPS persistence mice. BPS HDI mice remaining positive for serum HBV antigens for 4 weeks were injected with $2 \times 10^{11}$ geq of AAV-IL-21 ($n = 6$), AAV-Ctrl ($n = 3$), or left untreated ($n = 3$). Serum HBsAg was
tested at indicated time points. At 20 weeks post injection (w.p.i.) of AAV, mice were
sacrificed and liver samples taken (A). All 6 AAV-IL-21 treated mice had displayed
HBsAg negativity for over 4 weeks by the time of sacrifice. BPS plasmid in nuclear
dNA extracted from liver tissue samples were measured using quantitative realtime
PCR (B) and Southern blot (C). Serum HBsAg status of the mice are indicated (B and 
C). Data from each mouse and group means are presented (B). Statistical
significances are calculated using unpaired two-tailed t-test. **p < 0.05; ***p < 0.001. 
ns, not significant.
Figure S5. Liver HBsAg staining of BPS injected BALB/c mice and Ad/prcccDNA infected Cre Tg mice. (A) 10 μg of backbone pUC18 vector or pBPS were injected into male BALB/c mice aged 6-8 weeks through hydrodynamic injection via tail vein. (B) Male Cre Tg C57BL/6 mice aged 6-8 weeks, which are transgenic for liver-specific Cre recombinase, were left untreated or injected with 1.5 × 10⁹ plaque-forming units of Ad/prcccDNA via tail vein. At 1 week post injection (w.p.i.), liver sections were prepared from sacrificed mice and subjected to immunohistochemistry staining using anti-HBsAg, as well as H&E staining. Scale bars, 50 μm.
Figure S6. Extended follow-up confirmed that AAV-IL-21 treatment cleared intrahepatic rcccDNA from rcccDNA persistence mice. Cre Tg C57BL/6 mice positive for serum HBV antigen for 4 weeks after Ad/prcccDNA injection were
injected with $2 \times 10^{11}$ geq of AAV-IL-21 ($n = 6$), AAV-Ctrl ($n = 3$), or left untreated ($n = 3$). Serum HBsAg were tested as indicated. At 22 weeks post injection (w.p.i.) of AAV, mice were sacrificed and liver samples taken (A). All 6 AAV-IL-21 treated mice had been HBsAg-negative for over 4 weeks at sacrifice. RecccDNA in liver nuclear DNA was measured using quantitative realtime PCR and normalized against GAPDH genomic DNA in the same sample (B). Statistical significances are calculated using unpaired two-tailed $t$-test. **$p < 0.05$; ***$p < 0.001$. ns, not significant. Reaction mixtures from (B) were subjected to agarose electrophoresis for amplicon size check (C). Serum HBsAg status of respective mouse was indicated (B and C). naïve, Cre Tg C57BL/6 mice without Ad/preccDNA injection ($n = 2$).
Figure S7. ELISPOT analysis of HBsAg-specific spleen T cells in BPS persistence mice treated with recombinant AAV. (A) BPS HDI mice remaining positive for serum HBV antigens for 4 weeks were injected with $2 \times 10^{11}$ geq of AAV-IL-21 ($n = 3$), AAV-Ctrl ($n = 3$), or left untreated ($n = 3$). Serum HBsAg was tested at indicated time points and mice were sacrificed at 8 weeks post injection (w.p.i.) of AAV when
all three AAV-IL-21-treated mice had become negative for serum HBsAg, while all
mice in the other two groups remained positive. Splenocytes were prepared and
stimulated with 15 μg/ml recombinant HBsAg for 18 hours. (B) Gamma interferon
(IFN-γ)-secreting cells were measured using commercial IFN-γ ELISPOT assay kit
(eBioscience) and spots were analysed using iSpot ELISPOT Reader (AID, Germany).
Splenocytes from each mouse were analysed in duplicates. Group means and SEM
within group are presented. SFC: spot-forming cells. Statistical significance was
calculated using unpaired two-tailed t-test. **p < 0.05; ***p < 0.001. ns, not
significant.
Figure S8. Liver H&E and CD8 staining of BPS persistence mice treated with recombinant AAV. Male BALB/c mice aged 6-8 weeks were injected through hydrodynamic injection via tail vein with 10 μg of BPS replicon plasmid, and mice remaining positive for serum HBV antigens for 4 weeks were injected with $2 \times 10^{11}$ geq of AAV-IL-21 or AAV-Ctrl, or left untreated. Sera were collected at indicated time points and serum HBsAg was analyzed in ELISA. Randomly selected mice were sacrificed at 2 weeks post injection (w.p.i.) of AAV, and the remaining mice were sacrificed at 6 w.p.i. (A). Liver samples were taken from sacrificed mice, and H&E and anti-CD8 staining were performed (B). Treatment and serum HBsAg status at sacrifice time are indicated at the top. Representative images from at least 3 mice per group are shown. Arrows, infiltration foci. Scale bars, 50 μm.
Figure S9. Activation of JAK-STAT pathway in CD8+ T cells of BPS persistence mice receiving AAV-IL-21 treatment. Male BALB/c mice aged 6-8 weeks were injected through hydrodynamic injection via tail vein with 10 μg of BPS replicon
plasmid, and mice remaining positive for serum HBV antigens for 4 weeks were injected with $2 \times 10^{11}$ geq of AAV-IL-21 or AAV-Ctrl. Mice were sacrificed two weeks later and CD8$^+$ T cells were isolated from splenocytes to undergo RNA extraction and mRNA-targeted deep sequencing as described in Methods. Gene ontology (GO) enrichment analysis was performed on identified differentially expressed genes (DEG) (A). GO terms containing ‘T cell’ (27 in all) plus ‘Positive regulation of JAK-STAT cascade’ (GO:0046427, red) were significantly enriched in DEG, with 84 randomly selected GO terms plotted as control. (B) Volcano plot showing distribution of mRNA level ratios (AAV-IL-21 treated versus AAV-Ctrl treated BPS persistence mice) and adjusted $p$ values of genes identified in transcriptome sequencing. Significantly (adjusted $p < 0.05$) upregulated (red) and downregulated (blue) genes are highlighted. DEGs belonging to ‘Positive regulation of JAK-STAT cascade’ (GO:0046427) were highlighted (green arrows). Detailed sequencing analysis results are included in Table S1. (C) BPS persistence mice in groups of 3 were injected with $2 \times 10^{11}$ geq of AAV-IL-21 or AAV-Ctrl, or left untreated and 2 weeks later sacrificed for splenic CD8$^+$ T cell isolation. Isolated cells from each group were pooled and assayed for total and phosphorylated STAT1 and STAT3 in Western blot.
Figure S10. CD8 blocking delayed HBV clearance in AAV-IL-21-treated BPS persistence mice. BPS persistence mice were injected (solid arrows) with $2 \times 10^{11}$ geq of AAV-IL-21 or left untreated. Four hours earlier, AAV-IL-21-treated mice
received intraperitoneal injection (open arrows) with 10 µg CD8 monoclonal antibody or normal IgG, or no antibody injection. Antibody injections were repeated twice weekly for 6 weeks after AAV-IL-21 treatment. Sera collected at indicated time points were analyzed for HBsAg (A), HBsAb (B), HBeAg (C) and ALT (D). Means and SEMs within group are presented with the group size (n) indicated. Group positivity percentage data are presented for HBsAg, HBsAb and HBeAg (A-C, right panels). Dotted lines represent cut-off thresholds (A-C, left panels) or pre-treatment baseline (D). Statistical significances were calculated by comparing against untreated mice using log-rank (Mantel-Cox) test (A-C, right panels) and unpaired two-tailed t-test (D). **p < 0.01; ***p < 0.001. w.p.i., weeks post injection of AAV.
Figure S11. CD8 blocking by antibody injections delayed HBV clearance in AAV-IL-21-treated rcccDNA persistence mice. Male C57BL/6 mice transgenic for liver-specific Cre recombinase aged 6-8 weeks were injected with $1.5 \times 10^9$
plaque-forming units (PFU) of Ad/prcccDNA via tail vein, and mice remaining positive for serum HBV antigen for 4 weeks were left untreated, or injected (solid arrows) with $2 \times 10^{11}$ geq of AAV-IL-21, or injected (open arrows) intraperitoneally with 10 µg of CD8 monoclonal antibody or normal IgG 4 hours prior to AAV-IL-21 injection. Antibody injections were repeated (open arrows) twice a week for 6 weeks and then stopped. Sera were collected at indicated time points and analyzed for HBsAg (A), HBsAb (B), HBeAg (C), and ALT (D). Means and SEMs within group are presented with the group size ($n$) indicated. Group positivity percentage data are presented for HBsAg, HBsAb and HBeAg (A-C, right panel). Dotted lines represent cut-off thresholds (A-C, left panels) or pre-treatment baseline (D). Data are compared to that of the untreated mice and statistical significance calculated using log-rank (Mantel-Cox) test (A-C, right panels) and unpaired two-tailed t-test (D). **$p < 0.01$; ***$p < 0.001$. w.p.i., weeks post injection (w.p.i.) of AAV.
Figure S12. Liver H&E and CD8 staining of treatment-naïve BPS persistence mice receiving transfer of splenocytes stimulated with IL-21 and HBsAg.

Splenocytes from untreated BPS persistence mice were cultured in the presence of 100 ng/ml recombinant mouse IL-21 (rIL-21) with or without 15 μg/ml recombinant HBsAg, or without stimulation. After 60 hours, $1 \times 10^8$ splenocytes were injected into each BPS persistence mice via tail vein. Sera collected at indicated time points were analyzed for HBsAg. Randomly selected mice were sacrificed at 2 weeks post injection (w.p.i.) of splenocytes, and the remaining mice were sacrificed at 10 w.p.i. (A). Liver samples were taken from sacrificed mice, and H&E and anti-CD8 staining...
were performed (B). Treatment and serum HBsAg status at sacrifice time are indicated at the top. Representative images from at least 3 mice per group are shown.

Arrows, infiltration foci. Scale bars, 50 μm.
Figure S13. Detection of AAV-IL-21 DNA sequences in AAV-IL-21 treated BPS persistence mice. Male BALB/c mice aged 6-8 weeks were injected through hydrodynamic injection via tail vein with 10 μg of BPS replicon plasmid, and mice remaining positive for serum HBV antigens for 4 weeks were injected with $2 \times 10^{11}$ geq of AAV-IL-21 or AAV-Ctrl, or left untreated. At 4 or 20 weeks post injection (w.p.i.) of AAV, mice were sacrificed with liver samples and sera collected. (A) Nuclear DNA was extracted from liver tissue and tested for presence of AAV-IL-21-derived DNA in PCR using a primer set encompassing IL-21 and EGFP coding sequences. (B) Serum IL-21 levels were measured in ELISA. Each group contained three mice. Data are compared between groups and statistical significance calculated using unpaired two-tailed $t$-test. **$p<0.01$. ns, not significant.
Figure S14. AAV-IL-21 cured BPS persistence mice were protected against BPS re-challenge. Male BALB/c mice aged 6-8 weeks were injected through hydrodynamic injection (HDI) via tail vein with 10 μg of BPS replicon plasmid, and mice remaining positive for serum HBV antigens for 4 weeks were injected with $2 \times 10^{11}$ geq of AAV-IL-21 or AAV-Ctrl, or left untreated. Twenty weeks later, AAV-IL-21 treated BPS mice that achieved BPS clearance (serum HBsAg negative for at least 4 weeks) (C), as well as AAV-Ctrl treated (B) and untreated mice (A) were re-challenged with BPS via HDI (red arrow). Sera were collected at indicated time points and assayed for HBsAg, HBsAb, HBeAg and ALT. Dotted lines represent cut-off thresholds for HBsAg, HBsAb and HBeAg and pre-re-challenge baseline for ALT. d.p.i and w.p.i., days and weeks post BPS re-challenge injection. Mice were sacrificed at 5 w.p.i. of re-challenge and subjected to liver BPS plasmid DNA analysis (see Figure S15).
Figure S15. Clearance of intrahepatic BPS plasmid DNA from AAV-IL-21 cured BPS mice re-challenged with BPS. Mice from the experiment shown in Figure S14 were sacrificed at 5 w.p.i. of BPS re-challenge and total nuclear DNA extracted from liver samples. BPS plasmid DNA was then measured using quantitative realtime PCR (A) and Southern blot (B). Serum HBsAg status of the mice are indicated. Data are compared between groups and statistical significance calculated using unpaired two-tailed t-test. *p<0.05; **p<0.01. ns, not significant. naïve, male BALB/c mice without HDI of BPS.
Figure S16. Activation of JAK-STAT pathway in CD8⁺ T cells of AAV-IL-21 BPS persistence mice receiving treatment. Male BALB/c mice aged 6-8 weeks were injected through hydrodynamic injection (HDI) via tail vein with 10 μg of BPS replicon plasmid, and mice remaining positive for serum HBV antigens for 4 weeks were injected with 2 × 10¹¹ geq of AAV-IL-21 or AAV-Ctrl, or left untreated. Twenty weeks later, AAV-IL-21 treated BPS mice that achieved BPS clearance (serum HBsAg negative for at least 4 weeks), as well as AAV-Ctrl treated and untreated mice were re-challenged with BPS via HDI. Three days post re-challenge, mice were sacrificed for splenic CD8⁺ T cell isolation. Isolated cells from each group were pooled and assayed for total and phosphorylated STAT1 and STAT3 in Western blot. Each group contained 3 mice.
Figure S17. BPS persistence mice cured by adoptive transfer of T cells from AAV-IL-21-treated mice were protected against BPS re-challenge. BPS persistence mice were transferred with CD3⁺, CD4⁺, or CD8⁺ T cells from AAV-IL-21-cured BPS persistence mice (serum HBsAg negative for over 4 weeks), or left untreated. At about 15 weeks post transfer, CD3⁺ and CD8⁺ T cell recipient mice that displayed HBsAg negativity longer than 4 weeks as well as mice receiving no transfer or CD4⁺ T cells were re-challenged with BPS via HDI (red arrow). Sera were collected at indicated time points and assayed for HBsAg, HBsAb, HBeAg and ALT. Dotted lines represent cut-off thresholds for HBsAg, HBsAb and HBeAg and pre-re-challenge baseline for ALT. d.p.i and w.p.i., days and weeks post BPS re-challenge injection.
Figure S18. AAV-IL-21 cured rcccDNA persistence mice were protected against Ad/prcccDNA re-challenge. Cre Tg C57BL/6 mice positive for serum HBV antigen for 4 weeks after Ad/prcccDNA injection were injected with $2 \times 10^{11}$ geq of AAV-IL-21 or AAV-Ctrl, or left untreated. Fifteen weeks later, AAV-IL-21 treated BPS mice that achieved rcccDNA clearance (serum HBsAg negative for at least 4 weeks) (C), as well as AAV-Ctrl treated (B) and untreated mice (A) were re-challenged with Ad/prcccDNA (red arrow). Sera were collected at indicated time points and assayed for HBsAg, HBsAb, HBeAg and ALT. Dotted lines represent cut-off thresholds for HBsAg, HBsAb and HBeAg and pre-re-challenge baseline for ALT. d.p.i and w.p.i., days and weeks post Ad/prcccDNA re-challenge. Mice were sacrificed at 6 w.p.i. of re-challenge and subjected to liver rcccDNA analysis (see Figure S19).
**Figure S19. Clearance of intrahepatic rcccDNA from AAV-IL-21 cured rcccDNA mice re-challenged with Ad/prcccDNA.** Mice from the experiment shown in Figure S18 were sacrificed at 6 w.p.i. of Ad/prcccDNA re-challenge and total nuclear DNA extracted from liver samples. ReccDNA in liver nuclear DNA was measured using quantitative realtime PCR and normalized against GAPDH genomic DNA in the same sample (A). Reaction mixtures from (A) were subjected to agarose electrophoresis for amplicon size check (B). Serum HBsAg status of respective mouse was indicated. naïve, Cre Tg C57BL/6 mice without Ad/prcccDNA injection (n = 2).
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<th>Target</th>
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*, Targeted segment encompasses both BPS and vector backbone sequences.

**, targeted segment encompasses both IL-21 and vector EGFP sequences.