T Cells Expressing a Chimeric Antigen Receptor That Binds Hepatitis B Virus Envelope Proteins Control Virus Replication in Mice

Supplemental Figures

Figure S1

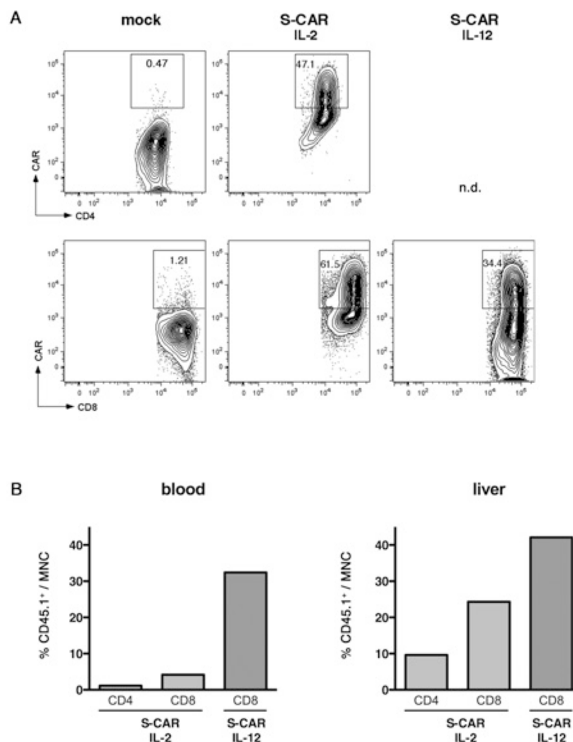

Figure S1. Comparison of IL-2 and IL-12 preconditioning of T cells before adoptive transfer. Stimulation and transduction was carried out either in the presence of IL-2 for total splenocytes or IL-12 for positively selected $CD8^+$ T cells. (*A*) Cell surface expression of the S-CAR on primary murine $CD4^+$ (upper row) and $CD8^+$ (lower row) T cells was quantified by flow cytometry. n.d.= not determined. (*B*) Recovery of transferred cells ($CD45.1^+$) from blood and liver 6 days after transfer of $3x10^6$ CAR^+ T cells into HBVtg mice. Cells from 4 mice per group were pooled and numbers of transferred cells are given relative to total isolated mononuclear cells.

18 **Figure S2**

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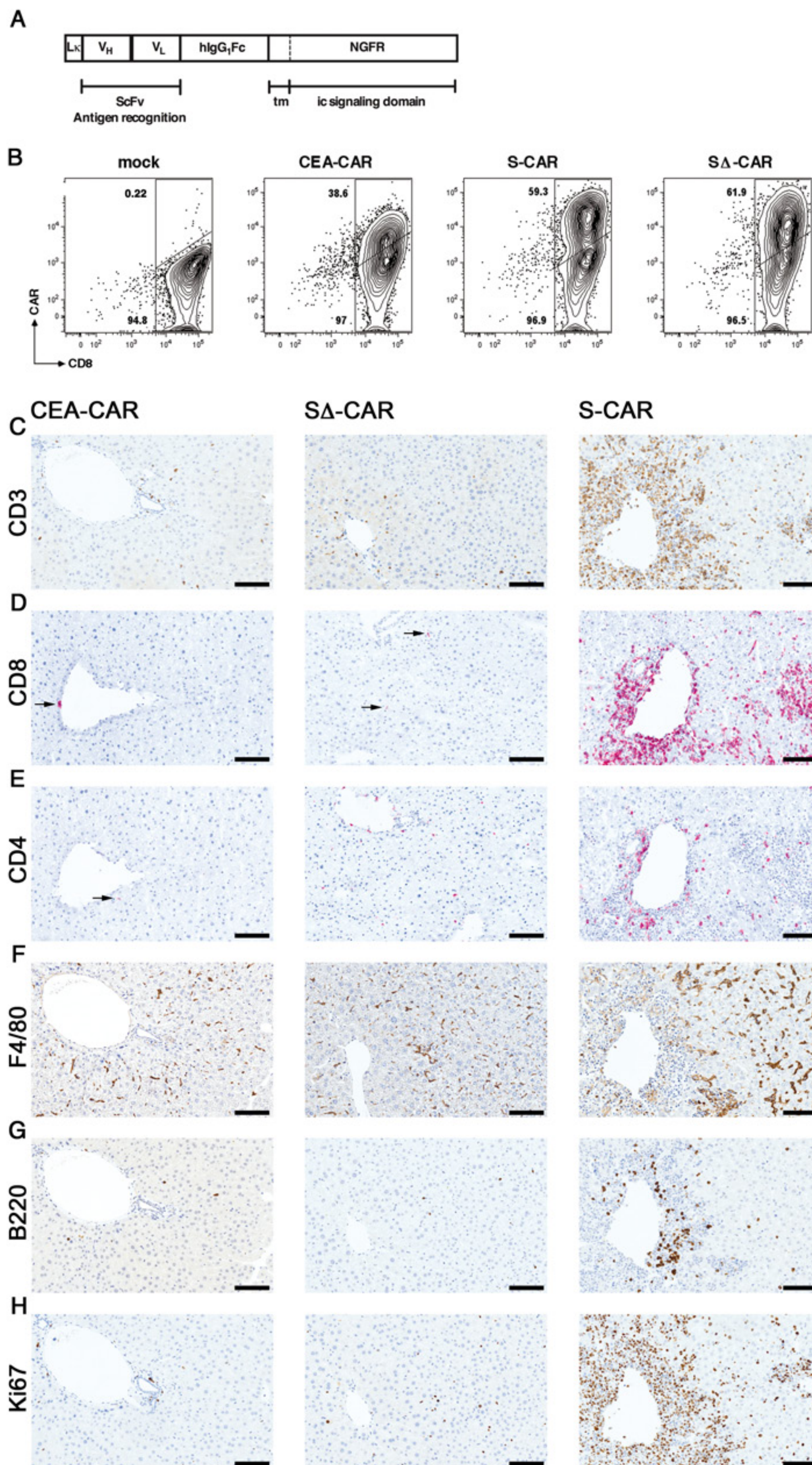

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21 **Figure S2.** Adoptive transfer of IL-12 stimulated and CAR-grafted CD8$^+$ T cells. (*A*)
22 Schematic representation of the SΔ-CAR, which combines the extracellular part of the S-
23 CAR with the intracellular domain of the nerve growth factor receptor (NGFR). (*B*) Cell
24 surface expression of CARs on murine CD8$^+$ T cells quantified by flow cytometry. Numbers
25 at the bottom indicate the percentage of CD8$^+$ T cells; numbers at the top indicate the
26 percentage of CAR-expressing cells. (*C-H*) Characterization of leukocytes in the liver
27 analyzed by histological staining on day 12 after adoptive T cell transfer. Arrows indicate
28 single positive cells. Tissue was cryo-preserved (*D,E*) or paraffin-embedded (*C,F,G,H*).
29 F4/80 stains macrophages, B220 stains B-cells, Ki-67 stains proliferating cells. Scale bars:
30 100 µm.

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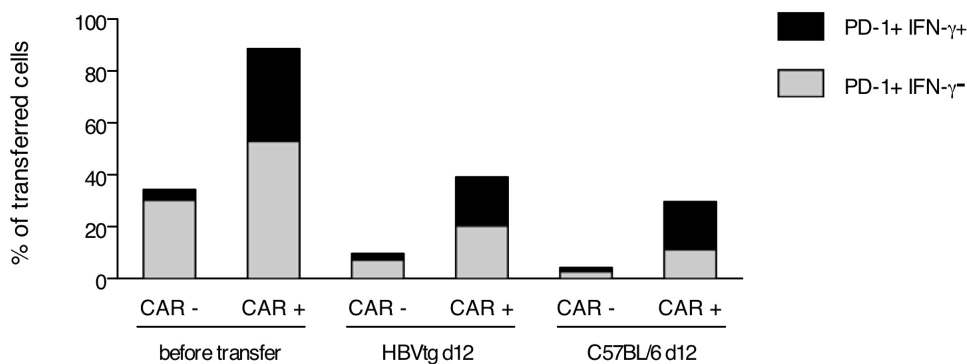
35 **Figure S3**



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37 **Figure S3**. PD-1 expression and IFN-γ response to *ex vivo* antigen stimulation of adoptively
38 transferred CD8$^+$ T cells. Cells were stimulated with plate-bound, recombinant HBsAg for 20h
39 and subsequently stained for CD8, CD45.1, S-CAR, PD-1 and IFN-γ. Left: CD8$^+$ T cells
40 before transfer. Right: Liver associated lymphocytes isolated at d12 post transfer from HBVtg
41 or wild-type C57BL/6 recipient mice. Cells were first gated on CD45.1$^+$ CD8$^+$ transferred T
42 cells and then on expression of S-CAR as indicated on the X-axis. Data represent mean
43 values, n=4. LAL from C57BL/6 mice were pooled for analysis.

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Figure S4

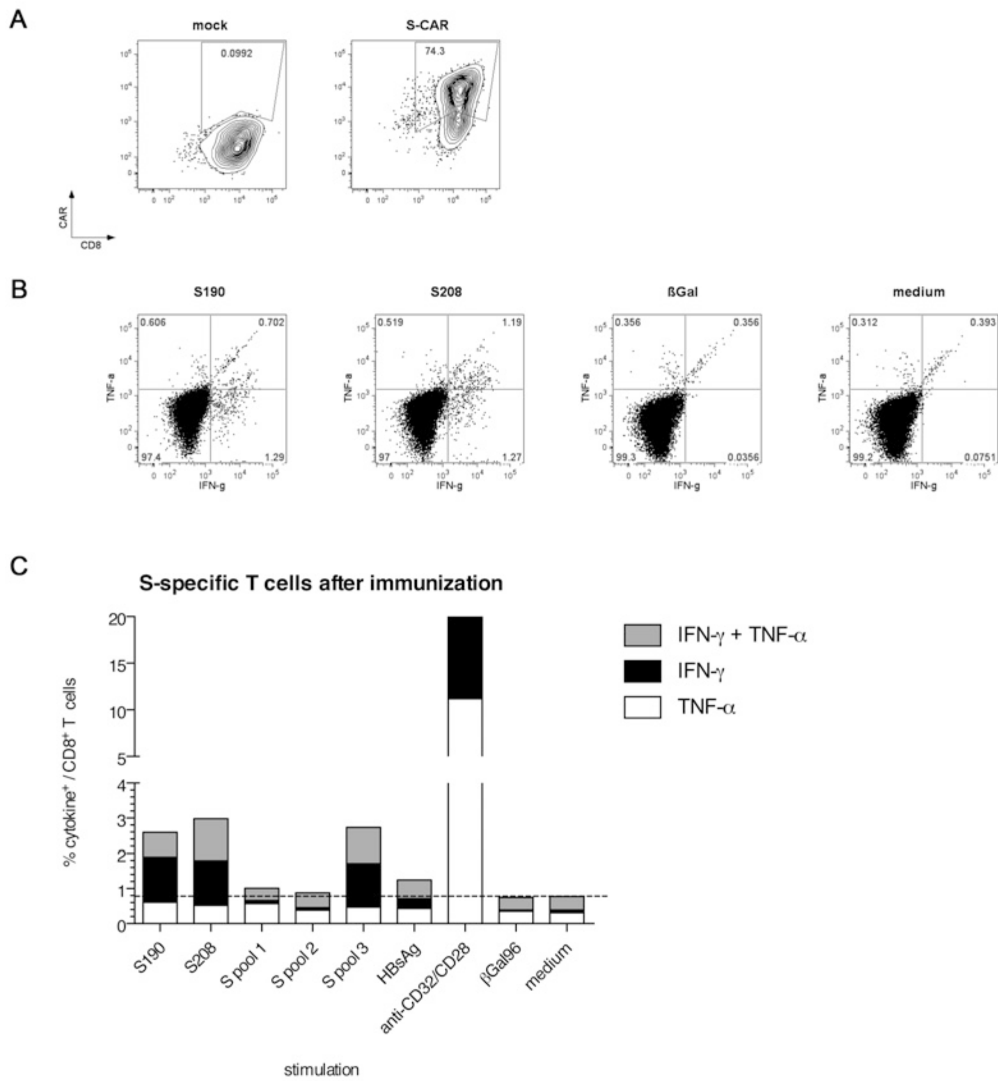

Figure S4. Preparation of S-CAR-grafted and natural HBV-specific $CD8^+$ T cells. (*A*) Cell surface expression of S-CAR on murine $CD8^+$ T cells quantified by flow cytometry. Numbers indicate the percentage of CAR-expressing cells. (*B*) $CD45.1^+$ mice were immunized with HBsAg and MVA-S and intracellular cytokine staining assessed numbers of S-specific T cells. Four representative FACS dot plots show IFN-γ and TNF-α production of $CD8^+$ T cells after stimulation with HBV S190 and S208 peptides, an irrelevant peptide or no peptide. (*C*) HBV-specific cytokine production of $CD8^+$ T cells after immunization.

62 ## Supplemental Methods

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64 **Cultivation of hepatoma cells.** Hepatoma cells were kept in full DMEM medium (DMEM, 10
65 % FCS, 1 % Pen/Strep, 1 % sodium pyruvate, 1 % NEAA; Life Technologies, Darmstadt,
66 Germany). For co-culture experiments $5x10^4$ target cells per well were seeded in collagen-
67 coated (Serva, Heidelberg, Germany) 96-well flat bottom plates. When cells had reached
68 confluence, differentiation medium was applied (Williams medium, 5 % FCS, 1 % Pen/Strep,
69 1 % sodium pyruvate, 1 % NEAA (Life Technologies) and 0.5 % DMSO (Sigma-Aldrich,
70 Taufkirchen, Germany). Target cells were used for experiments after 10 to 14 days of
71 differentiation.

72 **Cultivation of murine cells.** Primary murine cells were maintained in murine T cell medium
73 (RPMI dutch modified, 10 % FCS, 1 % glutamine, 1 % Pen/Strep, 1 % sodium pyruvate and
74 50 µM β-mercaptoethanol; Life Technologies).

75 **IL-12 production**. Murine recombinant IL-12 was produced by using a single polycistronic
76 vector containing IL12p40 and IL-12p35 obtained from Dr. W.J. Storkus (1) and subsequently
77 affinity purified.

78 **Co-culture with hepatoma cell lines and HBsAg stimulation.** The number of effector T
79 cells/well was adjusted according to the transduction efficiency of each receptor to identical
80 numbers of CAR-expressing cells. 5-10 µg /ml HBsAg (kindly provided by RheinBiotech-
81 Dynavax, Düsseldorf, Germany) was coated on 96-well flat bottom plates for 7 h at RT or
82 overnight at 4°C. Plates were washed with PBS before cells were added. Supernatants of
83 co-cultures and stimulation with recombinant antigen were subjected to a murine IFN-γ
84 ELISA (BD Biosciences).

85 **Proliferation assay.** S-CAR-grafted T cells were incubated with 2.5µM Carboxyfluorescein
86 succinimidyl ester (CFSE, Life Technologies) at 37°C for 10'. Cells were washed two times
87 and then $5x10^5$ T cells were co-cultured in a 24-well plate with HBV-replicating cell lines

88 (HepG2.2.15) or the parental HBV-negative cell line HepG2 for 3 days. Dilution of CFSE in
89 dividing cells was assessed by flow cytometry. Cells were additionally stained with anti-CD8
90 and anti-human IgG to discriminate between S-CAR positive and S-CAR negative cells.

91 **ALT measurement.** Serum alanin amino transferase activity was determined in 32 µl of
92 serum using the Reflotron system (Roche Diagnostics, Mannheim, Germany).

93 **Histopathology.** Liver tissue was either embedded in Tissue-Tek O.C.T. (Sakura, Staufen,
94 Germany) and frozen or fixed with 4 % paraformaldehyde and embedded in paraffin.
95 Cryo sections (5 µm) were fixed in aceton and stained with various antibodies: rabbit-anti-
96 CD3 (Neomarkers, Fremont, USA), rat-anti-B220, rat-anti-CD8a, rat-anti-CD4 (all BD
97 Biosciences, Heidelberg, Germany), mouse-anti-CD45.1 FITC, mouse-anti-CD45.2 FITC
98 (both eBioscience, Frankfurt, Germany), goat-anti-humanIgG FITC (Sigma-Aldrich), rat-anti-
99 CD68 (Serotec, Oxford, UK), goat-anti-rabbit Alexa488, goat-anti-rat Alexa594, goat-anti-
100 FITC Alexa488 (all Life Technologies), rabbit-anti-rat AP (Bond Polymer Refine Red
101 Detection, Leica, Newcastel Upon Tyne, UK). Paraffin sections (2 µm) were either stained
102 with haematoxylin / eosin or automated immunohistochemistry staining was performed using
103 rabbit-anti-HBcAg (Diagnostic Biosystems, Pleasanton, USA), rat-anti-F4/80 (BMA
104 Biomedicals, Augst, Switzerland), rabbit-anti-CD3, rat-anti-B220 (both BD Biosciences),
105 rabbit-anti-Ki67 (Neomarkers), rabbit-anti-cleaved caspase 3 (Cell Signaling, Danvers, USA),
106 rabbit-anti-rat HRP and anti-rabbit-poly HRP (Leica Bond Polymer Refine Detection
107 DS9800). Image acquisition was performed on the DotSlide BX51 (Olympus), SCN400
108 (Leica), Axio Z1 (Zeiss) or BX53 (Olympus) microscope.

109 **RNA extraction and quantification of mRNA levels.** Approximately 20 mg of liver tissue
110 was homogenized in 1 ml of Trizol (Life Technologies) using the Ultrathorax T10 (IKA,
111 Staufen, Germany). The RNA was extracted according to the manufacturer´s instruction.
112 Residual DNA was DNase digested (Roche Diagnostics, Mannheim, Germany) and 1 µg of
113 RNA was reverse transcribed into cDNA using the Superscript III Kit (Life Technologies).
114 Cytokine expression levels were determined relative to GAPDH expression by a quantitative

115 PCR on a LightCycler480 using SybrGreen (both Roche Diagnostics) and the primers
116 described before (2) or TNFα-fw 5'-ACGTCGTAGCAAACCAC-3' and TNFα-rev 5'-
117 AGATAGCAAATCGGCTG-3'. Expression levels were determined relative to GAPDH and
118 normalized to the CEA-CAR control group.

119 **DNA extraction and quantification of viral DNA.** Approximately 20 mg of liver tissue was
120 homogenized in 1 ml of Proteinase-K buffer (Tris pH 8.5 100 mM, EDTA 5 mM, SDS 0.2 %,
121 NaCl 200 mM) followed by a phenol-chloroform extraction of the DNA. Intracellular HBV
122 replicative intermediates were detected by Southern blot analysis of 75 µg of total liver DNA
123 digested with HindIII (Fermentas, St. Leon-Rot, Germany) and separated through a 0.85 %
124 agarose gel.
125 Quantitative PCR was performed using p-fwHBV 5'-TACTAGGAGGCTGTAGGCATA-3' and
126 p-revHBV 5'-GGAGACTCTAAGGCTTCCC-3'. Data were normalized to the cell number
127 determined by the single copy gene *Nid2*; mNid2Fw 5'-ATCCTACCGGGAAACAG-3' and
128 mNid2Rev 5'-CCCAAGGTCTTCGTTGA-3'. Viral DNA from serum was extracted using the
129 High pure viral nucleic acid Kit (Roche Diagnostics) and analyzed using fwHBV and revHBV
130 primers.

131 **Cytokines.** Serum cytokines were measured using the Cytometric Bead Array Mouse
132 Inflammation Kit (BD Biosciences). Briefly, 15 µl of undiluted or 1:4 diluted serum were
133 incubated with 15 µl of mixed capture beads and 15 µl of PE detector for 2 h at RT. 5000
134 beads were acquired on a BD FACS Canto II and analyzed with FCAP Array Software 3.0
135 (BD Biosciences).

136 **Immunoglobulin screening.** 100 µl of 1:1200 diluted serum were coated on Nunc Maxisorp
137 ELISA plates (Sigma) at 4 °C overnight. HRP-labeled detection antibodies (Southern
138 Biotech, Birmingham, USA) were diluted 1:1000 (anti-IgM and anti-IgG1), 1:500 (anti-IgA)
139 and 1:250 (anti-IgG2a, anti-IgG2b, anti-IgG3). ELISA was performed according to the manual
140 of the SBA Clonotyping System/HRP (Southern Biotech).

141 **Immunization.** CD45.1^{+} mice were immunized s.c. on day 0 with a volume of 50 µl vaccine
142 preparation containing 20 µg rHBsAg (Rheinbiotech, Düsseldorf, Germany), 0.1 mM CpG
143 1668 (Molbiol Syntheselabor GmbH, Berlin, Germany) and 50 µg Polyphosphazene (PCEP);
144 kindly provided by G. Mutwiri (Vaccine and Infectious Disease Organization/International
145 Vaccine Center, University of Saskatchewan, Saskatoon, Canada). On day 28 mice were
146 boosted i.p. with 1x10^{8} MVA pH5-S expressing HBs in 0.9 % NaCl. On day 35, splenocytes
147 were isolated and numbers of HBV-specific T cells were determined by intracellular cytokine
148 staining. For stimulation of cells from immunized mice, we used peptides ßGal 96-103, S190-
149 197 and S208-215 at a concentration of 1 µg/ml as well as three peptide pools of 18 15mers
150 overlapping by 11 amino acids covering the complete S protein (pool S1: S1-S83, S2: S73-
151 S155, S3: S144-S226). After overnight resting, CD8^{+} T cells were isolated using magnetic
152 cell sorting (Miltenyi) and processed as described for CAR-transduced T cells. The number
153 of HBV-specific CD8^{+} T cells was estimated to be approx. 4%.

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155 ## References

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157 1. Zitvogel, L., et al. Cancer immunotherapy of established tumors with IL-12. Effective
158 delivery by genetically engineered fibroblasts. *J Immunol*. 1995;155(3):1393-1403.
159 2. von Freyend, M.J., et al. Sequential control of hepatitis B virus in a mouse model of
160 acute, self-resolving hepatitis B. *J Viral Hepat*. 2010;18(3):216-226.

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