

**Efficient stabilization of therapeutic hepatitis B vaccine
components by amino-acid formulation maintains its potential to
break immune tolerance**

Julia Sacherl, Anna D. Kosinska, Kristina Kemter, Martin Kächele, Sabine Christine
Laumen, Hélène A. Kerth, Edanur Ates Öz, Lisa S. Wolff, Jinpeng Su, Sandra
Essbauer, Gerd Sutter, Martin Scholz, Katrin Singethan, Jens Altrichter, Ulrike
Protzer

Table of contents

| | |
|--------------------------------|----|
| Supplementary methods | 2 |
| Supplementary tables..... | 8 |
| Supplementary figures | 9 |
| Supplementary references | 14 |

Supplementary Methods

MVA-S/C production

Amplification and purification of the MVA-S/C was performed according to *Kremer et al.* with some modifications [1]. Confluent chicken fibroblast DF-1 cells (ATCC, CRL-12203) were infected with MVA-S/C and incubated until a cytopathic effect could be clearly seen. Remaining cells were scratched from the plates and harvested together with infected cells in the cell culture medium by centrifugation at 3853g for 5 min at 4°C. Cell pellets were resuspended in 10 mM Tris-HCl, pH 9.0 and stored at -80°C. After three freeze-thaw cycles, the virus suspension was sonicated three times for 30 s, spun-down by centrifugation and the pellet was resuspended in 10 mM Tris-HCl, pH 9.0. After repeating the sonication and centrifugation step three times, purification and concentration of the virus suspension was performed twice through a 36% sucrose cushion by ultracentrifugation (Optima L-90K Beckman Coulter GmbH, Krefeld, Germany) at 13500rpm for 1.5h at 4°C (SW32 Ti, SW41 Ti). After resuspending the pellet in 10 mM Tris-HCl, pH 9.0, the virus stock was stored at -80°C.

Titration of MVA-S/C by median tissue culture infection dose (TCID₅₀) assay

To determine the TCID₅₀ titer of MVA-S/C, the previously reported protocol by *Kremer et al.* [1] was used with some modifications. Confluent Baby hamster kidney cells (BHK-21; ATCC, CCL-10) were seeded on a 96-well plate in 10% RPMI medium and infected one day later with a series of 10-fold dilutions of the respective virus suspension in six steps in 2% RPMI medium and with 16 repetitions per dilution. At day seven post-infection, cells were analysed by light microscopy for a cytopathic effect induced by MVA-S/C. TCID₅₀/mL was calculated using the formula previously reported [2].

Stabilization, lyophilization, temperature exposure and reconstitution procedures

The LEUKOCARE database (LEUKOCARE AG, Martinsried, Germany) was applied to select excipients for stabilization of TherVacB components. It contains information about particular

physicochemical and structural properties of different kinds of biologics, formulations, and >100 excipients previously identified to be effective in stabilizing these biologics together with literature-known characteristic stabilization data. Based on this database we selected specifically tailored stabilizing amino acid (SAA)-based formulations successfully applied in former studies to stabilize proteins, viral vectors and other vaccine components in lyophilized formulations.

Stabilizing formulations were prepared by dilution of concentrated stock solutions of the HBV protein antigens (HBcAg, HBsAg) and MVA-S/C with the selected SAA-based formulation. As a negative control the vaccine components were diluted in PBS instead of an SAA-based buffer but otherwise underwent the identical treatment including lyophilization, storage and solubilization. Concentrations of the vaccine components and excipients used were the same in all studies. HBV protein antigens were formulated in combination if not stated otherwise. The MVA-S/C was formulated in a separate vial with the same SAA-based formulation as the antigens.

Lyophilization of the samples was performed in an Epsilon 2-6D freeze dryer (Martin Christ, Osterode am Harz, Germany) using the following protocol:

| Step | Target temperature (°C) | Slope (h) | Hold (h) | Pressure (mbar) |
|------------------|-------------------------|-----------|----------|-----------------|
| Introduction | 20 | 0 | 0 | 1000 |
| Freezing | -50 | 2:00 | 2:00 | 1000 |
| Sublimation | -50 | 0:01 | 0:30 | 0.045 |
| | -35 | 3:00 | 30:00 | 0.045 |
| Secondary drying | 20 | 3:00 | 7:00 | 0.009 |

After lyophilization samples were stored under specific defined temperature conditions according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines at 40°C/75 % relative humidity (RH),

25°C/60 % RH or 5°C in the refrigerator. Samples were reconstituted with the required volume of H₂O (*in vitro*) or PBS (*in vivo*) directly before *in vitro* analysis or *in vivo* immunization of mice.

Sandwich ELISA

ELISA was performed using NUNC MaxiSorp™ 96-well plates (Thermo Fisher Scientific, Waltham, USA). For HBcAg studies, the plate was coated with 1 µg/mL anti-IFA HepBCore (kindly provided by CIGB, Cuba) at 4°C overnight. After washing with PBS containing 0.05% Tween-20, blocking was performed with 5% bovine serum albumin (Roth, Karlsruhe, Germany) for 1h at room temperature (RT). Afterwards, 15 ng/mL sample was added to the well and incubated for 2h at RT. Following this, samples were incubated with anti HepBcore-HRP (diluted 1:7000; kindly provided by CIGB, Cuba) for 1h at RT. The absorbance was measured by Infinite F200 multiplate reader (Tecan Group AG, Männedorf, Switzerland) at a wavelength of 450 nm.

HBsAg was analysed using 1 µg/mL HBV-specific single-chain antibody for coating, 5 µg/well 5F9 as primary antibody and goat pAb to Hu IgG (HRP labeled; Abcam, Cambridge, UK) as secondary antibody diluted 1:1000 in 1% BSA. HBsAg samples were used at a concentration of 30 ng/mL. Coating, washing and detection was done as described above.

Native Agarose Gel Electrophoresis (NAGE)

To analyse integrity of HBcAg spontaneously forming capsids, 2.5 µg native HBcAg/lane was loaded on a 1% agarose gel (Peqlab, Erlangen, Germany) in 1x TAE buffer (50x: 2 M Tris-HCl, 1 M acetic acid, 50 mM EDTA, pH 8.0) and run for 90 min at 150V. Nucleic acid content was detected by UV-light in the Fusion FX7 (Peqlab, Erlangen, Germany) after Roti®-Safe GelStain staining. To visualize the protein content of the samples, gels were stained with Coomassie Staining Solution (0.1% Coomassie Brilliant Blue R250, 40% MeOH, 10% acetic acid, 50% H₂O) for 20 min at RT. For destaining at RT destain solution (10% acetic acid, 40% MeOH, 50% H₂O) was used.

Cell culture

BHK-21 were cultured in RPMI medium supplemented with 10% FCS (Thermo Fisher Scientific Inc., Waltham, USA) and 1% penicillin/streptomycin, 1% non-essential amino acids, 200 mmol/L L-glutamine and 1% of sodium pyruvate (all obtained from GIBCO, Thermo Fisher Scientific, Massachusetts, USA). DF-1 cells were cultured in DMEM+GlutaMAX™ medium (GIBCO, Thermo Fisher Scientific, Massachusetts, USA) containing the same supplements as described above without 2 mmol/L L-glutamine at 37°C, 5% CO₂.

AAV-HBV mouse model

Eight to ten weeks old wild-type male C57BL/6J mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Upon the transport or delivery, animals were always given 5-7 days' acclimatization period before the conducting of the experiments. Persistent HBV replication in wild-type C57BL/6J mice was established by intravenous injection of 4x10⁹ genome equivalents of adeno-associated virus (AAV)-HBV vector carrying a 1.2-fold overlength HBV genome (genotype D, ayw) [3]. AAV-HBV mice support replication of the HBV genome in mouse hepatocytes over months and develop HBV-specific immune tolerance. Moreover, the HBV genome remains episomal upon AAV-HBV infection which allows studying of the elimination of infected hepatocytes [3]. AAV-HBV mice were bled shortly before start of immunization and allocated into groups with comparable HBeAg and HBsAg serum levels. AAV-HBV infected or HBVtg mice which displayed ≥2-fold lower or higher HBV antigen levels than mean were excluded from the experiments. Grouping of mice was agreed upon all authors involved in the experiments and the supervisor. All experiments were designed as explorative orientation study and according to statistical analysis of the expected outcome (HBeAg/HBsAg drop, immune activation marker), 4-5 mice per group were used.

Intracellular cytokine staining (ICS)

For ICS, murine splenocytes and LALs were stimulated *ex vivo* with peptides in the presence

of brefeldin A (Sigma-Aldrich, Taufkirchen, Germany) at 37°C overnight. For antigen-specific stimulation 1 µg/mL MVA_{B8R}, OVA_{S8L}, HBV S₂₈₀, HBV C₉₃ or peptide pools covering HBV S (genotype D, aa 145-226) or core (genotype D, aa 70-157) containing the dominant CD8 as well as CD4 T-cell epitopes were used. On the next day, cells were stained with anti-CD4 APC (clone GK1.5, eBioscience™) and anti-CD8a Pacific blue (clone 53-6.7, BD Pharmingen™) at a volume of 50 µL/well for 20 min to determine the T-cell subtypes. Dead cells were excluded from analysis by staining with Fixable Viability Dye eFluor™ 780 (eBioscience, Frankfurt, Germany). Afterwards, cells were fixed and permeabilised using Cytofix/Cytoperm Kit (BD Biosciences, Heidelberg, Germany) according to manufacturer's protocol. Intracellular cytokine staining (ICS) was performed using anti-IFNγ (clone XMG1.2; BD Pharmingen™). Data were acquired on a CytoflexS flow cytometer (Beckmann Coulter, Brea, CA, USA). Analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA). HBV-specific responses are shown as relative values after subtraction of background using OVA_{S8L} peptide. Five mice per group were analysed. In the presented graphs of HBV-specific antibodies and CD4⁺ and CD8⁺ T cells values of individual mice are shown, horizontal lines indicate median. Time kinetics of serum HBsAg, HBeAg and ALT display the mean value of each experimental group.

Serological and virological analyses

DNA was extracted from 50 µL mouse serum using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) or 20 mg of liver tissue using a NucleoSpin Tissue DNA Kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions.

The quantification of HBV-DNA was performed through real-time PCR with SYBR green as previously described [4] using a LightCycler 480 PCR system (both: Roche, Mannheim, Germany) and the primers HBV-1745-Fw: 5'-GGAGGGATACATAGAGGTTCTTGA-3' and HBV-1844-Rev: 5'-GTTGCCCGTTTGTCTCTAATTC-3'. The results were normalized to a single copy prion protein (PrP) gene (primers: PrP-Fw 5'-TGCTGGGAAGTGCCATGAG-3' and

PrP Rev 5'-CGGTGCATGTTTTTCACGATAGTA-3'). The amplification conditions for both PCR reactions were 95°C for 300 s followed by 45 x (95°C for 15 s, 60°C for 10 s, and 72°C for 25 s).

Immunohistochemistry

Liver tissue samples were fixed in 4% buffered formalin for 48 h and were embedded in paraffin. Liver sections that were 2-µm-thin were then prepared with a rotary microtome (HM355S, ThermoFisher Scientific, Waltham, USA). Immunohistochemistry was performed according to the protocol described previously [4] using a Bond Max system (Leica Biosystems, Nussloch, Germany) with the anti-HBcAg primary antibody (Diagnostic Biosystems, Pleasanton, CA; 1:50 dilution) and a horseradish peroxidase coupled secondary antibody. Briefly, the slides were deparaffinized using deparaffinization solution pre-treated with epitope retrieval solution (corresponding to citrate buffer pH 6) for 20 min. Antibody binding was detected with a polymer refine detection kit without post primary reagent and was visualized with 3,3'-diaminobenzidine (DAB) as a dark brown precipitate. Counterstaining was done with haematoxylin. Slides were scanned using a SCN 400 slide scanner (Leica Biosystems). Determination of HBcAg-positive hepatocytes was performed based on the localization, intensity, and distribution of the signal in 10 random view fields (40x magnification). The mean numbers of the HBcAg-positive hepatocytes were quantified per mm².

Statistical analyses

To determine normal distribution of the data acquired, D'Agostino & Pearson omnibus normality test was applied. Data were analysed using GraphPad Prism version 5.01 or 9.0 (GraphPad Software Inc., San Diego, CA) using unpaired t-test or Mann-Whitney-U test if data were ordinal but not interval scaled. Statistical significance determined is indicated. P-values

Supplementary Tables

Supplementary Table 1. Excipients contained in SAA-based formulations F1.1-F1.4.

| Formulation | Excipients |
|-------------|--|
| F1.1 | Alanine, Arginine, Glutamic acid, Lysine, Histidine, Tryptophan, Citric acid, Trehalose, pH 7 |
| F1.2 | Alanine, Arginine, Glutamic acid, Histidine, Tryptophan, Methionine, Glutamine, Citric acid, Trehalose, Sorbitol, Mannitol, Polysorbate 80, pH 7 |
| F1.3 | Alanine, Arginine, Glutamic acid, Lysine, Histidine, Glycine, Sucrose, Mannitol, pH 7 |
| F1.4 | Alanine, Arginine, Glutamic acid, Histidine, Tryptophan, Methionine, Glutamine, Proline, Citric acid, Trehalose, Polysorbate 80, pH 7 |

Supplementary Figures

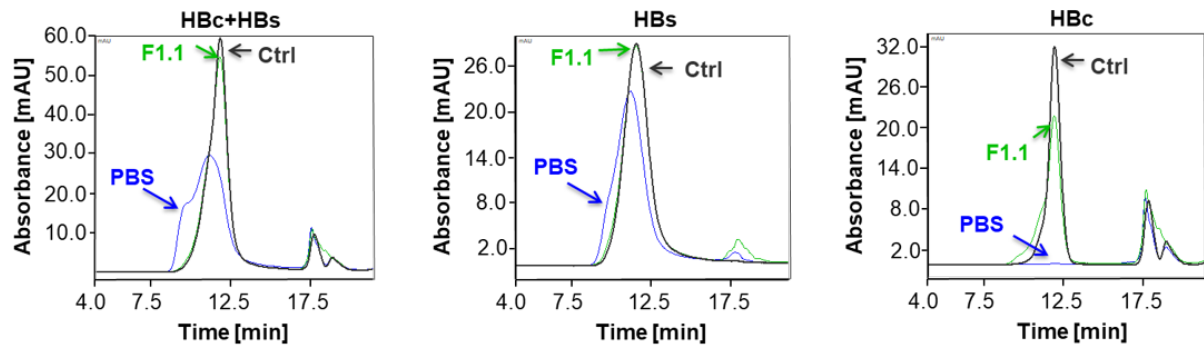


Fig. S1. Stability of F1.1-formulated HBV antigens after lyophilization. HBcAg and HBsAg were formulated in combination or as single components with SAA-based formulation F1.1 or PBS, lyophilized and analysed by SE-HPLC directly after lyophilization.

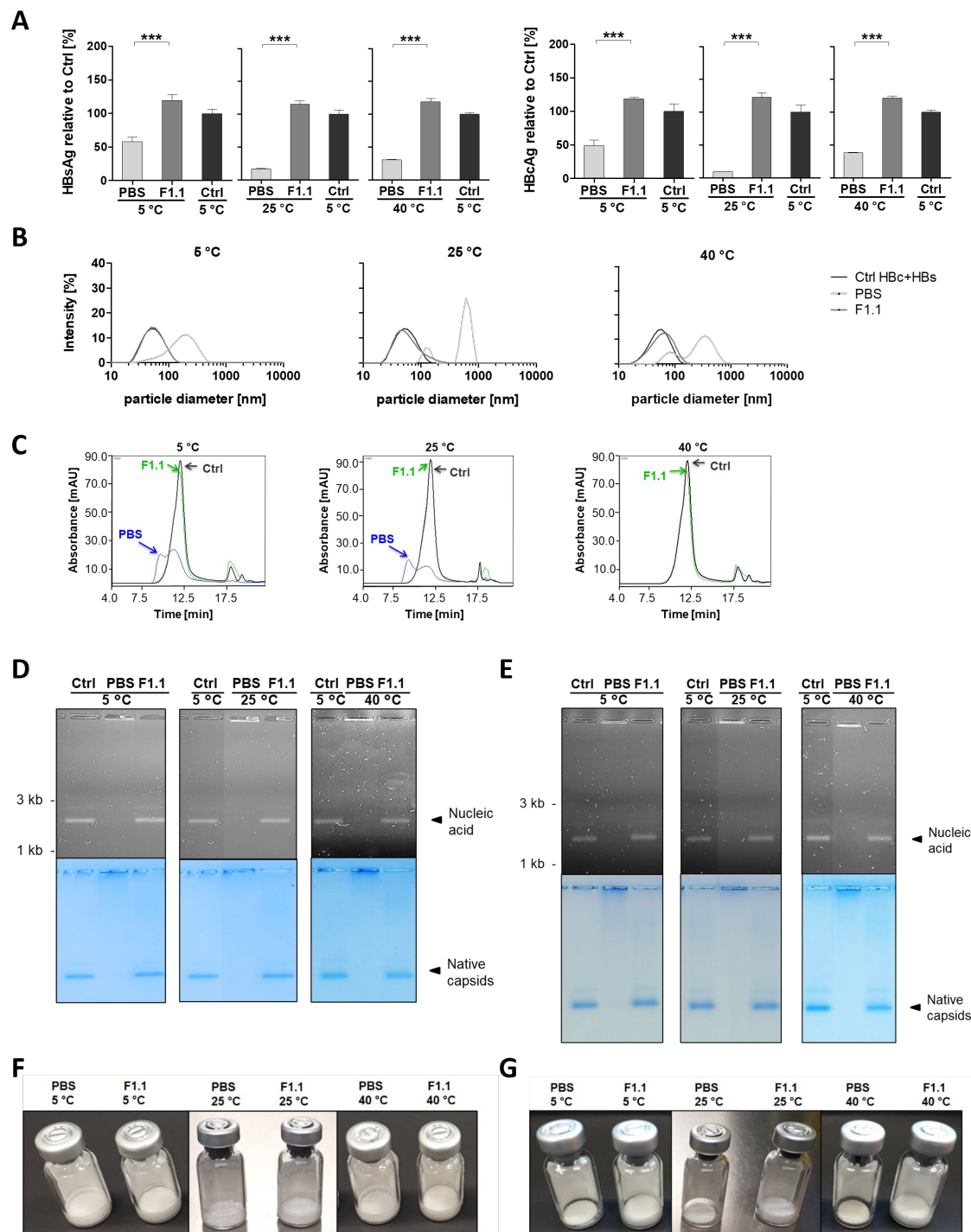


Fig. S2. Stability of *TherVacB* vaccine components after heat exposure for up to three months. Lyophilized F1.1- or PBS-formulated vaccine components were stored at 5°C, 25°C/60% RH and 40°C/75% RH for one (A-D) and three months (E-G). Control (Ctrl) vaccine components were without stabilization, lyophilization and temperature exposure. Antigen integrity was analysed by HBsAg- and HBcAg-specific ELISA relative to Ctrl set to 100%) (A),

DLS (B), SE-HPLC (C) of both protein antigens and NAGE of HBcAg after 1- (D) and 3-months storage (E). Cake structures of lyophilized antigens (F) and MVA-S/C (G) after 3-months storage. Data show mean±SD. Statistical analysis was performed using unpaired t-test. *** $p \leq 0.001$.

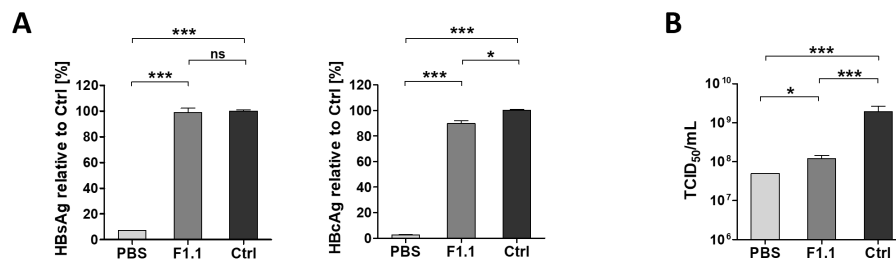


Fig. S3. Stability of *TherVacB* vaccine components after one month heat-exposure.

Lyophilized F1.1- or PBS-formulated vaccine components were stored at 40°C/75% RH for one month and vaccine potency was analysed *in vitro*. Control (Ctrl) vaccine components were without stabilization, lyophilization and temperature exposure. (A) HBsAg- and HBcAg-specific ELISA relative to Ctrl (set to 100%). (B) MVA-S/C titer determined by TCID₅₀. Data show mean±SD. Statistical analysis was performed using unpaired t-test. * $p < 0.05$; *** $p \leq 0.001$; ns - not significant.

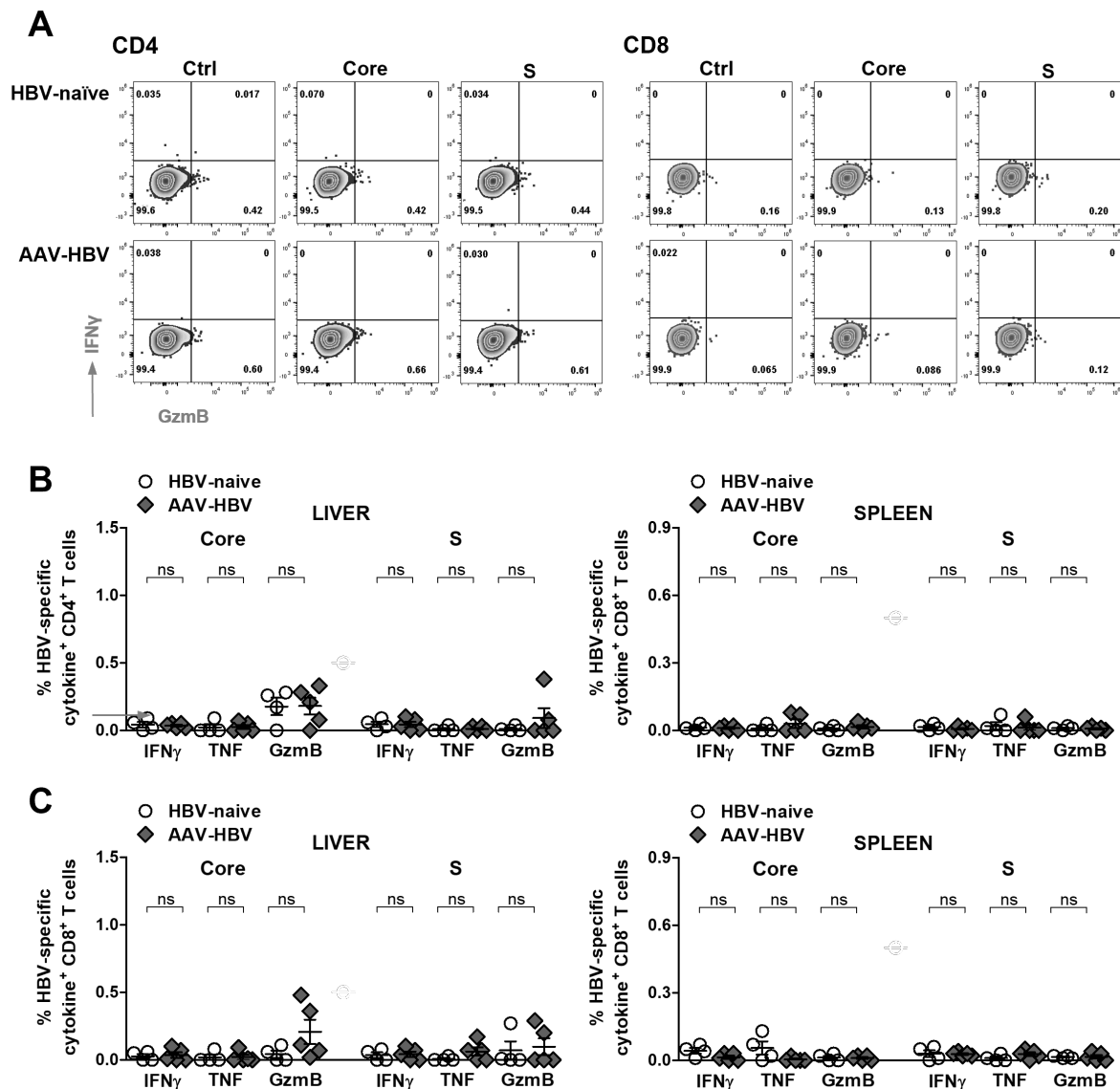


Fig. S4. HBV-specific T-cell response in AAV-HBV infected mice. (A) Exemplary results of HBV-specific T cells in livers of AAV-HBV infected and HBV-naïve mice determined by ICS four weeks after infection. HBV-specific CD4⁺ (B) and CD8⁺ (C) T cells were identified using sensitive staining with HBV core₉₃₋₁₀₀ (C₉₃) and S₁₉₀₋₁₉₇ (S₁₉₀) peptide-loaded MHC-multimers in AAV-HBV infected and HBV-naïve mice four weeks after infection. Data are given as mean±SEM. Statistical analysis applied unpaired Mann-Whitney test. ns - not significant.

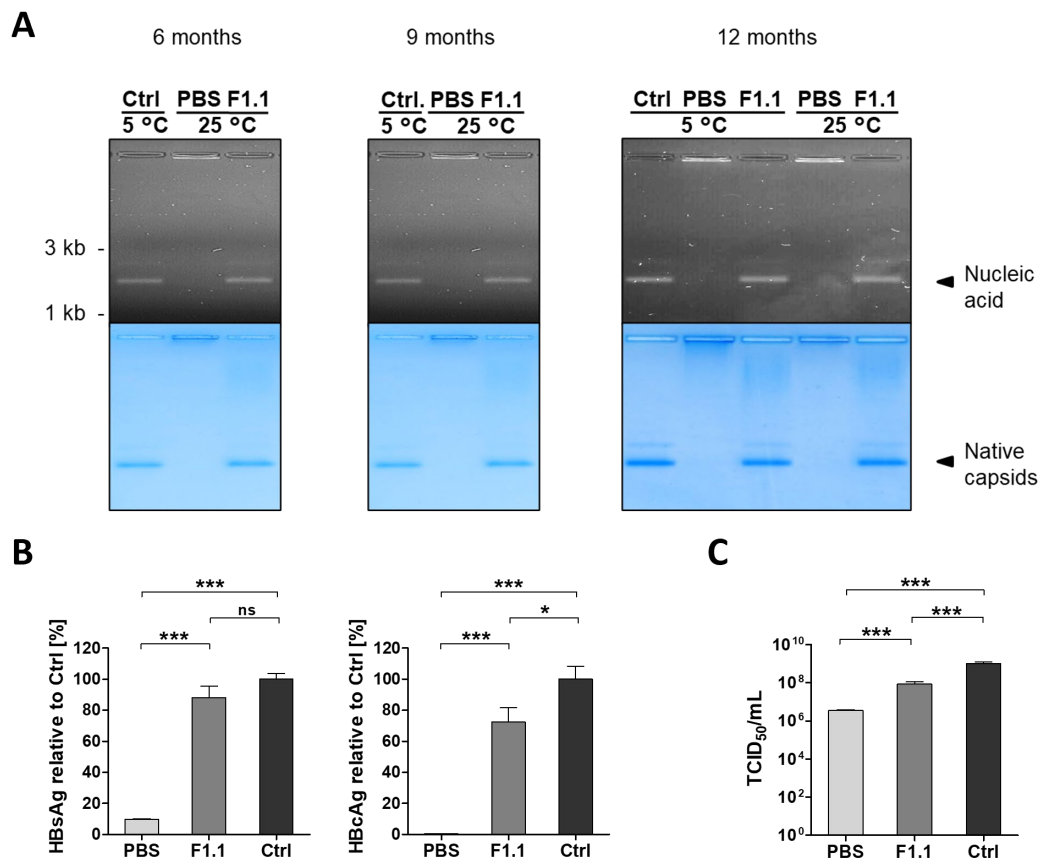


Fig. S5. Stability of *TherVacB* vaccine components after storage for up to one year at elevated room temperature. Lyophilized F1.1- or PBS-formulated vaccine components were stored at 5°C and 25°C/60% RH for 6, 9 and 12 months. Control (Ctrl) vaccine components were without stabilization, lyophilization and temperature exposure. (A) Antigen integrity was analysed by NAGE after 6-, 9- and 12 months of storage. After 12 months, vaccine potency was analysed by (B) HBsAg- and HBcAg-specific ELISA relative to Ctrl (set to 100%) and (C) titration of MVA-S/C titer using TCID₅₀ assay. Data show mean±SD. Statistical analysis was performed using unpaired t-test. * $p < 0.05$; *** $p \leq 0.001$; ns - not significant.

References

Author names in bold designate shared co-first authorship.

- [1] Kremer M, Volz A, Kreijtz JH, Fux R, Lehmann MH, Sutter G. Easy and efficient protocols for working with recombinant vaccinia virus MVA. *Methods Mol Biol* 2012;890:59-92.
- [2] Kärber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Naunyn-Schmiedebergs Archiv für experimentelle pathologie und pharmakologie* 1931;162:480-483.
- [3] Dion S, Bourguine M, Godon O, Levillayer F, Michel ML. Adeno-Associated Virus-Mediated Gene Transfer Leads to Persistent Hepatitis B Virus Replication in Mice Expressing HLA-A2 and HLA-DR1 Molecules. *Journal of Virology* 2013;87:5554-5563.
- [4] Kosinska AD, Festag J, Muck-Hausl M, Festag MM, Asen T, Protzer U. Immunogenicity and Antiviral Response of Therapeutic Hepatitis B Vaccination in a Mouse Model of HBeAg-Negative, Persistent HBV Infection. *Vaccines (Basel)* 2021;9.