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

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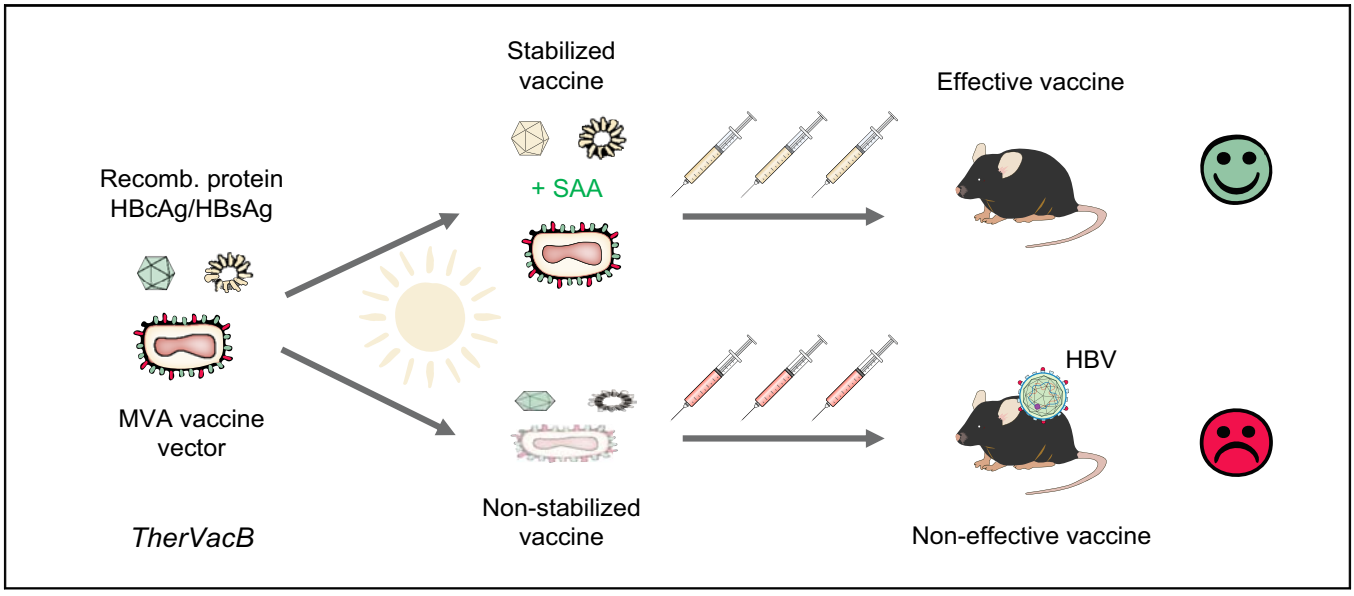
Graphical abstract



Highlights

- Heterologous protein-prime and MVA-vector boost can break immune tolerance to HBV.
- Exposure to higher temperature can damage protein- and vector-vaccine components.
- An amino acid-based formulation can preserve integrity of vaccine components.
- The formulated vaccine proves stable for 3 months at 40 °C and 1 year at 25 °C.
- Despite heat-exposure, stabilized *TherVacB* triggered strong HBV-specific immunity.

Impact and implications

Therapeutic vaccination is a promising therapeutic option for chronic hepatitis B that may enable its cure. However, its application requires functional cooling chains during transport and storage that can hardly be guaranteed in many countries with high demand. In this study, the authors developed thermostable vaccine components that are well tolerated and that induce immune responses and control the virus in preclinical mouse models, even after long-term exposure to high surrounding temperatures. This will lower costs and ease application of a therapeutic vaccine and thus be beneficial for the many people affected by hepatitis B around the world.

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

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Background & Aims: Induction of potent, HBV-specific immune responses is crucial to control and finally cure HBV. The therapeutic hepatitis B vaccine *TherVacB* combines protein priming with a Modified Vaccinia virus Ankara (MVA)-vector boost to break immune tolerance in chronic HBV infection. Particulate protein and vector vaccine components, however, require a constant cooling chain for storage and transport, posing logistic and financial challenges to vaccine applications. We aimed to identify an optimal formulation to maintain stability and immunogenicity of the protein and vector components of the vaccine using a systematic approach.

Methods: We used stabilizing amino acid (SAA)-based formulations to stabilize HBsAg and HBV core particles (HBcAg), and the MVA-vector. We then investigated the effect of lyophilization and short- and long-term high-temperature storage on their integrity. Immunogenicity and safety of the formulated vaccine was validated in HBV-naïve and adeno-associated virus (AAV)-HBV-infected mice.

Results: *In vitro* analysis proved the vaccine's stability against thermal stress during lyophilization and the long-term stability of SAA-formulated HBsAg, HBcAg and MVA during thermal stress at 40 °C for 3 months and at 25 °C for 12 months. Vaccination of HBV-naïve and AAV-HBV-infected mice demonstrated that the stabilized vaccine was well tolerated and able to brake immune tolerance established in AAV-HBV mice as efficiently as vaccine components constantly stored at 4 °C/−80 °C. Even after long-term exposure to elevated temperatures, stabilized *TherVacB* induced high titre HBV-specific antibodies and strong $CD8^+$ T-cell responses, resulting in anti-HBs seroconversion and strong suppression of the virus in HBV-replicating mice.

Conclusion: SAA-formulation resulted in highly functional and thermostable HBsAg, HBcAg and MVA vaccine components. This will facilitate global vaccine application without the need for cooling chains and is important for the development of prophylactic as well as therapeutic vaccines supporting vaccination campaigns worldwide.

Impact and implications: Therapeutic vaccination is a promising therapeutic option for chronic hepatitis B that may enable its cure. However, its application requires functional cooling chains during transport and storage that can hardly be guaranteed in many countries with high demand. In this study, the authors developed thermostable vaccine components that are well tolerated and that induce immune responses and control the virus in preclinical mouse models, even after long-term exposure to high surrounding temperatures. This will lower costs and ease application of a therapeutic vaccine and thus be beneficial for the many people affected by hepatitis B around the world.

Introduction

With more than 257 million chronic carriers and more than 880,000 HBV-related deaths worldwide, chronic hepatitis B (CHB) represents a major health problem.[1] The outcome of HBV infection is strongly dependent on the capability of the host to mount effective HBV-specific B- and T-cell responses. After infection, individuals either develop an acute, self-limiting HBV infection with strong antibody and effector T-cell responses, or a chronic infection characterized by scarce, partially dysfunctional

Keywords: Heat-stable vaccine; hepatitis B virus; HBsAg; HBcAg; MVA; stabilizing amino acid-based formulation; SPS®; heterologous prime/boost vaccination; lyophilization; formulation; stabilization; stabilizing excipients.

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T-cell responses and a lack of HBV-specific neutralizing antibodies.[2–4]

Available therapies for CHB include (pegylated) interferon (IFN) alpha and nucleos(t)ide analogues.[1,5] Widely used nucleos(t)ide analogues do not eliminate the persistent form of HBV, the covalently closed circular DNA (cccDNA), and therefore do not cure HBV resulting in reactivation of the virus once medication is stopped.[6] Thus, alternative treatment options are needed to achieve an HBV cure.

Therapeutic vaccination is a promising treatment approach. It aims at inducing HBV-specific immunity to combat the virus and cure the disease.[7] Recently, we have developed a heterologous protein-prime/MVA-boost vaccination strategy, termed *TherVacB,* that aims at inducing HBV-specific B-cell as well as helper and effector T-cell responses.[8,9] We have previously shown that *TherVacB* is able to break HBV-specific immune tolerance in HBV transgenic and adeno-associated virus (AAV)-HBV-infected mouse models.[8,10] In *TherVacB*, priming with particulate HBsAg and HBcAg leads to the induction of HBV-specific B- and helper $CD4^+$ T-cell responses, but also primes effector $CD8^+$ T cells. The following boost with an MVA-vector aims at amplifying the HBV-specific effector $CD8^+$ T cells. Neutralizing antibodies prevent viral spread and multifunctional and multispecific T-cell responses finally control HBV by eliminating virus-infected hepatocytes.[7]

A major challenge in vaccine application worldwide is a lack of functional cooling chains, especially in low- and middle-income countries or hard-to-reach areas, due to infrastructure gaps, high costs and logistical problems.[11–13] Consequently, vaccines may lose their potency due to thermal instability of the vaccine components.[12,14] Addressing this problem, the World Health Organization (WHO) has presented guidelines on "controlled temperature conditions" (CTC) and "extended controlled temperature condition".[13,15] The WHO CTC guideline requires vaccines to resist 40 °C for a minimum of three days without losing efficacy. This facilitates vaccine application worldwide, reduces the costs of vaccination campaigns and prevents administration of inactive vaccines.[12,13]

In this study we aimed to develop a lyophilized, thermostable version of the different vaccine components of *TherVacB*. For this purpose, a series of stabilizing amino acid (SAA)-based formulations were designed using the excipient database from LEUKOCARE. SAA-formulated vaccine components were heat-challenged to demonstrate compliance with WHO criteria. In addition, a storage period of up to one year at 25 °C was evaluated. Comprehensive *in vitro* analyses of antigen integrity and MVA-vector infectivity proved suitable SAA-formulations. In addition, the immunogenicity and efficacy of the SAA-formulated vaccine, despite heat-stress, was demonstrated in a preclinical model of HBV infection.

Materials and methods

For additional, detailed descriptions please refer to the supplementary information.

Vaccine components

HBcAg (HBV genotype D, subtype ayw) was expressed in *E. coli* (APP Latvijas Biomedicinas, Riga, Latvia), HBsAg (HBV genotype A, subtype adw) in yeast (Biovac, South Africa). MVA-S/C vector contains the coding sequences for the small envelop protein (S) of HBV genotype A2, subtype adw, and core protein of HBV genotype D, subtype ayw, connected by a P2A site and was produced as reported.[16]

Sample preparation

Vaccine components were diluted with SAA-formulations or PBS. After lyophilization, the samples were stored under specific defined temperature conditions according to the International Council for Harmonisation (ICH) guidelines at 40 °C/75% relative humidity (RH), 25 °C/60% RH or at 5 °C. Samples were reconstituted with the required volume of H_2O (*in vitro*) or PBS (*in vivo*).

Sandwich ELISA

HBcAg-specific ELISA: coating with 1 μg/ml anti-IFA HepBCore (CIGB, Havanna, Cuba), detection with HRP-labelled anti-HepBcore (1:7000; CIGB). HBsAg-specific ELISA: coating with 1 μg/ml HBV-specific single-chain antibody C8 (scFv C8),[17] detection with 5 μg/well HBs-specific murine antibody 5F9[18] and goat anti-human IgG Fc (HRP-labelled; Abcam, Cambridge, UK, 1:1000). 15 ng of HBcAg and 30 ng of HBsAg were analysed.

Native agarose gel electrophoresis

Native agarose gel electrophoresis (NAGE) was performed by running 2.5 μg of native protein sample per lane through a 1% agarose gel (Peqlab, Erlangen, Germany) at 150V for 90 min. Nucleic acid content was analysed by UV-light in the Fusion FX7 (Peqlab, Erlangen, Germany) after Roti®-Safe GelStain staining. Protein content was visualized by Coomassie staining.

Dynamic light scattering

Dynamic light scattering (DLS) was performed using a Malvern Zetasizer Nano ZS (Malvern Panalytical GmbH, Herrenberg, Germany) or DynaPro Nanostar DLS instrument (Wyatt Technology Europe GmbH, Dernbach, Germany) to analyse 5 μg of protein sample diluted in 10 mM NaCl. Per measurement, 50 individual scans, each 10 s long, were analysed.

Size exclusion-high performance liquid chromatography

Antigens were analysed by size exclusion-high performance liquid chromatography (SE-HPLC; UV-280 nm; UHPLC system UltiMate3000 Thermo Scientific, Germany). A size exclusion column TSK-gel® G5000 SW_{XL} 300 mm × 7.8 I.D. column (100 nm pore size; Tosoh Bioscience, Tokyo, Japan) with a flow rate of 0.6 ml/min at 25 °C and an injection volume of 100 μl was used. The mobile phase was a 50 mM phosphate buffer pH 7.0. Chromatograms were progressed using Chromeleon 7 Chromatography Data Software (Thermo Scientific, Germany).

Transmission electron microscopy

MVA-S/C inactivated with 2% paraformaldehyde was absorbed onto a copper grid with a formvar/carbon film (400 mesh) for 15 min, antigens for 5 min, at room temperature (RT), before being negatively stained with 1% phosphotungstic acid and imaged using a Libra 120 transmission electron microscope (Zeiss, Oberkochen, Germany).

Determination of MVA-S/C titre

MVA-S/C was titrated in serial dilutions on BHK-21 cells. The MVA-S/C titre was determined by half-maximal median tissue culture infection dose ($TCID_{50}$) of two repetitions.[17]

Animal experiments - ethical statement

Mouse experiments were performed according to the European Health Law of the Federation of Laboratory Animal Science Associations, the German regulations of the Society for Laboratory Animal Science and the 3R rules. Experiments were approved by the local Animal Care and Use Committee of Upper Bavaria (permission number: ROB-55.2-2532.Vet_02-18-24) according to the institution's guidelines. All animals received humane care. All experiments conform to ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and comply with the relevant institutional guidelines. Animals were maintained under pathogen-free conditions and experiments were performed during the light phase of the day. Male C57BL/6J mice were purchased from Janvier (Le Genest-Saint-Isle, France). To establish persistent HBV replication, mice were intravenously infected 4 weeks before vaccination with 4×10^9 genome equivalents of the AAV-HBV1.2 vector encoding 1.2-fold overlength HBV genome of genotype D.[19]

Heterologous protein-prime/MVA-boost vaccination

Mice were immunized i.m. using a heterologous protein-prime/MVA-boost vaccine as described.[8] Two protein immunizations consisted of a mixture of 10 μg particulate HBcAg, 10 μg particulate HBsAg and 10 μg bis-(3',5')-cyclic dimeric AMP (InvivoGen, San Diego, CA) 2 weeks apart. Booster immunization was performed with 5×10^7 $TCID_{50}$ of MVA-S/C 2 weeks after.

Intracellular cytokine staining

Single-cell suspensions of splenocytes and liver-associated lymphocytes were prepared as described.[10,20] For intracellular cytokine staining, lymphocytes were stimulated *ex vivo* with 1 μg/ml MVA_{B8R}, OVA_{S8L}, HBV S_{280} and C_{93} or peptide pools HBV S_{pool} or C_{pool}[10] (Table S1). After 1 h of stimulation, brefeldin A was added for 14 h before staining with Fixable-Viability-Dye eFluor™ 780 (eBioscience, Frankfurt, Germany), anti-CD4-APC (clone GK1.5, eBioscience™) and anti-CD8a-Pb (clone 53-6.7, BD Pharmingen™). For intracellular cytokine staining, we used anti-IFNγ (clone XMG1.2; BD Pharmingen™). Data were acquired on a CytoflexS flow cytometer (Beckmann Coulter, Brea, CA, USA) and analysed with FlowJo software (Tree Star, Ashland, OR, USA).

Multimer staining

HBV-specific $CD8^+$ T cells were detected through staining with MHC class I multimers conjugated with the H-$2K^b$-restricted HBV-derived peptides C_{93-100} (C_{93}, MGLKFRQL) and $S_{190-197}$ (S_{190}, VWLSAIWM), as described previously.[10,21] As a control, staining with multimer conjugated with the ovalbumin-derived peptide $S8L_{257}$ (OVA_{S8L}, SIINFEKL) was performed. Prior to use, C_{93} and OVA_{S8L} multimers (kindly provided by Dirk Busch, Technical University of Munich, Germany) were labelled with Streptactin-PE (IBA Lifesciences, Göttingen, Germany), as previously described.[10,21]

Serological and virological analyses

HBeAg, HBsAg and anti-HBs were quantified using the Architect™ platform (Abbott Laboratories, Wiesbaden, Germany), anti-HBc using Enzygnost® Anti-HBc monoclonal test (Siemens Healthcare Diagnostics, Erlangen, Germany). Alanine aminotransferase (ALT) activity was determined after 1:4 dilution in PBS by Reflotron® GPT/ALT tests (Roche Diagnostics, Mannheim, Germany).

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.[22]

Immunohistochemistry

Liver tissue samples were fixed in 4% buffered formalin for 48 h and embedded in paraffin. Immunohistochemistry was performed with anti-HBcAg antibody (Diagnostic Biosystems, Pleasanton, CA; 1:50 dilution) according to the protocol described previously.[22] HBcAg-positive hepatocytes were determined in 10 random view fields (40× magnification) and quantified per mm^2.

Statistical analysis

Data were analysed using GraphPad Prism version 5.01 (GraphPad Software Inc., San Diego, CA) using Mann-Whitney test or Student's *t* test. *p* values <0.05 were considered significant.

Results

Loss of functional integrity of protein and vector vaccine components after exposure to thermal stress

To analyse thermal stability of *TherVacB* components, antigens and MVA-S/C in solution were exposed to a temperature gradient ranging from 25 °C (representing RT) up to 40 °C (WHO CTC guideline[13]) for 3, 14 and 28 days. As a control, the antigens were stored at 4 °C, the MVA at −80 °C.

Antigen integrity was assessed by ELISA. HBsAg was stable for 3 days at 25 °C, but had lost 33% of the ELISA signal at day 14 and 80% at day 28 (Fig. 1A). Storage at 36 to 40 °C resulted in rapid loss of HBsAg detection by ELISA after 3 days (Fig. 1A). HBcAg did not show any obvious loss of antigen integrity for up to 14 days at 25 °C, but 25% of the ELISA signal was lost after 28 days (Fig. 1B). Storage at 36, 38 and 40 °C resulted in increasing loss of antigen integrity on ELISA; up to 60% loss at day 3, 87% at day 14 and an almost complete loss over time (Fig. 1B).

Integrity of MVA-S/C expressing HBV core and S proteins was determined by infectivity in cell culture. $TCID_{50}$ did not significantly drop until day 14 at 25 °C. Over 28 days $TCID_{50}$ dropped by approximately 1-log_{10} (Fig. 1C). However, increasing the temperature to 38 or 40 °C resulted in a >1-log_{10} reduction of MVA-S/C titres after only 3 days, and storage at 36, 38 and 40 °C resulted in a complete loss of MVA-S/C infectivity after 14 days (Fig. 1C). This indicated that stabilization of the vaccine components is crucial to allow for storage at RT or higher temperatures.

Selection of stabilizing excipients for the formulation of protein and vector vaccine components

Excipients for stabilizing the vaccine components were selected from a database established by LEUKOCARE AG, Martinsried, Germany, based on experience with thermal stabilization of lyophilized HBcAg and an MVA-vector expressing HBV core protein using a mixture of seven amino acids (alanine, arginine, glutamic acid, glycine, lysine, histidine and tryptophan) in combination with trehalose.

A first round of SAA-formulation optimization aimed at maintaining a pharmaceutically desired cake structure during

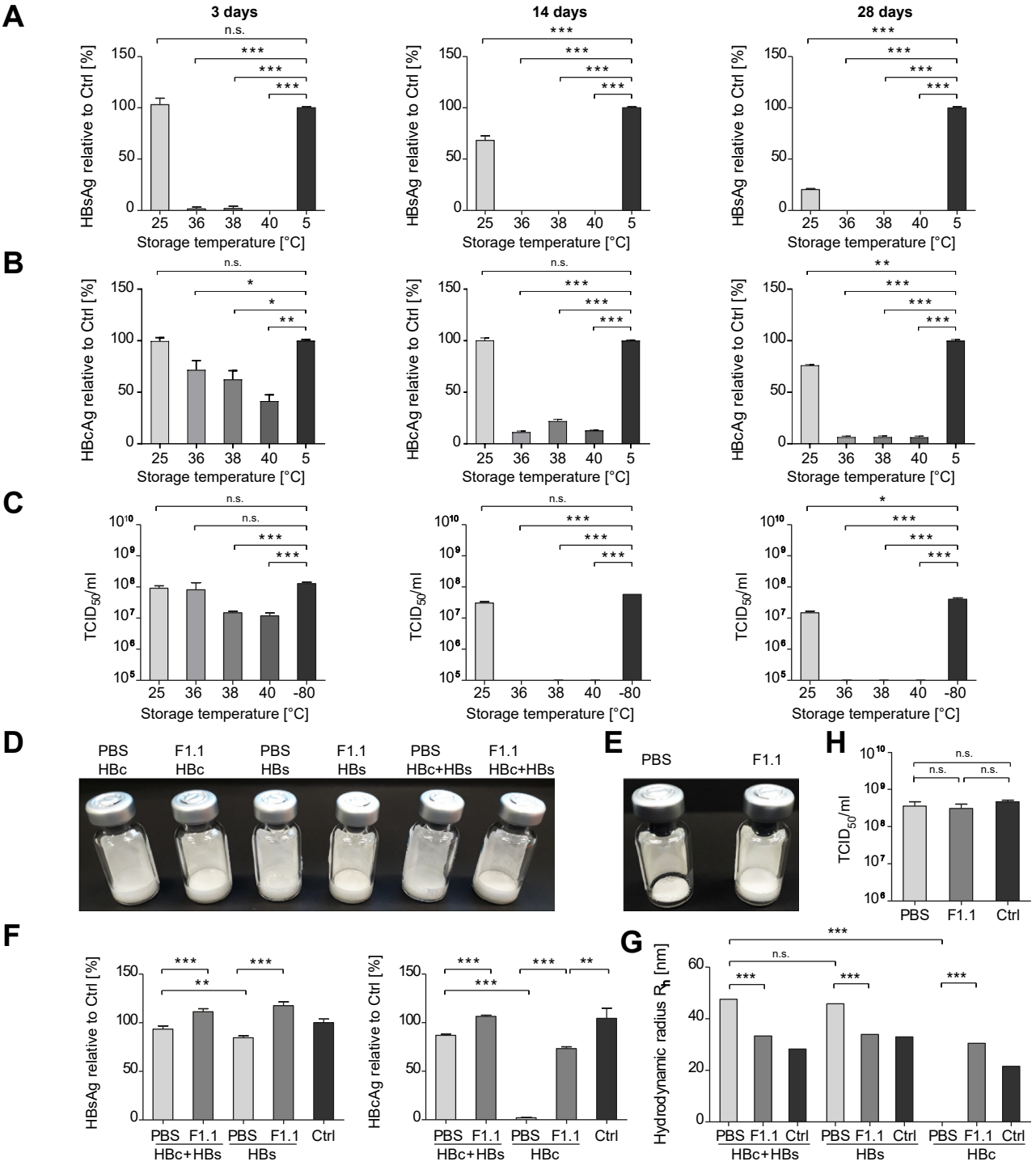

Fig. 1. Effect of heat-exposure on *TherVacB* vaccine components. Particulate HBsAg, HBcAg and MVA-S/C kept in solution were stored at 25 °C, 36 °C, 38 °C and 40 °C and as control at 5 °C or -80 °C, respectively, as indicated. (A) HBsAg- and (B) HBcAg-specific ELISA after 3 (left), 14 (middle) and 28 days of storage (right). (C) MVA-S/C titres were determined by $TCID_{50}$. (D-G) HBcAg, HBsAg and MVA-S/C were formulated with SAA-based formulation F1.1 or PBS, lyophilized and analysed directly after lyophilization. Cake structure of antigens (D) MVA-S/C (E) after lyophilization. (F-G) For control (Ctrl), antigens were stored at 5 °C, MVA-S/C at -80 °C without stabilization, lyophilization and temperature exposure. (F) Quantification of HBcAg- and HBsAg by ELISA relative to Ctrl (set to 100%). (G) Hydrodynamic radius of antigens determined by DLS. (H) $TCID_{50}$ of MVA-S/C. Data are given as mean ± SD. Statistical analysis applied unpaired *t* test. *p <0.05; **p ≤0.01; ***p ≤0.001; n.s., not significant.

lyophilization by addition of dextran and elimination of amino acids glycine and lysine. Antioxidative excipients, *e.g.* methionine, osmotic amino acids, *e.g.* proline, glutamine, metal-chelating excipients, *e.g.* citric acid, and a mixture of sugars and sugar alcohols, as well as small amounts of surfactant were assessed. Based on this, four SAA-based formulations (F1.1-F1.4, Table S1) were selected to stabilize *TherVacB* components.

Impact of lyophilization on particulate antigen and vector integrity

To facilitate distribution of *TherVacB* worldwide, without the necessity of a cooling chain, we chose to lyophilize the vaccine components. We therefore first determined the impact of thermal stress during lyophilization.

For stabilization, we used SAA-formulation F1.1 (Table S1) as an exemplary formulation for either individual HBsAg and HBcAg or a combination thereof and the MVA-vector vaccine component. After lyophilization, pharmaceutical cake structure remained intact for F1.1-formulated proteins in contrast to non-formulated proteins (Fig. 1D) and the MVA-vector (Fig. 1E). Antigen integrity was studied using an ELISA developed as a potency assay (Fig. 1F).

HBsAg integrity after lyophilization was reduced when formulated with PBS, but fully preserved by the F1.1-formulation (Fig. 1F, left panel). HBcAg lyophilized in PBS completely lost its integrity, which could to a large part be prevented by the F1.1.-formulation (Fig. 1F, right panel). Interestingly, combining HBcAg with HBsAg also preserved HBcAg integrity to a large extent.

DLS and SE-HPLC were used to evaluate the particulate structure of the antigens. High-speed centrifugation before SE-HPLC reduced larger particles as shown by a reduced peak absorbance if antigens were not SAA-formulated before lyophilization, and a shoulder before the elution of HBsAg, suggesting the presence of larger particles (Fig. S1). In line with this observation, DLS showed an increase in hydrodynamic radius of HBsAg particles. For HBcAg particles, the correlation function could no longer be defined, indicating the formation of larger aggregates if HBcAg was not formulated with F1.1 or combined with HBsAg (Fig. 1G). MVA-S/C infectivity remained high for all samples after lyophilization, indicating intrinsic stability of the vector (Fig. 1H).

Together, this indicated that homogeneity of HBcAg and HBsAg particles was lost during lyophilization if antigens were not formulated with SAA. From these data we concluded that the integrity of particulate protein-antigens, HBcAg and HBsAg, as well as the MVA-vector can be maintained during lyophilization by an SAA-based formulation, and that combining HBcAg and HBsAg is advantageous.

Stabilization of HBcAg and HBsAg during short-term thermal stress

To test if our lyophilized *TherVacB* vaccine components fulfill the requirements of the WHO CTC guideline, we thermally stressed them at 40 °C/75% RH for 3 days. By ELISA, NAGE, DLS and SE-HPLC we verified that formulation with SAA was able to maintain full antigen integrity for 3 days at 40 °C (Fig. 2A-D). PBS-formulated antigen samples lost their integrity, but SAA-formulation F1.1 prevented this, fulfilling the WHO CTC criteria for vaccine development. In contrast to the protein-antigens, the F1.1 formulation only showed a moderate effect on preventing temperature damage to MVA-S/C (Fig. 2E).

Selection of the SAA-based vaccine formulation for storage of lyophilized vaccine components

To challenge the SAA-formulation, we extended the recommended storage time of lyophilized vaccine antigens at 40 °C from 3 to 28 days and compared the modified formulations F1.1, F1.2, F1.3 and F1.4 (Table S1) *in vitro* and *in vivo*. Non-lyophilized, non-stressed and non-stabilized vaccine components constantly stored at either 4 °C (HBsAg, HBcAg) or −80 °C (MVA-S/C) served as control ($TherVacB_{Ctrl}$).

All four SAA-based formulations, in contrast to PBS, preserved HBcAg and HBsAg integrity at 40 °C/75% RH for 28 days and proved superior to storage in PBS at 5 °C in ELISA-based analyses (Fig. 3A). NAGE revealed the typical migration pattern of intact, particulate HBcAg in all SAA-formulated samples, in contrast to non-stabilized samples stored at 40 °C or 5 °C where protein aggregates prevented HBcAg from running into the gel (Fig. 3B). In addition, the loss of infectivity of MVA-S/C was prevented in the presence of all four selected SAA-based formulations (Fig. 3C).

To complete the analysis, we vaccinated naïve, male C57BL/6J mice with the SAA-formulated, heat-exposed vaccine components using a heterologous protein-prime/MVA-vector boost vaccination (Fig. 3D).[8] One week after boost vaccination, comparably high anti-HBs serum titres (Fig. 3E, left panel) and comparable activation of IFNγ-producing $CD4^+$ T cells (Fig. 3F) were detected in all mice irrespective of the vaccine formulation. In contrast, anti-HBc levels were significantly higher in mice immunized with stabilized vaccine components (Fig. 3E, right panel). *TherVacB* induced a weak S-specific, but a strong core-specific $IFN\gamma^+$ $CD4^+$ T-cell response which was comparable between all vaccinated groups (Fig. 3F). Importantly, stabilization of vaccine components with various SAA-formulations resulted in significantly higher HBV core- and S-specific $CD8^+$ T-cell responses (Fig. 3G). Although there was no statistically significant difference between the SAA-formulations evaluated, F1.1 seemed to elicit the most robust T-cell response among the different animals. As F1.1 also contained the least excipients, conferring an advantage for clinical vaccine development, it was selected for further evaluation.

Maintenance of antigen and MVA integrity during storage at high temperatures for up to 3 months

After successful stabilization of *TherVacB* components for 1 month, we wondered whether our SAA-formulation would enable exposure to 40 °C/75% RH for up to 3 months. ELISA analysis confirmed intact SAA-formulated HBcAg and HBsAg after 1-month (Fig. S2A) and 3-month storage (Fig. 4A) at 5 °C, 25 °C and 40 °C. In contrast, formulation with PBS led to >65% loss of structural and functional integrity (Fig. 4A, Fig. S2A). DLS and SE-HPLC indicated that SAA-formulated HBsAg and HBcAg did maintain their particulate appearance despite high-temperature exposure (Fig. 4B,C; Fig. S2B,C) while large, apparently aggregated particle-structures were detected after 1 month in non-stabilized samples (Fig. S2B) that did not run into the NAGE gel (Fig. S2D) and lost their cake structure (Fig. S2F). In contrast, even after 3 months at 40 °C, the particulate structure of SAA-formulated antigens remained intact (Fig. 4A-C; Fig. S2E,F). MVA-S/C lost infectivity over 3 months, with increasing storage temperature SAA-formulation could only partially prevent that loss (Fig. 4D,E, Fig. S2G). Transmission electron microscopy demonstrated that F1.1-formulated vaccine components preserved their morphology after heat-exposure while PBS-formulated protein-antigens attached to each other and formed aggregates (Fig. 4F,G).

Taken together, our results confirm the successful stabilization of all vaccine components by SAA-formulation despite a 25 °C or 40 °C exposure for up to 3 months.

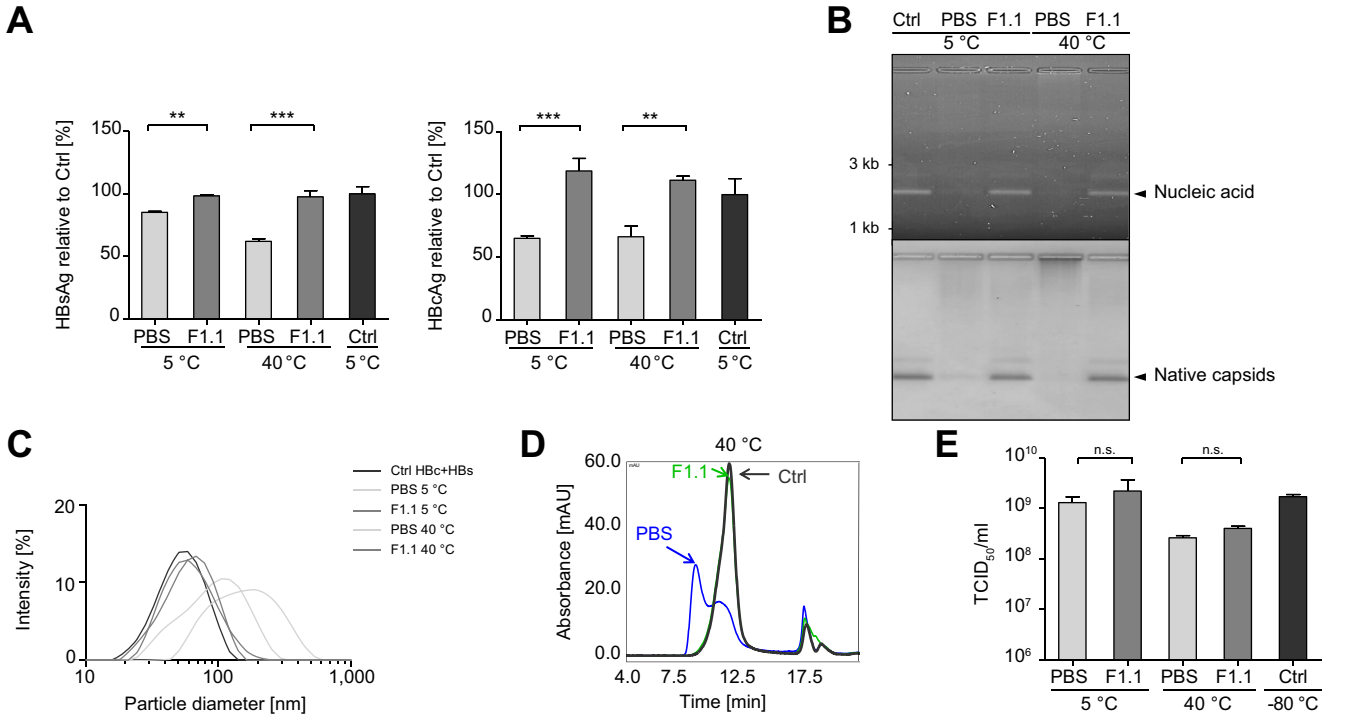

Fig. 2. Effect of short-term thermal stress exposure of *TherVacB* vaccine components. Vaccine components were formulated with SAA-based formulation F1.1 or PBS, lyophilized and stressed for 3 days at 40 °C/75% RH. Control (Ctrl) vaccine components were without stabilization, lyophilization and temperature exposure. (A) Quantification of HBcAg- and HBsAg by ELISA relative to Ctrl (set to 100%). (B) NAGE of HBcAg. (C) DLS and (D) SE-HPLC of both protein-antigens. (E) $TCID_{50}$ of MVA-S/C after 3 days of storage at 5 °C and 40 °C. Data are given as mean ± SD. Statistical analysis applied unpaired *t* test. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; n.s., not significant.

Immunogenicity of SAA-formulated *TherVacB* after one- and three-months storage

We next vaccinated naïve, male C57BL/6J mice to verify immunogenicity of thermally stressed *TherVacB* components. We used vaccine components that had been stored for 1 or 3 months at 40 °C/75% RH or 25 °C/60% RH. Non-lyophilized, non-stabilized and non-stressed vaccine components ($TherVacB_{Ctrl}$) served as a positive control. *In vitro* analysis confirmed preserved integrity of F1.1-formulated HBsAg and HBcAg and infectivity of MVA-S/C (Fig. 5A,B).

One week after MVA-boost vaccination (scheme in Fig. 3D), all vaccinated animals had developed high serum anti-HBc and anti-HBs titres (Fig. 5C). In addition, mice immunized with SAA-formulated *TherVacB* components or $TherVacB_{Ctr}$, but not with PBS-formulated vaccines elicited strong core-specific $CD8^+$ T-cell responses (Fig. 5D, right panel). S-specific $CD8^+$ T-cell responses were lost when vaccine components were not stabilized, but only slightly reduced in comparison to $TherVacB_{Ctr}$ when F1.1-formulated (Fig. 5D, left panel). These results clearly show that SAA-formulation maintains the immunogenicity of *TherVacB* despite exposure to up to 40 °C for 3 months.

Antiviral effect of stabilized *TherVacB* vaccine components after exposure to high temperature for 1 month

Following successful immunization of naïve mice, we asked whether *TherVacB* efficacy in breaking immune tolerance in HBV-carrier mice was preserved after exposure to 40 °C/75% RH for 1 month. For this, we took advantage of the AAV-HBV mouse model, in which AAV-HBV infection induces persistent replication of HBV in mouse hepatocytes over months without inducing HBV-specific immunity.[10,19,22]

9-week-old, male C57BL/6J mice were infected with 4×10^9 AAV-HBV genome equivalents known to reproducibly induce persistent HBV replication and were immunized with heat-exposed, SAA- or PBS-formulated vaccine components using the *TherVacB* vaccination scheme (Fig. 6A). *In vitro* analysis of the stored vaccine components confirmed antigen integrity was maintained and MVA-S/C infectivity improved with SAA-formulation (Fig. S3A,B).

Despite being fully immune competent, AAV-HBV-infected mice did not develop a detectable HBV-specific B- or T-cell response (Fig. 6B, Fig. S4). Four weeks after boost vaccination, however, high serum anti-HBc and anti-HBs titres were observed in all vaccinated mice (Fig. 6C). High anti-HBs antibody titres efficiently suppressed serum HBsAg over time in all vaccinated animals (Fig. 6D). At week 8, HBV-DNA in the sera of most of the vaccinated mice became undetectable (Fig. 6E). However, only immunization with SAA-formulated, but not with PBS-formulated vaccine components induced a strong S-specific $CD4^+$ T-cell response in the liver comparable to the "fresh" vaccine ($TherVacB_{Ctrl}$, Fig. 6F). Core-specific $CD4^+$ T-cell responses remained below the level of detection at the examined time point in all groups of mice. SAA-formulated vaccine components induced a strong core- and S-specific $CD8^+$ T-cell responses in the liver (Fig. 6G, upper panel) and spleen (Fig. 6G, lower panel) accompanied by a mild but sustained rise in serum ALT activity (Fig. 6H), while no $CD8^+$ T-cell response was detected without

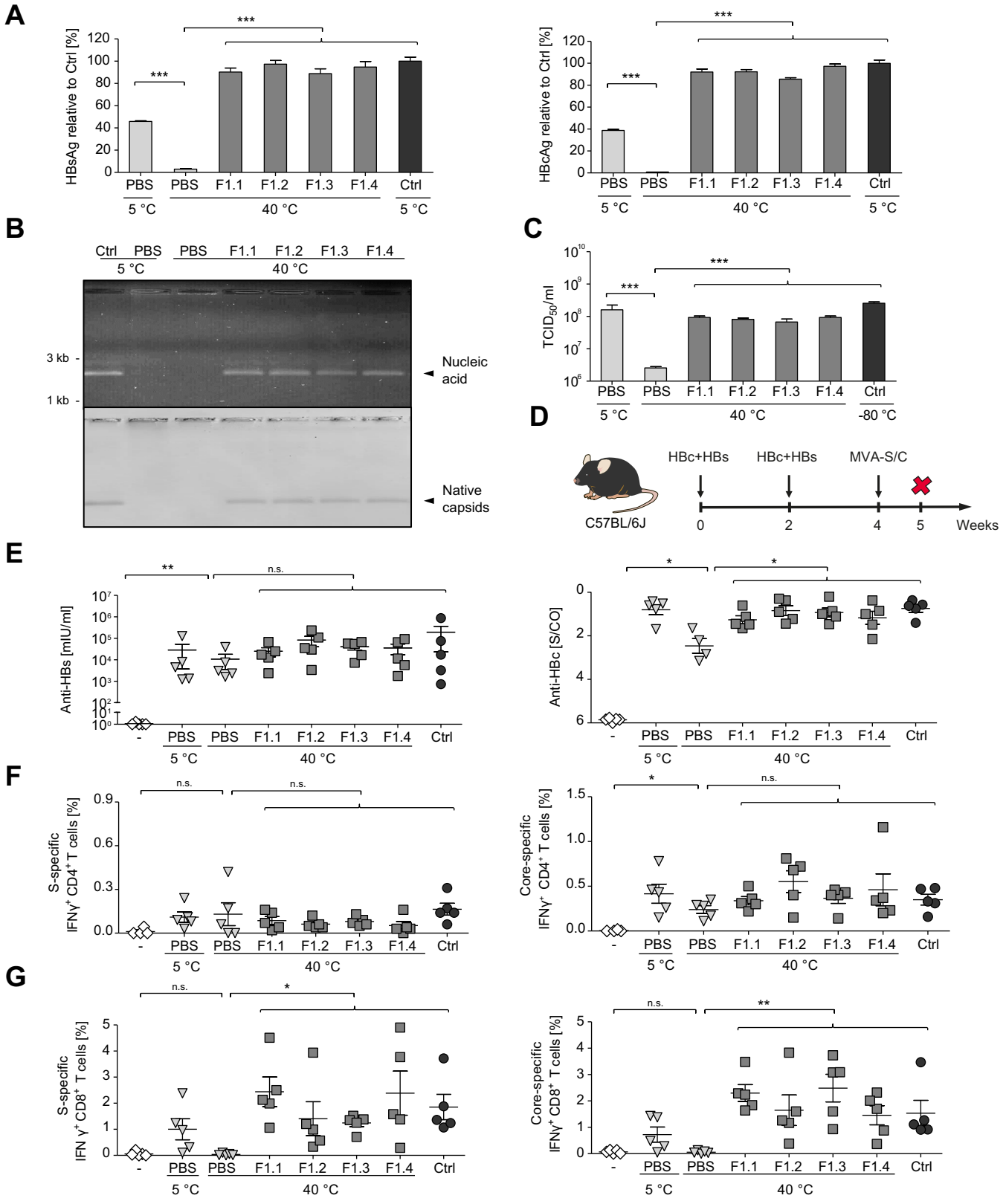

Fig. 3. Selection of the most suitable SAA-formulation. *TherVacB* was formulated with four different SAA-based formulations (F1.1-F1.4, see Table S1) or PBS only, lyophilized and stored at 5 °C or 40 °C/75% RH for 28 days as indicated. Control (Ctrl) vaccine components were without stabilization, lyophilization and temperature exposure (set to 100% in A). (A) HBsAg- and HBcAg-specific ELISA, (B) NAGE of HBcAg and (C) $TCID_{50}$ assay of MVA-S/C. Data are given as mean ± SD. (D-G) C57BL/6J mice (n = 5) were immunized with SAA-formulated or PBS-formulated *TherVacB* components. Non-vaccinated mice (-) or mice immunized with control (Ctrl) vaccine components not stabilized, lyophilized and constantly stored at 5 °C (protein) or -80 °C (MVA) served as controls. (D) Vaccination scheme. One week after boost, (E) serum anti-HBs (left panel) and anti-HBc (right panel) levels were quantified using the Architect™ platform. Frequencies of S-specific (left panel) and core-specific (right panel) IFNγ^+ splenic $CD4^+$ (F) and $CD8^+$ T cells (G) determined by flow cytometry after ICS. Data are given as mean ± SEM. Statistical analysis applied unpaired *t* test (A, B) and Mann-Whitney test (E, F). *p <0.05; **p ≤0.01; ***p ≤0.001; n.s., not significant.

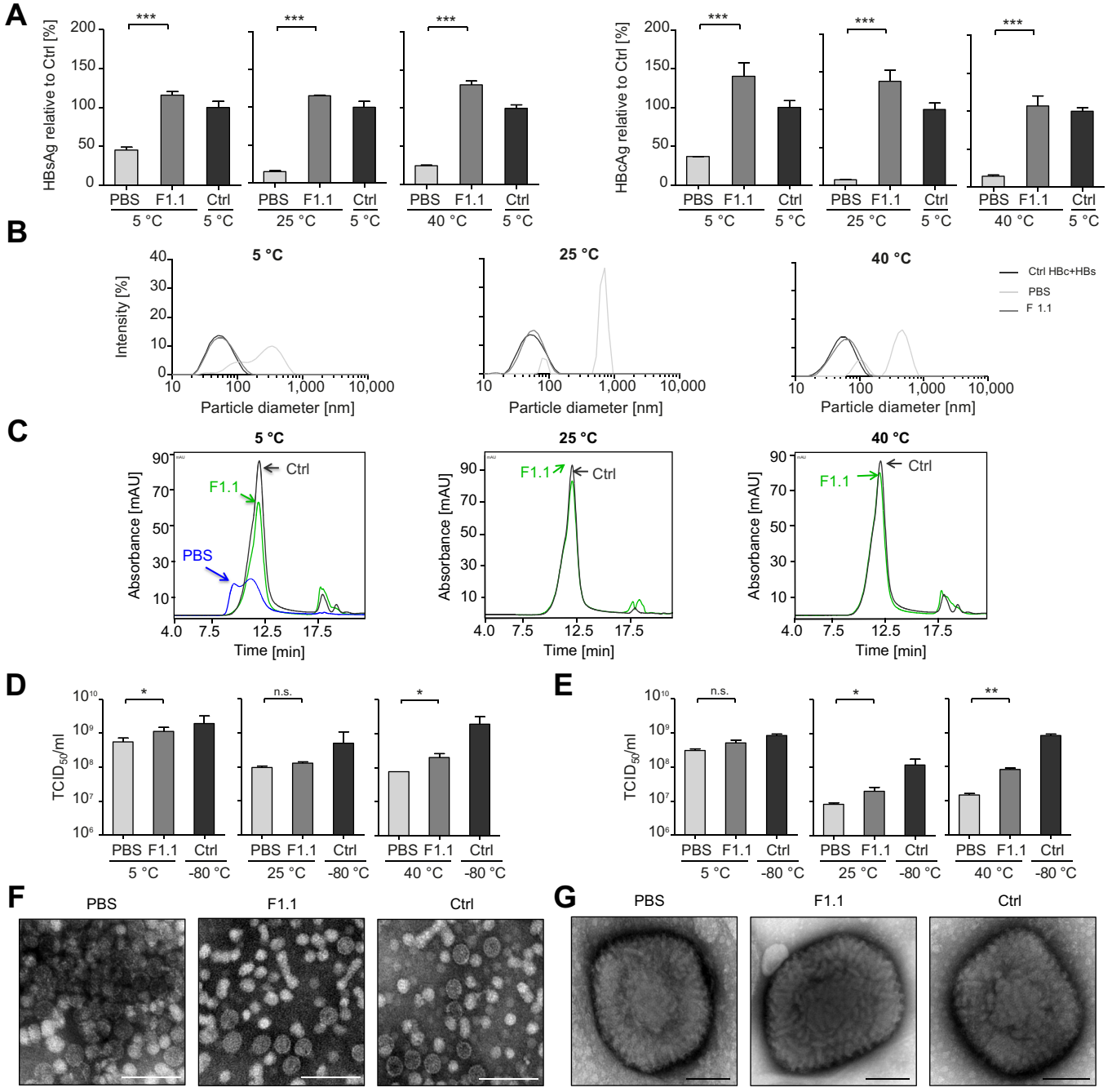

Fig. 4. Long-term thermal stress exposure of *TherVacB* vaccine components. Lyophilized F1.1- or PBS-formulated vaccine components were stored at 5 °C, 25 °C/60% RH or 40 °C/75% RH for 1 (D,F,G) and 3 months (A-C,E). Control (Ctrl) vaccine components were without stabilization, lyophilization and temperature exposure. Antigen integrity was analysed after 3 months (A-C). (A) HBsAg- and HBcAg-specific ELISA relative to Ctrl (set to 100%), (B) DLS and (C) SE-HPLC. (D,E) $TCID_{50}$ of MVA-S/C after storage for (D) 1 and (E) 3 months. Data are given as mean ± SD. (F) Transmission electron microscopy of negative-stained antigens and (G) MVA-S/C after 1 month of storage at 40 °C/75% RH. Magnification 40,000-fold, scale bars 100 nm. Statistical analysis applied unpaired *t* test. **p* <0.05; ***p* ≤0.01; ****p* ≤0.001; n.s., not significant.

stabilization. Consequently, a gradual reduction of HBeAg after immunization was detected (Fig. 6I), which was comparable to that after $TherVacB_{Ctrl}$ vaccination.

Taken together, this demonstrates that even after 1 month's storage at 40 °C, SAA-formulated *TherVacB* elicited high-titre HBV-specific antibodies and functional core- and S-specific T-cell responses in AAV-HBV-infected mice that efficiently suppressed HBsAg and HBeAg.

Stability and immunogenicity of vaccine components stored for 1 year at RT

The ICH protocol requires prolonged storage stability at elevated RT (25 °C/60% RH) for vaccines being applied in countries with climate zones 1 and 2.[23] Therefore, in further experiments we investigated whether SAA-formulation allows for long-term storage of *TherVacB* protein-antigens and the MVA-vector at RT for 1 year.

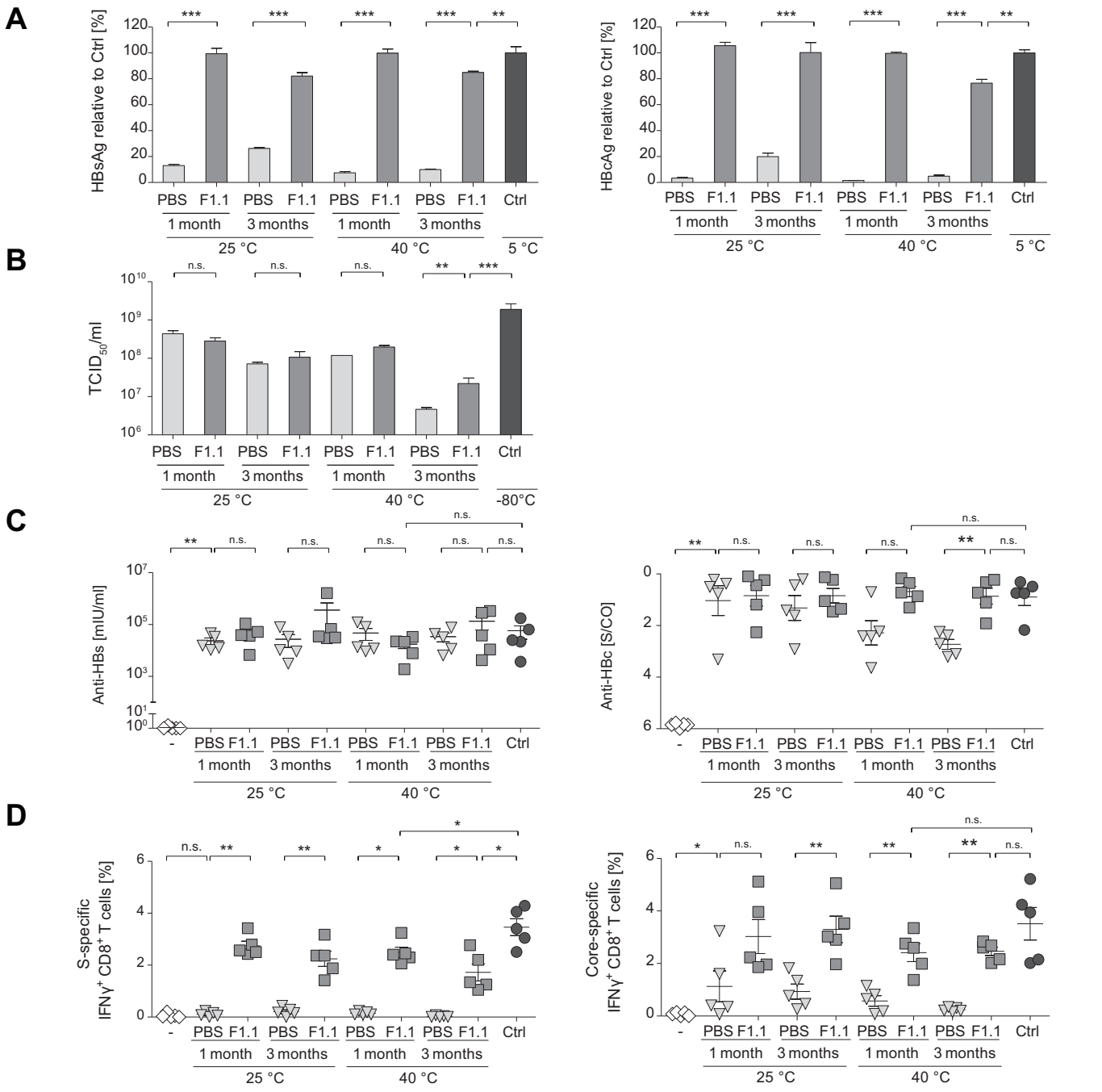

Fig. 5. *In vivo* immunogenicity of *TherVacB* after long-term thermal stress exposure. *TherVacB* vaccine components were formulated with F1.1 or PBS, lyophilized and stored at 25 °C/60% RH or 40 °C/75% RH for 1 or 3 months. Control (Ctrl) vaccine components were without stabilization, lyophilization and temperature exposure. (A) HBsAg- and HBcAg-specific ELISA relative to Ctrl (set to 100%). (B) $TCID_{50}$ of MVA-S/C. Data are given as mean ± SD. Male C57BL/6J mice (n = 5) were immunized with F1.1- or PBS-formulated, heat-exposed vaccine components as indicated and analysed 1 week after MVA-S/C boost. Non-vaccinated mice (-) or mice immunized with control (Ctrl) vaccine components not stabilized, lyophilized and constantly stored at 5 °C (protein) or -80 °C (MVA) served as controls. (C) Serum anti-HBs/anti-HBc levels. (D) Percentage of core- and S-specific $IFN\gamma^+$ splenic $CD8^+$ T cells. Data are given as mean ± SEM. Statistical analysis applied unpaired *t* test (A, B) and Mann-Whitney test (C,D). $^*p$ <0.05; $^{**}p$ ≤0.01; $^{***}p$ ≤0.001; n.s., not significant.

ELISA analysis demonstrated that both SAA-formulated HBsAg and HBcAg retained their antigenicity after storage for 6, 9 and 12 months at elevated RT (Fig. 7A,B). In contrast, non-stabilized antigens lost their integrity over time while similar results were obtained when the post-storage SAA-formulated samples were compared to the starting material (Fig. 7A-C). DLS (Fig. 7C) revealed an increased particle diameter and NAGE no longer enabled separation (Fig. S5A), again indicating an aggregation of the particulate antigens that could be prevented by SAA-formulation. SE-HPLC analysis performed after 9 and 12 months of storage confirmed that SAA-formulation was able to maintain HBcAg and HBsAg integrity for up to 1 year

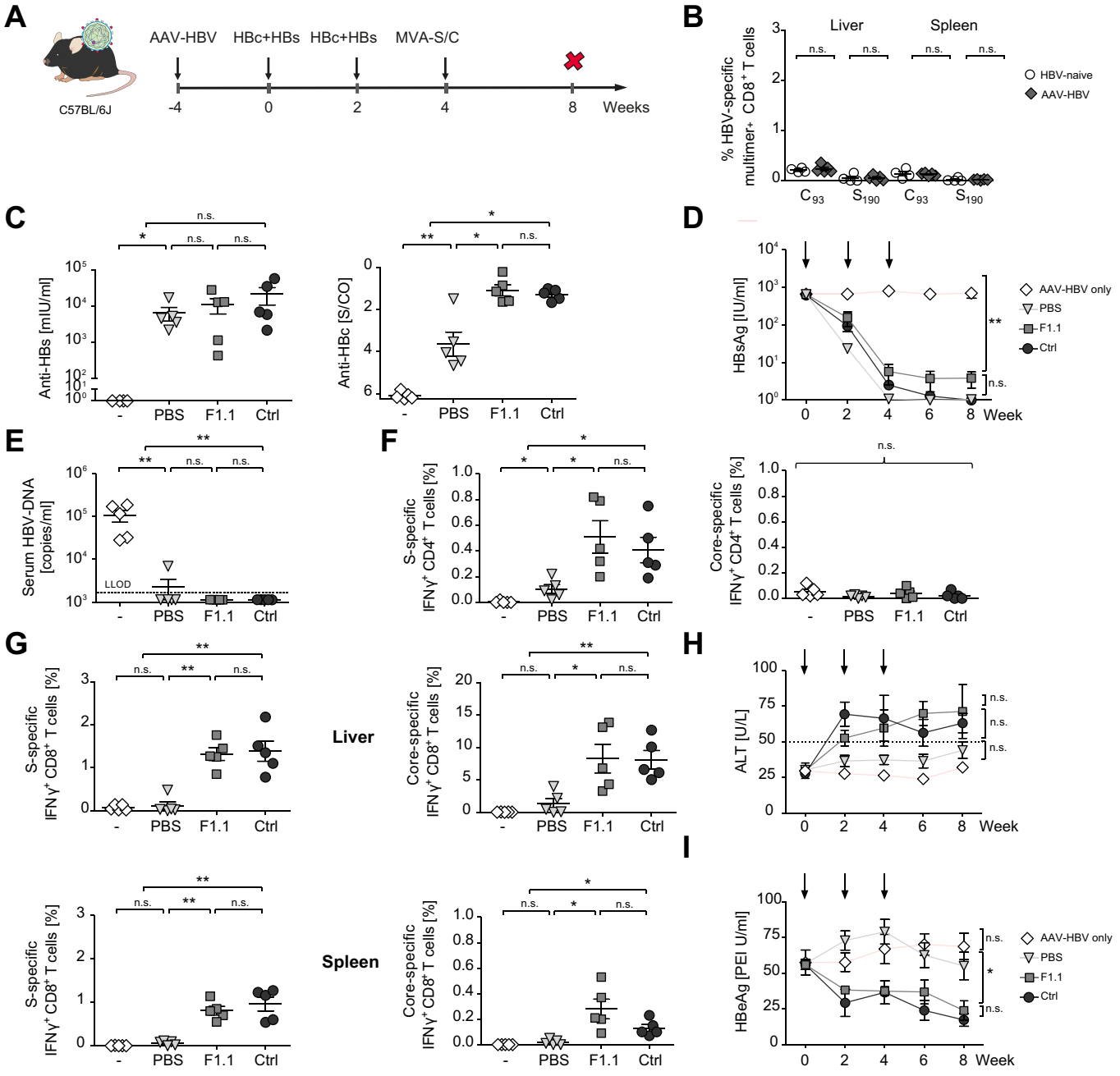

Fig. 6. Immunogenicity of SAA-formulated, heat-exposed *TherVacB* in a mouse model of chronic HBV infection. F1.1- and PBS-formulated vaccine components were lyophilized and stored at 40 °C/75% RH for 1 month. (A) Scheme of the experimental set-up. (B) Four weeks after infection, HBV-specific T cells in liver and spleen were identified using sensitive staining with HBV core$_{93\text{-}100}$ (C_{93}) and $S_{190\text{-}197}$ (S_{190}) peptide-loaded MHC-multimers in AAV-HBV-infected and HBV-naive control mice. (C–I) Male mice (n = 5) infected with AAV-HBV were immunized according to the *TherVacB* regimen. Non-vaccinated mice (-) or mice immunized with control (Ctrl) vaccine components not stabilized, lyophilized and constantly stored at 5 °C (protein) or -80 °C (MVA) served as controls. Final analyses were performed 4 weeks after boost vaccination. (C) Serum anti-HBs/anti-HBc titres. (D) Serum HBsAg kinetics. (E) Serum HBV-DNA levels at week 8. (F) Percentage of IFNγ$^+$ CD4$^+$ T-cell responses in liver. (G) IFNγ$^+$ CD8$^+$ T-cell responses in liver (upper panel) and spleen (lower panel). Left: HBV S-specific, right: HBV core-specific responses. (H) Serum ALT and (I) HBeAg kinetics. Data are given as mean ± SEM. Statistical analysis applied Mann-Whitney test. *p <0.05; **p ≤0.01; n.s., not significant.

(Fig. 7D). Although a loss of infectivity could not be completely prevented, SAA-formulated MVA-S/C retained significantly higher $TCID_{50}$ than non-stabilized samples after long-term storage at 25 °C (Fig. 7E). It is remarkable that MVA-S/C particles remained infectious at elevated RT for 1 year even without a stabilizing formulation, confirming their high intrinsic stability.

Using the SAA-formulated *TherVacB* components stored for 1 year at elevated RT (25 °C, Fig. S5B,C), we repeated the immunization (Fig. 6A) in AAV-HBV-infected mice as an HBV persistence model to determine the antiviral effect. Four weeks after boost vaccination, all vaccinated mice had high serum anti-HBs titres (Fig. 8A) irrespective of the vaccine formulation. Anti-HBc titres, in contrast, were significantly higher in mice vaccinated

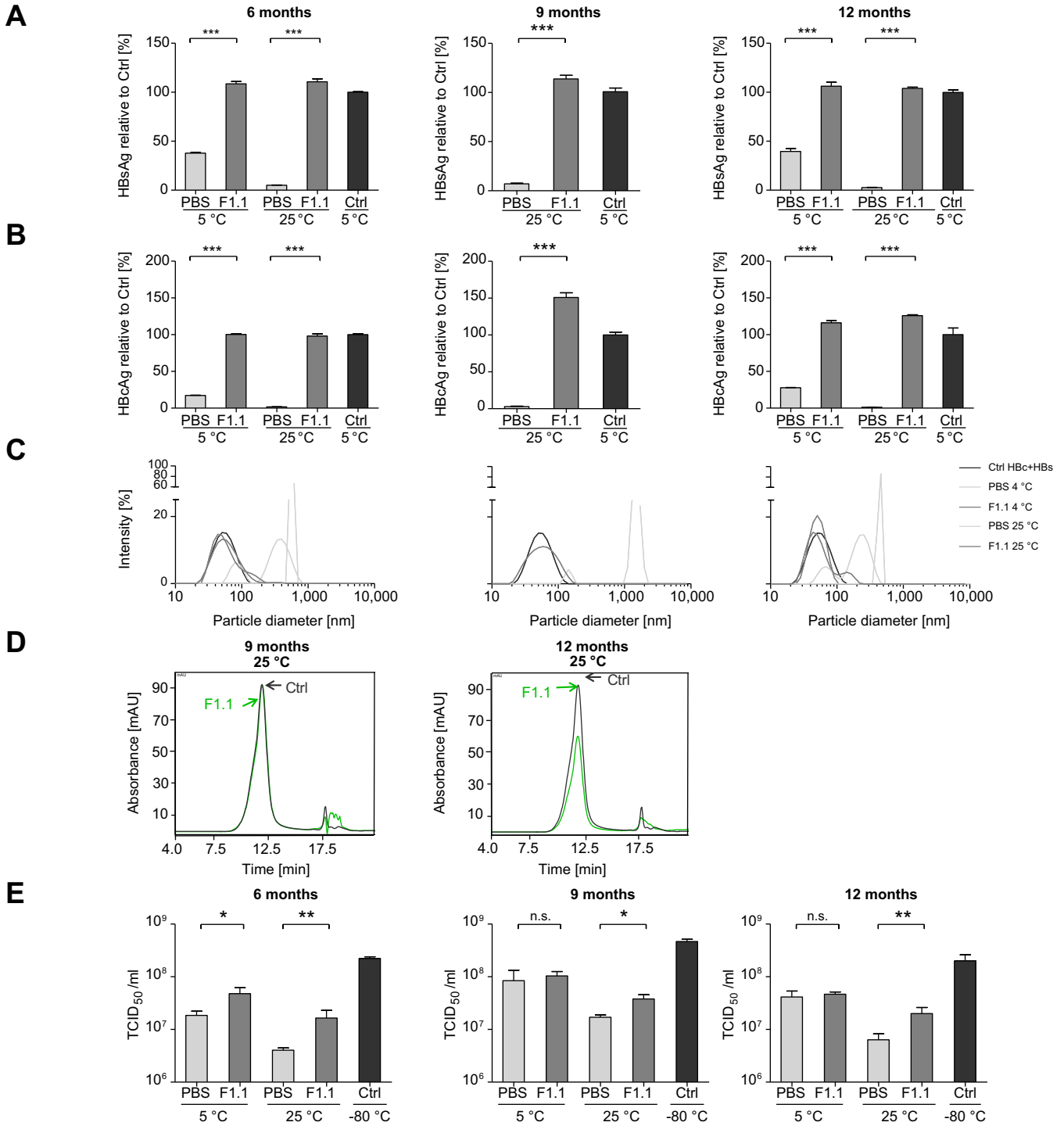

Fig. 7. *In vitro* analysis of vaccine components after storage at 25 °C for 12 months. *TherVacB* components were formulated using F1.1 or PBS, lyophilized and stored at 5 °C and 25 °C/60% RH for 6, 9 and 12 months. Vaccine components without lyophilization and stored at 5 °C (proteins) or -80 °C (MVA) were used as control (Ctrl). Stability of vaccine components was confirmed *in vitro* by (A) HBsAg- and (B) HBcAg-specific ELISA relative to Ctrl (set to 100%), (C) DLS and (D) SE-HPLC analysis of antigens and (E) $TCID_{50}$ assay of MVA-S/C. Data given are mean ± SD. Statistical analysis applied unpaired *t* test. *p <0.05; **p ≤0.01; ***p ≤0.001; n.s., not significant.

with SAA-formulated antigens (Fig. 8B). F1.1-formulated vaccine led to improved induction of S-specific and vigorous core-specific IFNγ^+ CD8$^+$ T-cell responses in spleen and liver (Fig. 8C,D). In particular, frequencies of HBV-specific granzyme B-positive hepatic CD8$^+$ T cells were higher in F1.1- compared to PBS-immunized mice (Fig. 8E), indicating an improved effector T-cell response elicited by stabilized *TherVacB*.

All vaccinated groups efficiently suppressed serum HBsAg and HBV-DNA (Fig. 8F,G). Hereby, the drop in HBsAg and in HBV-DNA was significantly stronger in the mice receiving the SAA-

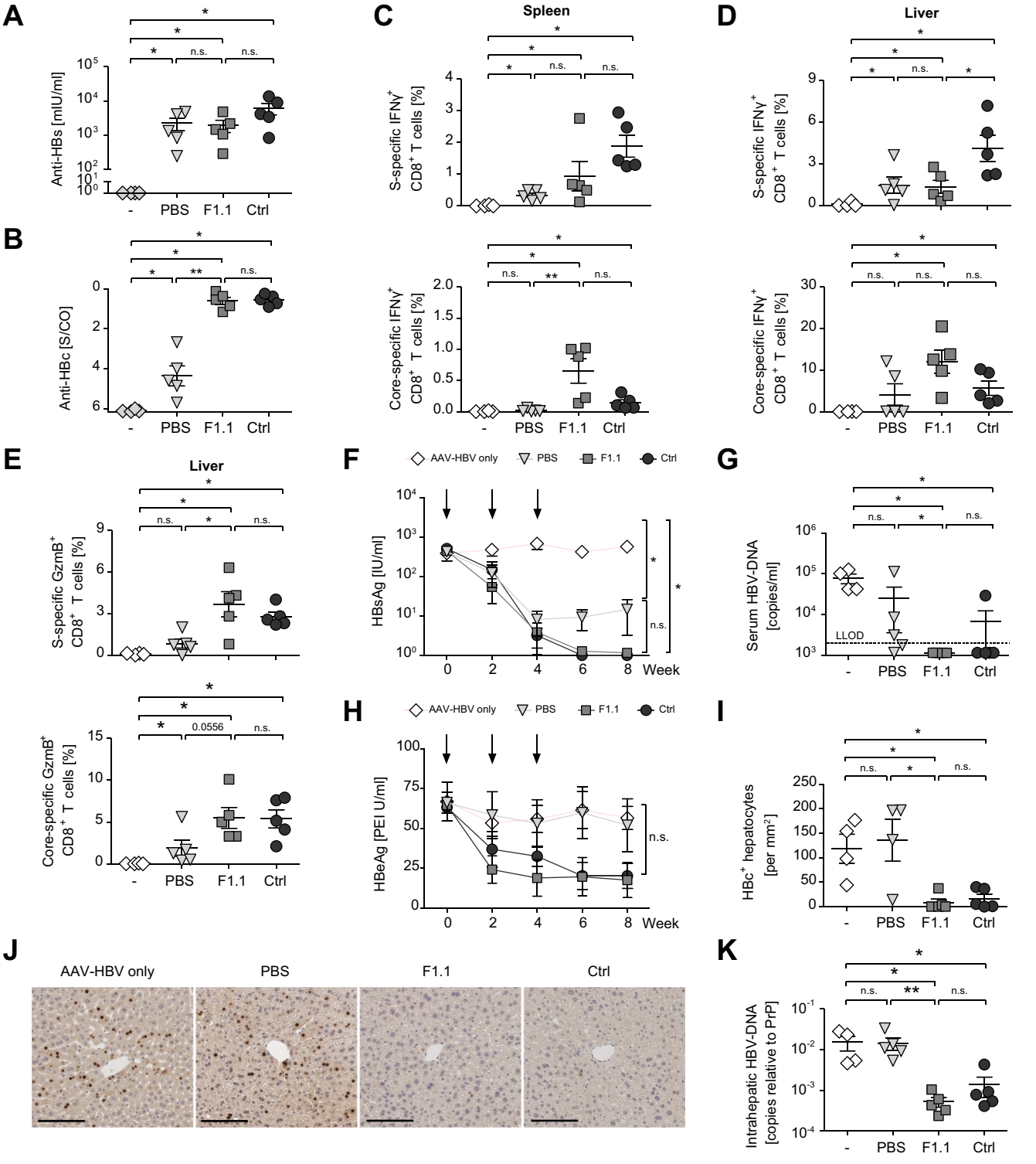

Fig. 8. Immunogenicity and antiviral efficacy of *TherVacB* in AAV-HBV mice after storage at 25 °C for 12 months. *TherVacB* components were formulated using F1.1 or PBS, lyophilized and stored at 25 °C/60% RH for 12 months. AAV-HBV-infected, male mice (n = 5) were immunized using the *TherVacB* scheme. Non-vaccinated mice (-) or mice immunized with control (Ctrl) vaccine components not stabilized, lyophilized and constantly stored at 5 °C (protein) or −80 °C (MVA) served as controls. Final analyses were performed 4 weeks after boost vaccination. (A) Serum anti-HBs and (B) anti-HBc levels. Percentage of splenic (C) and hepatic (D) IFNγ+ CD8+ T cells and hepatic GzmB+ CD8+ T cells (E). Upper panel: HBV S-specific, lower panel: core-specific responses. (F) Serum HBsAg kinetics. (G) Serum HBV-DNA levels at sacrifice. (H) Serum HBeAg kinetics. (I) Quantification of HBcAg-positive hepatocytes per mm^2; (J) corresponding representative immunohistochemistry, scale bars 100 μm; (K) intrahepatic HBV-DNA detected in liver tissue at final analysis. Data given are mean ± SEM. Statistical analysis applied Mann-Whitney test. *p <0.05; **p ≤0.01; n.s., not significant.

formulated vaccine than in those receiving the non-stabilized vaccine (Fig. 8F,G). The strong remaining immunogenicity of SAA-formulated *TherVacB* stored for 1 year at elevated RT was also reflected by a decrease in serum HBeAg levels (Fig. 8H) and a significant reduction of HBcAg-positive hepatocytes and intrahepatic HBV-DNA, indicating HBV clearance (Fig. 8I-K). This effect was as strong as the effect of $TherVacB_{Ctrl}$ that was not lyophilized and continuously stored at optimal conditions.

Taken together, SAA-formulated *TherVacB* components remained immunogenic and retained their potential to break immune tolerance in HBV-carrier mice. Even after 1 year of storage at elevated RT, SAA-formulated *TherVacB* protein and MVA-components remained active and elicited a potent, HBV-specific effector T-cell response, allowing TherVacB to efficiently control HBV replication and eliminate HBV-infected hepatocytes.

Discussion

To address the challenge of transport and storage of a vaccine at elevated temperatures, which is necessary for their application in many regions of the world, we formulated protein and vector vaccine components with SAA-based formulations. SAA-formulation resulted in a long-term thermostable therapeutic hepatitis B vaccine. The SAA-formulated vaccine components of *TherVacB* (HBsAg, HBcAg and the MVA-vector) fulfilled and exceeded the stability criteria of the WHO CTC guidelines as well as European Medicines Agency and ICH requirements.[23,24] Integrity of the particulate protein-antigens, HBsAg and HBcAg, as well as the MVA-vector was maintained despite a 40 °C heat-exposure for up to 3 months, as well as during long-term storage at 25 °C for 1 year (*i.e.* an elevated RT in warmer regions). SAA-formulated vaccine components were well tolerated and induced a strong HBV-specific antibody and T-cell response in HBV-naïve and AAV-HBV-infected mice even after long-term storage. Our results are not only important for the development of a therapeutic hepatitis B vaccine, but also provide a learning example for other vaccination campaigns currently running worldwide.

Thermal stability is essential for worldwide vaccination campaigns. Thus, the development of heat-stable vaccines has garnered significant interest over the past decade. *Ohtake et al.* reported on a spray-dried measles vaccine which was stable at 37 °C for approximately 2 months.[25] Lyophilized and stabilized herpes simplex virus type 2, Ebola and influenza vaccines proved heat-stable for 2 to 3 months at 40 °C.[26,27] Our SAA-formulated hepatitis B vaccine easily withstood these conditions by maintaining stability and immunogenicity *in vivo* for 3 months at 40 °C. This adds to the evidence that SAA-formulations can stabilize a broad range of various biologics, such as antibodies, adenoviral viral vectors or split-virion influenza vaccine.[28–30] In addition, we demonstrated that a single SAA-formulation is able to efficiently stabilize very different vaccine components such as the complex, particulate recombinant protein-antigens and the MVA-viral vector used in *TherVacB*. This will simplify vaccine manufacturing and clinical approval.

Lyophilization is a widely used drying method to improve the thermal stability of vaccine components during transport and storage. The advantage of HBsAg freeze-drying has already been demonstrated *in vitro* and *in vivo*.[31] We demonstrate that despite many advantages of lyophilization, the freeze-drying process can also cause significant damage. We show that the negative effects of lyophilization can be prevented by SAA-formulation and by combining *TherVacB* protein-antigens; we thereby confirm earlier reports that a clever selection of cryoprotectants and lyoprotectants helps to prevent vaccine damage.[32] Antigen combination provides an additional benefit for future clinical application as it allows a single "ready to use" formulation.

After thermal stressing, aggregated partially unstable vaccine antigens were still sufficient to induce HBV-specific antibodies. Electron microscopy revealed that even after prolonged stressing in high temperature the overall structure of the antigens remained intact. However, our potency ELISA indicated that some of the conformational epitopes had been lost. This confirms recent reports that aggregates with a native-like structure are more immunogenic than those consisting of fully degraded proteins[33,34] and are capable of inducing antibodies independently of vaccine formulation's particle diameter.[35] Interestingly, the thermal stress had less effect on antibody and $CD4^+$ T-cell responses than on $CD8^+$ T-cell responses. There are two potential reasons for this. First, infectivity of the MVA-vector which is essential to boost $CD8^+$ T-cell responses is affected by lyophilization and thermal stress. In addition, aggregation of the non-protected protein-antigens reduces the amount of antigen taken-up and presented by antigen presenting cells, and $CD8^+$ T-cell activation has been reported to critically depend on antigen amounts.[36] Accordingly, we also observed that immunization with lower amounts of antigens or MVA-vector significantly diminished vaccine-elicited $CD8^+$ T-cell responses but had a minor effect on the induction of HBV-specific antibodies (unpublished data).

Recently, MVA has gained considerable interest as a safe vaccine vector facilitating the induction of T-cell responses against encoded antigens.[37] The induction of potent, antigen-specific T-cell responses is of high importance for the development of vaccines against malaria, tuberculosis or human immunodeficiency virus infection,[38] which are also prevalent in countries with high outdoor temperatures. Other MVA-based prophylactic and therapeutic vaccines currently in clinical trials[38] may profit from our findings, as we clearly demonstrate that formulation with SAA retained infectivity and immunogenicity of MVA-S/C even after rather tough temperature stressing.

Very importantly, currently used prophylactic hepatitis B vaccines based on HBsAg must be stored at 2-8 °C to maintain vaccine efficacy.[14] We found a profound gradual loss of antigen integrity over time when non-stabilized HBsAg or HBcAg were exposed to elevated RT. Other studies reported that prophylactic hepatitis B vaccines containing HBsAg were stable even at higher temperatures.[14] Formulation with aluminium as adjuvants and other excipients may provide an explanation for the differences observed. Despite the potential advantage of alum for HBsAg stability, alum-based vaccine formulations are not suitable to support the induction of efficient $CD8^+$ T-cell responses required to break immune tolerance, eliminate chronic HBV infection[7,39,40] or prevent and control infections by a number of other viruses. Using bis-(3',5')-cyclic dimeric AMP as an adjuvant that allows for a balanced T helper 1/2- and subsequent effector T-cell response, our lyophilized HBsAg formulation demonstrated superior temperature and storage stability compared to the alum-based liquid HBsAg.[14,41,42]

During the course of CHB, HBV-specific T cells are scarce and progressively become dysfunctional.[2–4] We reason that a successful therapeutic vaccine must elicit highly functional virus-

specific B and T cells *de novo*. In the AAV-HBV mouse model that develops a strong immune tolerance, therapeutic vaccination is able to induce neutralizing antibodies and newly prime and activate functional effector T cells. In our study we have demonstrated that SAA-formulated *TherVacB* elicited high anti-HBs titres leading to undetectable circulating HBsAg and serum HBV-DNA levels in most of the mice, which represents functional cure of HBV.[5] Moreover, the stabilized vaccine resulted in high numbers of intrahepatic effector HBV-specific $CD8^+$ T cells expressing IFNγ and granzyme B which eliminated HBV-positive hepatocytes. However, restoration of effective HBV-specific immunity in chronic HBV carriers might be much harder due to the extremely long-term exposure to HBV antigens, in particular when HBV infection was acquired around birth.

In our experiments, clearance of HBV from the liver was accompanied by elevation in ALT levels suggesting an involvement of vaccine-induced, antigen-specific effector cells with cytolytic activity. Besides cytotoxic $CD8^+$ T cells, hepatic natural killer cells are known to contribute to elimination of HBV-infected cells and HBV-induced liver damage.[43] Our recent and previously published data[10,22] implied that *TherVacB* is capable of inducing hepatic HBV-specific CD8+ T cells with cytotoxic potential. However, the potential contribution of natural killer cells to the *TherVacB*-mediated antiviral effect observed in this study has not been addressed and cannot be excluded.

Taken together, we herein demonstrate that development of a thermostable, highly immunogenic, therapeutic vaccine for the treatment of chronic hepatitis B is feasible. The high thermostability of all vaccine components is of particular importance for future distribution of therapeutic as well as prophylactic vaccines, as hepatitis B is predominantly endemic in countries with high outdoor temperatures. Thus, the usage of thermostable vaccine components will be of the utmost importance for worldwide hepatitis B vaccination campaigns, as well as for other MVA-vector-based vaccines.

Abbreviations

AAV, adeno-associated virus; ALT, alanine aminotransferase; anti-HBc, hepatitis B core antibodies; anti-HBs, hepatitis B surface antibodies; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CTC, controlled temperature chain; Ctrl, control; DLS, dynamic light scattering; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; ICS, intracellular cytokine staining; IFNα, interferon alpha; MVA, Modified Vaccinia virus Ankara; NAGE, native agarose gel electrophoresis; RH, relative humidity; RT, room temperature; SAA, stabilizing amino acids; SEC-HPLC, size exclusion-high performance liquid chromatography; $TCID_{50}$, median tissue culture infection dose; $TherVacB_{Ctrl}$, non-lyophilized; non-stressed, non-stabilized *TherVacB*; WHO, World Health Organization.

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Conflict of interest

UP is a co-founder and shareholder of SCG Cell Therapy, and an *ad hoc* scientific advisor to Abbvie, Arbutus, Biontech, Gilead, GSK, J&J, Roche, Sanofi, Sobi, Vaccitech and VIR Biotechnology. UP and AK are named as inventors on a patent application describing the therapeutic vaccination scheme of *TherVacB* (PCT/EP2017/050553). KK, MS, JA are or were employees of LEUKOCARE AG. UP is member of the advisory board of LEUKOCARE AG.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

JSa, AK, KK, KS, JA, MS and UP designed the study; JSa, AK, KK and SL performed the experiments; MK, LW and GS produced critical components and reagents; HK, EAO and JSu supported mouse experiments; SE provided essential instruments and support; JSa, AK, KK and UP wrote the paper. All authors reviewed and confirmed the final version of the manuscript.

Data availability statement

All data supporting this study is provided in the results section or as supplementary information accompanying this paper.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2022.100603.

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Author names in bold designate shared co-first authorship.

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