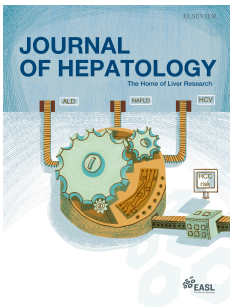

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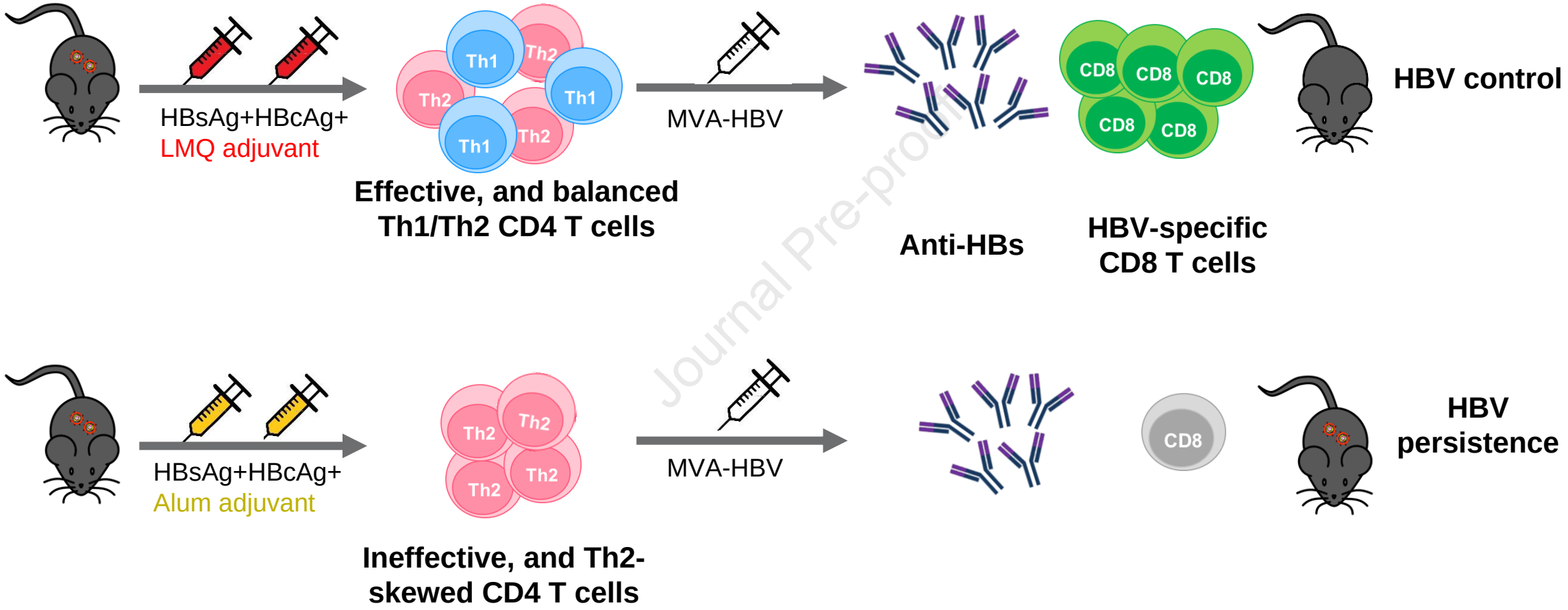

Activation of CD4 T cells during prime immunization determines the success of a therapeutic hepatitis B vaccine in HBV-carrier mouse models

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Authors' contributions

JSu, ADK, UP designed the study; JSu, ADK, LB, JSa, GF, EAO, HAK performed the experiment; CM evaluated liver histopathology; UP, PAK and NC provided supervision and funding; FT and MW contributed to planning and funding acquisition; LB, GF, NC provided key materials; JSu, ADK, UP wrote and finalized the manuscript. All authors read and approved the final version of the manuscript.

Data availability statement. All sequencing data were deposited in the Sequence Read Archive (SRA) under accession code number PRJNA874547 (available at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA874547).

Abstract

Background & Aims: Therapeutic vaccination represents a promising approach to cure HBV. We employ a heterologous therapeutic vaccination scheme (*TherVacB)* comprising a particulate protein-prime followed by modified vaccinia-virus Ankara (MVA) vector-boost. The key determinants required to overcome HBV-specific immune tolerance, however, remained unclear. Here, we unravel the essential role of CD4 T-cell activation during the priming phase for antiviral efficacy of *TherVacB*.

Methods: Recombinant hepatitis B surface (HBsAg) and core antigen (HBcAg) particles were formulated with different liposome-based or oil-in-water emulsion combination adjuvants containing saponin QS21 and monophosphoryl lipid A (MPL) and compared them to STING-agonist c-di-AMP and conventional alum formulation. Immunogenicity and antiviral effects of protein antigen formulations and the vector boost within *TherVacB* was evaluated in AAV-HBV infected and HBV-transgenic mice.

Results: Combination adjuvant formulations preserved HBsAg and HBcAg integrity for ≥ 12 weeks, promoted human and mouse dendritic cell activation and within *TherVacB* elicited robust HBV-specific antibody and T-cell responses in wild-type and HBV-carrier mice. Combination adjuvants priming a balanced HBV-specific type 1 and 2 T helper response induced high-titer anti-HBs antibodies, cytotoxic T-cell responses and long-term control of HBV. Lack of a T-cell booster by the MVA vector as well selective CD8 T-cell depletion allowed for a drop of HBsAg mediated mainly by anti-HBs antibodies but resulted in a lack of HBV control. Selective CD4 T-cell depletion during the priming phase of *TherVacB* resulted in a complete loss of vaccine-induced immune responses and its therapeutic anti-viral effect in mice.

Conclusions: Our results identify CD4 T-cell activation during the priming phase as a key determinant for HBV-specific antibody and CD8 T cell immunity using *TherVacB* as targeted therapeutic vaccine.

Impact and Implications

Therapeutic vaccination is a promising, potentially curative treatment for chronic hepatitis B, but the factors essential to break immune tolerance in HBV carriers and determinants of success remain unclear. Our study provides the first direct evidence that correct priming of HBV-specific CD4 T cells preparing a booster of CD8 T-cell responses determines the success of therapeutic hepatitis B vaccination. Understanding how to fine-tune therapeutic vaccines by the selection of appropriate vaccine components proved essential for its antiviral effect in two preclinical HBV-carrier mouse models and may help to guide the clinical development of therapeutic vaccines for chronic hepatitis B.

Lay summary

Therapeutic vaccination is a promising, potentially curative treatment for chronic hepatitis B. Which factors are essential for breaking immune tolerance in HBV carriers and determine a successful outcome of therapeutic vaccination, however, remains unclear. Our study provides the first direct evidence that efficient priming of HBV-specific CD4 T cells determines the success of therapeutic hepatitis B vaccination in two preclinical HBV-carrier mouse models. Applying an optimal formulation of HBV antigens that allows activating CD4 and CD8 T cells during prime immunization provided the foundation for an antiviral effect of therapeutic vaccination, while depletion of CD4 T cells lead to a complete loss of vaccine-induced antiviral efficacy. Boosting CD8 T cells was important to finally control HBV in these mouse models. Our findings provide important insights into the rational design of therapeutic vaccines to cure chronic hepatitis B.

Introduction

Over 290 million people worldwide are HBV carriers living with persistent infection or chronic hepatitis B (CHB), even though the World Health Organization committed to eliminating viral hepatitis as a public health threat by 2030 [1]. Current antiviral treatments with nucleos(t)ide analogues effectively suppress HBV replication, however, fewer than 1% of CHB patients per year achieve a functional cure defined as loss of HBsAg [2, 3]. Stimulation of anti-HBV immunity is regarded essential to achieve a cure [4].

Patients resolving acute HBV infection develop robust antibody, CD4 and CD8 T-cell responses against the virus. A number of studies indicated that an early priming of CD4 T cells is required for viral clearance during acute HBV infection [5-7]. CHB patients are characterized by a lack of neutralizing antibodies [8] and scarce, partially dysfunctional virus-specific T-cells resulting in HBV-specific immune tolerance [4]. Therapeutic vaccination represents a promising strategy to control and finally cure HBV if it was able to restore HBV-specific immunity resembling that observed in natural resolvers. However, therapeutic hepatitis B vaccines have only had limited success in clinical trials to date [9].

This demonstrates the necessity for more sophisticated approaches to overcome immune tolerance and induce functional immune responses to combat HBV in CHB patients. To this end, we have recently developed the heterologous prime-boost vaccine, *TherVacB*, employing a protein-prime with particulate HBsAg and HBcAg and a vector-boost with recombinant MVA expressing different HBV antigens [10]. Our results indicate that the antiviral potency of *TherVacB* depends on the induction of virus-specific helper and cytotoxic T cells to control and finally cure HBV in preclinical mouse models [10-12]. Because HBV does only infect humanoid primates and research using these animals has been banned, mouse models serve as surrogate preclinical models although the HBV genome has to be artificially introduced into the

hepatocyte nucleus, they do not allow to study persistence of an infection over decades as frequently observed in CHB, and immune stimulation using adjuvants may only partially mirror that observed in humans.

Protein-based vaccines usually require adjuvants to adequately stimulate and shape immune responses [13]. Most of the adjuvants in currently licensed human vaccines are optimized for prophylactic purposes that require eliciting humoral immunity through the induction of type 2 T helper (Th) cells [14]. Therapeutic vaccines on the other hand require a strong Th1-type response to allow development of an effector CD8 T-cell response, implying the need of Th1-inducing adjuvants. Combining several individual immunostimulatory compounds into a single formulation [15] has been shown to improve immunogenicity [13]. As an example, synthetic monophosphoryl lipid A (MPL) combined with a saponin or as a liposome formulation may be used to promote not only humoral, but also strong cellular immunity [16].

Given the strong virus-specific immune tolerance that develops during long-term chronic HBV infection, we reasoned that employing an optimized combination adjuvant in *TherVacB* would enhance HBV-specific immune responses. While previous studies pointed at an important role of CD4 T cells for viral clearance during acute HBV infection [5-7], the importance of CD4 T cells for therapeutic vaccination against CHB remained unclear. The aim of the present study was therefore to investigate a series of novel adjuvants that combine the saponin QS21 and synthetic MPL as immunostimulants with emulsions or liposomes as delivery systems adjuvants in comparison to nucleotide-based adjuvant c-di-AMP for their ability to activate potent CD4 T-cell responses and improve *TherVacB* efficacy in wild-type mice and HBV-carrier mice that persistently replicate HBV, the need for adding an MVA boost and to explore the contribution of CD4 and CD8 T cells for vaccine-mediated control of HBV.

Materials and methods

Antigen/adjuvant formulation and characterization. Recombinant HBsAg (genotype A, adw) was produced in yeast by Biovac, South Africa. Recombinant HBcAg (genotype D, ayw) produced in *E. coli* was kindly provided by APP Lativijas Biomedicinas, Latvia. Combination adjuvants based on either on liposomes or an oil-in-water (OiW) emulsion and containing different concentrations of MPL and QS21 (Table S1) were manufactured and provided by The Vaccine Formulation Institute, Switzerland and used in a volume ratio of 1:5 to formulate 10 µg HBsAg and HBcAg/100 µl per immunization dose. Stability and integrity of antigen/adjuvant formulations were characterized by Western blot, native agarose gel electrophoresis (NAGE), ELISA, transmission electron microscopy (TEM) and various physicochemical assays.

Stimulation of human monocyte-derived dendritic cells (hMoDCs). Human peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy volunteers. Monocytes were isolated with Pan Monocyte Isolation Kit (Miltenyi Biotec, Germany), differentiated into immature hMoDCs as described [17] and stimulated with 1/40 immunization dose, 1 µg/ml LPS (Sigma-Aldrich, Germany), or left unstimulated for 6，24 or 48 hours. Secretion of tumor necrosis factor alpha (TNFα) and interleukin (IL)-6 were determined in cell culture supernatants by ELISA (BD Biosciences, Germany / Invitrogen, USA). Concentrations of chemokines CCL3, CCL4, CXCL1, CXCL8 were determined using LEGENDplex™ HU Proinflam. Chemokine Panel 1 (Biolegend, USA). Cells were analyzed for expression of co-stimulatory molecules (CD86, CD80) by flow cytometry.

Ethical statements. Animal experiments were conducted in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA), and reported according to the ARRIVE guidelines. Experiments were

approved by the District Government of Upper Bavaria (permission number: 55.2-1-54-2532-103-12 and ROB-55.2-2532.Vet_02-18-24). Mice were kept in biosafety-level 2, specific pathogen-free animal facilities following institutional guidelines.

Animal models. HBV transgenic (HBVtg) mice (strain HBV1.3.32) carrying 1.3-fold overlength HBV genome (genotype D, ayw) were bred on C57BL/6J background [18]. Eight to ten weeks old wild-type C57BL/6J mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Persistent HBV replication in wild-type C57BL/6J mice was established by intravenous injection of 4-6×10^9 genome equivalents of adeno-associated virus (AAV)-HBV vector carrying a 1.2-fold overlength HBV genome (genotype D, ayw) [19]. HBVtg and AAV-HBV mice were bled shortly before start of immunization and allocated into groups with comparable HBeAg and HBsAg serum levels.

Therapeutic hepatitis B vaccine regimen. Mice received the therapeutic, heterologous protein prime / MVA boost hepatitis B vaccine (*TherVacB*) [10]. Briefly, mice were immunized intramuscularly twice with 10 µg each of particulate HBsAg and HBcAg formulated with the investigated adjuvants, followed by 3×10^7 infectious units of recombinant MVA expressing HBV S- or core- protein (MVA-S, MVA-core) in a 2-week interval.

Serological analyses. Serum HBsAg, HBeAg, and anti-HBs levels were quantified on an Architect™ platform (Abbott, Germany) as described previously [12]. anti-HBc was measured using the BEPIII platform (DiaSorin, Saluggia, Italy). IgG subclasses were detected by ELISA using anti-mouse IgG_1 and IgG_{2b} antibodies.

Characterization of HBV-specific T cells and murine DCs by flow cytometry. Murine splenocytes and liver-associated lymphocytes (LALs) were isolated and stained with MHC class I multimers as described previously [11, 20]. HBV-specific S_{190} (VWLSAIVM), C_{93} (MGLKFRQL), or ovalbumin-specific OVA_{S8L} (SIINFEKL) multimers

were labeled with APC- or PE-Streptactin (IBA Lifesciences, Germany).

For ICS, splenocytes and LALs were incubated overnight with HBsAg or HBcAg-derived overlapping peptide pools (Table S2 and S3), or OVA_{S8L} peptide (SIINFEKL) [12]. Alternatively, RNA was extracted from CD4+ splenocytes and analyzed by bulk RNA sequencing on an Illumina Novaseq platform (Novogene Technologies). After data clearance, differential gene expression analysis was performed using the DESeq2 R package (1.20.0). Up-regulated genes were identified based on *p*-adj <0.05 and (log2FoldChange)>1 and were used to identify significantly enriched pathways and for clustering analysis. All raw sequencing data were deposited at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA874547.

Cell surface staining was performed using anti-CD4, anti-CD8 antibodies (see Table S4). Dead cells were excluded from analysis by Fixable Viability Dye eF780 (eBioscience, Germany) staining. Intracellular cytokine staining (ICS) of IFNγ and TNFα was performed as described [11]. Data were acquired on a CytoFlexS flow cytometer (Beckmann Coulter, USA) and analyzed using FlowJo software (Tree Star, USA). Primary murine DCs were isolated from spleen via collagenase 4/DNase-I digestion and analyzed for expression of co-stimulatory molecules using anti-CD80 and anti-CD86 antibodies by flow cytometry (see Table S4).

Analyses of HBV parameters in liver tissue. For immunohistochemistry, livers were fixed in paraformaldehyde for 48 hours and then paraffin embedded. 2-µm-thin liver paraffin sections were subjected to core-specific immunohistochemistry as previously described [11]. Numbers of core-positive hepatocytes were determined in 10 random view-fields (20x magnification) and quantified per mm^2. Intrahepatic HBV DNA was analyzed with quantitative real-time PCR.

CD4 and CD8 T-cell depletion *in vivo*. CD4 and CD8 T cells were depleted using anti-CD4 GK1.5 or anti-CD8 RmCD8.2 monoclonal antibodies (mAb) kindly provided

by the Helmholtz Monoclonal Antibodies Core Facility. Mice were injected intraperitoneally with 300 µg of anti-CD4 GK1.5 mAb one day before and 150 µg on the day of first protein immunization, or with 50 µg of anti-CD8 RmCD8.2 mAb on the day of first protein immunization. On the day of second protein immunization, mice received 150 µg of GK1.5 or 25 µg of RmCD8.2 mAb. The levels of CD4 and CD8 T cells in blood were monitored by flow cytometry.

Statistical analyses. In all graphs, mean ± SEM is given. Data were analysed using GraphPad Prism version 5.01 or 9.0 (GraphPad Software Inc., San Diego, CA) using one- or two-way ANOVA or Mann-Whitney test depending on normal distribution of data. *P*-values <0.05 were considered significant. Only statistically significant differences are indicated.

For details of adjuvant preparation and formulation and all methods applied see Supplementary Material.

Results

Liposome- and oil-in-water (OiW) emulsion-based adjuvants preserve structural integrity and antigenicity of HBsAg and HBcAg and activate dendritic cells.

We investigated different combination adjuvant formulations: liposomes, OiW, and water-in-oil emulsion Montanide ISA720. Liposomes (L) and squalene-based (S) OiW-emulsions were combined with synthetic MPL and QS21 at various doses (Table S1) and used to formulate HBsAg and HBcAg. Western blot analysis (Fig.S1A) and native agarose electrophoresis (Fig.S1B) of HBcAg formulations proved intact capsid-particles except for Montanide ISA720. Antigenicity of HBsAg or HBcAg determined by ELISA was preserved (Fig.S1C), and electron microscopy of Liposome-3 adjuvant formulation confirmed intact HBsAg and HBcAg particles and liposomes after 12 weeks of storage at 4°C (Fig.S1D). Particle size (Fig.S2A), poly-dispersity index, zeta potential, and pH were preserved (Fig.S2B-E). This demonstrated that HBsAg and

HBcAg in L- and S-formulations [21] remained intact and stable for at least 12 weeks. To test their efficacy within the *TherVacB* regimen (Fig.1A), we selected six representative adjuvants: L and S combined with QS21, referred to as LQ and SQ, and synthetic MPL (LMQ and SMQ) or a formulation containing a lower QS21 dose (LMQ^{low} and SMQ^{low}) (Fig.1B).

We stimulated human monocyte-derived dendritic cells (hMoDCs) with these combination adjuvants to characterize their immunostimulatory properties *in vitro* compared to LPS-stimulated and mock-treated hMoDCs. Stimulation of hMoDCs with combination adjuvants or LPS, but not with L or S alone, induced secretion of cytokines like TNFα (Fig.1C) and IL-6 (Fig.S3A) as well as chemokines CCL3, CCL4 CXCL1, CXCL8 (Fig.1D), in particular when synthetic MPL was added. Consistent with activation of DCs, CD86 and CD80 co-stimulatory molecules were upregulated strongest by LMQ and SMQ formulations (Fig.1E,F).

To compare this with the effect of the combination adjuvants *in vivo*, C57BL/6J mice were immunized at week 0 and 2 with HBsAg and HBcAg formulated with either of the adjuvants or without adjuvant (no adj). According to the *in vitro* stimulation, primary murine DC isolated from the spleen directly *ex vivo*, upregulate of CD80 and CD86 when antigen formulations with LQ, LMQ, SQ and SMQ were used (Fig.1G,S3B). Thus, the combination adjuvants showed potent immune stimulatory effects on DCs *in vitro* and *in vivo*.

TherVacB prime immunization using liposome- and OiW-based combination adjuvants demonstrates strong immunogenicity _in vivo_

To investigate the immunogenicity *in vivo*, mice were, according to the *TherVacB* regimen (Fig.1A), vaccinated with adjuvanted protein twice, week 0 and 2, and boosted with MVA expressing S and core at week 4. Immunization of mice with the combination adjuvant formulations activated CD4 T cells (Fig.2A) and elicited high levels of serum

anti-HBs of up to 10^6 mIU/ml as well as anti-core antibodies (Fig.2B), both primarily of the IgG_{2b} subclass (Fig.2C). All adjuvant formulations allowed to prime CD4 and CD8 T cells, and MVA-vaccination boosted robust and multifunctional S- and core-specific, $IFN\gamma^+$ and $TNF\alpha^+$ CD4 and CD8 T-cell responses (Fig.2D,E). Hereby, SMQ^{low}, proved inferior to the other adjuvant combinations. Of note, mice primed with HBV antigens without adjuvant failed to develop any S-specific CD4 or CD8 T-cell responses (Fig.2D,E). Taken together, formulation of HBsAg and HBcAg with adjuvants LQ, LMQ, SQ and SMQ induced strong HBV-specific humoral and cellular immune responses and were selected for studies in mouse models of persistent HBV infection.

TherVacB **using liposome- or OiW-combination adjuvants breaks immune tolerance and induces strong HBV-specific antibody responses**

HBV-transgenic (HBVtg) and AAV-HBV infected, HBV carrier mice were used to evaluate whether *TherVacB* using either c-di-AMP or LQ, LMQ, SQ or SMQ formulated antigens can break HBV-specific immune tolerance. Mice were immunized at week 0 and 2 (protein with combination adjuvants) and 4 (MVA) and sacrificed at week 5 (HBVtg mice) or week 10 (AAV-HBV mice) to analyze S-specific (left panels) and core-specific (right panels) immunity.

TherVacB priming with the five adjuvant formulations induced mean anti-HBs levels $\geq 10^5$ mIU/ml as well as anti-HBc in both, HBVtg (Fig.3A) and AAV-HBV mice (Fig.3B). Immunization with HBV antigens without any adjuvant only elicited low anti-HBs responses in HBVtg mice (Fig.3A), but no detectable response at all in AAV-HBV mice (Fig.3B). This correlates with the observation that HBVtg mice tend to undergo spontaneous HBsAg seroconversion [22]. In both HBVtg and AAV-HBV mice, comparable anti-HBc responses were detected irrespective of whether HBcAg was adjuvanted or not (Fig.3A,B) indicating that the particulate HBcAg, which contains large amounts of bacterial RNA, serves itself as potent immunogen [23].

All vaccine formulations induced predominantly IgG_{2b}-type anti-HBc, but comparable levels of IgG_1 and IgG_{2b} anti-HBs indicating the induction of balanced Th1- and Th2-type S-specific helper T-cell responses (Fig.3C). To confirm this finding, we analyzed the production of the characteristic Th1 cytokine IFNγ and the Th2 cytokine IL-5 in splenocytes isolated from AAV-HBV mice, and found a predominant Th1-type CD4 T-cell response when SMQ and LMQ formulations were employed for priming (Fig.3D,E) although we cannot exclude that IFNγ at least partially stemmed from HBV-specific CD8 T cells.

Taken together, formulations of HBsAg and HBcAg with adjuvants c-di-AMP or LQ, LMQ, SQ and SMQ were able to break immune tolerance and activate CD4 T-cell responses. While c-di-AMP mainly activated an S-specific response, in particular the combination adjuvants LMQ and SMQ promoted a Th1-type S- and core-specific helper T-cell response in HBV-carrier mice.

TherVacB immunization with combination adjuvants stimulates vigorous intrahepatic HBV-specific effector T-cell responses.

We next evaluated HBV-specific effector CD4 and CD8 T-cell responses in the livers of HBVtg mice one week (Fig.4A,B) and AAV-HBV infected mice six weeks after MVA-boost vaccination (Fig.4C,D) by flow cytometry after ICS.

In all vaccinated HBV carrier mice, we detected intrahepatic S-specific, but no core-specific $IFN\gamma^+$ CD4 T-cell responses after the MVA-boost vaccination (Fig.4A,C), but all adjuvants allowed for the induction of S- and core-specific CD8 T-cell responses (Fig.4B,D). It was expected that CD4 T-cell responses can hardly be detected at this time points anymore as they form a small-sized memory pool. The induction of anti-HBc antibodies (Fig.3A,B), IL5 secretion upon HBcAg stimulation (Fig.3E) and the induction of core-specific CD8 T cells (Figs.4B,D, S4), however, strongly suggests generation of an effective CD4 helper T-cell response. More specifically, over 90% of

S-specific CD8 T cells induced by *TherVacB* using combination adjuvant formulations were polyfunctional and simultaneously produced lFNγ and TNFα (Fig.S4). Without adjuvant low-level core-specific CD8 T-cell responses were detected in the liver, but not in the spleen indicating (i) an adjuvant effect of the RNA-containing HBcAg particles and (ii) that the T cells remained in the liver to control HBV infection. Among the adjuvants analyzed, the LMQ formulation tended to elicit the most robust, multifunctional HBV-specific CD4 and CD8 T-cell responses in HBV carrier mice.

Using combination adjuvants within *TherVacB* results in sustained immune control of persistent HBV infection

To evaluate the long-term effect on HBV control and cure, we monitored HBV replication in AAV-HBV infected mice after *TherVacB* using the different adjuvants. Immunization with all adjuvant formulations resulted in an almost 3-log decline of serum HBsAg, whereas the decline was moderate in control mice that were immunized with protein without adjuvant (Fig.5A). Already during the priming phase of *TherVacB*, we detected a marked decline in serum HBsAg levels pointing towards a role of the recombinant protein immunization in inducing anti-HBs antibodies. Immunization with c-di-AMP, LQ, SQ, and particularly LMQ adjuvanted formulation also induced a significant decline of HBeAg in serum (Fig.S5A), while inducing only a max. 2-fold increase of ALT activity, a moderate increase of the histopathology score and no weight loss (Fig.S5B-D).

As HBeAg decline in serum indicated clearance of AAV-HBV infected hepatocytes, we stained liver sections for HBV core protein positive hepatocytes and quantified intrahepatic HBV-DNA. Indeed, immunization with c-di-AMP, LQ, LMQ and SQ adjuvanted formulations led to a significant reduction in the numbers of core-positive hepatocytes and intrahepatic HBV-DNA. SMQ formulation only induced anti-HBs and reduced HBsAg levels (Fig.5A), but failed to reduce intrahepatic HBV-DNA and HBV-positive hepatocytes (Fig.5B-D,S5) – consistent with the poor induction of core-specific

CD8 T-cells (Fig.4B,D). Taken together, these findings indicate that prime immunization with a properly adjuvanted protein antigen is key to allow for a long-term immune control of persistent HBV infection, while being well tolerated.

Priming with particulate HBV antigens formulated with LMQ, but not with aluminum hydroxide (alum) enables antiviral activity of *TherVacB*

To compare the efficacy of combination adjuvants with the classical alum adjuvant formulation for the immune priming step of *TherVacB*, we immunized AAV-HBV infected mice with *TherVacB* regimens comparing LMQ and alum formulated antigens for priming.

Immunization with alum- or LMQ-adjuvanted antigen formulations induced significantly higher levels of anti-HBs compared to non-adjuvanted antigens while comparable anti-HBc levels were detected in all groups receiving antigen - even in the absence of adjuvant (Fig.6A). LMQ adjuvanted formulation induced predominantly IgG_{2b}- (Fig.6B), whereas alum predominantly induced IgG_1-type anti-HBs. Importantly, *TherVacB* immunization using LMQ-adjuvanted antigens stimulated strong intrahepatic, S-specific CD4 and CD8 T-cell responses, in contrast to alum that only induced minor CD4 and no CD8 T-cell responses (Fig.6C,S6A). Anti-HBc antibodies were predominantly IgG_{2b} subclass (Fig.6B), and core-specific CD8 T cells were detected irrespective of the antigen formulation (Fig.6C) - again indicating that bacterial RNA contained in recombinant HBV core antigen has an adjuvant effect. HBsAg- and HBcAg-stimulated splenocytes from mice immunized with alum-adjuvanted antigens secreted predominantly IL-5, while those from mice immunized with LMQ-adjuvanted antigens secreted both IL-5 and IFNγ (Fig.S6B,C), although we cannot exclude that IFNγ may also be derived from HBV-specific CD8 T cells.

Injection of LMQ adjuvant without antigen followed by recombinant MVA immunization did not stimulate any S- or core-specific antibody, CD4, or CD8 T-cell responses

(Fig.6A-C, S6A). This confirmed that a heterologous protein prime with adjuvanted antigen is essential and neither MVA alone nor the circulating antigen are sufficient to break immune tolerance in mice with persistent HBV-replication.

To characterize the differences in CD4 T cell subsets induced, we isolated HBV-specific CD4 T cells *ex vivo* after two vaccinations with LMQ- or alum-adjuvanted protein antigens to perform a more detailed analysis. Three days after the second vaccination, HBV-specific CD4 T cells expressing IL-2 upon stimulation with recombinant HBsAg or HBcAg were readily detected (Fig.6D). At day 5, CD4 T cells were increased in numbers (Fig.S7A) and showed the pattern of activated T cells when LMQ but not when alum was used to formulate the protein antigens (Fig.S7B). Bulk RNA sequencing revealed a distinct activation pattern when LMQ-formulated antigens had been used (Fig.6E,F) with - compared to alum or no vaccination - pathways involved cell proliferation and regulation of Th-1 type immune responses being induced (Fig.S7C).

Boosting T-cell responses using an MVA-vector is essential to achieve sustained antiviral effects.

To determine whether an MVA boost is required or whether protein vaccination alone would be sufficient to break immune tolerance and control HBV if a potent adjuvant is used, we immunized mice either - according to the *TherVacB* regimen – twice with LMQ-formulated HBsAg and HBcAg and once with recombinant MVA expressing S and core or only with LMQ-formulated antigens. There was no difference in the levels of anti-HBs and anti-HBc between the groups with and without MVA boost vaccination (Fig.7A) proving that the HBV-specific antibody responses are mainly induced by the protein prime vaccination. However, the mice that had received an MVA boost showed significantly stronger hepatic S- and core-specific CD8 T cell responses (Fig.7B,C). Both groups of mice showed a comparable drop on HBsAg, but only the group of mice receiving the MVA-boost demonstrated a significant decrease in HBeAg levels

418 indicating control of HBV replication in the liver (Fig.7D). These data indicate that anti-
419 HBs induced by protein immunization can neutralize HBsAg but the boost of T cell
420 responses by the MVA vector is necessary to achieve long-term antiviral control of
421 HBV even if a potent adjuvant is used for the protein immunization.

422 **CD4 T cells play a crucial role in initiating *TherVacB*-mediated antiviral immunity**
423 **against HBV.**

424 As all our data on protein antigen priming shown above indicated that CD4 T cells
425 serve as master regulator of HBV-specific immune responses during therapeutic
426 vaccination, we decided to selectively deplete either CD4 or CD8 T cells during the
427 protein-priming phase of *TherVacB.*

428 Mice were infected with AAV-HBV and either CD4 or CD8 T-cells were depleted using
429 anti-mouse CD4 (αCD4) or CD8 (αCD4) mAb before each prime vaccination with LMQ-
430 formulated HBsAg and HBcAg at week 0 and 2 (Fig.8A, grey arrows). Flow cytometry
431 analysis confirmed efficient depletion of CD4-positive T cells and conventional DCs 2
432 (cDC2) using αCD4-mAb, but only CD8-positive T cells using αCD8-mAb (Fig.S8).
433 Since cDC2 have been shown to specifically prime CD4 T cells [24], their co-depletion
434 still allows to explore the impact of a loss of CD4 T-cell functionality. At week 6, when
435 T cells were at least partially restored (Fig.8B), mice were boosted with recombinant
436 MVA. *TherVacB* immunization of αCD4-mAb treated mice induced significantly lower
437 anti-HBs and anti-HBc levels (Fig.8C) as well as intrahepatic S- and core-specific CD4
438 and CD8 T-cell responses (Fig.8D-E), while depletion of CD8 T cells did not show a
439 significant effect. Interestingly, the effect on HBsAg loss was lost upon CD4 but not
440 CD8 T-cell depletion, while the overall antiviral effect indicated by the loss of HBeAg
441 was lost when either T-cell population was depleted (Fig.8F-G). These data
442 demonstrate that CD4 T-cell activation during the priming phase of therapeutic
443 vaccination is key to induce HBV-specific immunity and achieve an antiviral effect, but

proper priming of CD8 T cells is essential to finally execute the antiviral effect of *TherVacB*.

Discussion

Although therapeutic vaccination is considered a promising, potentially curative treatment for chronic hepatitis B, the determinants of successful vaccination are poorly understood [2-4]. Here, we show that an HBV-specific CD4 T-cell response is indispensable to induce the type of immunity observed in individuals resolving HBV infection and initiate immune control of HBV in mouse models. We demonstrate that priming with particulate HBV antigens formulated with a potent adjuvant activating a Th1-type CD4 T-cell response lays the foundation for the MVA boost vaccination to elicit potent CD8 T-cell responses, break immune tolerance in HBV-carrier mice and control HBV infection.

Virus-specific CD8 T cells represent the key cellular effectors responsible for HBV clearance during acute infection [25]. Induction of an effective HBV-specific CD8 T-cell response, however, depends on early priming of CD4 T cells, and insufficient HBV-specific CD4 T-cell responses may therefore contribute to the dysfunctional virus-specific T-cell immunity observed during CHB [5]. We demonstrate that depletion of CD4 T cells during the priming phase of *TherVacB* led to a complete loss of vaccine-mediated therapeutic effects against HBV, and therefore provide direct evidence that efficient priming of HBV-specific CD4 T cells is essential for successful therapeutic vaccination to break virus-specific immune tolerance during persistent HBV infection in mice. Although a selective depletion of helper T cells is not possible, the co-depletion of cDC2, which play an unique role in priming CD4 T cells [24], substantiates our observation. We also demonstrate that the MVA-vector mediated boost of T-cell immunity [10, 26] is indispensable for the antiviral effect of *TherVacB* in mice, and that the depletion of CD8 T cells during the priming phase results in a loss of antiviral efficacy and control of HBV comparable to what has been described for HBV control

in chimpanzees [25]. This strongly supports the use of a heterologous prime-boost regimen for therapeutic vaccination although it obviously is much harder to translate into the clinics than using only a single vaccine component.

To explore the determinants of efficient CD4 T-cell responses and to improve vaccine-mediated antiviral efficacy, we investigated the impact of different combination adjuvant protein formulations for the immunogenicity of *TherVacB.* As expected, we found that protein priming with combination adjuvants such as LMQ induces a stronger Th1-type response than alum known to mainly activate Th2-type responses [13-16]. Of note, antigen formulation with alum enhanced HBV-specific antibody and CD4 T-cell responses but did neither improve virus-specific CD8 T-cell responses nor achieve HBV control. Interestingly, HBV core-specific immunity elicited by *TherVacB* did not benefit from combination adjuvant formulation confirming the previously described immunogenic properties of HBcAg itself [27]. Nevertheless, non-adjuvanted antigens failed to activate S-specific immune responses and – as adjuvants alone - resulted in poor therapeutic efficacy in both mouse models of chronic HBV infection. Of note, we found a significant difference particularly in core-specific T-cell responses between naïve and HBV-carrier mice after vaccination, indicating the importance of inducing core-specific immunity to achieve an HBV cure [25].

HBV-specific B- and T-cell tolerance observed in CHB patients could be due to a lack of activation of crucial immune cells such as DCs [28]. Therefore, a successful immunotherapeutic approach against CHB should overcome not only adaptive, but also mechanisms of innate immune tolerance against HBV to mount an effective antiviral immune response. Employing combination adjuvants that activate various immune signaling pathways [29], induced superior adaptive immune responses. The combination adjuvants investigated, especially the ones containing both synthetic MPL and QS21 saponin, exhibited strong immune stimulatory effects on human DCs *in vitro* and murine DC *in vivo* and – as well as the nucleotide-based adjuvant c-di-AMP -

allowed to achieve long-term reduction or even complete loss of HBV antigens in HBV-carrier mice.

HBV-transgenic and AAV-HBV mice employed in this study represent the most suitable models to proof preclinical efficacy of a therapeutic hepatitis B vaccine or other therapies stimulation adaptive immunity. Although they don't support HBV spread, these models allow for establishment of HBV-specific immune tolerance which can be broken by an effective therapeutic vaccine. The AAV-HBV model in addition allows to study the elimination of HBV-positive hepatocytes. Overall, mouse models proved useful to explain mechanisms of adjuvant action [30], but may only partially predict vaccine immunogenicity in humans. Relevant obstacle are differences between mice and humans, such as the immune cell and receptor repertoire and expression of pattern-recognition receptors. Thus, systematic approaches using human DCs are now being increasingly used to assess immune responses to vaccination in humans, to identify molecular signatures that can predict vaccine efficacy and to obtain mechanistic insights [30]. In our study, activation of human DC *in vitro* accurately predicted the activation of murine DC *in vivo* confirming that human DC cultures may help to identify immune signatures predicting clinical vaccine responses.

In conclusion, our study shows that activation of HBV-specific CD4 T cells during the priming phase of the heterologous prime-boost *TherVacB* vaccination scheme is essential for the antiviral potency of therapeutic hepatitis B vaccination. However, a multispecific HBV-specific CD8 T-cell immunity boosted by the MVA-vector was equally important to achieve long-term control of HBV in our preclinical mouse models. These insights need to be taken into account when developing therapeutic vaccines for the urgently needed clinical application.

Abbreviations

AAV, adeno-associated Virus; alum, aluminum hydroxide; ANOVA, Analysis of Variance; Ag, antigen; conventional DCs 2 (cDC2); CHB, chronic hepatitis B; DCs, Dendritic cells; DEGs, differentially expressed genes; HBV, hepatitis B virus; HBcAg, HBV core antigen; HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBVtg mice, HBV transgenic mice; hMoDCs, human monocyte-derived dendritic cells; ICS, intracellular cytokine staining; IFN, interferon; IL-6, interleukin 6; mAb, monoclonal antibody; MVA, modified vaccinia virus Ankara; LALs, liver-associated lymphocytes; Lipo, Liposomes; synthetic MPL, synthetic monophosphoryl lipid A; NAGE, native agarose gel electrophoresis; No vac, non-vaccinated; No adj, no adjuvant; OiW, oil-in-water emulsions; TEM, transmission electron microscopy; Th1 cells, type 1 helper T cells; *TherVacB*, therapeutic hepatitis B vaccine; TNF, tumor necrosis factor; PBMCs, peripheral blood mononuclear cells; PCA, principal component analysis (PCA).

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Author names in bold designate shared co-first authorship

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Figure legends

Fig. 1. Activation of dendritic cells by liposome- and oil-in-water combination adjuvants. (A) Schematic depiction of the *TherVacB* vaccination scheme. (B) Selected liposome (L) or oil-in-water (S) combination adjuvants. (C-E) Human monocyte-derived dendritic cells (hMoDCs) were stimulated with the indicated adjuvants. Cells stimulated with LPS or unstimulated hMoDCs (mock) served as controls. (C) TNFα and (D) Chemokine CCL3, CCL4, CXCL1, CXCL8 secreted into the supernatants of stimulated hMoDCs within 6- or 24-hours. Data are presented as fold-change compared to mock. (E,F) Expression of CD86 and CD80 on the surface of hMoDCs detected by flow cytometry after 48-hour stimulation with (E) liposome- or (F) OiW-based combination adjuvants. (G) C57BL/6J mice received two immunizations (day 0 and 14) with HBsAg and HBcAg formulated with the indicated adjuvant or were left untreated (no vac). Expression levels of CD86 and CD80 on the surface of primary spleen-derived DCs (mDCs) detected by flow cytometry 2 days after the 2nd protein vaccination. Statistical analyses using Mann-Whitney test, **p*<0.05.

Fig. 2. Immunogenicity of combination adjuvant formulations in wild-type C57BL/6J mice. At 2-week intervals each, C57BL/6J mice received two protein-prime vaccinations containing HBsAg and HBcAg formulated with the indicated adjuvant each and an MVA-boost. (A) Frequencies of total CD69+ and CD44+ splenic CD4 T cells detected by flow cytometry 2 days after the 2nd protein vaccination. (B-E) HBV-specific antibody and T-cell responses were analyzed one week after the MVA boost. (B) Levels and (C) IgG_{2b}/IgG_1 ratios of anti-HBs and anti-HBc in serum. Frequencies of S- and core-specific IFNγ$^+$ and TNFα$^+$ CD4 (D) and CD8 T cells (E) determined by flow cytometry after intracellular cytokine staining (ICS) following stimulation with respective S- and core-specific peptide pool. Statistical analyses using Mann-Whitney test, **p*<0.05.

Fig. 3. HBV-specific humoral immune responses induced in HBV-transgenic and AAV-HBV mice by *TherVacB* using different adjuvants.

(A) HBV-transgenic (HBVtg) mice or (B-E) C57BL/6J mice transduced with AAV-HBV six weeks prior to vaccination were immunized at week 0 and 2 with HBsAg and HBcAg adjuvanted with either c-di-AMP or indicated combination adjuvants, with MVA at week 4 and analyzed at (A) week 5 and (B-E) week 10. Levels of serum anti-HBs and anti-HBc in (A) HBVtg and (B) AAV-HBV mice. (C) IgG_{2b}/IgG_1 ratio of serum anti-HBs and anti-HBc. IFNγ and IL-5 secretion after 48-hour stimulation of splenocytes with particulate (D) HBsAg or (E) HBcAg. no vac: non-vaccinated; no adj: no adjuvant; n.a., not applicable; n.d., not detectable. Statistical analyses using Mann-Whitney test, *p<0.05, n.s., not significant. n.a. non applicable.

Fig. 4. HBV-specific effector T-cell responses induced by *TherVacB* in HBV-carrier mice using different adjuvants during protein prime. Vaccination as in Fig. 3 using the indicated adjuvants. (A-D) Analysis of HBV-specific T-cell responses in liver-associated lymphocytes at week 5 in HBVtg mice (A, B) and at week 10 in AAV-HBV mice (C, D). Frequencies of intrahepatic S- and core-specific $IFN\gamma^+$ CD4 (A, C) and CD8 T cells (B, D) following stimulation with an S- (left column) or core-specific peptide pool (right column). no vac: non-vaccinated; no adj: no adjuvant. Statistical analyses using Mann-Whitney test, *p<0.05.

Fig. 5. Long-term immune control of HBV following *TherVacB* immunization with different adjuvants. End-point analyses of AAV-HBV infected mice was performed at week 10 after start of *TherVacB* using indicated adjuvants. (A) Time kinetics of serum HBsAg levels (left: liposome-based, right: OiW adjuvants, each compared to c-di-AMP). Arrows indicate the vaccination time points. (B) Representative images and (C) quantification of HBV core-positive hepatocytes (brown) detected by liver immunohistochemistry staining. Scale bar: 100µm. (D) Intrahepatic HBV-DNA

679 detected in liver tissue lysates by qPCR. no vac: non-vaccinated; no adj: no adjuvant.
680 Statistical analyses using Mann-Whitney test, *p<0.05.

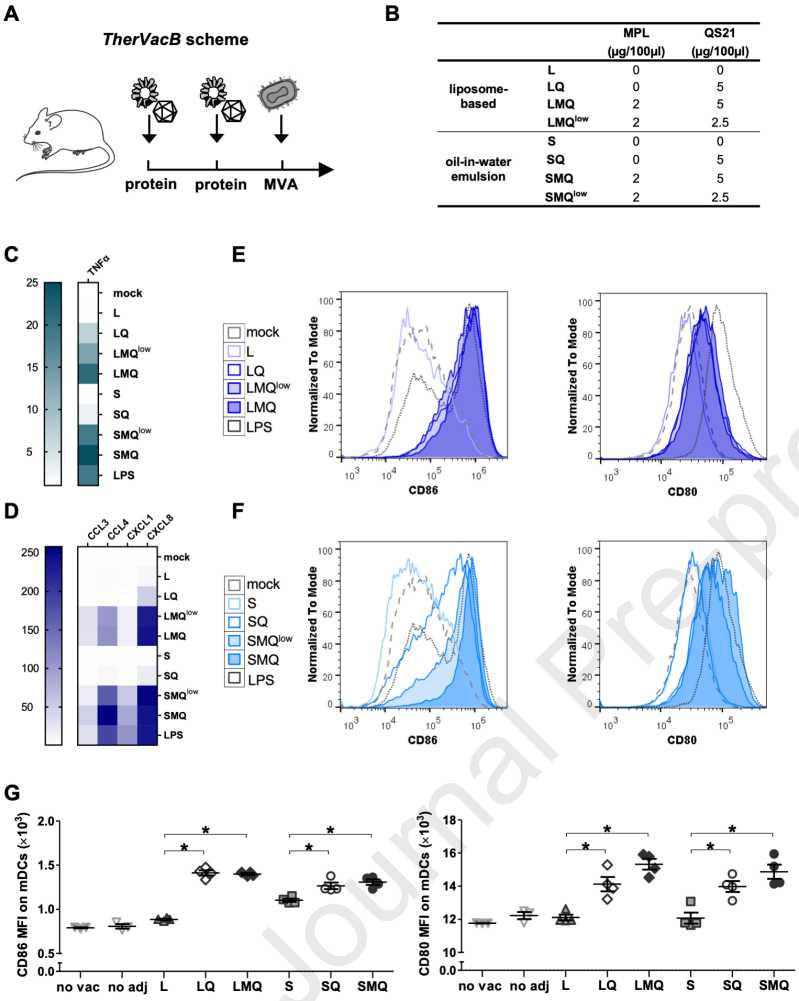
681 **Fig. 6. Comparison of combination adjuvant LMQ to alum.** Six weeks after AAV-
682 HBV infection, mice were immunized with *TherVacB* using LMQ without antigen (w/o
683 Ag), HBsAg and HBcAg only (no adj), or alum- and LMQ-adjuvanted antigen
684 formulations for prime vaccination. AAV-HBV infected mice receiving no vaccination
685 (no vac) served as controls. HBV-specific antibody and T-cell responses were
686 analyzed at week 10 after the start of *TherVacB*. (A) Levels and (B) IgG_{2b}/IgG_1 ratio of
687 serum anti-HBs and anti-HBc. (C) Frequencies of $IFN\gamma^+$ CD8 T cells determined by
688 flow cytometry after ICS following stimulation with corresponding peptide pools. (D-F)
689 Mice infected with AAV-HBV were immunized twice with LMQ or alum adjuvanted
690 HBsAg/HBcAg twice. No vaccination (no vac) served as controls. (D) Frequencies of
691 splenic S- and core-specific IL-2+ CD4 T cells determined at day 3 thereafter following
692 stimulation with recombinant HBsAg or HBcAg. Statistical analyses using Mann-
693 Whitney test, *p<0.05. n.a., not applicable; n.s., not significant. (E,F) Total RNA was
694 extracted from splenic CD4 T cells and bulk RNA sequencing was performed. (E)
695 Principal component analysis (PCA) plot and (F) heatmap of differentially expressed
696 genes in no vac, alum and LMQ groups.

697 **Fig. 7. The role of MVA-boost vaccination in therapeutic vaccination using LMQ-**
698 **adjuvanted proteins.** AAV-HBV infected mice received the full *TherVacB* scheme
699 (Fig.1A; LMQ-adjuvanted antigens week 0 and 2, MVA boost week 4) or protein only
700 but no MVA-boost (LMQ w/o MVA). HBV-specific antibody and T-cell responses were
701 analyzed 6 weeks after the last vaccination. (A) Levels of serum anti-HBs and anti-
702 HBc. (B) Frequencies of intrahepatic S- and core-specific $IFN\gamma^+TNF\alpha^+$ CD8 T cells
703 after stimulation with corresponding peptide pools. (C) Frequencies of intrahepatic S-
704 specific CD8 T cells stained directly *ex vivo* using S_{190} multimers (S_{190}). (D) Serum
705 HBsAg and HBeAg levels detected before and six weeks after the last vaccination

(week 10 for LMQ and no vac groups; week 8 for LMQ w/o MVA). Statistical analyses using Mann-Whitney test *p<0.05, **p<0.01, n.s., not significant.

Fig. 8. Impact of T-cell subset depletion on efficacy of therapeutic vaccination. (A) Mice infected with AAV-HBV were immunized with *TherVacB* after six weeks (black arrows) using LMQ formulated HBsAg and HBcAg with or without prior treatment with an αCD4 or αCD8-depleting mAb (grey arrows). (B) Dynamics of $CD4^+$ $CD45^+$ and $CD8^+$ $CD45^+$ T cells in blood. (C-G) End-point analyses at week 12. (C) Serum anti-HBs and anti-HBc levels. (D) Frequencies of intrahepatic S-specific $IFN\gamma^+$ CD4 T cells determined by flow cytometry after ICS. (E) Frequencies of intrahepatic S- and core-specific CD8 T cells detected by S_{190}- or C_{93}-multimer staining. Time kinetics of serum HBsAg (F) and HBeAg (G) levels. Statistical analyses using Mann-Whitney test (C-E) or two-way ANOVA (F-G), *p<0.05.

Figure 1



		MPL (μg/100μl)	QS21 (μg/100μl)
liposome-based	L	0	0
	LQ	0	5
	LMQ	2	5
	LMQ^{low}	2	2.5
oil-in-water emulsion	S	0	0
	SQ	0	5
	SMQ	2	5
	SMQ^{low}	2	2.5

Figure 2

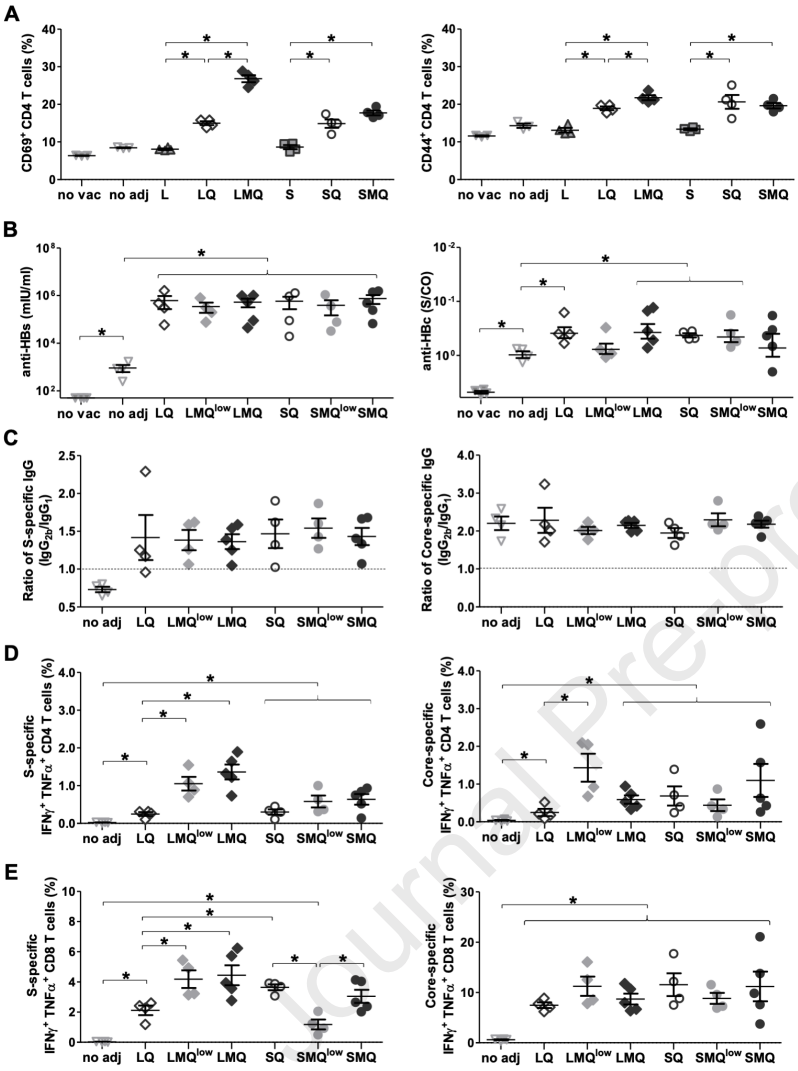


Figure 3

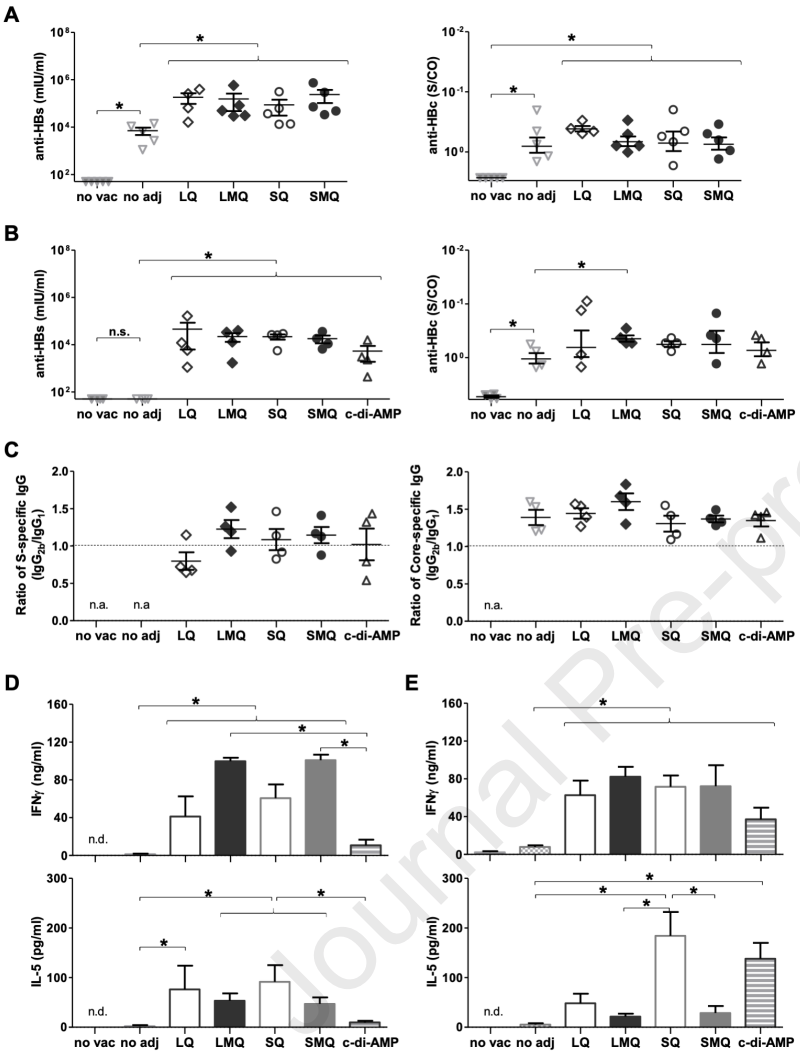


Figure 4

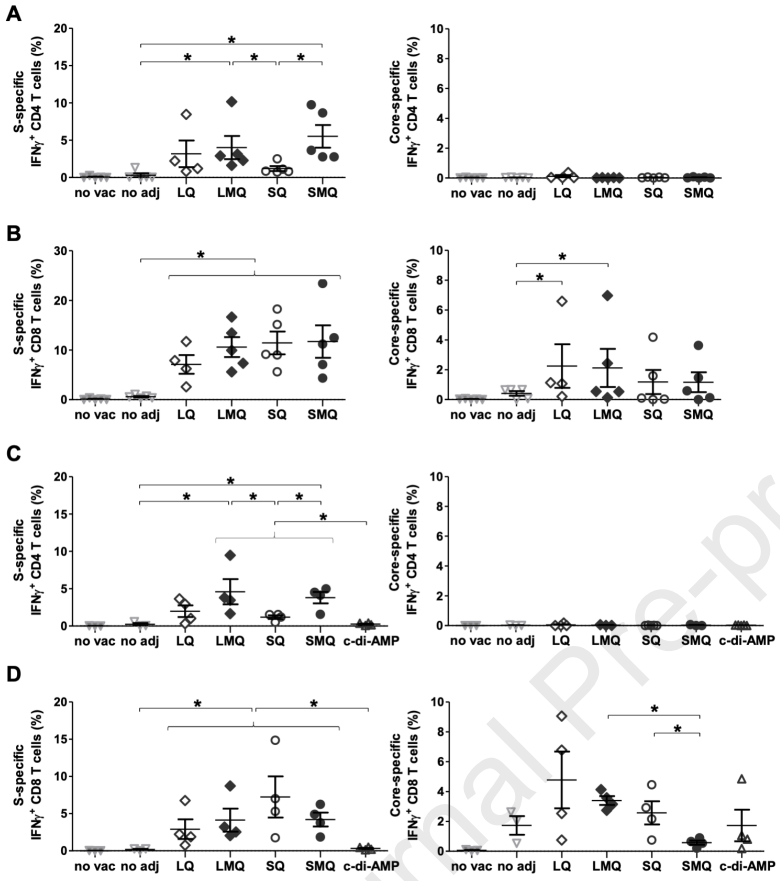


Figure 5

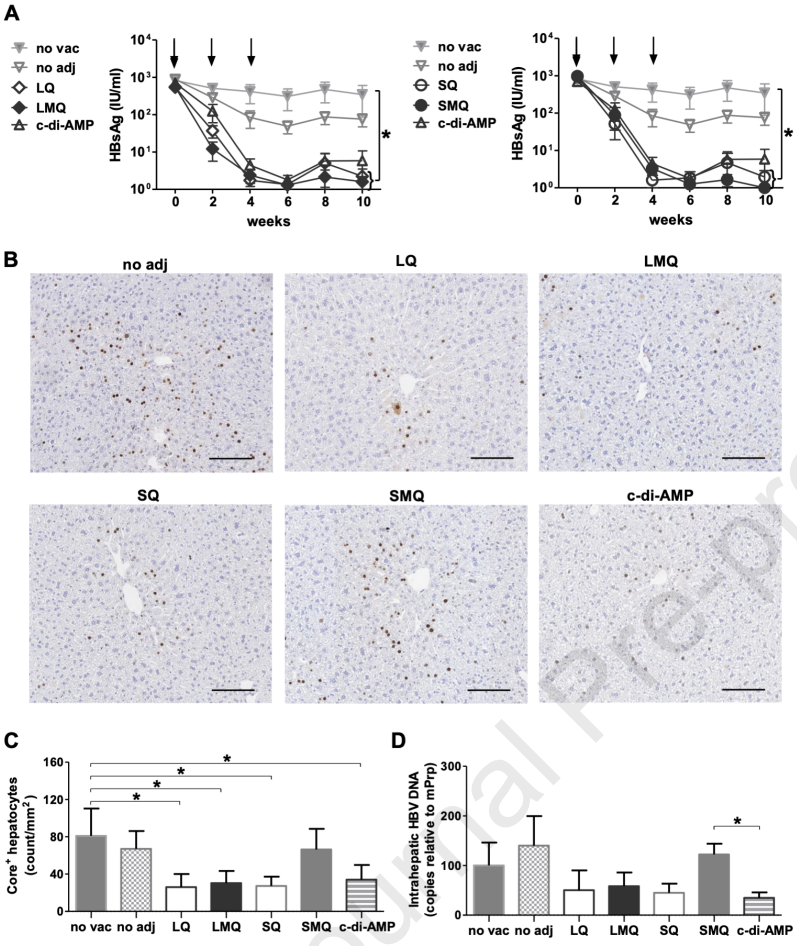
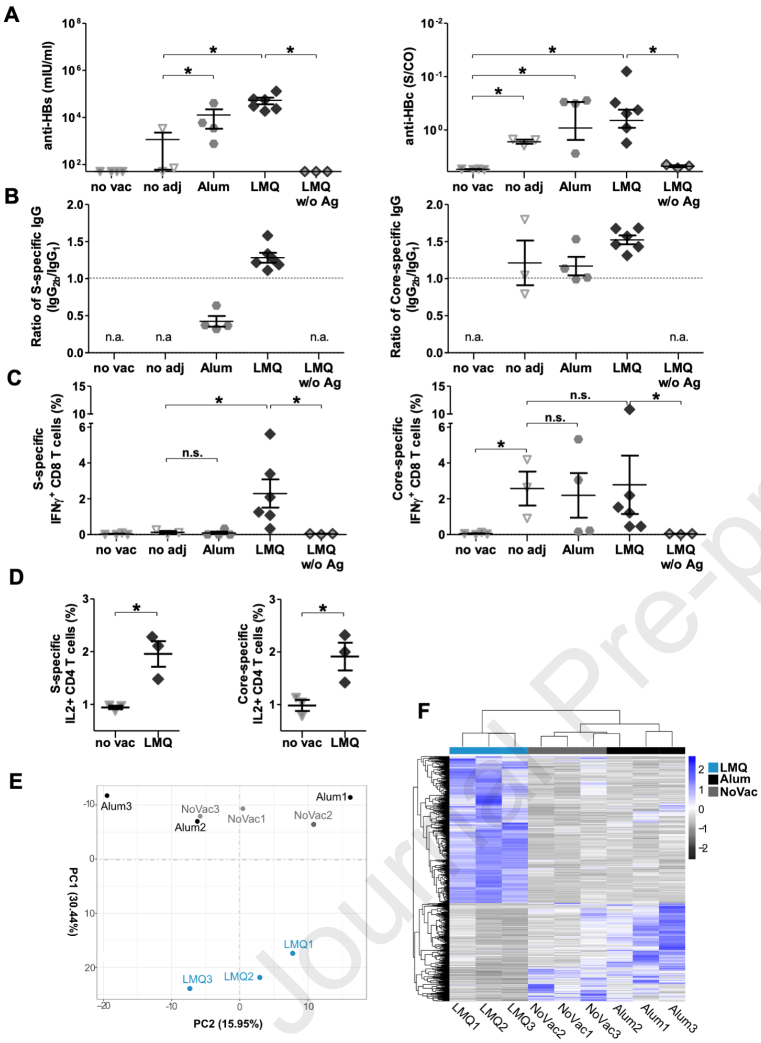


Figure 6


A
anti-HBs (mIU/ml)
anti-HBc (S/CO)
no vac
no adj
Alum
LMQ
LMQ w/o Ag
B
Ratio of S-specific IgG (IgG2b/IgG1)
Ratio of Core-specific IgG (IgG2b/IgG1)
n.a.
C
S-specific IFNγ+ CD8 T cells (%)
Core-specific IFNγ+ CD8 T cells (%)
n.s.
D
S-specific IL2+ CD4 T cells (%)
Core-specific IL2+ CD4 T cells (%)
E
Alum3
Alum2
NoVac3
NoVac1
Alum1
NoVac2
LMQ1
LMQ3
LMQ2
PC1 (30.44%)
PC2 (15.95%)
F
LMQ
Alum
NoVac
LMQ1
LMQ2
LMQ3
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NoVac3
Alum2
Alum1
Alum3

Figure 7

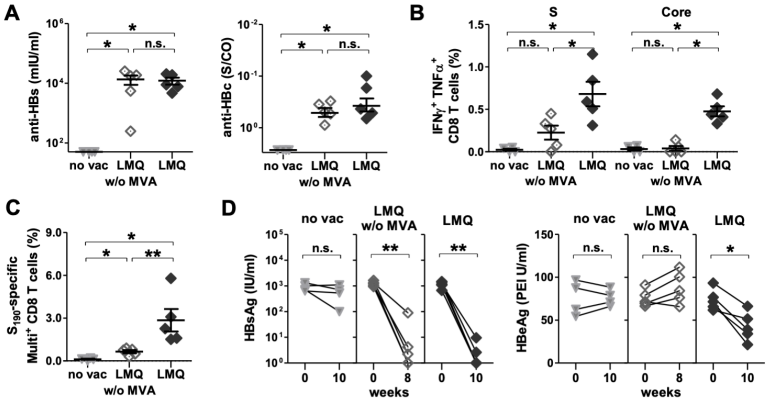
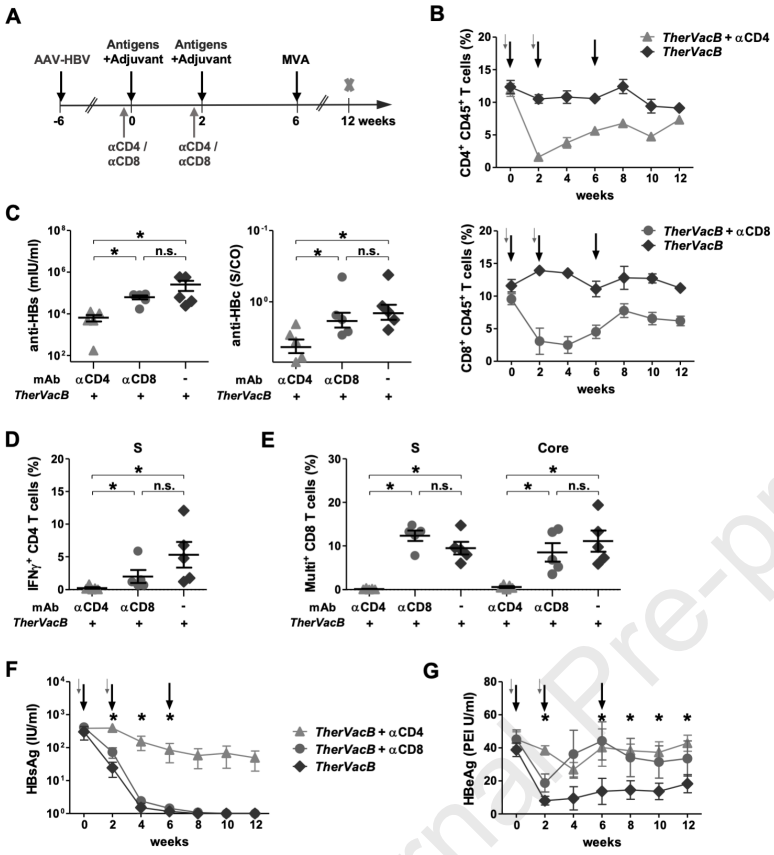


Figure 8



Highlights

- Rational design of therapeutic hepatitis B vaccines is key to improve their antiviral effect
- A heterologous prime-boost vaccine with particulate antigen, an optimized adjuvant and a vector-boost is most promising
- Activation of CD4 T-cells during prime vaccination is the key to break immune tolerance and control HBV.
- HBV antigens formulated with potent adjuvants activating Th1 type CD4 T cells promote antibody and CD8 T-cell responses
- Antiviral efficacy is lost when CD4 or CD8 T cells are depleted during prime vaccination.

(3 to 5 bullet points, max **120 characters** including spaces/point)