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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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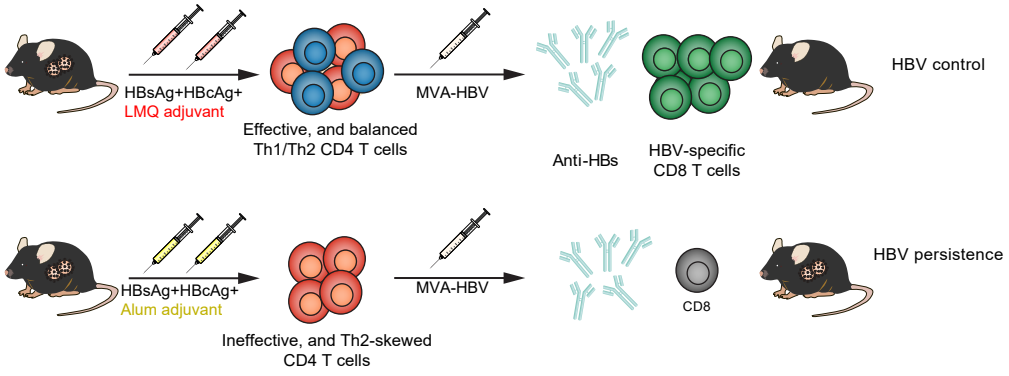
Graphical abstract



Highlights

- Rational design of therapeutic hepatitis B vaccines is key to improving 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.

Impact and implications

Therapeutic vaccination is a potentially curative treatment option for chronic hepatitis B. However, it remains unclear which factors are essential for breaking immune tolerance in HBV carriers and determining successful outcomes. 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 activates CD4 and CD8 T cells during prime immunization provided the foundation for an antiviral effect of therapeutic vaccination, while depletion of CD4 T cells led 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 for the cure of chronic hepatitis B.

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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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Background & Aims: We recently developed a heterologous therapeutic vaccination scheme (*TherVacB)* comprising a particulate protein prime followed by a modified vaccinia-virus Ankara (MVA)-vector boost for the treatment of HBV. However, the key determinants required to overcome HBV-specific immune tolerance remain unclear. Herein, we aimed to study new combination adjuvants and unravel factors that are essential for the antiviral efficacy of *TherVacB*.

Methods: Recombinant hepatitis B surface and core antigen (HBsAg and HBcAg) particles were formulated with different liposome- or oil-in-water emulsion-based combination adjuvants containing saponin QS21 and monophosphoryl lipid A; these formulations were compared to STING-agonist c-di-AMP and conventional aluminium hydroxide formulations. Immunogenicity and the antiviral effects of protein antigen formulations and the MVA-vector boost within *TherVacB* were evaluated in adeno-associated virus-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 that prime 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. In the absence of an MVA-vector boost or following selective CD8 T-cell depletion, HBsAg still declined (mediated mainly by anti-HBs antibodies) but HBV replication was not controlled. Selective CD4 T-cell depletion during the priming phase of *TherVacB* resulted in a complete loss of vaccine-induced immune responses and its therapeutic antiviral effect in mice.

Conclusions: Our results identify CD4 T-cell activation during the priming phase of *TherVacB* as a key determinant of HBV-specific antibody and CD8 T-cell responses.

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 patients with CHB per year achieve a functional cure defined as loss of HBsAg.[2,3] Stimulation of anti-HBV immunity is regarded as 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] The immune response in patients with CHB is 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 can restore HBV-specific immunity to a state 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 patients with CHB. 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 modified vaccinia-virus Ankara (MVA) expressing different HBV antigens.[10] Our results indicate that the antiviral

Keywords: Chronic hepatitis B; immunotolerance; therapeutic vaccine; *TherVacB*; combination adjuvant; CD4 T cells.
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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 only infects humanoid primates and research using these animals has been banned, mouse models have been used as surrogate preclinical models. However, the HBV genome has to be artificially introduced into the hepatocyte nucleus and they do not enable the study of viral persistence over decades as frequently observed in CHB, while 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 for 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 to 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 compare a series of novel adjuvants that combine the saponin QS21 and synthetic MPL as immunostimulants, with emulsions or liposomes as delivery systems, to the 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. We also investigated the need to add an MVA boost and explored the contribution of CD4 and CD8 T cells to 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 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 at 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, ELISA, transmission electron microscopy and various physicochemical assays.

Stimulation of hMoDCs

Human peripheral blood mononuclear cells were isolated from blood of healthy volunteers. Monocytes were isolated with Pan Monocyte Isolation Kit (Miltenyi Biotec, Germany), differentiated into immature human monocyte-derived dendritic cells (hMoDCs) (as described[17]), and stimulated with a 1/40 immunization dose, 1 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich, Germany), or left unstimulated for 6, 24 or 48 h. 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, C–C motif chemokine ligand (CCL)3, CCL4, C-X-C motif chemokine ligand (CXCL)1, 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 GV-SOLAS (German regulations of the Society for Laboratory Animal Science) and the European Health Law of FELASA (the Federation of Laboratory Animal Science Associations), 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 a C57BL/6J background.[18] Eight-to ten-week-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 $4\text{-}6\times10^9$ 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) at 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 (Siemens Healthcare, Germany). 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 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 intracellular cytokine staining (ICS), splenocytes and liver-associated lymphocytes 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). Upregulated genes were identified based on *p*-adj <0.05 and log2(fold-change) >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. 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 dendritic cells (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 h and then paraffin embedded. 2 μm-thick 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 (mAbs) 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 analyzed 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 further details regarding adjuvant preparation and formulation, and all other materials and methods, please refer to the CTAT table and supplementary information.

Results

Liposome- and OiW emulsion-based adjuvants preserve structural integrity and antigenicity of HBsAg and HBcAg and activate dendritic cells

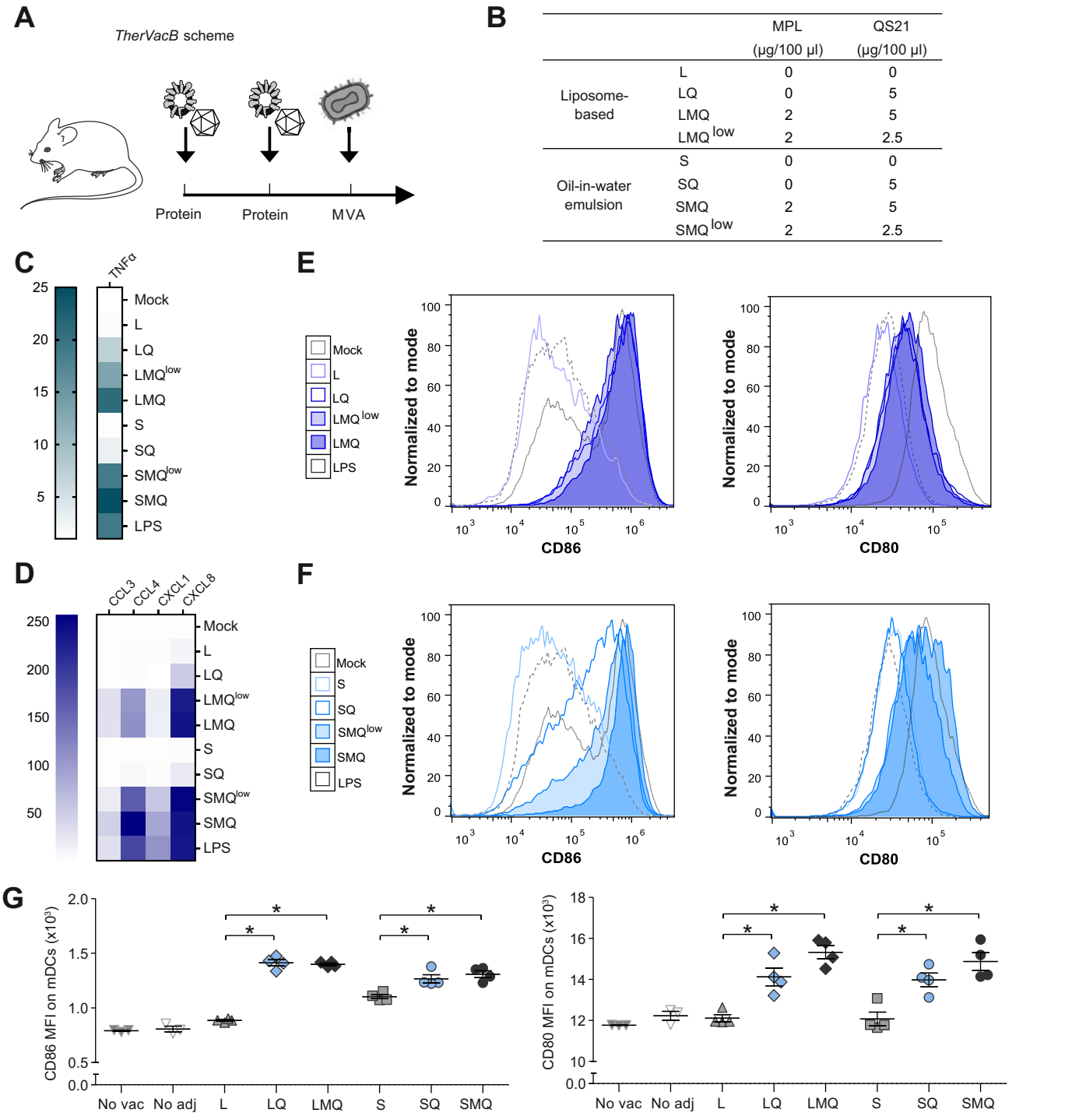
We investigated different combination adjuvant formulations: liposomes, oil-in-water emulsion (OiW), and water-in-oil emulsion Montanide ISA720. Liposome (L) and squalene (S)-based OiW-emulsions were combined with synthetic MPL and QS21 at various doses (Table S1) and used to formulate HBsAg and HBcAg. 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 most strongly 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). In line with *in vitro* stimulation, in primary murine DCs isolated from the spleen directly *ex vivo*, antigen formulations with LQ, LMQ, SQ and SMQ led to upregulation of CD80 and CD86 (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 immunogenicity *in vivo*, mice were, vaccinated with adjuvanted protein twice, week 0 and 2, and boosted with MVA-S and MVA-core at week 4, according to the *TherVacB* regimen (Fig. 1A). 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-HBc antibodies (Fig. 2B), both primarily of the IgG_{2b} subclass (Fig. 2C). All adjuvant formulations enabled priming of 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



		MPL (μg/100 μl)	QS21 (μg/100 μl)
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

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) hMoDCs were stimulated with the indicated adjuvants. Cells stimulated with LPS or unstimulated hMoDCs (mock) served as controls. (C) TNFα and (D) chemokines CCL3, CCL4, CXCL1, CXCL8 secreted into the supernatants of stimulated hMoDCs within 6 or 24 h. 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) L- or (F) S-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 two days after the 2nd protein vaccination. Statistical analyses using Mann-Whitney test, *p <0.05. hMoDCs, human monocyte-derived dendritic cells; LPS, lipopolysaccharide; mDCs, mouse dendritic cells; no adj, no adjuvant; no vac, non-vaccinated. (This figure appears in color on the web.)

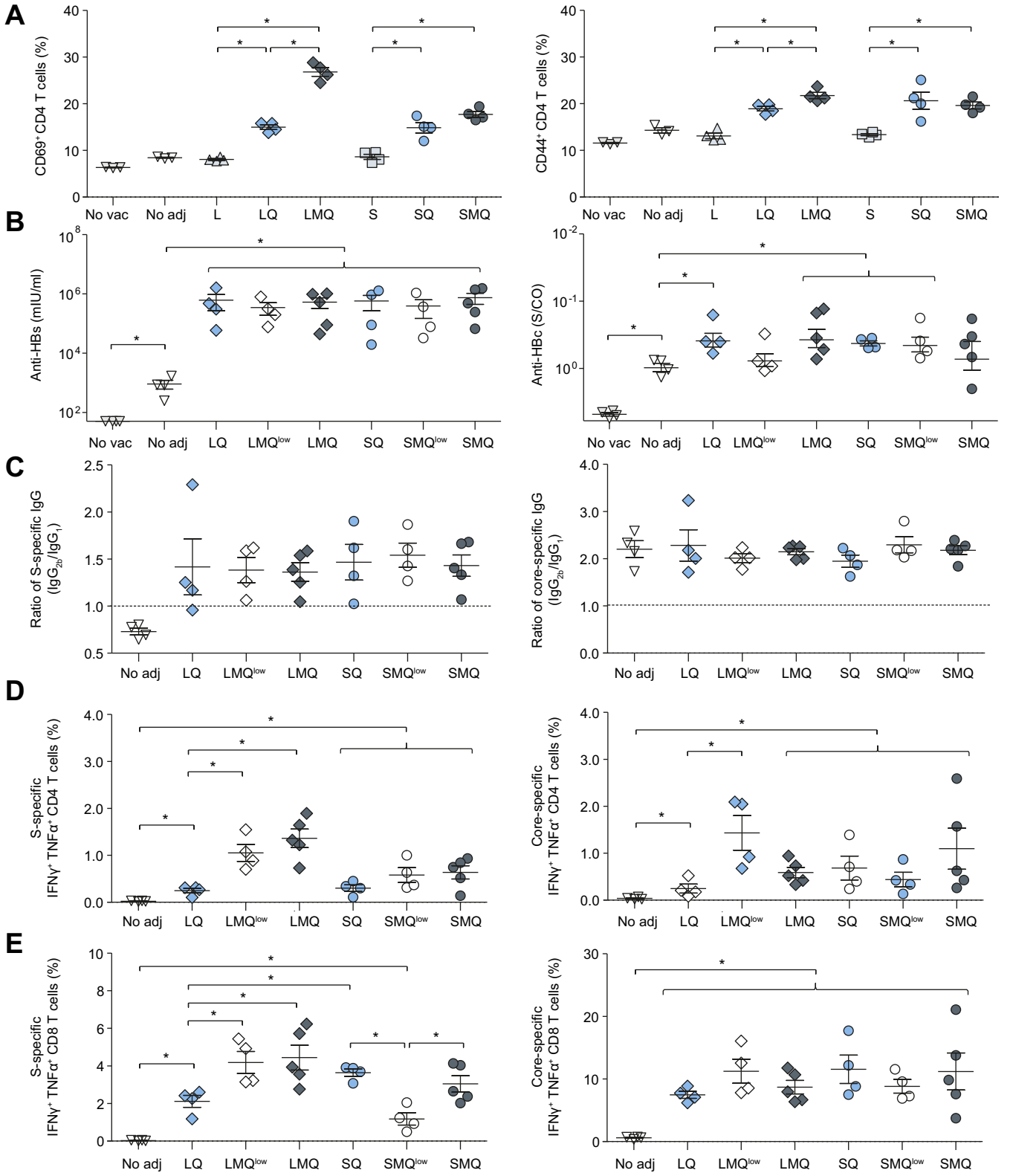


Fig. 2. Immunogenicity of combination adjuvant formulations in wild-type C57BL/6J mice. At two-week intervals, C57BL/6J mice received two protein-prime vaccinations containing HBsAg and HBcAg each formulated with the indicated adjuvant and an MVA-boost. (A) Frequencies of total CD69+ and CD44+ splenic CD4 T cells detected by flow cytometry two days after the 2nd protein vaccination. (B-E) HBV-specific antibody and T-cell responses were analysed 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 $IFN\gamma^+$ and $TNF\alpha^+$ CD4 (D) and CD8 T cells (E) determined by flow cytometry after ICS following stimulation with respective S- and core-specific peptide pool. Statistical analyses using Mann-Whitney test, *$p<0.05$. ICS, intracellular cytokine staining; MVA, modified vaccinia-virus Ankara.

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, formulations 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

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, is a potent immunogen *per se*.[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 (Fig. 4A, B) and AAV-HBV-infected mice (Fig. 4C, D), one week and six weeks after MVA-boost vaccination, respectively, by flow cytometry after ICS.

In all vaccinated HBV-carrier mice, we detected intrahepatic S-specific but not core-specific IFNγ^+ 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 would be hardly detectable at this time point as they form a small 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 IFNγ 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 recombinant protein immunization in inducing anti-HBs antibodies. Immunization with c-di-AMP-, LQ-, SQ-, and particularly LMQ-adjuvanted formulations also induced a significant decline of HBeAg in serum (Fig. S5A), while inducing only a max. two-fold increase of alanine aminotransferase 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 formulations only induced anti-HBs and reduced HBsAg levels (Fig. 5A), but failed to reduce intrahepatic HBV DNA and HBV-positive hepatocytes (Figs. 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 for long-term immune control of persistent HBV infection, while being well tolerated.

Priming with particulate HBV antigens formulated with LMQ, but not with alum, confers antiviral activity within *TherVacB*

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

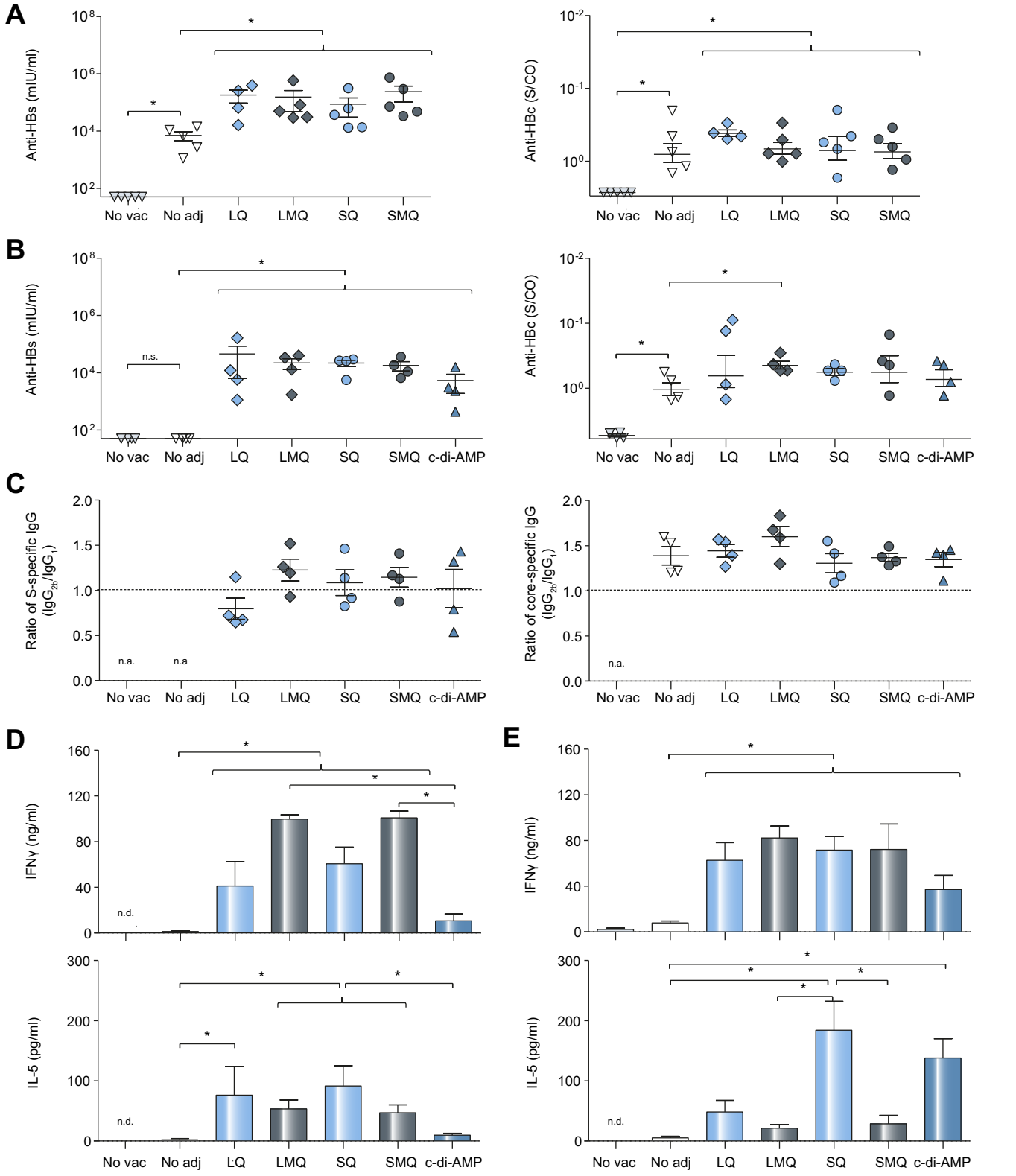


Fig. 3. HBV-specific humoral immune responses induced in HBVtg and AAV-HBV mice by *TherVacB* using different adjuvants. (A) HBVtg mice or (B-E) C57BL/6J mice transduced with AAV-HBV six weeks prior to vaccination were immunized at week 0 and 2 with HBsAg and HBcAg adjuvanted with either c-di-AMP or 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. Statistical analyses using Mann-Whitney test, *$p<0.05$, n.s., not significant. AAV, adeno-associated virus; HBcAg, HBV core antigen; HBsAg, HBV surface antigen; HBVtg, HBV-transgenic; MVA, modified vaccinia-virus Ankara; n.a., not applicable; n.d., not detectable; no adj, no adjuvant; no vac, non-vaccinated.

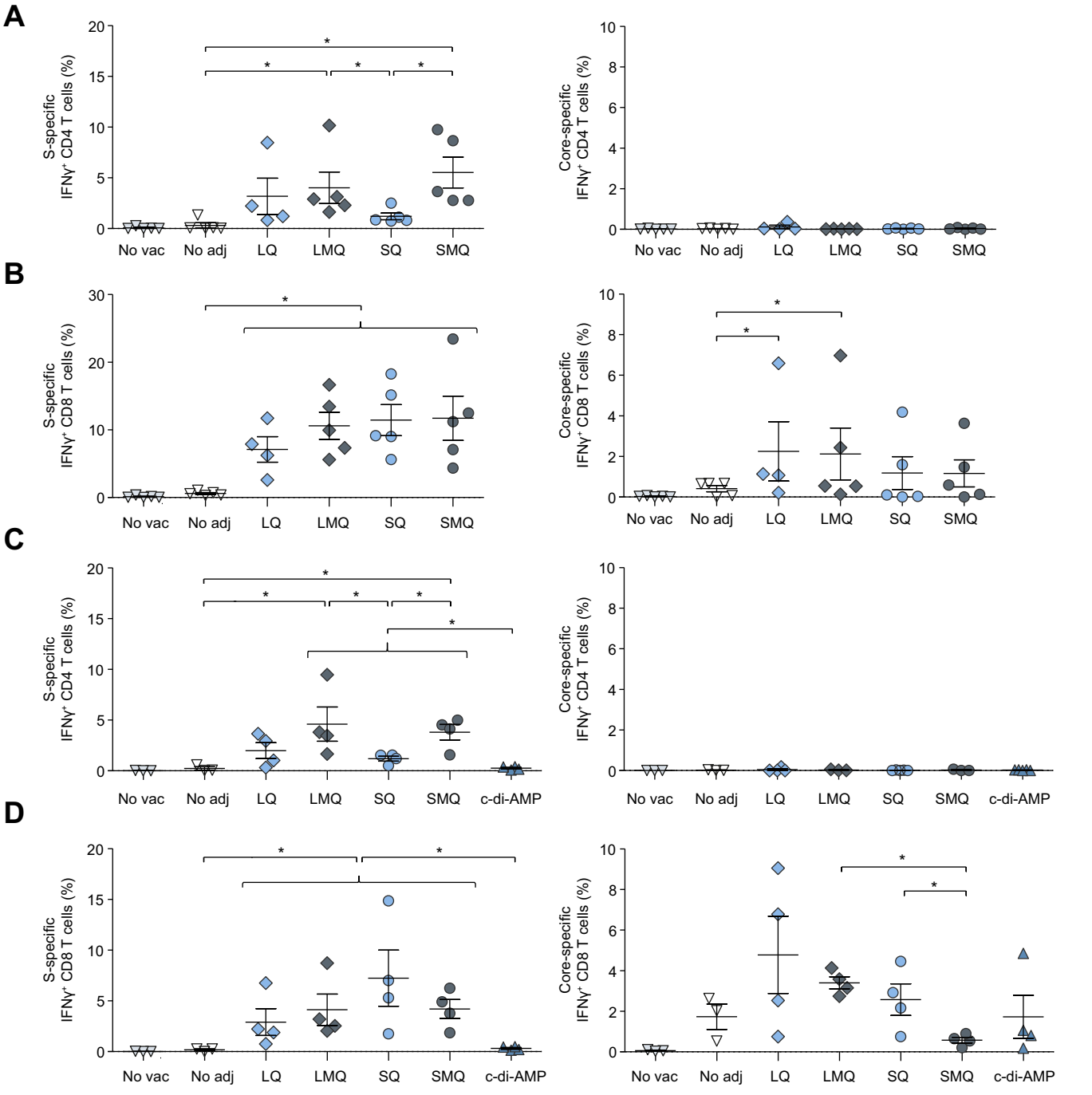


Fig. 4. HBV-specific effector T-cell responses induced by *TherVacB* in HBV-carrier mice using different adjuvants during protein prime. Vaccination as in Fig. 3 using the indicated adjuvants. (A-D) Analysis of HBV-specific T-cell responses in liver-associated lymphocytes at week 5 in HBVtg mice (A,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

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). The 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 (Figs. 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

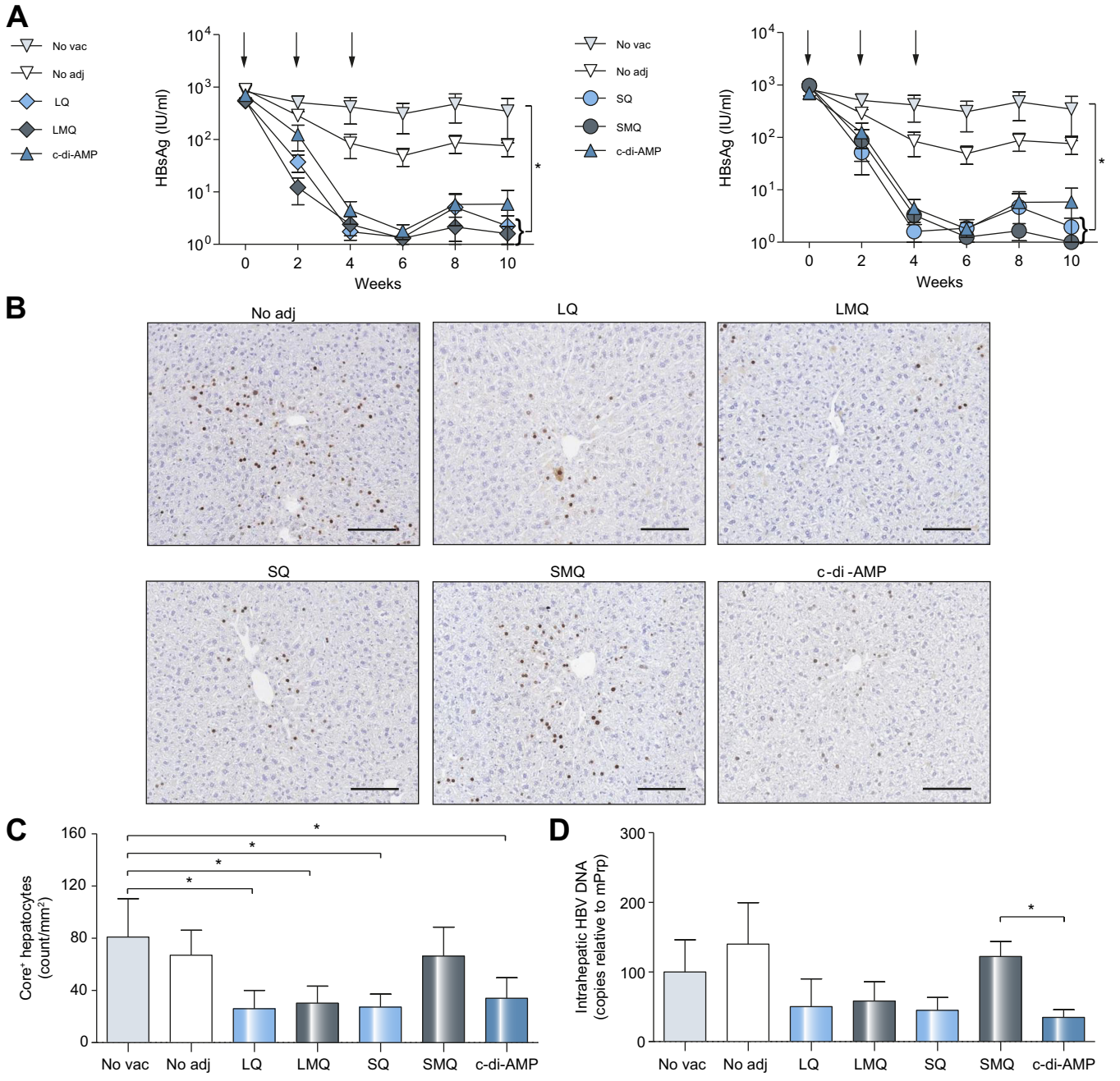


Fig. 5. Long-term immune control of HBV following *TherVacB* immunization with different adjuvants. End-point analyses of AAV-HBV-infected mice were performed at week 10 after start of *TherVacB* using indicated adjuvants. (A) Time kinetics of serum HBsAg levels (left: liposome-based, right: oil-in-water 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: 100 μm. (D) Intrahepatic HBV DNA detected in liver tissue lysates by qPCR. Statistical analyses using Mann-Whitney test, *p<0.05. AAV, adeno-associated virus; no vac, non-vaccinated; no adj, no adjuvant.

in recombinant HBcAg 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 (Figs. 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. 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

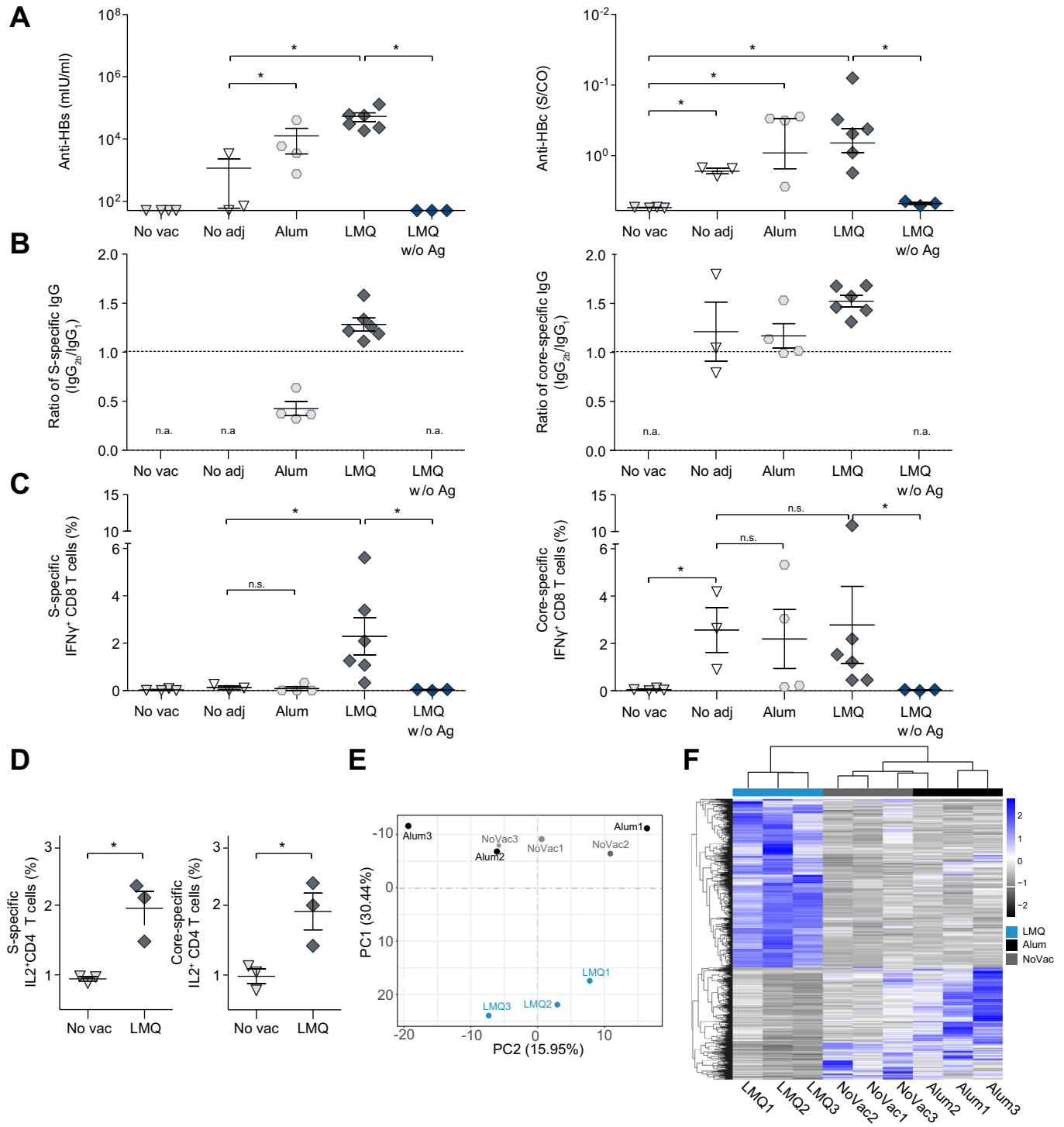


Fig. 6. Comparison of combination adjuvant LMQ to alum. Six weeks after AAV-HBV infection, mice were immunized with *TherVacB* using LMQ without antigen (w/o Ag), HBsAg and HBcAg only (no adj), or alum- and LMQ-adjuvanted antigen formulations for prime vaccination. AAV-HBV-infected mice receiving no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

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) – compared to alum or no vaccination – with pathways involved in 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-S and MVA-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 in HBsAg, but only the group of mice receiving the MVA-boost demonstrated a significant decrease in HBeAg levels indicating control of HBV replication in the liver (Fig. 7D). These data indicate that anti-HBs induced by protein immunization can neutralize HBsAg but the boost of T-cell responses by the MVA-vector is necessary to achieve long-term antiviral control of HBV even if a potent adjuvant is used for the protein immunization.

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

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

Mice were infected with AAV-HBV and either CD4 or CD8 T cells were depleted using anti-mouse CD4 (αCD4) or CD8 (αCD4) mAb before each prime vaccination with LMQ-formulated HBsAg and HBcAg at week 0 and 2 (Fig. 8A, grey arrows). Flow cytometry analysis confirmed efficient depletion of CD4-positive T cells and type 2 conventional DCs (cDC2) using αCD4-mAb, but only CD8-positive T cells using αCD8-mAb (Fig. S8). Since cDC2 have been shown to specifically prime CD4 T cells,[24] their co-depletion still allows for exploration of the impact of a loss of CD4 T-cell functionality. At week 6, when T cells were at least partially restored (Fig. 8B), mice were boosted with recombinant MVA. *TherVacB* immunization of αCD4-mAb-treated mice induced significantly lower anti-HBs and anti-HBc levels (Fig. 8C) as well as intrahepatic S- and core-specific CD4 and CD8 T-cell responses (Fig. 8D-E), while depletion of CD8 T cells did not show a significant effect.

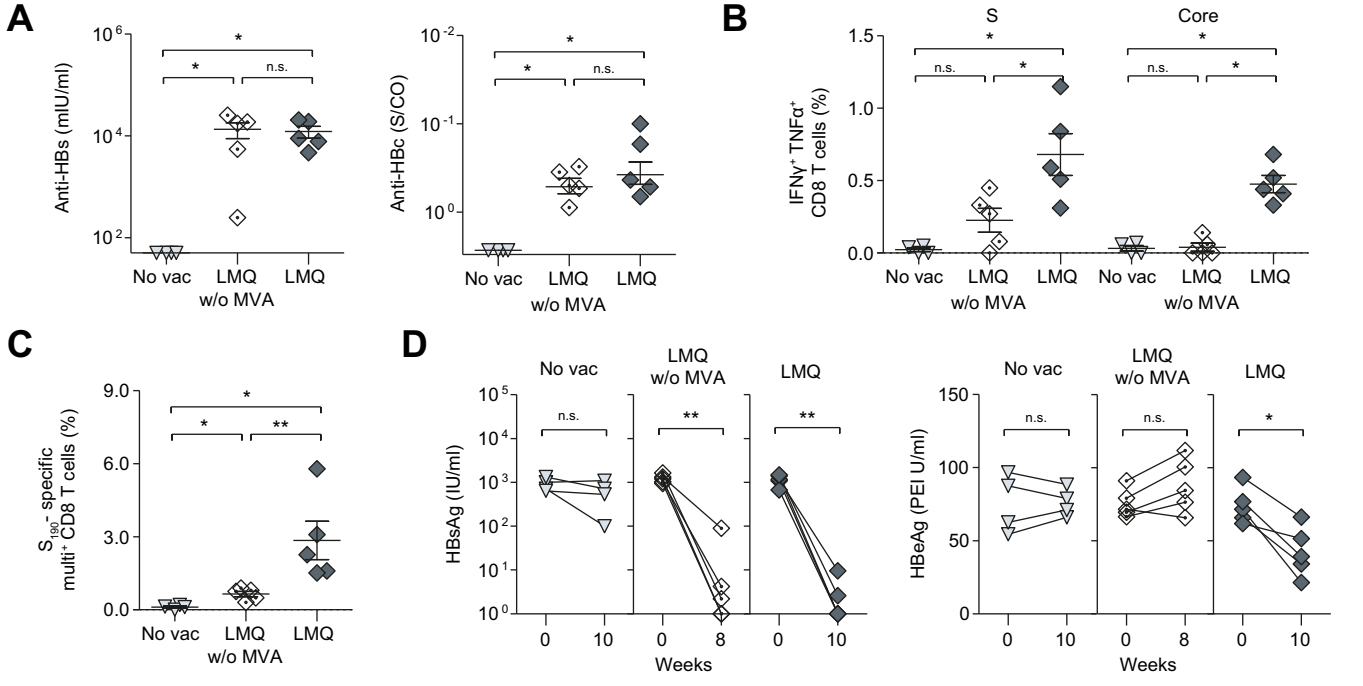


Fig. 7. The role of MVA-boost vaccination in therapeutic vaccination using LMQ-adjuvanted proteins. AAV-HBV-infected mice received the full *TherVacB* scheme (Fig. 1A; LMQ-adjuvanted antigens week 0 and 2, MVA boost week 4) or protein only but no MVA-boost (LMQ w/o MVA). HBV-specific antibody and T-cell responses were analyzed 6 weeks after the last vaccination. (A) Levels of serum anti-HBs and anti-HBc. (B) Frequencies of intrahepatic S- and core-specific $IFN\gamma^+TNF\alpha^+$ CD8 T cells after stimulation with corresponding peptide pools. (C) Frequencies of intrahepatic S-specific CD8 T cells stained directly *ex vivo* using S_{190} multimers. (D) Serum 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. AAV, adeno-associated virus; MVA, modified vaccinia-virus Ankara; no vac, non-vaccinated; w/o, without.

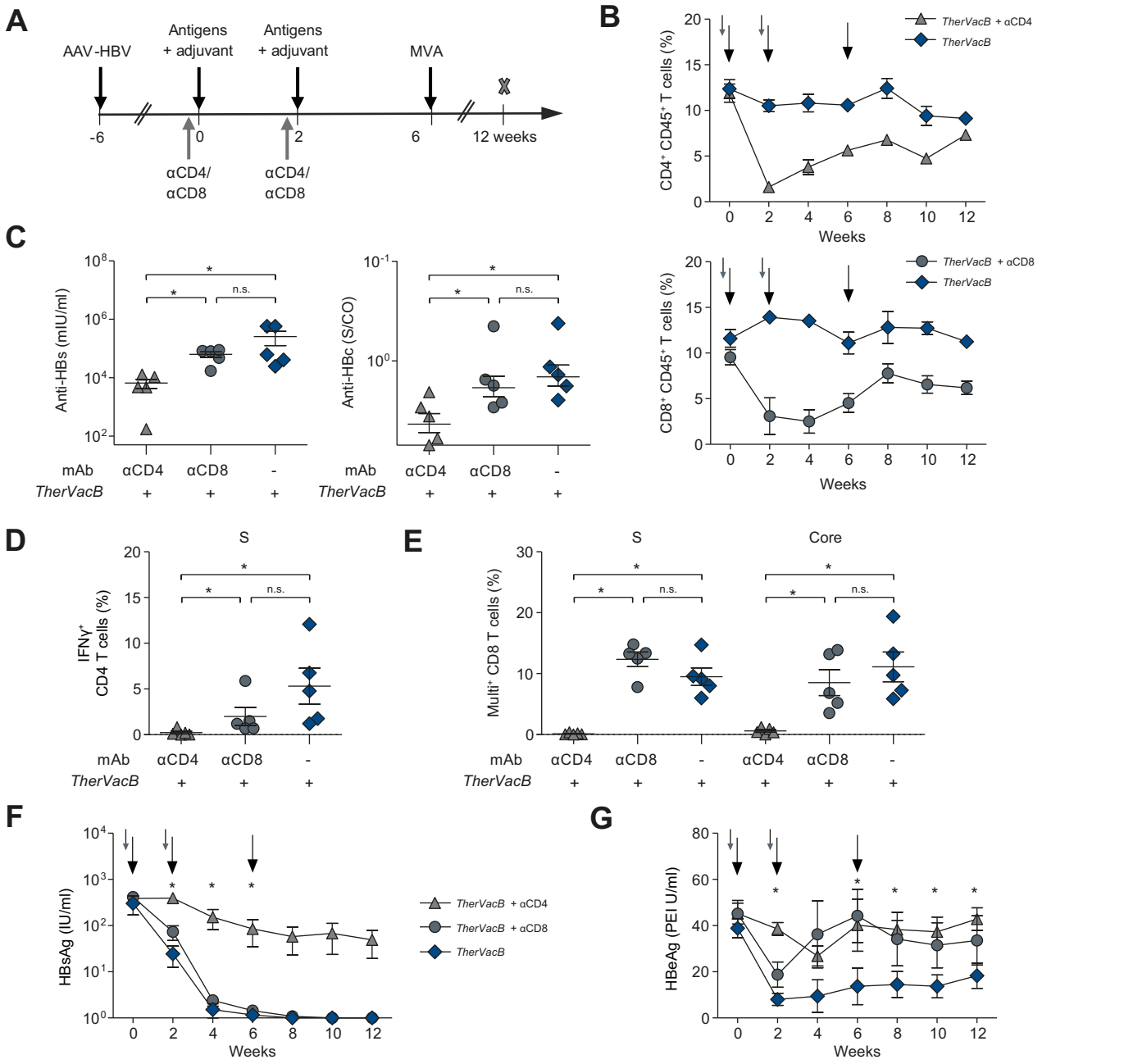


Fig. 8. Impact of T-cell subset depletion on efficacy of therapeutic vaccination. (A) Mice infected with AAV-HBV were immunized with *TherVacB* after six weeks (black arrows) using LMQ-formulated HBsAg and HBcAg with or without prior treatment with an αCD4 or αCD8-depleting mAb (grey arrows). (B) Dynamics 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. AAV, adeno-associated virus; ICS, intracellular cytokine staining; mAb, monoclonal antibody; MVA, modified vaccinia-virus Ankara.

Interestingly, the effect on HBsAg loss was lost upon CD4 but not CD8 T-cell depletion, while the overall antiviral effect indicated by the loss of HBeAg was lost when either T-cell population was depleted (Fig. 8F-G). These data demonstrate that CD4 T-cell activation during the priming phase of therapeutic 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 potentially curative treatment option for chronic hepatitis B, the determinants of successful vaccination are poorly understood.[2–4] Herein, 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 to 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] However, induction of an effective HBV-specific CD8 T-cell response 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, providing direct evidence that efficient priming of HBV-specific CD4 T cells is essential for successful therapeutic vaccination and 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 a 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 is obviously 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 formulations on 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, which is known to mainly activate Th2-type responses.[13–16] Of note, vaccine formulation- that used alum as an adjuvant enhanced HBV-specific antibody and CD4 T-cell responses but did not 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 formulations, 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 patients with CHB 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 mechanisms of adaptive but also of innate immune tolerance to 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 DCs *in vivo* and – as well as the nucleotide-based adjuvant c-di-AMP – led to long-term reductions or even complete loss of HBV antigens in HBV-carrier mice.

The HBVtg and AAV-HBV-infected mice employed in this study represent the most suitable models to prove the preclinical efficacy of a therapeutic hepatitis B vaccine or other therapies that stimulate adaptive immunity. Although they do not support HBV spread, these models allow for establishment of HBV-specific immune tolerance which can be broken by an effective therapeutic vaccine. In addition, the AAV-HBV model allows for the study of 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 obstacles 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 increasingly being 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 DCs *in vitro* accurately predicted the activation of murine DCs *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, 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 considered when developing therapeutic vaccines for this urgently needed clinical application.

Affiliations

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Abbreviations

AAV, adeno-associated virus; alum, aluminum hydroxide; cDC2, type 2 conventional DCs; DCs, dendritic cells; 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; MPL, monophosphoryl lipid A; MVA, modified vaccinia-virus Ankara; OiW, oil-in-water emulsions; Th1 cells, type 1 helper T cells; *TherVacB*, therapeutic hepatitis B vaccine; TNF, tumor necrosis factor.

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Conflict of interest

UP is a co-founder, shareholder and board member of SCG Cell Therapy, serves as ad hoc advisor for Abbott, Aligos, Arbutus, Gilead, GSK, Merck, Sanofi, Roche and VirBiotech. UP and ADK are named as inventors on a patent application describing the therapeutic vaccination scheme of *TherVacB* (PCT/EP2017/050553). The remaining authors declare no competing interests.

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

Authors' contributions

JSu, ADK, UP designed the study; JSu, ADK, LB, JSa, GF, EAO, HAK performed the experiment; CM evaluated liver histopathology; UP, PAK, ADK 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).

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.12.013.

References

Author names in bold designate shared co-first authorship

[1] Organization WH. Combating hepatitis B and C to reach elimination by 2030: advocacy brief. World Health Organization; 2016.
[2] Trépo C, Chan HL, Lok A. Hepatitis B virus infection. The Lancet 2014;384:2053–2063.
[3] Lok AS, Zoulim F, Dusheiko G, Ghany MG. Hepatitis B cure: from discovery to regulatory approval. Hepatology 2017;66:1296–1313.
[4] Gehring AJ, Protzer U. Targeting innate and adaptive immune responses to cure chronic HBV infection. Gastroenterology 2019;156:325–337.
[5] Chisari FV, Isogawa M, Wieland SF. Pathogenesis of hepatitis B virus infection. Pathol Biol (Paris) 2010;58:258–266.
[6] Asabe S, Wieland SF, Chattopadhyay PK, Roederer M, Engle RE, Purcell RH, et al. The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. J Virol 2009;83:9652–9662.
[7] Yang PL, Althage A, Chung J, Maier H, Wieland S, Isogawa M, et al. Immune effectors required for hepatitis B virus clearance. Proc Natl Acad Sci 2010;107:798–802.
[8] Burton AR, Pallett LJ, McCoy LE, Suveizdyte K, Amin OE, Swadling L, et al. Circulating and intrahepatic antiviral B cells are defective in hepatitis B. J Clin Invest 2018;128:4588–4603.
[9] Kosinska AD, Bauer T, Protzer U. Therapeutic vaccination for chronic hepatitis B. Curr Opin Virol 2017;23:75–81.
[10] **Backes S**, **Jager C**, Dembek CJ, Kosinska AD, Bauer T, Stephan AS, et al. Protein-prime/modified vaccinia virus Ankara vector-boost vaccination overcomes tolerance in high-antigenemic HBV-transgenic mice. Vaccine 2016;34:923–932.
[11] Kosinska AD, Moeed A, Kallin N, Festag J, Su J, Steiger K, et al. Synergy of therapeutic heterologous prime-boost hepatitis B vaccination with CpG-application to improve immune control of persistent HBV infection. Sci Rep 2019;9:10808.
[12] **Michler T**, **Kosinska AD**, Festag J, Bunse T, Su J, Ringelhan M, et al. Knockdown of virus antigen expression increases therapeutic vaccine efficacy in high-titer hepatitis B virus carrier mice. Gastroenterology 2020;158:1762–1775 e1769.
[13] Bowen WS, Svrivastava AK, Batra L, Barsoumian H, Shirwan H. Current challenges for cancer vaccine adjuvant development. Expert Rev Vaccin 2018;17:207–215.
[14] Nanishi E, Dowling DJ, Levy O. Toward precision adjuvants: optimizing science and safety. Curr Opin Pediatr 2020;32:125–138.
[15] Garcon N, Di Pasquale A. From discovery to licensure, the Adjuvant System story. Hum Vaccin Immunother 2017;13:19–33.
[16] Rivera-Hernandez T, Rhyme MS, Cork AJ, Jones S, Segui-Perez C, Brunner L, et al. Vaccine-induced Th1-type response protects against invasive group A Streptococcus infection in the absence of opsonizing antibodies. Mbio 2020:11.
[17] Kaebisch R, Mejías-Luque R, Prinz C, Gerhard M. Helicobacter pylori cytotoxin-associated gene A impairs human dendritic cell maturation and function through IL-10–mediated activation of STAT3. J Immunol 2014;192:316–323.
[18] Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus replication in transgenic mice. J Virol 1995;69:6158–6169.
[19] Dion S, Bourgine M, Godon O, Levillayer F, Michel ML. Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules. J Virol 2013;87:5554–5563.
[20] Festag MM, Festag J, Fräßle SP, Asen T, Sacherl J, Schreiber S, et al. Evaluation of a fully human, hepatitis B virus-specific chimeric antigen receptor in an immunocompetent mouse model. Mol Ther 2019;27:947–959.
[21] Younis SY, Barnier-Quer C, Heuking S, Sommandas V, Brunner L, Dubois P, et al. Down selecting adjuvanted vaccine formulations: a comparative method for harmonized evaluation. BMC Immunol 2018;19:1–11.
[22] Fumagalli V, Di Lucia P, Venzin V, Bono EB, Jordan R, Frey CR, et al. Serum HBsAg clearance has minimal impact on CD8+ T cell responses in mouse models of HBV infection. J Exp Med 2020:217.
[23] Riedl P, Stober D, Oehninger C, Melber K, Reimann J, Schirmbeck R. Priming Th1 immunity to viral core particles is facilitated by trace amounts of RNA bound to its arginine-rich domain. J Immunol 2002;168:4951–4959.
[24] Durai V, Murphy KM. Functions of murine dendritic cells. Immunity 2016;45:719–736.
[25] Thimme R, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol 2003;77:68–76.
[26] Acres B, Bonnefoy J-Y. Clinical development of MVA-based therapeutic cancer vaccines. Expert Rev Vaccin 2008;7:889–893.
[27] Billaud JN, Peterson D, Schodel F, Chen A, Sallberg M, Garduno F, et al. Comparative antigenicity and immunogenicity of hepadnavirus core proteins. J Virol 2005;79:13641–13655.
[28] van der Molen RG, Sprengers D, Binda RS, de Jong EC, Niesters HG, Kusters JG, et al. Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. Hepatology 2004;40:738–746.
[29] Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity 2010;33:492–503.
[30] Pulendran B, Arunachalam PS, O'Hagan DT. Emerging concepts in the science of vaccine adjuvants. Nat Rev Drug Discov 2021;20:454–475.