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A novel therapeutic hepatitis B vaccine induces cellular and humoral immune responses and breaks tolerance in hepatitis B virus (HBV) transgenic mice☆

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ABSTRACT

Therapeutic vaccines are currently being developed for chronic hepatitis B and C. As an alternative to long-term antiviral treatment or to support only partially effective therapy, they should activate the patient's immune system effectively to fight and finally control the virus. A paradigm of therapeutic vaccination is the potent induction of T-cell responses against key viral antigens – besides activation of a humoral immune response. We have evaluated the potential of a novel vaccine formulation comprising particulate hepatitis B surface (HBsAg) and core antigen (HBcAg), and the saponin-based ISCOMATRIX™ adjuvant for its ability to stimulate T and B cell responses in C57BL/6 mice and its ability to break tolerance in syngeneic HBV transgenic (HBVtg) mice. In C57BL/6 mice, the vaccine induced multifunctional HBsAg- and HBcAg-specific CD8+ T cells detected by staining for IFNγ, TNFα and IL-2, as well as high antibody titers against both antigens. Vaccination of HBVtg animals induced potent HBsAg- and HBcAg-specific CD8+ T-cell responses in spleens and HBcAg-specific CD8+ T-cell responses in livers as well as anti-HBs seroconversion two weeks post injection. Vaccination further reduced HBcAg expression in livers of HBVtg mice without causing liver damage.

In summary, this study demonstrates therapeutic efficacy of a novel vaccine formulation in a mouse model of immunotolerant, chronic HBV infection.

1. Introduction

HBV infects the livers, where it establishes either transient or persistent infection and may cause necroinflammatory liver disease – hepatitis B. Vertical transmission from mothers to their neonates, or infection during the first year of life, results in persistent, often life-long infection in >90%. In contrast, infection during adulthood is cleared in most cases, and results in life-long protective immunity [1]. A polyclonal and multispecific T-cell response is characteristic for cleared acute infection [2,3], while a weak and oligoclonal response is associated with chronic infection [4,5].

Despite the availability of an effective prophylactic vaccine, worldwide more than 350 million humans are chronically infected with HBV being at risk to develop liver cirrhosis or hepatocellular carcinoma. Current treatment options for chronic hepatitis B depend on interferon α or nucleos(t)ide analogs, which efficiently control virus replication but rarely eliminate the virus. HBV covalently closed circular DNA (cccDNA) persists in the host cell nucleus and drives a viral rebound and recurrent disease once therapy is discontinued. Therefore, cost-intensive long-term treatment is required.

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; IFNγ, interferon γ; anti-HBs, antibodies against HBsAg; anti-HBc, antibodies against HBcAg.

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Immunotherapies designed to activate either nonspecific or HBV-specific immune responses could achieve sustained viral control after timely limited treatment [6]. Support for the efficacy of T-cells has been gained from the clinical observation that chronic HBV infection may resolve in bone marrow transplant patients receiving bone marrow from an HBV immune donor [7,8]. Therapeutic vaccination can induce T cell responses [9], and preclinical studies in chimpanzees had a promising outcome [10].

First clinic attempts based on available prophylactic vaccines, alone or in combination with interferon-α and/or antiviral compounds [11–13] were not able to induce an immune response in chronic hepatitis B [14].

Failure was mainly attributed to the fact that the aluminum adjuvanted vaccines induce a pronounced Th2 type immune response and do not stimulate cytotoxic T lymphocytes (CTL). Hence, novel therapeutic vaccine formulations were developed to induce a CTL response. The lipopeptide Theradigm®, containing an HBcAg 18–27 HLA-A2 peptide epitope and an universal tetanus toxoid helper T cell epitope induced a CTL response in healthy volunteers [15], but had no therapeutic effect in chronic hepatitis B patients [16]. HBsAg adjuvanted with MPL, QS21 and an oil-in-water emulsion induced HBsAg-specific T cells and antibodies in healthy volunteers [17], but in patients with HBeAg positive chronic hepatitis B under lamivudine treatment failed to increase HBeAg seroconversion rates [18]. HBsAg complexed with anti-HBs also showed only minor effects in clinical studies [19].

All these approaches had in common that a single HBV antigen was included in the vaccine. In addition, particulate antigens (*e.g.* virus-like particles) combined with a potent adjuvant seem better suited to induce robust CD4+ and CD8+ T-cell responses. To induce multispecific CTL responses, which are key to resolution of HBV infection [20,21], we tested a combination of particulate HBcAg and HBsAg adjuvanted with saponin-based ISCOMATRIX™ adjuvant for its immunogenicity in naïve as well as in HBVtg mice.

2. Materials and methods

2.1. Antigens, adjuvant, vaccine formulation

HBcAg particles (lacking the nucleic acid binding region) of genotype D subtype ayw (ay) were produced in *Escherichia coli* and purified as described [22]. Endotoxin concentration was 65 endotoxin units/mg corresponding to 0.65 units per dose. Recombinant HBsAg particles of genotype A/subtype adw, and genotype C/subtype adr were produced in the yeast H. polymorpha [23]. Saponin-based ISCOMATRIX™ adjuvant capable of inducing both humoral and cellular immune responses was obtained from CSL Limited (Parkville, Victoria, Australia). The vaccine formulation contained 10 μg HBsAg, 10 μg HBcAg, and 4 μg ISCO™ Units ISCOMATRIX™ adjuvant, respectively, in a volume of 100 μl per mouse dose, if not otherwise stated.

2.2. Mice and immunizations

All animals received human care and study protocols were in compliance with institutional guidelines. Mice were immunized s.c. on day 1 and day 22 unless otherwise indicated. Spleens, livers and sera were collected day 14 after the last immunization for subsequent analysis. Liver associated lymphocytes (LAL) were prepared as described [24,25]. C57BL/6 HBV1.3.32 transgenic mice (line 1.3.32 [26]), kindly provided by F. Chisari (The SCRIPPS Institute, La Jolla, CA, USA), carry a 1.3-fold overlength HBV genome. HBsAg(adw) and HBsAg(adr), both heterologous to HBV genotype D/subtype ay expressed in HBVtg mice, proved to be equally immunogenic (data not shown). Therefore, HBsAg(adr) was used in most experiments.

2.3. Stimulation and intracellular cytokine staining of HBV-specific T cells

Freshly isolated splenocytes ($2–6 \times 10^6$ cells per well) or LAL were incubated with 1 μg/ml or 0.25 μM HBcAg 93–100 MGLKFRQL, HBsAg (ay) 208–215 ILSPFLPL, HBsAg (adw) 208–215 IVSPFIPL or control peptide SIINFEKL (jpt Peptide Technologies, Berlin, Germany) and 5 μg/ml brefeldin A (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) for 4 h. After stimulation, cells were washed and incubated with Ethidium monoazide to assess viability. After stained for CD8 and CD4 cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Kit (BD Bioscience, Heidelberg, Germany). Fluorochrome conjugated antibodies were added for intra cellular cytokine staining: IFNγ-FITC, TNFα-PECy7 (BD Bioscience) and IL-2-AF647 (eBioscience, Eching, Germany). Cells were analyzed by flow cytometry to determine the number of responding cells per 10^5 CD8+ T cells. Background levels of cytokine production from control peptide stimulated samples were subtracted from the corresponding responses.

2.4. Enzyme linked ImmunoSpot

1.5×10^6 splenocytes per well were plated on 96-well plates for ELISpot assays for IL-4 or IL-5 release using ELISpot plus for Mouse Kits (MABTECH, Nacka Strand, Sweden). Cells were unstimulated or stimulated with 2 μg/ml PMA/Ionomycin (Sigma–Aldrich, Taufkirchen, Germany) or with 5 μg/ml recombinant HBsAg or HBcAg for 48 h at 37 °C. After washing and counterstaining, spot forming cells/well (SFC) were counted on a CTL ImmunoSpot S5 UV Analyser. Positive results were defined as ≥5 SFC. In unstimulated samples, SFC background was <2. Negative results were defined as <5 SFC when positive controls were ≥50 SFC.

2.5. Detection of serological and biochemical parameters

Serum alaninaminotransferase (ALT) activity was determined using bioreaction strips on a Reflovet® Plus reader (Roche Diagnostics, Mannheim, Germany). For C57BL/6 mice, anti-HBsAg antibodies were quantified using IMx AUSAB reagents (Abbott Laboratories, Abbott Park, IL, USA). For HBVtg mice, HBsAg and hepatitis B e antigen (HBeAg) as well as anti-HBs and anti-HBc were measured in 1:20 diluted sera using standardized assays (AXSYM™, Abbott). IgG1 and IgG2b antibody isotypes were determined by ELISA. HBV DNA was quantified in the serum by quantitative real time PCR as described [19].

2.6. Liver histology

Liver tissue specimens were fixed in 4% buffered formalin for ≥24 h and embedded in paraffin. Four μm sections were stained with hematoxylin and eosin (HE). Ten vision fields per mouse were randomly chosen and inflammatory foci per visual field were counted in a 100-fold magnification. Desmet scores: 0 = no fibrosis (0–2 inflammatory foci), 1 = mild fibrosis (periportal fibrous expansion or 2–5 foci); 2 = moderate fibrosis (porto-portal septa or 6–10 foci); 3 = severe fibrosis (portcentral septa or >10 foci); 4 = cirrhosis were applied [27]. Immunohistochemistry was performed using polyclonal rabbit anti-hepatitis B core antigen (HBcAg) (Dako Hamburg, Germany, dilution 1:500). Positive HBcAg staining was evaluated separately for nuclei and cytoplasm of hepatocytes.

2.7. Adoptive transfer of HBV-specific T cells

Splenocytes from immunized C57BL/6 mice were enriched by magnetic depletion of non-T cells using the Pan T cell isolation kit on an auto-MACS apparatus (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. 2.5×10^7 T cells (CD4+ and CD8+, purity 94%) were injected i.v. per HBVtg recipient mouse.

3. Results

3.1. Induction of humoral and cellular immune responses directed against HBsAg and HBcAg

Different vaccine formulations were tested for their ability to induce HBV-specific CD4+ and CD8+ T cell as well as humoral immune responses (Fig. 1). The most potent vaccine candidate formulation contained two particulate structural HBV antigens, HBcAg and HBsAg, as well as the novel ISCOMATRIX™ adjuvant (Fig. 1, group 9). After two immunizations with HBsAg/HBcAg/ISCOMATRIX™ in C57BL/6 mice, HBcAg-specific CD8+ T-cell

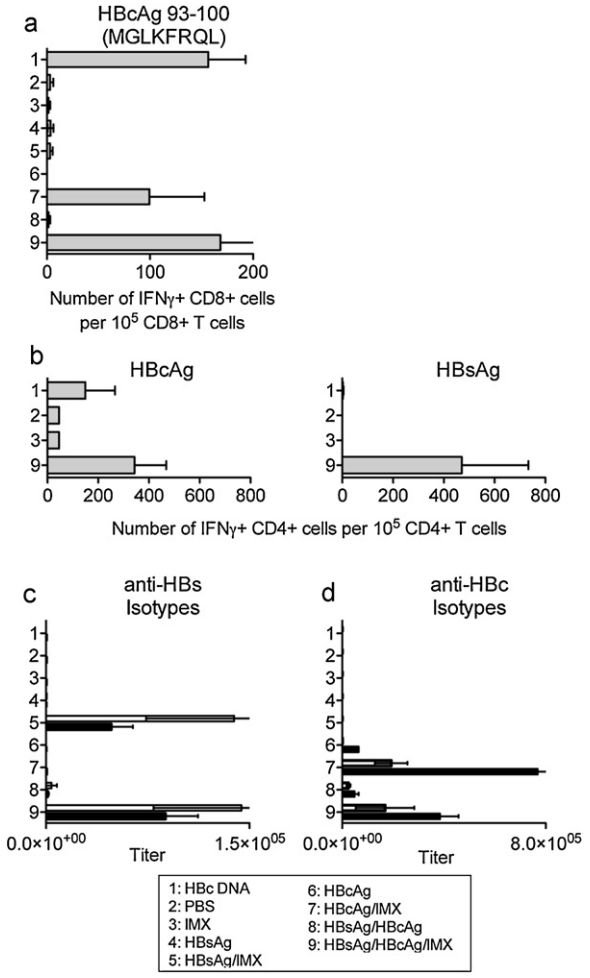

Fig. 1. A protein based vaccine formulation induces HBV-specific immune responses of a balanced Th1/Th2 type in C57BL/6 mice. C57BL/6 mice were immunized twice with the indicated vaccine formulations containing 5 μg HBsAg, 10 μg HBcAg, and 5 μg IMX. Splenocytes were analyzed for (a) CD8+ T cells or (b) CD4+ T cells producing intracellular IFNγ after antigen specific stimulation with (a) indicated K^b binding peptide epitope or (b) recombinant antigens. (c) Anti-HBs and (d) anti-HBc of IgG1 (white bars) and IgG2 (black bars) isotypes. Mean values of 2–4 mice per group were analyzed (with exceptions of one mouse per group in (b), group 2 and 3). Error bars = S.E.M. IMX = ISCOMATRIX™ adjuvant.

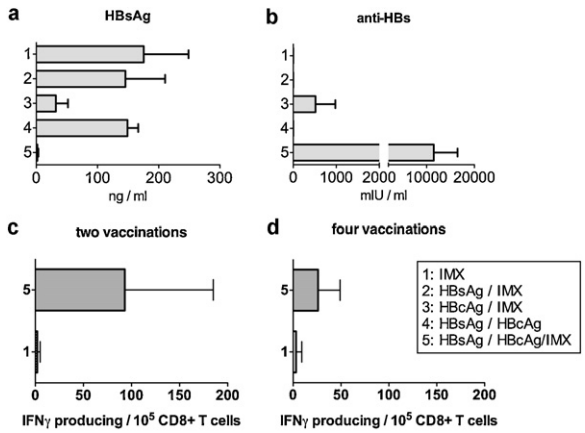

Fig. 2. A protein based vaccine formulation induces HBsAg seroconversion in HBVtg mice. HBVtg mice were immunized twice with indicated formulations (1–5). (a) HBsAg levels and (b) anti-HBs antibody titers were determined in mouse sera by immunoassays. T cell responses against peptide $HBsAg_{208–215}$ were determined in splenocytes at d14 after (c) two vaccinations (d1 and d22; analysis d36) or (d) four vaccinations (d1, d15, d29, d57; analysis d71) with either the HBsAg/HBcAg/ISCOMATRIX™ vaccine formulation or ISCOMATRIX™ adjuvant alone. Mean values of 7–8 mice per group are given with error bars = S.E.M.; IMX = ISCOMATRIX™ adjuvant.

responses (Fig. 1a) were as high as those obtained with HBcAg/ISCOMATRIX™ or with DNA vaccine pCI-core – a construct known to induce HBcAg-specific CD8+ T-cell responses in mice [28]. It also induced HBV-specific CD4+ T-cell responses detected after *ex vivo* stimulation with HBsAg or HBcAg protein (Fig. 1b). Immunization achieved a balanced Th1/Th2 response indicated by anti-HBs and anti-HBc antibodies of IgG1 and IgG2 isotypes (Fig. 1c and d). By ELISPOT analysis of splenocytes, IL-5 and to a lesser extend IL-4 secretion were observed in response to HBcAg and HBsAg (data not shown). These data indicate a balanced Th1/Th2 response against HBsAg and HBcAg. We therefore considered the combination HBsAg/HBcAg/ISCOMATRIX™ valuable for potential therapeutic use.

3.2. HBsAg/HBcAg/ISCOMATRIX™ vaccine breaks tolerance in HBVtg mice

HBsAg/HBcAg/ISCOMATRIX™ vaccine formulation also proved superior in the HBV tg mouse model of chronic HBV infection. HBsAg/HBcAg/ISCOMATRIX™ induced complete anti-HBs seroconversion in HBVtg mice two weeks after the second injection, while HBsAg/ISCOMATRIX™ only reduced HBsAg levels (Fig. 2a and b). In addition, high titers of anti-HBc antibodies were induced by two immunizations with HBcAg/ISCOMATRIX™ and HBsAg/HBcAg/ISCOMATRIX™ vaccine and lower titers by non-adjuvanted HBsAg/HBcAg (data not shown).

To estimate kinetics of vaccine responses, we compared two vaccinations at day 1 and 22 with four vaccinations at day 1, 15, 29 and 57. In 7/8 animals, anti-HBs titers raised >10,000 IU/l after 4 vaccinations, while after 2 vaccinations only 2/8 animals had a titer >10,000 IU/l. HBV-specific CD8 T-cell responses were already detected after two vaccinations but did not further increase after four vaccinations (Fig. 2c and d). $HBsAg_{208–215}$-specific CD8 T cells were detected in 6/7 animals after two and in 5/8 animals after four vaccinations by intracellular cytokine staining for IFNγ. Titers of circulating virus decreased in HBsAg/HBcAg/ISCOMATRIX™ vaccinated HBVtg mice after two and four vaccinations although this did not reach statistical significance (data not shown).

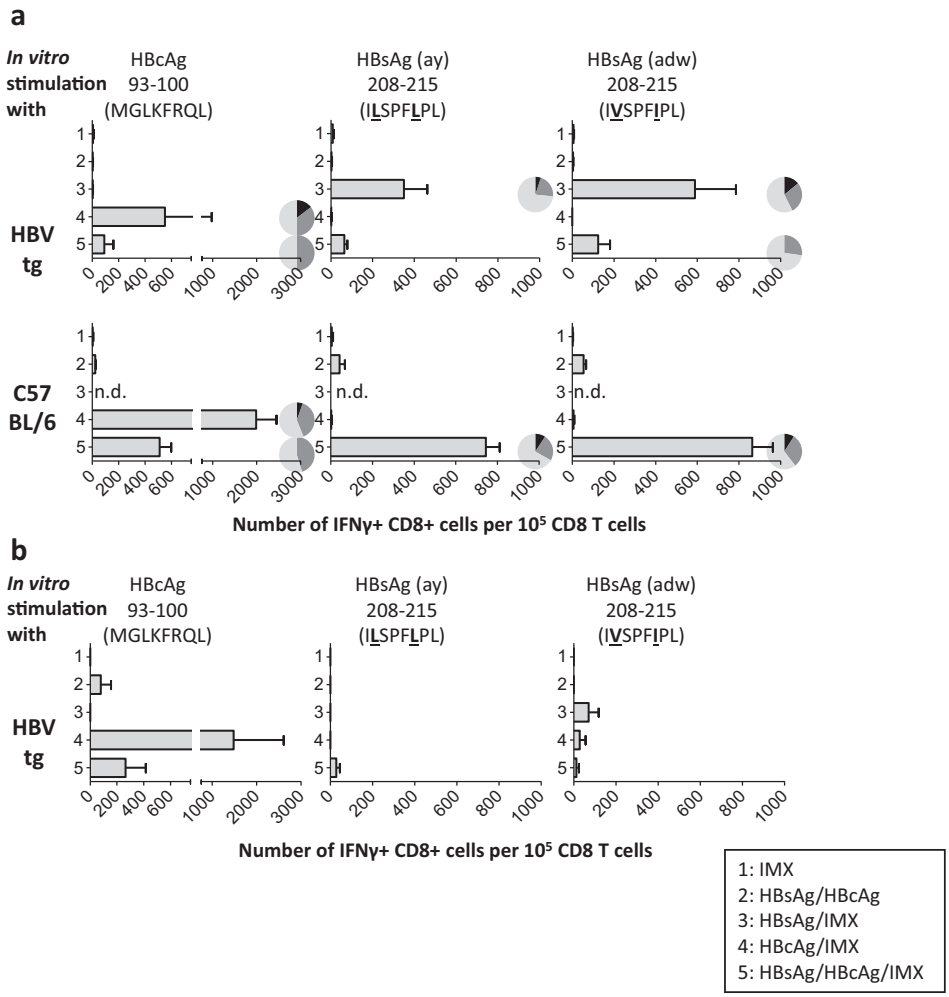

Fig. 3. Vaccination induces HBV-specific CD8+ T-cell responses in spleen and liver of HBVtg mice. HBVtg mice or C57BL/6 mice were immunized three times with indicated vaccine formulations (1–5). According to HBeAg levels transgenic mice were equally distributed among groups. CD8+ splenocytes (a) or liver associated lymphocytes (b) were analyzed for intracellular IFNγ production (bars) after antigen specific *in vitro* stimulation with indicated K^b binding peptide epitopes. Mean values of three to four mice per group are given with one exception (group 3, C57BL/6), where no data were evaluated (n.d.). Error bars = S.E.M. The capacity of specific CD8+ T cells to produce several cytokines simultaneously was investigated in moderate responses with >100 IFNγ+ CD8+ cells per 10^5 CD8+ T cells. The quality of total response implies IFNγ, IL-2 and TNFα production. Pie charts represent the proportion of tri- (black), bi- (dark gray) and monofunctional (light gray) CD8+ T cells. Before vaccination, HBV DNA in sera of HBVtg mice was median 4.3×10^5 copies/ml serum (range 4.7×10^3–2.8×10^6) and HBsAg levels were median 2.2 S/CO in a 1:20 serum dilution (range 0–4.8); Underlined amino acids indicate sequence variations between HBV subtypes. IMX = ISCOMATRIX™ adjuvant.

3.3. *HBsAg/HBcAg/ISCOMATRIX™ vaccine induces multispecific and multifunctional T cells infiltrating the liver*

HBVtg and C57BL/6 mice were immunized three times, at day 1, 15 and 29, with ISCOMATRIX™ adjuvant alone, particulate HBsAg (subtype adw), HBcAg alone or with vaccine formulations containing the respective combinations. To evaluate HBV-specific CD8+ T cells we restimulated splenocytes of vaccinated mice with K^b binding epitope $HBcAg_{93–100}$ and $HBsAg_{208–215}$, which is presented on cell surfaces after exogenous uptake of HBsAg [29]. The HBsAg/HBcAg/ISCOMATRIX™ vaccine elicited HBc- and HBs-specific, multifunctional CD8+ T-cell responses in naïve C57BL/6 mice (Fig. 3a, lower panel). Importantly, HBsAg-specific T-cell responses were directed against $HBsAg_{208–215}$ epitopes of both HBV subtypes IVSPFIPL (adw) and ILSPFLPL (ay), the latter being expressed in HBVtg mice. While vaccination of HBVtg mice with HBsAg and/or HBcAg without adjuvant was not immunogenic, the inclusion of ISCOMATRIX™ adjuvant allowed induction of HBV-specific, polyfunctional CD8+ T-cells capable of producing IFNγ as well as TNFα and IL-2 (5–17%) or IFNγ and TNFα (25–50%) (Fig. 3a, upper panel). Combining both antigens induced lower numbers of HBcAg- and HBsAg-specific CD8+ T-cells than the respective single antigens. CD8+ T cells were reactive to $HBsAg_{208–215}$ of HBV subtype adw (IVSPFIPL) and cross-reactive to subtype ay (ILSPFLPL) expressed in HBVtg mice.

Having shown that vaccination with HBsAg/HBcAg/ISCOMATRIX™ induces polyfunctional T-cells in HBVtg mice, we investigated if CD8+ T-cells were functional in the tolerogenic microenvironment of the liver (Fig. 3b). High numbers of IFNγ-secreting HBc-specific, but only low numbers of functional HBs-specific CD8+ T-cells were detected in LAL from HBVtg mice. HBV-specific CD4+ T-cell responses in vaccinated HBVtg remained below detection limit (data not shown).

Taken together, after immunization with HBsAg/HBcAg/ISCOMATRIX™ multispecific, multifunctional HBV-specific CD8+ T cells were detected in spleen and liver of HBVtg mice.

3.4. *HBV-specific T cells induced by the HBsAg/HBcAg/ISCOMATRIX™ vaccine do not cause liver damage in HBVtg mice*

Having demonstrated that immunization with HBsAg/HBcAg/ISCOMATRIX™ vaccine induced HBV-specific CD8+ T-cell

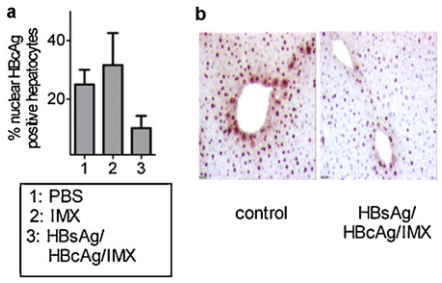

Fig. 4. Vaccination reduces HBcAg in hepatocytes of HBVtg mice. HBVtg mice were immunized with HBsAg/HBcAg/IMX. Liver sections were immuno-stained with anti-HBcAg. IMX = ISCOMATRIX™ adjuvant. (a) The frequency of hepatocytes with HBcAg positive nuclei was determined. Mean values of 8 mice per group (2 mice in PBS group) are given with error bars = S.E.M. (b) Representative picture of anti-HBc-immune stained liver sections of immunized and non-immunized HBVtg mice.

responses in spleen and liver of vaccinated HBVtg mice, we next investigated the efficacy of these responses. Immunostaining of liver sections for HBcAg demonstrated a clear trend for reduction in the percentage of HBcAg positive hepatocytes in HBVtg mice immunized with HBsAg/HBcAg/ISCOMATRIX™ vaccine in comparison to PBS or ISCOMATRIX™ adjuvant injected control animals (Fig. 4). However this was not statistically significant ($p = 0.07$).

An obvious question was whether an immunization inducing humoral and cellular immune responses and reducing HBcAg in livers of HBVtg mice would result in inflammation or damage of the HBV replicating liver. For this, HBVtg mice were immunized with the combination HBsAg/HBcAg/ISCOMATRIX™ vaccine or appropriate controls (Fig. 5a). ALT activity (indicating hepatocyte damage) was determined in the sera of immunized mice and appeared not to be influenced by the vaccine. Next, HBVtg and C57BL/6 mice vaccinated with HBsAg/HBcAg/ISCOMATRIX™ vaccine or injected with ISCOMATRIX™ or PBS alone were analyzed for necroinflammatory liver damage according to the Desmet grading system [27] (Fig. 5b–d). However, analysis of the grade of liver inflammation demonstrated that induction of cellular and humoral immune responses against HBV did neither lead to increased

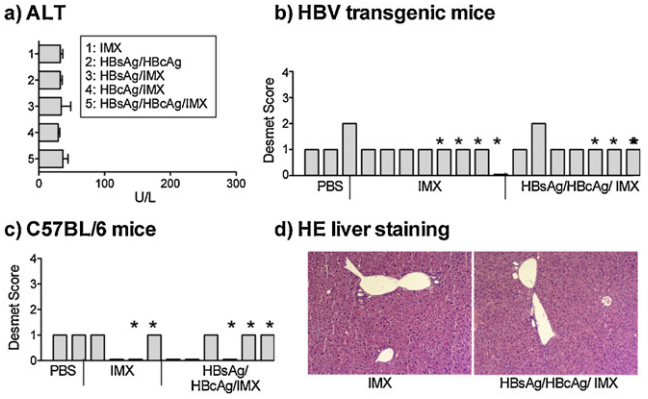

Fig. 5. Vaccination does not cause liver damage in HBVtg mice. (a) HBVtg mice were immunized with indicated vaccine formulations (1–6) and alaninaminotransferase (ALT) levels were determined in sera 2 weeks after the last immunization. Mean values of 3–4 mice per group are given with error bars = S.E.M. (b) HBVtg mice or (c) C57BL/6 mice were immunized 2 or 3 (*) times with the vaccine combination HBsAg/HBcAg/IMX. Liver sections were stained with hematoxylin/eosin and the grade of hepatitis was evaluated according to the Desmet Score System in a blinded fashion. Desmet scores indicates the stages of hepatitis with 0 = no fibrosis, 1 = mild fibrosis, 2 = moderate fibrosis, 3 = severe fibrosis and 4 = cirrhosis. (d) Representative hematoxylin/eosin staining of livers obtained from IMX only and HBsAg/HBcAg/IMX immunized mice. IMX = ISCOMATRIX™ adjuvant.

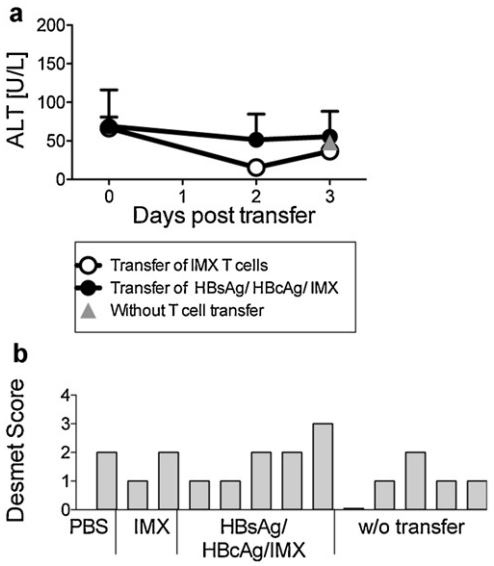

Fig. 6. Vaccine induced HBV-specific T cells adoptively transferred into HBVtg mice did not cause severe liver damage. C57BL/6 mice were immunized twice with the vaccine formulation HBsAg/HBcAg/IMX, containing 5 μg HBsAg, 16 μg HBcAg and 2.5 μg IMX. Isolated splenocytes obtained from three immunized C57BL/6 mice were adoptively transferred into one HBVtg mouse each. (a) On day 0, 2 and 3 post transfer serum ALT levels (mean values of 2–5 HBVtg mice are given with error bars = S.E.M.) were determined. (b) Desmet scores (indicating the stages of hepatitis with 0 = no fibrosis, 1 = mild fibrosis, 2 = moderate fibrosis, 3 = severe fibrosis and 4 = cirrhosis) of hematoxylin/eosin stained liver sections of individual mice 3 days after adoptive T cell transfer. IMX = ISCOMATRIX™ adjuvant.

inflammatory scores nor necroinflammatory tissue damage of the HBV expressing livers.

As induction of liver inflammation with concomitant tissue damage is a major concern in HBV therapeutic vaccination and the high level of T cell tolerance in HBVtg animals may prevent liver damage, we investigated the impact of high numbers of HBV reactive T cells induced in non-tolerant animals and adoptively transferred into HBVtg mice (Fig. 6). For this, we immunized C57BL/6 mice twice with the HBsAg/HBcAg/ISCOMATRIX™ vaccine and isolated splenocytes at day 12 after the second immunization. CD4+ and CD8+ T cells from three mice each were pooled and adoptively transferred into one syngeneic, HBVtg animal. HBV-specificity of the T cells to be used for adoptive transfer was confirmed before transfer (data not shown). As control, T cells were isolated from PBS or ISCOMATRIX™ adjuvant injected C57BL/6 donors. ALT levels of recipient HBVtg mice were neither elevated on day 2 nor day 3 after adoptive T cell transfer and were comparable to those observed in control mice, which received no T cells or T cells from ISCOMATRIX™ adjuvant only injected mice (Fig. 6a). Necroinflammatory liver damage was assessed 3 days after adoptive T cell transfer – a time point when transferred effector T cells are expected to be active and before being eliminated. Histopathological analysis of livers from the recipient HBVtg mice at this time point revealed signs of mild liver inflammation but did not reveal any severe liver damage (Fig. 6b). As expected, liver inflammation in animals that had received adoptively transferred T cells (Fig. 6b, groups PBS, IMX and HBsAg/HBcAg/IMX) was slightly increased due to injection of a substantial number of activated T cells that generally home to the liver, compared to mice that did not receive adoptively transferred T cells (Fig. 6b, group w/o transfer; Fig. 5b).

Taken together, the HBsAg/HBcAg/ISCOMATRIX™ vaccine induced HBV-specific T cells adoptively transferred into HBVtg mice did not damage the HBV replicating liver.

4. Discussion

Therapeutic vaccination aims at inducing an efficient multi-specific anti-HBV immune response to resolve chronic HBV infection. If successful, it may avoid long-term therapy and possible treatment failure due to antiviral resistance or side effects. In this study, a novel protein-based vaccine formulation comprising two major HBV antigens, HBsAg and HBcAg, in a particulate form, and the ISCOMATRIX™ adjuvant was tested. This therapeutic hepatitis B vaccine candidate induced potent HBsAg- and HBcAg-specific IFNγ+ CD8+ and CD4+ T cells as well as high antibody titers against both antigens in naïve C57BL/6 mice. In HBVtg mice used as a surrogate model for persistent HBV infection after neonatal transmission, the multi-antigen vaccine formulation HBsAg/HBcAg/ISCOMATRIX™ adjuvant fulfilled the major requests to a therapeutic vaccine: inducing anti-HBs seroconversion and eliciting HBcAg-specific T cells in spleen and liver without causing obvious liver damage.

The vaccine composition was designed to induce multispecific, multifunctional cellular and humoral responses, mimicking the characteristics of self-limiting HBV infection. Particle forming HBsAg and HBcAg antigens were included in the vaccine formulation to offer a large epitope repertoire to the immune system to induce high antibody titers against HBsAg and HBcAg. This was effective in C57BL/6 as well as in HBVtg mice, which are tolerant to HBV antigens due to transgene expression early in life [26].

For a therapeutic vaccine it is most important to induce potent T-cell responses. Induction of a CD8+ T-cell response requires cross-presentation of the exogenous vaccine antigens by professional antigen-presenting cells mediated by antigen uptake into distinct endosomal compartments [30]. Our vaccine candidate proved to induce CD4+ and CD8+ T-cell responses against multiple epitopes of HBsAg and HBcAg - in line with the described action of the saponin-based ISCOMATRIX™ adjuvant [31,32] already after two vaccinations. Importantly, vaccine induced cross-reactive CD8+ T cells reacted not only against HBsAg epitopes of subtype adw corresponding to the vaccine, but also against epitopes of HBsAg subtype ay expressed in HBVtg mice. HBV-specific T cells produced IFNγ, which is considered to be key in controlling HBV replication [33,34], and to a lesser extend also TNFα and IL-2. These "polyfunctional" T cells have been associated with protection in human viral infections [35,36]. We therefore concluded, that this novel vaccine combination may initiate the critical immune responses to achieve sustained control or clearance of HBV in chronic infection.

Using two antigens, HBsAg and HBcAg, antigenic competition may be a problem and we noticed some in HBVtg mice on the CD8+ T cell level [37,38]. The combination of HBsAg and HBcAg, however, was needed to induce a broad T cell response, and even had an additive effect in inducing antibody responses leading to anti-HBs seroconversion in HBVtg mice. This additive effect may be related to the ability of HBcAg particles to directly activate B cells to take up the antigen, process and present it [39].

A major concern in therapeutic vaccination strategies is the potential risk of overt immune responses leading to an exacerbation of chronic hepatitis B or even liver decompensation [14]. We neither observed significant liver damage nor severe inflammatory responses in vaccinated animals. To exclude that the vaccine did not induce fully functional T cells in the HBVtg mice, we adoptively transferred T cells from vaccinated naïve, *i.e.* non-tolerant, mice into HBV-transgenic animals, but also observed no liver damage. This is in line with clinical experience transferring bone marrow transplants from donors with naturally acquired immunity to HBV into chronically infected recipients [7].

T cells induced in vaccinated mice remained functional in the HBV expressing liver. We found polyfunctional HBcAg-specific CD8+ T cells in the liver, and HBcAg expression in liver sections of HBVtg mice was reduced. The absence of obvious liver damage indicated a prominent antiviral effect of cytokines such as IFNγ [33].

In summary, we have described and evaluated a therapeutic vaccine candidate in a mouse model of chronic HBV infection that demonstrates the importance of both antigen components, HBcAg and HBsAg, in inducing anti-HBs seroconversion as well as multi-specific T cells and highlights the need for a potent adjuvant activity in order to break tolerance in this chronic viral disease.

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References

[1] Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005;5(March (3)):215–29.
[2] Bertoletti A, Ferrari C, Fiaccadori F, Penna A, Margolskee R, Schlicht HJ, et al. HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. Proc Natl Acad Sci USA 1991;88(December (23)):10445–9.
[3] Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995;181(March (3)): 1047–58.
[4] Penna A, Chisari FV, Bertoletti A, Missale G, Fowler P, Giuberti T, et al. Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. J Exp Med 1991;174(December (6)):1565–70.
[5] Tan AT, Loggi E, Boni C, Chia A, Gehring AJ, Sastry KS, et al. Host ethnicity and virus genotype shape the hepatitis B virus-specific T-cell repertoire. J Virol 2008;82(November (22)):10986–97.
[6] Protzer U, Maini MK, Knolle PA. Living in the liver: hepatic infections. Nat Rev Immunol 2012;12(March (3)):201–13.
[7] Lau GK, Suri D, Liang R, Rigopoulou EI, Thomas MG, Mullerova I, et al. Resolution of chronic hepatitis B and anti-HBs seroconversion in humans by adoptive transfer of immunity to hepatitis B core antigen. Gastroenterology 2002;122(March (3)):614–24.
[8] Ilan Y, Nagler A, Adler R, Tur-Kaspa R, Slavin S, Shouval D. Ablation of persistent hepatitis B by bone marrow transplantation from a hepatitis B-immune donor. Gastroenterology 1993;104(June (6)):1818–21.
[9] Schirmbeck R, Reimann J. Enhancing the immunogenicity of exogenous hepatitis B surface antigen-based vaccines for MHC-I-restricted T cells. Biol Chem 1999;380(March (3)):285–91.
[10] Pancholi P, Lee DH, Liu Q, Tackney C, Taylor P, Perkus M, et al. DNA prime/canarypox boost-based immunotherapy of chronic hepatitis B virus infection in a chimpanzee. Hepatology 2001;33(February (2)):448–54.
[11] Heintges T, Petry W, Kaldewey M, Erhardt A, Wend UC, Gerlich WH, et al. Combination therapy of active HBsAg vaccination and interferon-alpha in interferon-alpha nonresponders with chronic hepatitis B. Dig Dis Sci 2001;46(April (4)):901–6.
[12] Dahmen A, Herzog-Hauff S, Bocher WO, Galle PR, Lohr HF. Clinical and immunological efficacy of intradermal vaccine plus lamivudine with or without interleukin-2 in patients with chronic hepatitis B. J Med Virol 2002;66(April (4)):452–60.
[13] Dienstag JL, Stevens CE, Bhan AK, Szmuness W, Hepatitis B. vaccine administered to chronic carriers of hepatitis b surface antigen. Ann Intern Med 1982;96(May (5)):575–9.
[14] Hilleman MR. Critical overview and outlook: pathogenesis, prevention, and treatment of hepatitis and hepatocarcinoma caused by hepatitis B virus. Vaccine 2003;21(December (32)):4626–49.
[15] Vitiello A, Ishioka G, Grey HM, Rose R, Farness P, LaFond R, et al. Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. J Clin Invest 1995;95(January (1)):341–9.
[16] Heathcote J, McHutchison J, Lee S, Tong M, Benner K, Minuk G, et al. A pilot study of the CY-1899 T-cell vaccine in subjects chronically infected with hepatitis B virus. The CY1899 T Cell Vaccine Study Group. Hepatology 1999;30(August (2)):531–6.
[17] Vandepapeliere P, Horsmans Y, Moris P, Van Mechelen M, Janssens M, Koutsoukos M, et al. Vaccine adjuvant systems containing monophosphoryl lipid A

and QS21 induce strong and persistent humoral and T cell responses against hepatitis B surface antigen in healthy adult volunteers. Vaccine 2008;26(March (10)):1375–86.
[18] Vandepapeliere P, Lau GK, Leroux-Roels G, Horsmans Y, Gane E, Tawandee T, et al. Therapeutic vaccination of chronic hepatitis B patients with virus suppression by antiviral therapy: a randomized, controlled study of co-administration of HBsAg/AS02 candidate vaccine and lamivudine. Vaccine 2007;25(December (51)):8585–97.
[19] Xu DZ, Zhao K, Guo LM, Li LJ, Xie Q, Ren H, et al. A randomized controlled phase IIb trial of antigen–antibody immunogenic complex therapeutic vaccine in chronic hepatitis B patients. PLoS One 2008;3(7):e2565.
[20] 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(January (1)):68–76.
[21] Webster GJ, Reignat S, Brown D, Ogg GS, Jones L, Seneviratne SL, et al. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 2004;78(June (11)):5707–19.
[22] Birnbaum F, Nassal M, Hepatitis B. virus nucleocapsid assembly: primary structure requirements in the core protein. J Virol 1990;64(July (7)):3319–30.
[23] Janowicz ZA, Melber K, Merckelbach A, Jacobs E, Harford N, Comberbach M, et al. Simultaneous expression of the S and L surface antigens of hepatitis B, and formation of mixed particles in the methylotrophic yeast, Hansenula polymorpha. Yeast 1991;7(July (5)):431–43.
[24] Stross L, Gunther J, Gasteiger G, Asen T, Graf S, Aichler M, et al. Foxp3+ regulatory T cells protect the liver from immune damage and compromise virus control during acute, experimental hepatitis B virus infection. Hepatology 2012;56(April (3)):873–83.
[25] Huang LR, Gabel YA, Graf S, Arzberger S, Kurts C, Heikenwalder M, et al. Transfer of HBV genomes using low doses of adenovirus vectors leads to persistent infection in immune competent mice. Gastroenterology 2012;142(June (7)):1447e3–50e3.
[26] Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus replication in transgenic mice. J Virol 1995;69(October (10)):6158–69.
[27] Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19(June (6)):1513–20.
[28] Kuhober A, Pudollek HP, Reifenberg K, Chisari FV, Schlicht HJ, Reimann J, et al. DNA immunization induces antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2b mice. J Immunol 1996;156(May (10)): 3687–95.
[29] Schirmbeck R, Melber K, Reimann J. Adjuvants that enhance priming of cytotoxic T cells to a Kb-restricted epitope processed from exogenous but not endogenous hepatitis B surface antigen. Int Immunol 1999;11(July (7)):1093–102.
[30] Burgdorf S, Kautz A, Bohnert V, Knolle PA, Kurts C. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. Science 2007;316(April (5824)):612–6.
[31] Boyle J, Eastman D, Millar C, Camuglia S, Cox J, Pearse M, et al. The utility of ISCOMATRIX adjuvant for dose reduction of antigen for vaccines requiring antibody responses. Vaccine 2007;25(March (14)):2541–4.
[32] Pearse MJ, Drane D. ISCOMATRIX adjuvant for antigen delivery. Adv Drug Deliv Rev 2005;57(January (3)):465–74.
[33] Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity 1996;4(January (1)):25–36.
[34] Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science 1999;284(April (5415)):825–9.
[35] Kosinska AD, Zhang E, Lu M, Roggendorf M. Therapeutic vaccination in chronic hepatitis B: preclinical studies in the woodchuck. Hepat Res Treat 2010;2010:817580.
[36] Pantaleo G, Harari A. Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. Nat Rev Immunol 2006;6(May (5)):417–23.
[37] Kastenmuller W, Gasteiger G, Gronau JH, Baier R, Ljapoci R, Busch DH, et al. Cross-competition of CD8+ T cells shapes the immunodominance hierarchy during boost vaccination. J Exp Med 2007;204(September (9)):2187–98.
[38] Palmowski MJ, Choi EM, Hermans IF, Gilbert SC, Chen JL, Gileadi U, et al. Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. J Immunol 2002;168(May (9)):4391–8.
[39] Milich DR, Chen M, Schodel F, Peterson DL, Jones JE, Hughes JL. Role of B cells in antigen presentation of the hepatitis B core. Proc Natl Acad Sci USA 1997;94(December (26)):14648–53.