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Woodchuck hepatitis virus core antigen-based DNA and protein vaccines induce qualitatively different immune responses that affect T cell recall responses and antiviral effects

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ABSTRACT

T helper type 1 (Th1) immunity was considered to play a dominant role in viral clearance of hepadnaviral infection. However, pre-primed Th2 type responses were able to efficiently control hepadnaviral infection in animal models. We investigated how pre-primed Th1/2 responses control hepadnaviral replication using the newly established mouse models. DNA (pWHcIm, pCTLA-4-C) and protein vaccines based on the nucleocapsid protein (WHcAg) of woodchuck hepatitis virus (WHV) primed specific immune responses with distinct features. The pre-primed responses determined the characteristics of recall responses if challenged with a WHcAg-expressing adenoviral vector. Vaccination with pWHcIm and pCTLA4-C facilitated viral control in the hydrodynamic injection model and reduced WHV loads by about 3 and 2 logs in WHV-transgenic mice, respectively, despite of different kinetics of specific CD8+ T cell responses. Thus, pre-primed Th2-biased responses facilitate the development of CD8+ T cell responses in mice compared with naïve controls and thereby confer better viral control.

Introduction

Hepatitis B virus (HBV) infection is a global public health problem, as more than 350 million people worldwide are chronically infected with HBV and at high risk for developing severe liver diseases, such as cirrhosis and hepatocellular carcinoma. The prophylactic vaccine based on HBV surface antigen (HBsAg) has been successfully used since 1980 and can effectively induce protective anti-HBs antibodies, which neutralize HBV particles. Current research focuses on the development of therapeutic vaccines for the treatment of chronic HBV infection. HBV clearance following primary infection requires the appropriate host immune responses, including both humoral and cellular immune responses (Chisari and Ferrari, 1995; Rehermann and Nascimbeni, 2005). HBV-specific cytotoxic T cells (CTLs) targeting HBsAg, HBV core protein (HBcAg), and the viral polymerase have been detected in HBV-infected individuals and are associated with HBV clearance (Phillips et al., 2010; Thimme et al., 2003). Chronically infected patients show immunotolerance to the HBV proteins, with no production of anti-HBs antibodies and very low or undetectable HBV-specific CTL responses. Restoration of the HBV-specific immune response in chronic HBV carriers is a potential strategy for achieving control of the infection or even for curing patients (Kosinska et al., 2010; Maini and Schurich, 2010). However, clinical trials with various vaccine formulations including DNA based vaccines and the HBsAg-HBs antibody immunogenic complexes were not as successful as initially anticipated (Mancini-Bourgine et al., 2004; Michel and Deng and Mancini-Bourgine, 2011; Xu et al., 2013, 2008; Yang et al., 2006).

One major uncertainty for the immunotherapeutic approach is the suitability of used vaccines. In general, vaccines that prime Th1-dominant immune responses to HBV were considered as better candidates for prophylactic and immunotherapeutic approaches. However, an early publication reported that HBcAg immunizations conferred partial protection against HBV challenge in chimpanzees (Iwarson et al., 1985). Later, Schodel et al. (1993a) showed that vaccination of woodchucks with WHcAg protein

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protected them against WHV challenge. Menne et al. (1997) used a WHcAg-derived peptide to immunize woodchucks, achieving efficient protection against WHV challenge. We previously found that a CTLA-4-WHcAg fusion plasmid induced Th2-dominant immune responses but was similarly protective when compared to a plasmid that induces Th1-dominant responses to WHcAg (Lu et al., 2005). Furthermore, we demonstrated that pre-primed Th2-dominant immune responses to HBcAg efficiently cleared HBV from the host in the HBV mouse model (Yin et al., 2011). Thus, different vaccine types should not only be judged on the basis of primed immune responses and needs to be tested for their ability to control HBV infection in *in vivo* models. In addition, the mode of protection after the vaccinations was not analysed in details in the previous studies.

Therefore, it is essential to analyze the host immune responses post challenge to understand how vaccine-induced immune responses determine the process of viral clearance. The woodchuck model is a useful animal model for studies on HBV infection. Woodchucks can be infected with woodchuck hepatitis virus (WHV), a genetically closely related virus to HBV. The woodchuck model has been used to test different approaches for immunomodulation and immunotherapy of chronic HBV infection. As mentioned above, a number of protein- and DNA-based vaccines were tested in challenge studies in the woodchuck model and showed the ability to protect. However, due to the lack of required immunological reagents for the woodchuck model, it is yet not possible to characterize the fine features of vaccine-induced immune responses and the mechanisms of viral control in woodchucks. Recently, we established two new mouse models to studies on WHV replication and WHV-specific immune responses. In this way, the features of WHV-specific immune responses could be determined more precisely, for example, by distinguishing Th types. In this study, we explored WHV-specific vaccines for the experimental approaches in the novel mouse models. We characterized the different types of specific immune responses primed by DNA and protein vaccines to WHcAg and tested the combinations of the different vaccine types. Viral challenges with adenoviral vectors expressing WHcAg and by hydrodynamic injection with WHV were performed to examine the recall responses and viral clearance. Two different WHcAg DNA vaccines were also tested in WHV transgenic (tg) mice, demonstrating that both vaccines were able to induce specific immune responses and to suppress WHV replication, though many features of the induced specific immune responses may differ strongly.

Results

Priming of qualitatively distinct WHcAg-specific immune responses using DNA and WHcAg protein vaccines

Previous experiments demonstrated that DNA and WHcAg protein vaccines were able to stimulate WHcAg-specific immune responses in mice and to confer a certain degree of protection against WHV challenge in woodchucks (Kosinska et al., 2012, 2010; Lu et al., 2005; Menne et al., 1997). To accurately define the different features of WHcAg-specific immune responses primed by these vaccines, C57BL/6 mice were immunized three times with each vaccine. The DNA vaccines pWHcIm and pCTLA-4-C and the WHcAg protein vaccine induced anti-WHcAg antibodies (Fig. 1A). As IL-4 from T helper 2 CD4 cells induces IgG1 while IFN-γ from T helper 1 CD4 cells induces IgG2a production in mice, antigen-specific IgG subtype IgG1 and 2a were used to approximately classify the specific response (Stevens et al., 1988). The WHcAg-specific immune responses were classified as Th1 or Th2 type according to the WHcAg-specific IgG subtype IgG1 and 2a. Consistent with previous findings, pWHcIm preferentially induced antibodies of the IgG2a subtype, while both IgG2a and IgG1 antibodies were found in mice immunized with pCTLA-4-C and WHcAg protein. Furthermore, the IFN-γ secreting CD4 and CD8 cells, which were belonging to Th1 type response were characterised to precisely identify the types of WHcAg-specific response. WHcAg-specific T cell responses in immunized mice were assessed by detection of IFN-γ+ T cells after a 7-day *in vitro* stimulation of splenocytes with the appropriate WHcAg-derived peptides (Fig. S1A, Fig. 1B). Immunizations with pWHcIm elicited strong WHcAg-specific CD8+ and lower but clearly detectable CD4+ T cell responses. Mice immunized with pCTLA-4-C had very limited CD8+ T cell responses, but significant CD4+ T cell responses were detected. In contrast, no detectable CD4+ or CD8+ T cell response was induced in WHcAg-immunized mice (Fig. 1B). Antigen-specific CD8+ T cells in pWHcIm-immunized mice showed both IFN-γ production and degranulation, indicating that these antigen-specific T cells were functional CTLs (Fig. 1C). The *in vivo* cytotoxicity assay demonstrated that killing of target cells loaded with WHcAg-derived peptide p13-21 only occurred in mice immunized with pWHcIm but not in the other vaccination groups (Fig. S1B, Fig. 1D). These results indicated that pWHcIm induced a typical Th1-dominant immune response, while immunization with pCTLA-4-C shifted the WHcAg-specific immune response towards a Th2-dominant type. WHcAg protein induced a Th2 type response without a detectable T cell component (Table 1).

WHcAg-specific recall responses after administration of a WHcAg-expressing adenoviral vector were strongly influenced by the pre-primed responses

Next we addressed the question about the specific recall responses in mice received different DNA and protein vaccines after re-exposure to WHcAg. First, the mice were primed with pWHcIm or WHcAg twice and then boosted with heterologous vaccines WHcAg or pWHcIm, respectively (see supplementary text). The results showed that the specific immune responses and the function of CTLs in vaccinations with heterologous priming and boost could be complex but were strongly determined by the type of vaccines applied for the boost (Figs. S2 and S3). The application of WHcAg protein in our experiments significantly inhibited the induction of WHcAg-specific CD8+ T cells, likely due to the priming of the Th2 dominance. These results hint that the way of the latest encounter with WHcAg may influence the recall responses and thereby modulate the specific T and B responses.

To achieve intrahepatic WHcAg expression and study the recall responses after intrahepatic challenge that is relevant for hepadnaviral infection, a replication-incompetent adenoviral vector genotype 5 expressing WHcAg (Ad5WHc) was constructed (Kosinska et al., 2012). We showed that Ad5WHc induces strong T cell and antibody responses to WHcAg in mice if applied by intramuscular injection. Intravenous injection of Ad5WHc led to the accumulation mainly in the liver within 48 h and only a very low level in the other organs (Fig. 2A). This fact allows us to characterize the recall response after intrahepatic expression of WHcAg. Mice were immunized three times with pWHcIm, pCTLA-4-C, or WHcAg as described above. Two weeks later, the immunized mice received intravenously 1×10^{10} pfu Ad5WHc and were sacrificed on days 4, 7, and 10 (Fig. 2B). Ad5WHc disappeared from the liver with similar kinetics in mice of different groups, indicating that its clearance was not related to the pre-existing immune responses (Fig. 2C).

Ad5WHc administration in immunized mice induced rapid and vigorous T cell responses to WHcAg, allowing the analysis of WHcAg-specific T cell recall responses using *ex vivo* assays. The functionality of specific T cells to WHcAg p13-21 was evaluated by

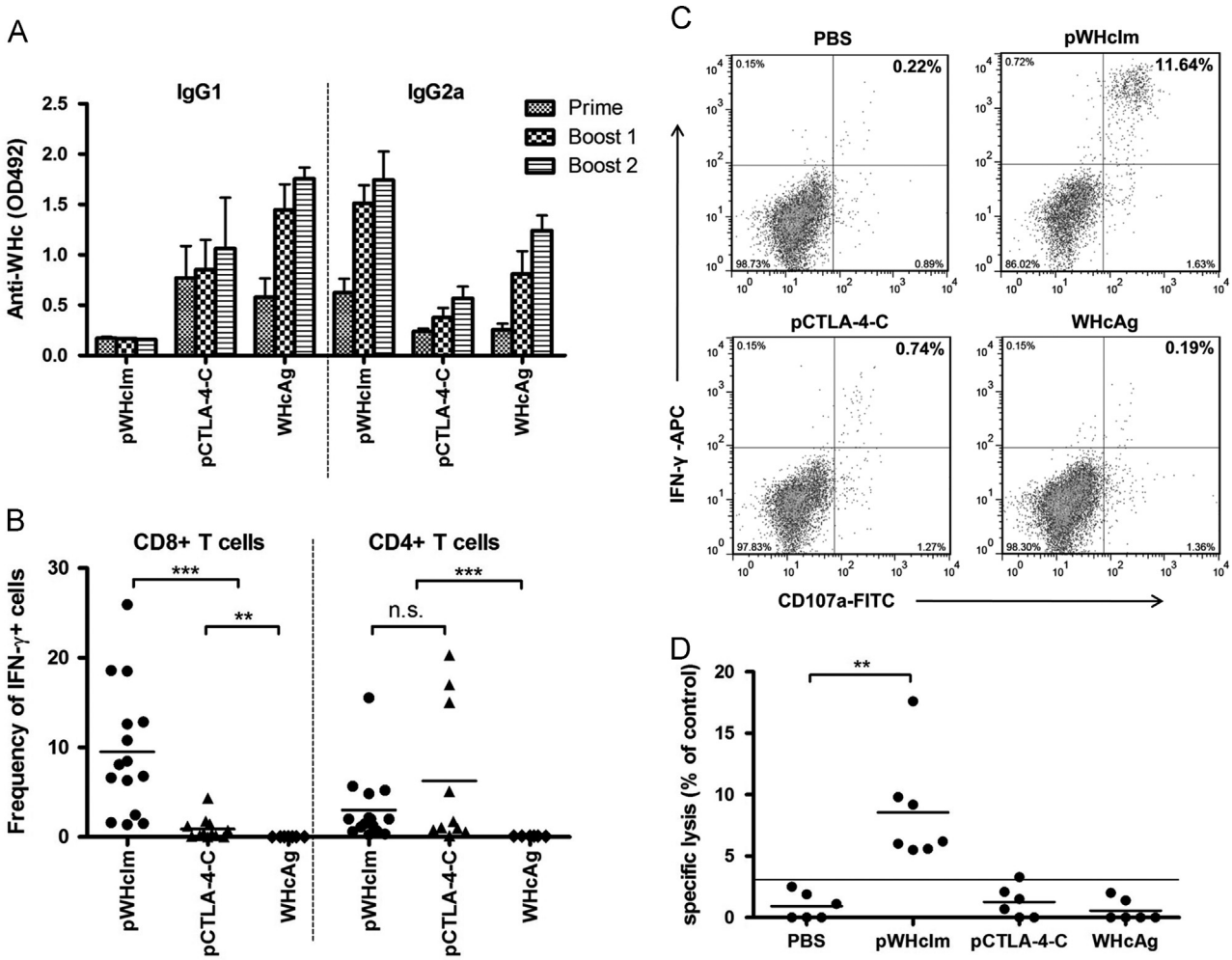

Fig. 1. WHcAg-specific immune responses in C57BL/6 mice primed by pWHclm, pCTLA-4-C, and WHcAg. Mice were immunized three times with indicated vaccines by i.m. or s.c. routes and sacrificed on day 14 after the final immunization. Splenocytes were separated and stimulated with p2 or p27 (2 μg/ml) for 7 days. (A) Serum IgG1 and IgG2a anti-WHcAg antibody responses 14 days after each immunization. The error bar represents SEM from the mean value. (B) Frequencies of IFN-γ+ CD8+ T cells to p2 or CD4+ T cells to p27 induced by pWHclm, pCTLA-4-C, and WHcAg. For each group 5–8 mice were included in three independent experiments. Data from each experiement were pooled for A and B. (C) Degranulation and cytokine production in antigen-specific CD8+ T cells. (D) pWHclm, pCTLA-4-C, and WHcAg induced different WHcAg-specific CD8+ T cell cytotoxicity in peripheral blood on day 7–10 post the final immunization. Six mice per group were analyzed, and data are one representative of two independent experiments. Hair line represents the cut-off value of spontaneous killing.

Table 1
Summary and characteristics of pWHclm-, pCTLA-4-C-, and WHcAg-induced WHcAg-specific antibody and T cell immune responses.

Vaccine	pWHclm	pCTLA-4-C	WHcAg
Application route	i.m.	i.m.	s.c.
IgG subtype			
IgG1	−	+	+
IgG2a	+	+	+
CD8+IFN-γ+	++	±	−
CD4+IFN-γ+	+	+	−
***In vivo* killing**	+	−	−
Th bias	Th1 dominant	Th2 dominant	Th2 type

measuring degranulation and cytokine production using *ex vivo* staining to detect CD107a, IFN-γ, TNF-α, and IL-2. A rapid and strong CD8+ T cell recall response to WHcAg p13-21 developed by day 4 in mice immunized with pWHclm and decreased by days 7 and 10. The majority of WHcAg-specific CD8+ T cells were double positive for CD107a and IFN-γ (Fig. 2D). In mice immunized with pCTLA-4-C or WHcAg, WHcAg-specific CD8+ T cells were initially absent and appeared at lower levels with delayed kinetics after Ad5WHc administration. Detectable CD8+ T cell responses to WHcAg p13-21 arose on day 7 at a low frequency and decreased on day 10. Consistent with the results of the prime-boost immunization described above, mice that received the pCTLA-4-C DNA vaccine had higher levels of CD8+IFN-γ+ T cells than mice immunized with WHcAg. In all conditions, CD8+ T cells expressing IFN-γ, TNF-α, and IL-2 were found following Ad5WHc challenge (Fig. 2E), however, the phenotypes of specific CD8+ T cells could not be analysed in details due to the low frequencies.

Taken together, the nature and magnitude of WHcAg-specific recall T cell responses following Ad5WHc challenge in mice was strongly influenced by the preceding vaccinations. The strongest WHcAg-specific CD8+ T cell responses were detected in mice immunized with pWHclm.

Primed Th1 or Th2 type immune responses to WHcAg facilitated viral reduction after WHV challenge by HI

To assess the protective values of different types of immune responses, we established the HI model of WHV challenge in immunized mice. A plasmid, pBS/WHV-Sa1.3, contains a 1.3-fold overlength WHV genome was used (Pan et al., manuscript under

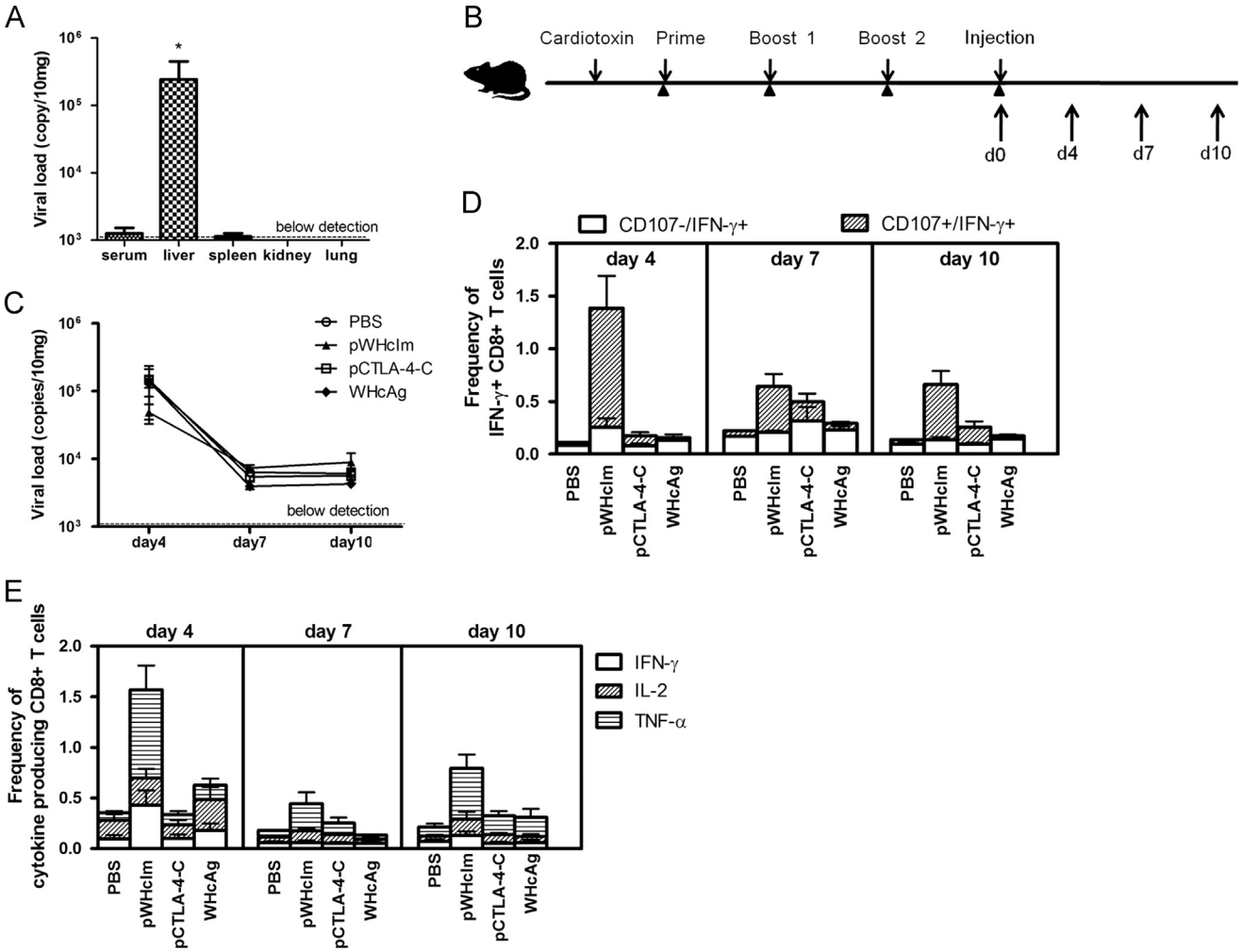

Fig. 2. Challenge with Ad5WHc, viral clearance, and CD8 T cell recall responses. (A) The distribution of Ad5WHc vector in different organs in naive mice 48 h post intravenously injection with 1×10^{10} pfu of Ad5WHc. (B) Experimental design of vaccination and challenge. Mice were immunized 3 times and then challenged with 1×10^{10} pfu of Ad5WHc on day 14 after the final immunization. Splenocytes were isolated on days 4, 7, and 10 and stimulated with p13-21 (2 μg/ml) for 5 h. (C) Kinetics of Ad5WHc clearance from the liver after challenge. (D) CD8+ T cells with respect to IFN-γ secretion and degranulation on days 4, 7, and 10 after Ad5WHc challenge. (E) Functional analysis of CD8+ T cells with respect to IFN-γ, IL-2, and TNF-α production on days 4, 7, and 10 after Ad5WHc challenge. Six mice per group were analyzed, and the error bar represents SEM from the mean value of duplicated experiments.

consideration). In this construct, the "a" determinant domain of WHsAg was replaced in the WHV genome with the corresponding region of HBsAg, resulting in a chimeric WHsAg that can be detected with HBsAg ELISA. HI of mice with 10 μg plasmid via the tail vein resulted in persistence of WHV for over 8 weeks, based on HBsAg assays (Fig. 3). Over 75% of mice of the control group were persistently positive for HBsAg and showed a very low or undetectable WHcAg-specific CD8+ T cell response until the end of experiment over 8 weeks. HBsAg and WHcAg were steadily expressed in the liver during the whole experimental period (data not shown).

Immunizations with pWHcIm and pCTLA4-C led to rapid decrease of HBsAg levels in mice after HI, though they primed two different types of WHcAg-specific immune responses (Fig. 3A and B). The majority of immunized mice were negative for serum HBsAg at day 3. Only one mouse in the pWHcIm group was positive for HBsAg on day 63. This mouse had low anti-WHcAg responses prior to HI (S/N of IgG1=1, IgG2a=2.3, no detectable CD8+ T cells in PBMCs). Thus, both Th1- and Th2-dominant immune responses to WHcAg led to efficient viral reduction and prevented the prolonged WHV persistence in mice, consistent with the previous results with HBV. The recall responses in the mice were monitored until week 5, and analyzed by detection of WHcAg-specific, IFN-γ secreting CD8+ T cells in PBMCs after short term *ex vivo* stimulation (Fig. 3C). The WHcAg-specific CD8+ T cell responses were variable across the individual mice. However, increased numbers of WHcAg-specific CD8+IFN-γ+ T cells were mainly detected on days 7–14 and days 28–35 (Fig. 3D). WHcAg-specific CD8+IFN-γ+ T cells appeared earlier in pWHcIm-immunized mice than in pCTLA-4-C immunized mice and were still detectable in the spleen or among infiltrating hepatic lymphocytes in some animals throughout the follow-up period, although the response were already very low on day 63. A summary of the data is given in Table 2. Thus, effective WHcAg-specific CD8+ T cell responses could be induced independently on the Th1/Th2 dominance of pre-primed immune responses, resulting finally in decrease of WHV replication.

Both pWHcIm and pCTLA-4-C vaccines reduced viral replication in WHV tg mice

DNA vaccination may induce specific CD8+ T cells and suppress WHV replication in WHV tg mice (Kosinska et al., 2013; Meng et al., 2014). We asked whether vaccination with pCTLA-4-C could also reduce WHV replication in WHV tg mice. WHV tg mice strain 1217 used in the experiments contains a wild-type WHV

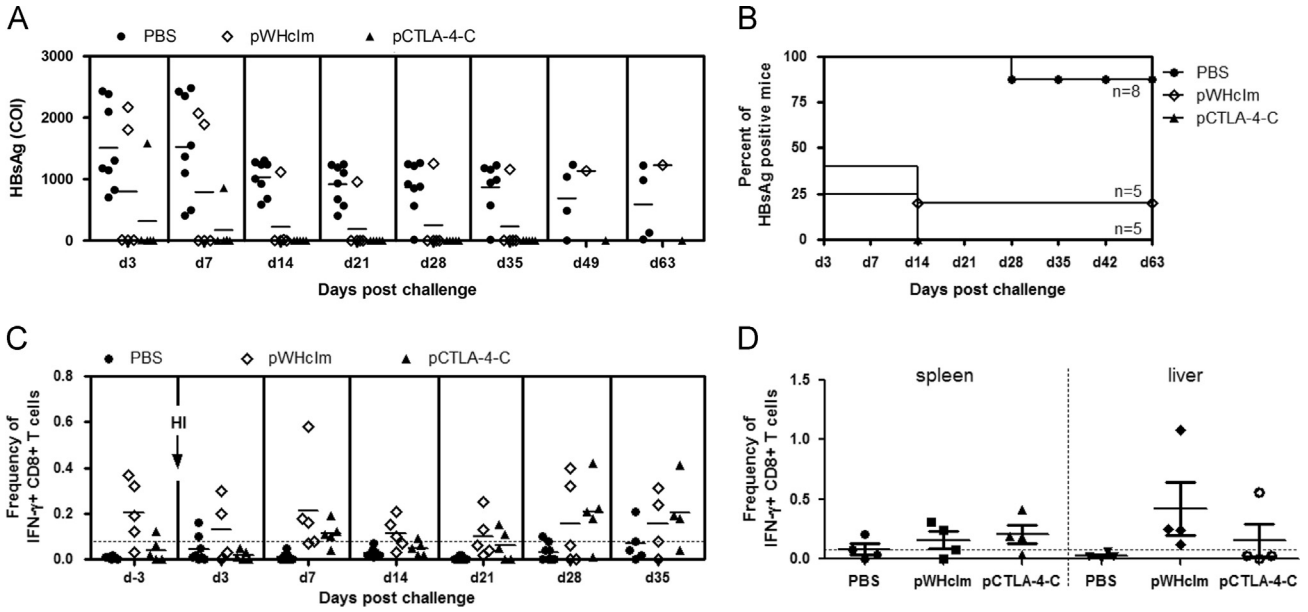

Fig. 3. Both pre-existing Th1- and Th2-dominant responses accelerated viral clearance in mice after HI challenge. Mice were immunized three times and then challenged with pBS/WHV-Sa1.3 replication-competent plasmid by HI on day 14 after the final immunization. Serum samples were taken from mice on the indicated time points to monitor serum HBsAg or CD8+ T cell response. PBMCs and intrahepatic lymphocytes were isolated on day 35. Splenocytes were analyzed following *in vitro* stimulation with p2 (2 μg/ml) for 7 days, while intrahepatic lymphocytes were stimulated with p13-21 (2 μg/ml) for 5 h. (A) and (B) Kinetics of HBsAg clearance following challenge of different plasmid pre-treated animals. (C) Kinetics of CD8+ T cell responses in PBMCs post challenge by HI. (D) CD8+ T cell responses in the spleen and liver on day 35 post challenge by HI. The error bar represents SEM from the mean percentage of CD8+ IFN-γ+ T cells. For each group 5–8 mice were included. Data are one representative of two independent experiments.

Table 2
Summary of immunological and virological parameters in mice after immunization and WHV HI challenge.

Vaccine group		PBS	pWHclm	pCTLA-4-C
Pre-challenge	anti-WHc IgG	IgG1 −, IgG2a −	IgG1 −, IgG2a +	IgG1+IgG2a +
	WHcAg-specific CD8 T cell response	−	+	±
Post-challenge	WHcAg-specific CD8 T cell response (PBMCs)	Gradual increase and peak at day 35	Peak at days 7–14 and 28–35	Peak at days 7–14 and 28–35
Follow-up days 35–63 post-challenge	Splenic WHcAg-specific CD8 T cell response	1/7*	2/5	3/5
	intrahepatic WHcAg-specific CD8 T cell response	0/7	4/5	1/5
	anti-HBs	0/7	0/5	0/5
	anti-WHc	0/7	5/5	5/5
	HBsAg in the liver	5/7	1/5	0/5
	WHcAg in the liver	5/7	0/5	0/5

* Indicates number of positive animals in total animals.

genome and showed serum WHV DNA loads of 10^6–10^7 genome equivalents/ml and were negative for anti-WHcAg antibody. They received three immunizations with pCDNA3, pWHclm, or pCTLA-4-C.

DNA vaccinations induced similar anti-WHcAg antibody profiles in WHV tg mice like in C57BL/6 mice shown in Fig. 1A (Fig. 4A). In contrast, the magnitude of the WHcAg-specific CD8+ T cell response in WHV tg mice after pWHclm immunization was significantly lower than that in wild type C57BL/6 mice shown in Fig. 1B (Fig. 4B). Interestingly, WHV tg mice immunized with pCTLA-4-C developed WHcAg-specific CD8+ T cells and, in part of mice, WHcAg-specific CD4+ cells (Fig. 4B). These data indicated that pCTLA-4-C-primed Th2-biased immune responses in WHV tg mice are similar to those induced in wild type C57BL/6 mice. WHcAg-specific T cells in WHV tg mice primed with pWHclm could also be detected by dimer staining and were able to produce IFN-γ and degranulate as shown by CD107a assay (Fig. 4C). In contrast, only few to no IFN-γ+ or CD107a+ T cells could be detected in WHV tg mice that received pCTLA-4-C, indicating that this DNA vaccine does not prime multifunctional CD8+ T cells in mice.

Finally, serum WHV DNA loads in WHV tg mice were determined before and after DNA vaccination. Application of plasmid DNA alone led to a slight decrease of serum WHV DNA (pcDNA3 group), likely due to unspecific triggering of host innate responses. WHV DNA was strongly reduced (> log3) and became undetectable in 3 of 10 WHV tg mice after pWHclm vaccination (Fig. 4D). Mice in the pCTLA-4-C group showed a weaker but significant decrease in WHV DNA loads (≈ log2, Fig. 4D). The WHV DNA and RNA levels in WHV tg mice were relatively low but were also slightly reduced after vaccination. These results indicated that both pWHclm and pCTLA-4-C DNA vaccines were able to elicit WHcAg-specific antibody and T cell responses that reduced WHV replication in WHV tg mice.

Discussion

In the present study, we tested three different vaccines to WHcAg and their combinations in the mouse models. Protein or DNA vaccines base on WHcAg were used according to their most compatible doses and routes of immunization according to the

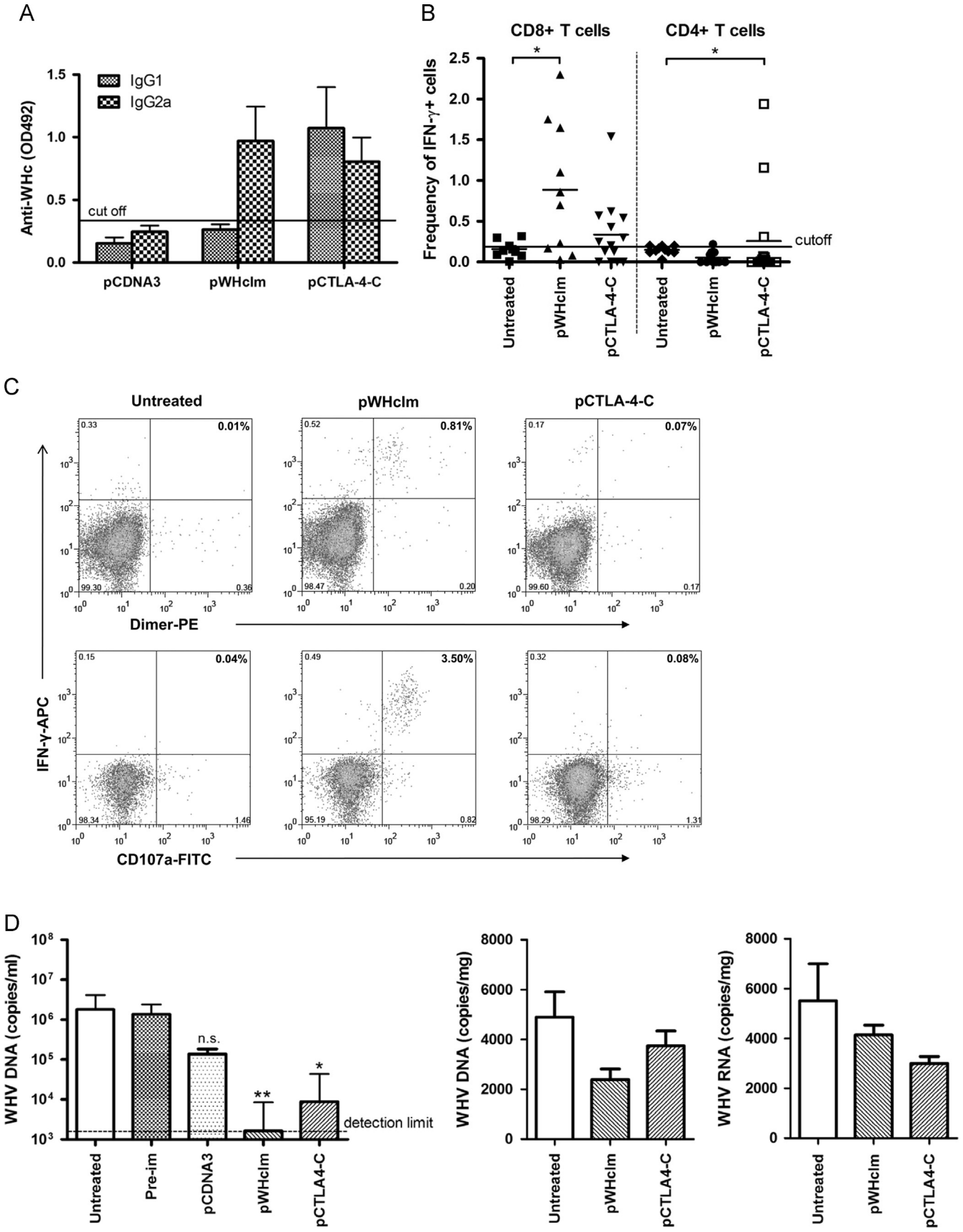

Fig. 4. pWHclm and pCTLA-4-C induced antigen-specific immune responses in WHV transgenic mice. WHV transgenic mice (strain 1217) were immunized three times and sacrificed 14 days after the final immunization. Splenocytes were isolated and stimulated with p2 or p27 (2 μg/ml) for 7days. Cells were restimulated with corresponding peptides and analysed following staining with antibodies to CD8, IFN-γ, CD107a, or p13-21 loaded H-2Db dimers. (A) Anti-WHc IgG subtypes in serum. (B) Frequency of IFN-γ-secreting CD8+ T cells to p2 and CD4+ T cells to p27. (C) Functional analysis of WHcAg-specific CD8+ T cells with respect to IFN-γ secretion and degranulation. (D) Comparison of serum WHV DNA viral loads, hepatic WHV DNA, and RNA levels before and after three immunizations with pWHclm or pCTLA-4-C. Pre-im, pre-immuization; dashed line, detection limit. The error bar represents SEM from the mean value. For each group 6–8 mice were included. Data were pooled from two independent experiments with similar results.

previous experiments. The vaccines used in this study induced WHcAg-specific immune responses with significant differences in the strength of CD8+ T-cell responses and antibody subtype ratios. Clearly, pWHcIm primed a typical Th1 dominant immune response, while pCTLA4-C and WHcAg protein induced immune responses with Th2 bias. The different features of the immune responses induced by protein and DNA vaccines may be partly a result of the route of immunization and the way of antigen presentation, as plasmid and protein vaccines are differently presented by DC and thereby promote different types of T helper cells. As shown in the previous studies, all three vaccines conferred protection against WHV challenge in woodchucks (Lu et al., 2005; Menne et al., 1997; Schodel et al., 1993b). Our results showed that WHcAg protein based vaccine did not induce any detectable CD8+ T cell response, thus, the recall response after WHV challenge may be critical to control viral infection in immunized woodchucks.

Our immunizations were carried out in mice without pre-existing anti-WHc antibodies. In chronically HBV-infected patients or WHV-infected animals, anti-HBc and anti-WHc antibodies are present, respectively. In such cases, therapeutic vaccines may boost the pre-existing antibody responses, similarly to the situation to boost vaccination in individuals who received initial doses of vaccines and developed antibody responses. In our experiments, mice also developed anti-WHc after initial vaccination, so all boost vaccinations were carried out under the conditions with pre-existing anti-WHc antibodies. Our results presented in Supplement Fig. 3 showed clearly that the pre-existing anti-WHc antibody responses may influence the late vaccinations while the late vaccinations could also change the nature of anti-WHc antibody responses.

We carefully analysed recall responses in our mouse models. Challenge with a replication-incompetent adenoviral vector expressing WHcAg allowed for a more detailed characterization of antigen-specific T cell responses after re-exposure to WHcAg. In all mice vaccinated against WHcAg, WHcAg-specific CD8+ T cells were expanded after challenge. This expansion occurred rapidly in mice with pre-existing Th1-dominant responses but with delayed kinetics in mice with Th2-dominant and Th2 type responses. Moreover, WHcAg-specific CD8+ T cells from the three groups displayed different phenotypes. The majority of WHcAg-specific CD8+ T cells produced IFN-γ but did not show degranulation. Thus, the pre-existing Th2-biased immune responses do not prevent induction of the CD8+T cell response but influence its kinetics and functionality. Despite these differences in the recall responses, both Th1- and Th2-dominant responses were protective against WHV challenge by HI. These results provide an explanation for how a vaccine that induces Th2-biased responses could protect against hepadnaviral infection, as has been observed in chimpanzees and woodchucks (Menne et al., 1997; Murray et al., 2005).

Previously, we assessed the protective values of the immune responses primed by two different DNA vaccines pHBc and pCTLA-4-HBc that are the equivalents to pWHcIm and pCTLA4-C. Mice immunized with pHBc and pCTLA-4-HBc developed Th1- and -2 biased immune responses to HBcAg, respectively. Challenge by HI of pAAV/HBV1.2 showed that HBsAg clearance was completed within 16 days in immunized mice, while HBsAg still remained to be detectable in more than 50% of the control mice on day 22. Stronger HBcAg-specific T-cell responses were primed by pHBc correlating with a more rapid decline of intrahepatic HBcAg expression, while anti-HBsAg antibody response developed rapidly in the mice immunized with pCTLA-4-HBc. Therefore, the Th1/Th2 bias of vaccine-primed immune responses did not significantly change the kinetic but influences the mode of viral clearance. Consistent with the published study, we showed that both pWHcIm and pCTLA-4-C immunizations led to a decrease of WHV replication after HI challenge in mice. Here, we further studied the relationship between the pre-primed immune responses and the mode of viral clearance. We refined the analysis of the recall responses to determine the specific CD8+ T cell responses to WHcAg after the challenge. WHcAg-specific CD8+IFN-γ+ T cells were present in peripheral blood of pWHcIm-immunized mice but decreased shortly after HI, likely due to the recruitment to the liver. Such T cells reappeared usually at day 7 or 14 and remained detectable through the experimental period. Interestingly, the pCTLA-4-C immunized mice also developed significant WHcAg-specific CD8+IFN-γ+ T cells showed at later time points. WHcAg-specific CD8+IFN-γ+ T cells were detectable in the liver of some mice of both immunized groups. Again, the Th2 bias of pCTLA-4-C primed immune responses in mice did not delay viral control after HI but change the kinetics of WHcAg-specific CD8+IFN-γ+ T cell responses.

Surprisingly, immunization of WHV tg mice with pCTLA-4-C induced significant WHcAg-specific CD8+ T cell responses. Clearly, these CD8+ T cells were functionally limited compared to those induced by pWHcIm. They may only be able to produce IFN-γ or to degranulate but are not multifunctional. Nevertheless, a significant reduction in WHV DNA loads occurred after immunizations of mice with pCTLA-4-C. In future experiments, it would be interesting to examine the properties of the antigen-specific T cells and their antiviral functions in detail. Such studies may help us to understand why the functions of antigen-specific CD8+ T cells in chronic HBV and WHV infections are limited. In some mice, pCTLA-4-C induced CD4+ T cells, which may contribute to the therapeutic effect.

In this study, we tested different prime-boost immunizations combining DNA and protein vaccines. The results were rather discouraging as such combinations did not show obvious advantage. In this experiment, Th1-biased vaccines could boost antigen-specific CD8+ T cell immune responses in the presence of pre-primed Th2-biased immune responses, but with significantly reduced effectiveness. In a recent study, we used DNA vaccination-adenoviral vector boost and observed great improvement of specific CD8+ T-cell responses (Kosinska et al., 2012). Certainly, the usefulness of combined DNA and protein vaccines needs to be tested from case to case. The findings in the present study do not have an immediate impact on clinical practice but add some data to our understanding about the viral control by vaccination.

In conclusion, we primed defined antigen-specific immune responses with Th1 and Th2 biases. We demonstrated that pre-existing immune responses strongly influenced the functionality and kinetics of the antigen-specific T cells in recall responses. Clearly, vaccines that prime Th1-dominant immune responses induced more efficiently multifunctional CD8+ T cells in immune-tolerant hosts and are therefore suitable as therapeutic vaccines. However, vaccines that prime Th2-biased immune responses to HBV proteins could be equally effective as prophylactic vaccines. As such vaccines are able to induce stronger CD4+ T cell responses and provide so call “intermolecular help” to support broad immune responses against other viral components, its value in the therapeutic setting needs more attention in the future. Nevertheless, both types of vaccines are able to induce specific immune responses and contribute to the antiviral actions.

Materials and methods

Plasmids, protein, and peptides

The pWHcIm and pCTLA-4-C plasmids used for DNA vaccination were described previously (Lu et al., 2005). Plasmid pWHcIm

contains the WHV C region of WHV8 in pcDNA3.1 vector, while pCTLA-4-C contains the coding regions for the extracellular domain of woodchuck CTLA-4 fused with WHV C coding sequence. WHcAg protein was expressed in E. coli and purified by chromatography with Sepharose 6 and sucrose gradient centrifugation (Zhang et al., 2006). WHcAg-derived peptides were purchased from EMC microcollections (Tubingen, Germany) and were described previously (Kosinska et al., 2012). For stimulation of murine lymphocytes, the following two 15-mer and single 9-mer peptides were used: p2 (aa 6–20, YKEFGSSYQLLNFLP), containing a CD8+ T cell epitope; p27 (aa131–145, PYRPPNAPILSTLPE), containing a CD4+ T cell epitope; and p13-21 (YQLLNFLPS), the precise D^b-restricted CD8+ T cell epitope present within p2. An unrelated cytomegalovirus (CMV)-derived peptide (YILEETSVM) served as a negative control.

Animal experiments

C57BL/6 mice were purchased from Harlan Laboratories Inc., Germany, or the Animal Center of Beijing province, China. WHV transgenic mice (strain 1217) were generated and bred in the Central Animal Laboratory of University Hospital Essen (Meng et al., 2014). The Tg mice strain 1217 harbors a wild-type WHV genome and show sex- and age-dependent viral replication in the liver. The 1217 tg mice were also partially immune tolerant to WHV antigens. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the local Animal Care and Use Committee (Central Animal Laboratory, University of Duisburg-Essen, Essen, Germany, and the district government of Düsseldorf, Germany; or Tongji Medical College, Wuhan, China). All mice were females and at 8–10 weeks of age at the time the experiments were initiated.

Immunization of mice

For plasmid DNA immunization, mice were pretreated with 50 μl cardiotoxin (10 μM) via intramuscular injection into M. tibialis anterior. After 1 week, 50 μg of plasmids in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml was injected into the same muscle of both legs. The control mice received 100 μl PBS. For WHV transgenic mice, the controls received 100 μl of the empty vector pCDNA3 using the technique described above. For WHcAg protein immunizations, 10 μg protein was diluted into 50 μl PBS and emulsified in an equal volume of Freund's incomplete adjuvant. The protein emulsion was administered by subcutaneous injection at 4–5 points on the back. The immunization was repeated twice at 2-week intervals. The mice were sacrificed 2 weeks after the final immunization. A minimum of 8 mice were included in each group.

Serology and detection of WHV DNA

Antibodies to WHcAg (anti-WHcAg) of the IgG, IgG1, and IgG2a subtypes were detected in mouse serum samplesby specific enzyme linked immunosorbent assays (ELISAs) as described previously (Lu et al., 2005). Serum HBsAg and antibodies to HBsAg (anti-HBsAg) were detected by ELISA using commercial diagnostic kits (Kehua, Shanghai, China). The cut-off value was set as 2.1-fold the mean value of the negative controls. For detection of serum WHV DNA, total DNA was then extracted using the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Germany). Each serum sample was pretreated with 30 units of DNase I (TAKARA, Japan) at 37 °C overnight to eliminate residual plasmid DNA used for HI before total DNA was extracted. WHV DNA was detected by real-time PCR as described previously (Lu et al., 2005; Yin et al., 2011). The primers used for PCR are listed in Table 3 (Frank et al., 2007; Kosinska et al., 2012; Lu et al., 2005).

Detection of WHV DNA and RNA in liver tissue

Mice were sacrificed and liver tissue was frozen in liquid nitrogen. Total liver DNA was isolated using the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Germany). Total RNA was extracted from liver tissue samples with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. WHV RNA and DNA were detected by real time-PCR and real time RT-PCR as described previously (Frank et al., 2007).

Preparation and stimulation of mouse PBMCs and splenocytes

Splenocyte suspensions were prepared by homogenization and cultured as described previously (Dietze et al., 2011). Up to $1\text{-}2 \times 10^6$ splenocytes per well were plated in 96-well plates. For isolation of mouse PBMCs, each 100 μl sample of anticoagulant-treated blood was mixed with 1 ml Buffer EL (Qiagen, Germany) for 5 min to lyse red blood cells, washed twice with 50 ml PBS, resuspended in 100 μl complete RPMI1640 medium, and then seeded in a single well of a 96-well plate. Individual peptides were added to the wells at a final concentration of 2 μg/ml. For *in vitro* stimulation, splenocytes were incubated with peptides p2 or p27 and recombinant mouse IL-2 (10 U/ml) added 3 days after plating and then restimulated after 6–7 days. For *ex vivo* analysis, PBMCs or splenocytes were stimulated with p13-21 (2 μg/ml) in the presence of Brefeldin A (BFA, 4 μg/ml) for 5 h.

Flow cytometry with cell surface and intracellular cytokine staining

Cell surface staining was performed using BD Biosciences or eBioscience reagents (Dietze et al., 2011; Zelinskyy et al., 2011). The T cell antibodies used were anti-CD4 (H129.19), anti-CD8 (53-6.7), anti-CD43 (eBioR2/60), and anti-CD107a (1D4B). Dead cells were excluded from the analysis by 7-AAD staining (BD Biosciences). Next, cells were washed, permeabilized using the Cytofix/Cytoperm intracellular staining kit (BD Biosciences), and stained with cytokine-specific mAbs including IFN-γ (XMG1.2), IL-2 (JES6-5H4), and TNF-α (MP6-XT22).

Mouse DimerX (Recombinant Soluble Dimeric H-2Db:Ig Fusion Protein)was purchased from BD Biosciences. DimerX was preloaded with p13-21 at 37 °C overnight and incubated with splenocytes or PBMCs at 4 °C for 1 h to facilitate its binding to the p13-21-specific CD8+ T cells. Afterwards, cells were stained with an anti-mouse IgG-PE (BD Biosciences) that specifically recognises DimerX.

Flow cytometry data were acquired on FACS Caliburor or FACS LSR II flow cytometers (BD Biosciences), with 100,000–150,000 lymphocyte-gated events collected per sample. Analyses were performed using FlowJo (Tree Star) software.

In vivo cytotoxicity assay

A modified version of the *in vivo* CTL assay described by Barber et al. was used to measure cytotoxicity in vaccinated mice (Barber andWherry and Ahmed, 2003; Dietze et al., 2011). Splenocytes from naïve mice were loaded with 4 μM p13-21 and stained with 36 nM 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen). As a control, unloaded splenocytes were stained with 9 nM CFSE. Each of 1×10^7 loaded or unloaded splenocytes were mixed and transferred intravenously into naïve or vaccinated mice. Eight hours after the transfer, spleens and blood were harvested from recipient mice and cell suspensions prepared from the tissues. Target cells were distinguished from recipient cells

Table 3
Primers for real-time-PCR detection.

		Sequence	Position	Refs.
WHV	Sense	5′-TGGGGCCATGGACATAGATCCTTA-3′	nt2015[a]	[15,18,29]
	Antisense	5′-AAGATCTCTAAATGACTGTATGTTCCG-3′	nt2467[a]	
Adenoviral	AQ1	5′-GCCACGGTGGGGTTTCTAAACTT-3′	nt18993[b]	[22,30]
	AQ2	5′-GCCCCAGTGGTCTTACATGCACATC-3′	nt18862[b]	
	Probe	5′-GFAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-TMR-3′	nt18930[b]	

[a] Numbering of the Sequence is according to J04514.
[b] Numbering of the Sequence is according to M73260.

based on CFSE staining. The percentage of killing was calculated using the following equation: $100-([(\%$ peptide-loaded cells in vaccinated mice/% unloaded cells in vaccinated mice)/(% peptide-loaded cells in naïve mice/% unloaded cells in naïve mice)$]\times 100$).

Challenge with a recombinant adenoviral vector expressing WHcAg

A recombinant adenoviral vector expressing WHcAg (Ad5WHc) was generated previously (Kosinska et al., 2012). To infect mice, 1×10^{10} pfu of Ad5WHc were injected intravenously. For detection of adenoviral DNA, total DNA was extracted from 200 µl of mouse serum samples or 10 µg of liver tissues with the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Adenoviral DNA was detected by TaqMAN real-time PCR using the primers listed in Table 3 (Berciaud et al., 2012; Heim et al., 2003) with the LightCycler® FastStart DNA Master HybProbe kit (Roche, USA).

WHV challenge by hydrodynamic injection (HI) of pBS/WHV-Sa1.3 plasmid

The plasmid pBS/WHV-Sa1.3 containing a 1.3-fold overlength WHV genome was constructed in our laboratory (Pan et al., under consideration). The "a"-determinant of WHsAg was replaced with the corresponding fragment from HBsAg, resulting in a chimeric WHsAg that is reactive in an HBsAg-specific ELISA. On day 14 after the final immunization, 10 µg of pBS/WHV-Sa1.3 diluted in a 0.9% NaCl solution was injected into the tail veins of mice in a volume equivalent to 8% of each mouse's body weight (Berciaud et al., 2012). The total volume was injected within 8 s. Serum specimens were collected and assayed for HBsAg, anti-HBs and anti-WHcAg antibodies, and WHV DNA at the indicated times. Mice were sacrificed 8 weeks after HI. The splenocytes and liver-infiltrating lymphocytes were isolated for intracellular cytokine staining (Yang et al., 2010).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA). Statistical differences were analyzed with ANOVA One Way analysis of variance followed by Bonferroni's Multiple Comparison Test. The *P*-values <0.05 were considered significant.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.11.004.

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