Prophylactic and therapeutic HBV vaccination by an HBs-expressing cytomegalovirus vector lacking an interferon antagonist in mice

Hongming Huang*1, Meike Rückborn*2, Vu Thuy Khanh Le-Trilling2, Dan Zhu1, Shangqing Yang1, Wenging Zhou1, Xuecheng Yang1, Xuemei Peng1, Yiming Lu1, Mengji Lu2, Ulf Dittmer2, Dongliang Yang1, Mirko Trilling*2 and Jia Liu*1

1 Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
2 Institute for Virology, University Hospital of Essen, University of Duisburg-Essen, Essen, Germany

Cytomegalovirus (CMV)-based vaccines show promising effects against chronic infections in nonhuman primates. Therefore, we examined the potential of hepatitis B virus (HBV) vaccines based on mouse CMV (MCMV) vectors expressing the small HBsAg. Immunological consequences of vaccine virus attenuation were addressed by either replacing the dispensable gene m157 (“MCMV-HBs”) or the gene M27 (“ΔM27-HBs”), the latter encodes a potent IFN antagonist targeting the transcription factor STAT2. M27 was chosen, since human CMV encodes an analogous gene product, which also induced proteasomal STAT2 degradation by exploiting Cullin RING ubiquitin ligases. Vaccinated mice were challenged with HBV through hydrodynamic injection. MCMV-HBs and ΔM27-HBs vaccination achieved accelerated HBV clearance in serum and liver as well as robust HBV-specific CD8+ T-cell responses. When we explored the therapeutic potential of MCMV-based vaccines, especially the combination of ΔM27-HBs prime and DNA boost vaccination resulted in increased intrahepatic HBs-specific CD8+ T-cell responses and HBV clearance in persistently infected mice. Our results demonstrated that vaccines based on a replication competent MCMV attenuated through the deletion of an IFN antagonist targeting STAT2 elicit robust anti-HBV immune responses and mediate HBV clearance in mice in prophylactic and therapeutic immunization regimes.

Keywords: cytomegalovirus · HBsAg · hepatitis B virus · interferon · vaccine

Introduction

More than 2 billion individuals have been infected with hepatitis B virus (HBV) worldwide. The prophylactic HBV vaccine is a tremendous medical success, which saved countless lives. However, due to its inability to confer therapeutic protection, chronic HBV infections remain a major public health issue affecting approximately 250 million individuals [1]. Persisting
HBV predisposes to end-stage liver diseases, such as liver cirrhosis and hepatocellular carcinoma. According to the WHO, HBV is responsible for more than 850,000 deaths per year (https://www.who.int/news-room/fact-sheets/detail/hepatitis-b) [2]. Two types of antiviral strategies are currently available for chronic hepatitis B (CHB): PEGylated IFN alpha 2 (PEG-IFN-α2) and nucleos(t)ide analogues (NUC), such as entecavir and tenofovir. However, both are suboptimal. The treatment with PEG-IFNα2 is associated with significant side effects (e.g., flu-like symptoms, mood disorders, and depression), and long-term virus clearance is limited to approximately one-third of treated patients [3]. NUC medication selects for viral resistance mutations and is hampered by frequent episodes of rebounding viremia after cessation of antiviral therapy [4]. Therefore, alternative strategies for the treatment of chronic HBV infection are urgently needed.

The clearance of HBV by the immune system relies on a potent and broad T-cell immune response, which usually becomes dysregulated during chronic HBV infection [5–7]. This exhaustion of HBV-specific T cells is believed to be one major reason for the inability of the host to eliminate the persisting pathogen. Aiming to enhance the patient’s own antiviral cellular immune response by therapeutic vaccination is considered a promising strategy. During the last two decades, countless attempts have been made to establish an effective therapeutic vaccine against CHB [8]. For example, existing protein-based prophylactic vaccines have been given to chronically infected patients in order to restore HBV-specific immunity. Unfortunately, it turned out to be unsuccessful [9, 10]. An antigen–antibody (HBsAg–HBIG) immune complex termed YIC initially showed promising results in preclinical models and in phases IIA and IIB clinical trials [11, 12]. However, the results of a phase III clinical trial enrolling 450 patients were disappointing [13]. A number of DNA-based vaccine regimens have shown promising efficacy in preclinical animal models, but also failed to generate effective therapeutic responses in humans [8, 14]. Therefore, replicating virus-based vectors, which stimulate a broad range of immune responses including T-cell-mediated immunity, gained increasing attention in the field of therapeutic HBV vaccine development. In particular, vectors based on adenoviruses [15], modified vaccinia virus Ankara [16], and recombinant vesicular stomatitis virus [17] are under investigation for treatment of CHB.

Recently, cytomegalovirus (CMV)-based vectors emerged as exciting platform for vaccines against infectious diseases and cancer [18]. Rhesus CMV (RhCMV)-based vaccines provided protective immunity to rhesus macaques against simian immunodeficiency virus (SIV), ebolavirus [19], and tuberculosis [20]. Successfully RhCMV-vaccinated animals, even a CD8+ T-cell depletion did not result in detectable SIV rebound, indicating very efficient virus control [21, 22]. Vaccination with RhCMV vectors elicits robust and long-lasting cellular immune responses against pathogens mediated by effector-memory CD8+ T cells [23]. Importantly, RhCMV-based vaccines demonstrated their efficacy even in the presence of preexisting immunity against CMV [24]. These findings prompted us to evaluate the potential of CMV as platform for prophylactic and therapeutic vaccines against CHB in the established HBV hydrodynamic injection (HDI) mouse model. Our recent findings indicate that the interferon (IFN) antagonism of CMVs relies on viral proteins targeting the transcription factor STAT2 for proteasomal degradation by exploiting the adapter protein DDB1 and cellular Cullin RING ubiquitin ligases [25–27]. Virus mutants lacking these immune evasins are replication competent but highly attenuated in vivo [28]. The protein pM27 mediates STAT2 degradation in the MCMV context, while human CMV (HCMV) encodes the protein pUL145 which acts as a functional analog of pM27 [29]. The proteins pM27 and pUL145 share a functionally relevant H-box motif present in viral and cellular DDB1 Cullin-associated factors. Based on these findings, we reasoned that the attenuation of CMVs through the loss of STAT2 antagonists may represent a promising approach to establish novel platforms for the vaccination against viruses such as HBV.

Results

Construction and characterization of a recombinant MCMV expressing the HBV small surface antigen

In order to evaluate the vaccine performance of CMV-based vaccine vectors in small animal models, we inserted an expression cassette comprising the coding sequence of the small HBsAg (sHBsAg; YP_009173871.1; corresponding to serotype “ayw” HBV genotype D) under the control of the strong eukaryotic EF1 promoter into the mouse CMV (MCMV) genome by site-specific recombination. The expression cassette was introduced into an MCMV bacterial artificial chromosome (BAC) harboring a deletion of the coding sequences of m157 as schematically depicted in Fig. 1A and described in the Methods section. The gene m157 is dispensable for MCMV replication in vitro and in vivo [30]. To generate a live-attenuated vaccine virus, the expression cassette was also inserted into an MCMV mutant genome lacking the gene coding for the IFN antagonist pM27. The protein M27 interferes with IFN signaling by inducing ubiquitination and proteasomal degradation of STAT2 [26]. M27-deficient MCMVs (“ΔM27-Ctrl”) exhibit a slightly impaired (approximately tenfold) replication in the absence of exogenous IFN treatment in vitro, but are severely attenuated in vivo [31, 32] in a STAT2-dependent manner [28]. Correct mutagenesis was verified and HBsAg expression was confirmed by immunoblotting (Fig. 1B). As expected from previous work [33, 34] and the presence of an N-glycosylation site within the S domain of sHBsAg (Asn-146; sequence NcT), we observed two sHBsAg protein forms (Fig. 1B), most likely reflecting the previously described nonglycosylated p24 and the glycosylated gp27. The insertion of sHBsAg did not impair the replication of MCMV in cell culture (Fig. 1C). In vivo, we observed a trend toward reduced MCMV replication upon insertion of the sHBsAg expression cassette (Fig. 1D). As expected from our previous work, the deletion of M27 resulted in a reduced replication in vitro (Fig. 1C) and a pronounced attenuation in vivo (Fig. 1D). Taken together, we generated two MCMV-based vectors expressing sHBsAg, one of
Figure 1. Generation and validation of HBsAg-expressing MCMV vectors. (A) Schematic overview of the cloning strategy for the generation of the HBsAg-expressing MCMV vectors. Please see the Materials and Methods section for details. (B) HBsAg expression was confirmed by immunoblot. The housekeeping protein β-Actin served as loading control. The expression of HBsAg was confirmed by western blot analysis in more than three independent experiments. (C) The in vitro replication competence of MCMV vectors as well as corresponding parental viruses was determined by classic plaque titration. Mouse newborn cells were infected (0.05 PFU/cell) with indicated MCMVs. At indicated time points, cells and supernatants were frozen and stored at −80°C until all samples were titrated simultaneously. Virus titer determinations were performed in three independent experiments each titrated in triplicates. Data are shown as mean ± SD. (D) The in vivo replication competence of the HBsAg-expressing MCMVs and wt-MCMV was determined in salivary glands at 21 days post i.p. infection of C57BL/6 mice (n = 6 mice per group) by classic plaque titration of organ homogenates. Data are pooled from two independent in vivo experiments. Data are shown as geometric mean ± SD. Statistical significance was tested using a one-way ANOVA test. *p < 0.05.

which is severely attenuated in vivo due to the deletion of the MCMV-encoded STAT2 antagonist M27.

Vaccination with a recombinant MCMV expressing HBsAg protects mice against HBV challenge

We explored the protective effects of MCMV-HBs vaccination against an HBV challenge in the established HBV HDI mouse model. As schematically depicted in Fig. 2A, C57BL/6 mice were inoculated twice with MCMV-HBs, and then challenged with an HBV-expressing plasmid through HDI (see the Methods section for experimental details). Since the MCMV infection itself can negatively influence other infections [35] including HBV [36], the parental MCMV ("MCMV") devoid of the HBsAg expression cassette was included as negative control. While mice infected with empty MCMV exhibited continuous HBV viremia, the clearance of serum HBsAg, HBeAg, and HBV DNA was significantly accelerated in mice that received the MCMV-HBs vaccine (Fig. 2B). All MCMV-HBs-vaccinated mice became negative for serum HBsAg and HBeAg. Eighty percent of mice even became HBV DNA-negative at 9 days post HDI (dpi), while all control mice remained viremic for HBsAg, HBeAg, and HBV DNA (Fig. 2B and C). The MCMV-HBs-vaccinated mice also cleared HBsAg and HBcAg from the liver at 10 dpi, while the control mice expressed high levels of HBsAg and HBcAg, as evident by immune-histochemical staining (Fig. 2D and E).

Vaccination with a recombinant MCMV expressing HBsAg raises potent CD8+ T-cell responses

Next, we examined the impact of MCMV-HBs vaccination on immune responses. No anti-HBsAg antibodies were detectable in serum until day 7 after HDI for MCMV-HBs vaccination and day 9 after HDI for wt-MCMV vaccination (data not shown).
Figure 2. An HBsAg-expressing MCMV protects mice against HBV challenge. (A) Schematic overview of the experimental setup: C57BL/6J naïve mice were immunized intraperitoneally with $2 \times 10^5$ PFU of wt-MCMV or MCMV-HBs at day 0 and day 21 and were challenged with HBV plasmid pSM2 via hydrodynamic injection (HI) at day 42. The mice were sacrificed after HBV clearance in serum at 52 dpi, 10 days after HI. (B) The concentrations and positive rates of serum HBsAg and HBeAg levels were monitored by ELISA at indicated time points. (C) The concentrations and positive rates of serum HBV DNA levels were monitored by real-time PCR at indicated time points. The quantification of HBV DNA was performed by using the standard curve created with the negative control and four HBV DNA standards provided by the diagnostic kit (Sansure, Changsha, China) according to the manufacturer’s instructions. (D) Immunohistochemical staining of HBsAg in the livers of wt-MCMV- and MCMV-HBs-immunized mice. Left: Calculation of the average numbers of HBsAg-positive hepatocytes per field of vision. Right: Representative staining of a liver section (original magnification: $200 \times$, scale bar = 50 μm). (E) Immunohistochemical staining of HBcAg in the livers of wt-MCMV- and MCMV-HBs-immunized mice. Left: Calculation of the average numbers of HBcAg-positive hepatocytes per field of vision. Right: Representative staining of a liver section (original magnification: $200 \times$, scale bar = 50 μm). Data are depicted as arithmetic means ± SEM, and experiments were repeated three times ($n = 5$ mice per group). The statistical analysis was performed by log-rank test (B and C) or unpaired t test (D and E). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. dpi, days postinfection.

However, since the HBV challenge is administered in form of a DNA molecule in the HDI mouse model, we reasoned that protection cannot be explained by neutralizing antibodies. Therefore, we focused our attention on T-cell responses. We addressed intrahepatic T-cell infiltration, activation, and HBV-specific CD8 T-cell immune responses. In terms of frequency and absolute numbers, MCMV-HBs-vaccinated mice showed significantly higher CD8$^+$ T-cell numbers in the liver, while CD4$^+$ T cells remained largely unaffected (Fig. 3A). A phenotypic analysis revealed that PD-1, but not CD43, expression on CD8$^+$ T cells in the liver of MCMV-HBs-vaccinated mice was significantly increased compared to control mice (Fig. 3B and C). Since PD-1 is an activation marker for CD8$^+$ T cells during acute virus infection [37], this result suggests that MCMV-HBs vaccination enhanced the CD8$^+$ T-cell activation in the liver of HBV-challenged mice. In the liver, the MCMV-HBs-vaccinated mice showed a significant increase in absolute numbers and frequencies of CD8$^+$ T cells specific for HBsAg, but not HBcAg, compared to control mice, as indicated by cyometry using Env190- and Core93-dimers, respectively (Fig. 3D and E). Accordingly, significantly higher percentages
Figure 3. MCMV-HBs vaccination enhances the intrahepatic HBsAg-specific CD8 T-cell responses. (A) Numbers, frequencies, and representative flow cytometry plots of CD4+ and CD8+ T cells among liver infiltrating lymphocytes in mice immunized with MCMV-HBs or wt-MCMV. (B) Numbers, frequencies, and representative flow cytometry plots of PD1+ cells among CD8+ T cells in mice immunized with MCMV-HBs or wt-MCMV. (C) Numbers, frequencies, and representative flow cytometry plots of CD43+CD8+ T cells among CD8+ T cells in mice immunized with MCMV-HBs or MCMV. (D) Numbers, frequencies, and representative flow cytometry plots of HBV Env190-specific CD8+ T cells. (E) Numbers, frequencies, and representative flow cytometry plots of HBV Core93-specific CD8+ T cells. (F) Numbers, frequencies, and representative flow cytometry plots of HBV Env190-specific cytokine-positive CD8+ T cells after in vitro stimulation with Env190 peptide. (G) Numbers, frequencies, and representative flow cytometry plots of HBV Core93-specific cytokine-positive CD8+ T cells after in vitro stimulation with Core93 peptide. Data were replicated in at least three independent experiments (*n* = 5 mice per group). Data are depicted as arithmetic mean ± SEM.*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. A unpaired *t*-test was used to assess statistical significance; Core93, core93-100; Env190, env190-197.

and absolute numbers of CD8 T cells in the livers of MCMV-HBs-vaccinated mice produced IFN-γ or IL-2 in response to stimulation with the HBsAg epitope peptide (Env190), but not HBcAg epitope peptide (Core93) (Fig. 3F and G), suggesting that MCMV-HBs specifically enhanced HBsAg-specific CD8+ T-cell responses.

**Vaccination with ΔM27-HBs protects mice against HBV challenge**

Even if cytomegaloviral vectors may be excellent vectors based on their ability to induce very strong T-cell responses against foreign antigens, the use of replication competent HCMV-based vectors in humans is limited by their pathogenicity especially for immunocompromised individuals. One option to circumvent this safety issue is the use of live-attenuated CMV mutants [38], which may be achieved by different means. Therefore, we explored the potential of an HBsAg-expressing MCMV vector, which is replication competent in cell culture, but highly attenuated in vivo due to the inability to counteract STAT2-dependent IFN signaling resulting from the lack of the gene product pM27 [28, 31, 32]. C57BL/6 mice were inoculated twice with either empty ΔM27-MCMV (“ΔM27-Ctrl”) or ΔM27-MCMV expressing HBsAg, for convenience denoted “ΔM27-HBs” thereafter, and then challenged with an HBV-expressing plasmid through HDI. We also included PBS-treated mice as negative control to evaluate the protective effect of ΔM27-HBs (Fig. 4A). The clearance of serum HBsAg, HBcAg, and HBV DNA was significantly accelerated in mice vaccinated with ΔM27-HBs as compared to those which received PBS or the empty ΔM27-Ctrl (Fig. 4B and C). All ΔM27-HBs-vaccinated mice became serum HBsAg negative at 9 dpi, while the PBS-treated and ΔM27-Ctrl-infected mice remained 100% positive for serum HBsAg at this time point (Fig. 4B). Eighty percent of ΔM27-HBs-vaccinated mice also became HBcAg negative and 60% became serum HBV DNA negative at 9 dpi. In contrast, none of the mice from the PBS-treated or ΔM27-Ctrl-infected control groups cleared HBcAg or HBV DNA (Fig. 4C). The ΔM27-HBs-vaccinated mice were also the only animals that cleared HBsAg and HBcAg from the liver as evident by diminished immunohistochemical staining (Fig. 4D and E).
Figure 4. ΔM27-HBs vaccination protects mice against HBV challenge in mice. (A) Schematic overview of the experimental setup: C57BL/6J naïve mice were immunized intraperitoneally with $2 \times 10^5$ PFU of ΔM27-Ctrl, ΔM27-HBs or PBS in a volume of 100 μL at days 0 and 21, and were challenged with the HBV plasmid pSM2 via hydrodynamic injection (HI) at day 42. The mice were sacrificed after HBV clearance in serum at 52 dpi, 10 days after HI. (B) The concentrations and positive rates of serum HBsAg and HBeAg levels were monitored by ELISA at indicated time points. (C) The concentrations and positive rates of serum HBV DNA levels were monitored by real-time PCR at indicated time points. The quantification of HBV DNA was performed by using the standard curve created with the negative control and four HBV DNA standards provided by the diagnostic kit (Sansure, Changsha, China) according to the manufacturer’s instructions. (D) Immunohistochemical staining of HBsAg in the livers of PBS-, ΔM27-Ctrl-, and ΔM27-HBs-immunized mice. Left: Calculation of the average numbers of HBsAg-positive hepatocytes per field of vision. Right: Representative staining of a liver section (original magnification: 200×, scale bar = 50 μm). (E) Immunohistochemical staining of HBcAg in the livers of PBS-, ΔM27-Ctrl-, and ΔM27-HBs-immunized mice. Left: Calculation of the average numbers of HBcAg-positive hepatocytes per field of vision. Right: Representative staining of a liver section (original magnification: 200×, scale bar = 50 μm). Data are depicted as arithmetic mean ± SEM, and experiments were repeated three times (n = 5 mice per group). The statistical analyses were performed by log-rank test (B and C) or one-way ANOVA (D and E). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Vaccination with ΔM27-HBs induces potent CD8+ T-cell responses

Compared to the PBS-treated control mice, the livers of ΔM27-HBs-vaccinated mice showed significantly higher percentages and absolute numbers of CD8 T cells, but not CD4+ T cells (Fig. 5A). Interestingly, the ΔM27-Ctrl-infected mice also showed a significant increase in both percentages and absolute numbers of CD8+ T cells in the liver as compared to PBS-treated control mice (Fig. 5A, right panel), but the effect was more pronounced in ΔM27-HBs-vaccinated mice. Consistent with the above-mentioned observation in the MCMV-HBs-vaccinated mice, PD-1 expression on CD8+ T cells in the liver of ΔM27-HBs-vaccinated mice was significantly increased compared to the PBS-treated or ΔM27-Ctrl-infected mice (Fig. 5B). Both ΔM27-Ctrl and ΔM27-HBs led to an increase in absolute numbers of CD43+ CD8+ T cells infiltrating the liver as compared to PBS-treated mice (Fig. 5C). These results suggest that the MCMV infection by itself results in enhanced infiltration of activated CD8+ T cells into the liver. Compared to the controls, the ΔM27-HBs-vaccinated mice showed a significant increase in both absolute numbers and frequencies of HBsAg-specific CD8+ T cells (Fig. 5D).

The core-specific CD8+ T cells were significantly increased in numbers but not in percentages in the liver of ΔM27-HBs vaccinated mice 10 days after the challenge (Fig. 5E). When C57BL/6 mice were inoculated only once with MCMV-HBs or ΔM27-HBs, mice were also protected against a challenge with an HBV-expressing plasmid applied by HDI 3 weeks later (data not shown). Like in case of the prime-boost vaccination regime, single round vaccination with MCMV-HBs or ΔM27-HBs induced enhanced intrahepatic anti-HBV CD8+ T-cell responses in vivo (data not shown).

Significantly higher percentages and absolute numbers of CD8+ T cells in the livers of ΔM27-HBs-vaccinated mice produced IFN-γ in response to stimulation with the Env190 peptide than stimulated cells from the control groups (Fig. 5F). Additionally, the absolute numbers of IL-2-producing CD8+ T cells in the liver of ΔM27-HBs-vaccinated mice were also significantly increased in response to both Env190 and Core93 peptide stimulations (Fig. 5G).

Taken together, these results demonstrate that an HBsAg vaccination based on an MCMV vector incapable to interfere with STAT2-dependent IFN signaling enhances intrahepatic anti-HBV CD8 T-cell responses in vivo and prophylactically protects mice against HBV challenges.
MCMV-based HBsAg vaccination enhances anti-HBV responses in HBV-persistent mice

After these promising results in prophylactic vaccination regimens, we examined the therapeutic capacity of MCMV-based HBsAg vaccination for the treatment of a previously established persistent HBV infection. The efficacy of MCMV-based HBV vaccine vectors was investigated by applying the established pAAV/HBV1.2 HDI mouse model, which mimics persistent HBV infections in humans [39]. C57BL/6 mice were hydrodynamically injected with pAAV/HBV1.2. We chose a strategy, which combines an MCMV-based priming with a DNA-based booster immunization. The experimental setup is depicted in Fig. 6A. Treatment with MCMV-HBs prime and DNA boost resulted in a substantial reduction in HBsAg and HBV DNA levels in the serum (Fig. 6B and C). Eighty percent of MCMV-HBs-vaccinated mice became serum HBsAg negative and 60% became HBV DNA negative at 42 dpi. In contrast, only 20% of the wt-MCMV-infected control mice became serum HBsAg negative, and none cleared the HBV DNA viremia (Fig. 6B and C). The MCMV-HBs-vaccinated mice also cleared HBsAg and HBcAg from the liver at 43 dpi, while the wt-MCMV-infected control mice still harbored high levels of HBsAg and HBcAg in the liver (Fig. 6D and E).

Next, we examined the impact of MCMV-HBs vaccination on the intrahepatic anti-HBV CTL response. The percentages and absolute numbers of HBsAg-specific CD8+ T cells were significantly increased in the liver of MCMV-HBs-vaccinated mice as compared to mice, which received the empty wt-MCMV (Fig. 6F). Moreover, significantly higher percentages and absolute numbers of liver CD8+ T cells of MCMV-HBs-vaccinated mice were capable of producing IFN-γ and TNF-α in response to Env190 peptide stimulation than cells derived from control treated mice (Fig. 6G).

The same experimental setup was also applied to explore the therapeutic effect of ΔM27-HBs for the treatment of already-established persistent HBV replication (Fig. 7A). Interestingly, the attenuated ΔM27-HBs demonstrated even superior effects on accelerating HBV clearance than the more virulent and higher titer replicating MCMV-HBs in the HBV-persistent pAAV/HBV1.2 HDI mouse model. All ΔM27-HBs-vaccinated mice became serum HBsAg and HBV DNA negative at 42 dpi, while the ΔM27-Ctrlr-infected and PBS-treated mice remained 100% positive for serum HBsAg and HBV DNA at the time point (Fig. 7B and C). The ΔM27-HBs-vaccinated mice also cleared HBsAg and HBcAg from the liver at 43 dpi, while mice from the three control groups exhibited high levels of HBsAg and HBcAg in liver sections (Fig. 7D and E). Accordingly, ΔM27-HBs vaccination resulted in significantly increased infiltration of HBsAg-specific CD8+ T cells in the liver as compared to PBS treatment and ΔM27-Ctrlr infection (Fig. 7F). Significantly higher percentages and absolute numbers of liver CD8+ T cells of ΔM27-HBs-vaccinated mice were capable of producing IFN-γ in response to Env190 peptide stimulation than cells of control mice (Fig. 7G).

Taken together, our results suggest that HBsAg expression by an MCMV-based vector attenuated through the loss of the STAT2-specific IFN antagonist pM27 combined with a plasmid-based booster immunization could overcome HBV-specific CD8+ T-cell dysfunction and induce HBV clearance in mice that previously established a persistent infection.

Discussion

HCMV is a betaherpesvirus that usually causes subclinical infections in healthy adults. However, HCMV elicits and sustains extraordinarily high numbers of antigen-specific T cells and is emerging as an exciting platform for vaccines against infectious diseases and cancers. To our knowledge, CMV-based vaccines against HBV have not been reported so far. Colleagues previously constructed an HBsAg-expressing MCMV [40], but seemingly did not approach the immunogenicity or vaccine potential. In the current study, we constructed two different recombinant MCMV vectors expressing HBsAg and explored their ability to induce HBV-specific CD8+ T-cell responses and to inhibit HBV replication in HBV HDI mouse models. Our findings reveal that vaccination with the virulent MCMV-HBs and the attenuated ΔM27-HBs provided protection to mice against HBV challenges. The vaccinated mice quickly cleared HBV antigens and DNA from the serum and the liver, and generated robust intrahepatic HBsAg-specific CD8 T-cell responses. Importantly, MCMV prime DNA boost vaccination strategies based on both recombinant MCMVs could even break HBsAg tolerance, elicit intrahepatic anti-HBV CD8+ T-cell responses, and lead to viral clearance in the serum and liver in persistently HBV-infected mice, suggesting that CMV-based vaccines possess therapeutic potential for treatment of chronic HBV infection in patients. In this respect, HCMV mutants lacking the pM27 analogous STAT2 antagonist pUL145 may constitute interesting candidates for live-attenuated vaccine platforms.

Prophylactic HBV vaccines that are currently in use rely on the induction of humoral immune responses against HBsAg, and thus neutralize infectious HBV particles before viral entry into hepatocytes. While antibodies are indispensable for prophylactic vaccination against HBV, this immune mechanism alone is most likely insufficient to eliminate HBV-infected cells in case vaccines are applied therapeutically, as indicated by the failures of approved prophylactic vaccines in therapeutic vaccination attempts. This assumption is consistent with our finding that the prophylactic HBV vaccine Engerix-B showed no protective effects in the acute HBV HDI mouse model (data not shown). However, this model is particularly biased against neutralizing antibodies since they are bypassed by unpackaged HBV genomes during direct transfection into hepatocytes through HDI. In case of a previously established infection, a potent cellular immune response mainly based on CD8+ T cells is essential for the elimination of infected cells [41]. CMV infection causes an extraordinary strong T-cell response. It has been estimated that CMV-specific T-cell populations comprise an average 10% of the memory CD8+ T lymphocytes of infected individuals [42]. In some cases, even up to 50% of all CD8+ T cells recognize a single epitope derived from the HCMV antigen pp65/pUL83 [43]. Certain CMV-induced CD8+
T-cell responses even show a so-called memory inflation, which means that their frequency continuously increases over time [44, 45]. These inflammatory CD8+ T cells are maintained as effector or effector-memory T cells, retaining the ability to produce inflammatory cytokines and to kill target cells [46, 47]. In line with these reports, our findings demonstrate that vaccination with HBsAg encoded by MCMV vectors induced significantly increased numbers of activated HBsAg-specific CD8+ T cells infiltrating the livers of HBV-infected mice. These cells were capable of producing antiviral cytokines and led to accelerated HBV clearance. Interestingly, control MCMVs also induced increased infiltration of activated total, but not HBV-specific, CD8+ T cells in the liver.
Figure 7. ΔM27-HBs vaccination accelerates HBV clearance and enhances the intrahepatic anti-HBV CD8+ T-cell responses in HBV persistent mice. (A) C57BL/6j naïve mice were hydrodynamically injected with pAAV-HBV1.2 (day 0) and immunized (primed) 1 week later (day 7) intraperitoneally with $2 \times 10^5$ PFU of ΔM27-Ctrl, ΔM27-HBs, PBS in a volume of 100 μL, and subsequently boosted with HBV plasmid pSM2 at week 3 (day 21). Mice were sacrificed after HBV clearance in serum at week 6, 42 days after HI ($n = 5$). (B) The concentrations and positive rates of serum HBsAg levels were monitored by ELISA at indicated time points. (C) The concentrations and positive rates of serum HBV DNA levels were monitored by real-time PCR at indicated time points. The quantification of HBV DNA was performed by using the standard curve created with the negative control and four HBV DNA standards provided by the diagnostic kit (Sansure, Changsha, China) according to the manufacturer’s instructions. (D) Immunohistochemical staining of HBsAg in the livers of PBS-, ΔM27-Ctrl-, or ΔM27-HBs-immunized mice. Left: Calculation of the average numbers of HBsAg-positive hepatocytes per field of vision. Right: Representative staining of a liver section (original magnification: 200×, scale bar = 50 μm). (E) Immunohistochemical staining of HBcAg in the livers of PBS-, ΔM27-Ctrl-, or ΔM27-HBs-immunized mice. Left: Calculation of the average numbers of HBcAg-positive hepatocytes per field of vision. Right: Representative staining of a liver section (original magnification: 200×, scale bar = 50 μm). (F) Numbers, frequencies, and representative flow cytometry plots of HBV Env190-specific CD8+ T cells. (G) Numbers, frequencies, and representative flow cytometry plots of Env190-specific cytokine-positive CD8+ T cells after in vitro stimulation with Env190 peptide. Data are depicted as arithmetic mean ± SEM, and experiments were repeated three times ($n = 5$ mice per group). Statistical analyses were performed by log-rank test (B and C) or one-way ANOVA (F–G). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

MCMV replicates efficiently in primary hepatocytes in vitro (see, e.g., [48]) and the liver in vivo (see, e.g., [28]), these results most likely reflect the effector nature of CMV in activating T cells and eliciting robust T-cell responses against endogenous antigens. Such MCMV-specific T-cell responses may explain why MCMV-HBs increases the absolute number of core-specific CD8+ T cells, but not the frequency (Fig. SE). In addition, CMV possesses other features favorable for a vaccine vector. First, HCMV can superinfect preimmune hosts [23, 49]. Thus, CMV-based vaccines may be applicable regardless of preexisting immune responses raised by naturally acquired CMVs, which would be favorable due to the high sero-prevalence of HCMV in countries with high incidences of CHB. Second, the large genome of CMVs allows CMV to take up large segments of foreign DNA at several different genomic loci. Another advantage might be the prolonged period of productive CMV replication, which can last several months in case of MCMV in mice [50], resulting in continuous antigen expression. The ability of herpesviruses to reactivate locally and spontaneously [51], leading to sequential rounds of immune stimulation, might further enhance potent immunogenicity.
A significant increase of PD-1 expression on CD8+ T cells was observed in the livers of MCMV-HBs-vaccinated mice after resolving intrahepatic HBV replication. Functional properties of the PD-1/PD-L1 pathway during acute virus infections are a matter of debate. Some studies suggest that PD-1 expression causes T-cell dysfunction even during the initial phase of infection [52–54]. While PD-1 expression did not induce exhaustion of virus-specific CD8+ T cells during acute infection in other models [37, 55, 56]. It was demonstrated that virus-specific PD-1-high CTLs efficiently kill Friend retrovirus (FV)-infected target cells, and were critical for reducing acute viral loads [37]. PD-1 is indeed an activation rather than an exhaustion marker for CD8+ T cells during the initial phase of FV infection, as highlighted by the fact that CD8+ T cells with the highest PD-1 levels simultaneously exhibited the highest cytolytic potential [37]. Later during FV infection (>10 dpi), T cells start to become dysfunctional, and signaling through the PD-1 receptor contributes to this exhaustion. FV infection experiments with PD-1/PD-L1 KO mice supported the notion that this pathway did not facilitate T-cell exhaustion during the early phase of infection, but rather controls the magnitude of virus-specific CD8+ T-cell responses [57].

Although most primary and recurrent HCMV infections progress subclinically in healthy adults, severe cases do occur [58]. Additionally, HCMV causes morbidity and mortality in immune-immature, immune-compromised, and immune-senescence individuals. Therefore, it is hard to envisage that authorities such as the FDA would approve vaccines based on virulent HCMVs. Thus, it will be necessary to increase the safety by attenuation. One appealing strategy for the generation of immunogenic and safe vaccines is the use of mutants lacking immune antagonists. Such viruses are by definition attenuated and therefore less likely to cause disease. Due to the inability to counteract a given aspect of immunity, such vectors may even induce superior immune responses. Here, we evaluated the effect of the lack the IFN antagonist pm27. ΔM27-MCMV is replication competent in vitro in the absence of IFN treatment but highly attenuated in vivo [28, 31, 32]. Interestingly, the vacinia with the attenuated ΔM27-HBs showed superior protection compared to MCMV-HBs in terms of breaking the HBV tolerance in HBV persistent mice. Thus, the residual and temporary replication of the attenuated ΔM27-HBs vaccine generated sufficient numbers of HBsAg-specific CD8+ T cells to protect mice from the HBV challenge. IFNs stimulate the expression of several proteins involved in antigen processing and MHC presentation (see, e.g., [59]). In conjunction with TCR activation and co-stimulatory molecules, IFNs can also serve as third signal for T-cell activation [60]. Thus, it is tempting to speculate that the inability of the MCMV vector to counteract STAT2-dependent IFN signaling is beneficial in terms of raising HBsAg-specific CD8 responses.

Altogether, our data corroborate the concept that CMV-based vectors are promising candidates for the development of new therapeutic vaccines for CHB treatment. The results of our study warrant more detailed investigations of HCMV-based vaccines to induce protective CD8 T-cell responses against chronic HBV infection in patients.

Materials and methods

Genetic engineering of MCMV vaccine vectors

MCMV vectors were constructed by BAC technology as described [61, 62]. The coding sequence of the sHBsAg was amplified by PCR using the following primers, which introduce a C-terminal HA tag and harbor flanking restriction sites: MR-Nhel-sHBsAg-fw (5’-tag gct aag gag aac atc tca gga ttc c-3’) and MR-sHBsAgHA-Xhol-rev (5’-ata ctc ggt tta aac gta atc tgg aac atc gta tgg gta aat gta tca tcc aac aca aac gaa tat ggg-3’). The PCR product was cloned into the insertion plasmid pFRTZ-hEF1. The resulting pFRTZ-hEF1-sHBsAgHA vector harbors the HA-tagged sHBsAg gene under the control of the human elongation factor-1α (hEF1) promoter. Correct cloning was verified by restriction fragment analysis and sequencing (data not shown). pFRTZ-hEF1-sHBsAg was cleaved by Xhol that cleaves the two frt sites flanking the expression cassette and the ZeoR gene. This fragment was ligated, giving rise to a circular DNA element harboring one frt site, the ZeoR gene, and hEF1-sHBsAg, but lacking the plasmid backbone. The element was introduced into the MCK-2 repaired MCMV-BACs [63] either harboring an frt site instead of the gene m157 or M27 [28] by FLP-mediated homologous recombination in Escherichia coli DH10B using the FLP-expressing plasmid pCP20. Correct mutagenesis was confirmed by restriction digest analysis, Southern blot, and PCR analysis (data not shown). MCMV mutants were reconstituted by transfection of BAC DNA into permissive mouse newborn cells [64].

Western blot analysis

Western blotting was performed as described previously [65] using the following antibodies: α-β-actin, α-HA (both from Sigma-Aldrich), and α-pp89-IE1 (GROMA101, generously provided by Stjepan Jonjić, Rijeka, Croatia). Proteins were visualized using peroxidase-coupled secondary antibodies and an ECL system (Cell Signaling Technology).

Mouse newborn cells

Mouse newborn cells (MNCs) were prepared as described previously [64]. In all experiments, MNCs were used in passage 3. Cell culture media and supplements were obtained from Gibco/Life technologies and HyClone (Logan, UT). MNCs were used to propagate the MCMV viruses.

MCMV propagation, titration, and replication analysis

MCMV stocks were generated as described [66]. In vitro infections and titrations were enhanced by centrifugation (900g for 30 min). For the in vivo MCMV replication analysis, mice were infected
intraperitoneally (i.p.). Organs of infected mice were harvested, snap-frozen in liquid nitrogen, and stored at –80°C until titration was performed.

Mice

Male, 6- to 8-week-old wild-type C57BL/6J mice and timed pregnant 10- to 20-week-old female mice were purchased from Hunan Slack King Laboratory Animal Co., Ltd. (Changsha, China) and housed under specific pathogen-free conditions in the Animal Care Center of Tongji Medical College or obtained from Charles River or Harlan and housed in the animal facility of the Institute for Virology of the University Hospital Essen. Timed pregnant 10- to 20-week-old female mice were used for generation of MNCs. Mice were sacrificed on the indicated days after vaccination and livers were harvested for analysis by IHC and flow cytometry. Procedures in Essen were conducted in accordance with regulations of the European Union and with permission of local authorities (the Landesamt für Natur, Umwelt, und Verbraucherschutz) in North Rhine Westphalia, Germany (permit numbers 84-02.04.2014.A390 & 84-02.04.2013.A414).

Intraperitoneal injection in mice

Intraperitoneal injection in mice was performed using 2 × 10^5 tissue culture infective dose affecting 50% of wells (TCID50) 50/mL of recombinant virus in 200 μL (Δm157-MCMV ["MCMV"], Δm157-MCMV: EF1-HBsAg ["MCMV-HBs"], ΔM27-MCMV ["ΔM27-Ctrl"], or ΔM27-MCMV: EF1-HBsAg ["ΔM27-HBs"]). Identical volume of phosphate-buffered saline (PBS) served as control.

Hydrodynamic injection in mice

We used pSM2 which is a pUC19 vector-based plasmid harboring a head-to-tail-oriented HBV genome, which has been shown by us previously to cause acute-resolving HBV replication in mice after HDI. Following administration of pSM2 by HDI, viremia uniformly becomes undetectable before day 21 in immune competent mice [67]. Conversely, pAAV/HBV1.2 causes chronic HBV replication in mice during which viremia persists longer than 6 months in 40% of mice [68, 69]. Hydrodynamic injection was performed as described previously using the replicating HBV plasmids pSM2 (generously provided by Dr. Hans Will, Heinrich-Pette-Institute, Hamburg, Germany) or pAAV/HBV1.2 (generously provided by Professor Pei-Jer Chen, National Taiwan University College of Medicine, Taipei, Taiwan) to establish HBV replication in mice [69–71]. In brief, male mice (6–8 weeks of age) were injected with 10 μg pSM2 or AAV/HBV1.2 in a volume of normal saline solution equivalent to 0.1 mL/g of the mouse body weight through the tail vein within 8 s [72, 73].

Detection of serological HBV markers

Sera were prepared from blood collected at the indicated time points from the retro-orbital sinus of mice. Serum levels of HBsAg and HBeAg were measured by the corresponding ELISA kits (Kehua, Shanghai, China) according to manufacturer’s instructions. HBV DNA copies were determined by a diagnostic kit (Sansure, Changsha, China) using a quantitative real-time PCR according to manufacturer’s instructions.

Cell isolation

Preparation of single-cell suspensions of murine intrahepatic lymphocytes was performed as described previously [73, 74].

Flow cytometry

We have adhered to the “Guidelines for the use of flow cytometry and cell sorting in immunological studies” [75]. Surface and intracellular staining for flow cytometry analysis were performed as described previously [67]. The following antibodies were used for surface and intracellular staining: FITC-anti-CD3, APC-Cy7-anti-CD4, Pacific Blue-anti-CD8, PE-anti-PD1, and FITC-anti-IgG1 (all BD Biosciences, USA). For intracellular cytokine staining, we used the following antibodies: APC-anti-IFN-γ, PerCP-Cy5.5-anti-IL-2, or FITC-anti-TNF-α (BD Biosciences, USA). For HBV-specific CD8⁺ T-cell detection, soluble DimerX H-2Kb: Ig fusion protein technology (BD Biosciences, USA) was applied. In brief, cells were incubated with CD16/CD32 anti-mouse antibody (clone 2.4G2; BD Pharmingen) to block FcRs. After washing, dimer staining was performed by incubation of dimer (which has been loaded with HBV Cor93-100 or Env190-197 peptide) and cells for 1 h at 4°C. The cells were washed and incubated with anti-IgG1 antibody (BD Biosciences, USA) for 30 min at 4°C. Data were acquired on a BD FACs Canto II flow cytometer. Cell debris and dead cells were excluded from the analysis based on scatter signals and Fixable Viability Dye eFluor 560 (eBioscience). Isolated murine intrahepatic lymphocytes were used for all assays, and approximately 20 000–40 000 T cells were acquired for each sample using a BD FACs Canto II flow cytometer. Data analysis was performed using FlowJo software V10.0.7 (Tree Star, Ashland, OR, USA). Gating strategy is shown in Supporting Information Fig. S1.

Statistical analysis

Statistical data were derived by using the GraphPad Prism software (GraphPad Software). Data were analyzed using log-rank test, unpaired t test, and one-way ANOVA as described in each figure legend.
Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee Tongji Medical College, Huazhong University of Science and Technology in accordance with the recommendations in the National Advisory Committee for Laboratory Animal Research (NALCAR) guidelines: IACUC Number 2019-S1016. The experiments were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Acknowledgements: We thank Professor Pei-Jer Chen for providing the pAAV/HBV1.2 plasmid. This work was supported by the National Natural Science Foundation of China (91742114, 91642118, 81861138044, and 91742114), the National Scientific and Technological Major Project of China (2017ZX10202203), and the Sino-German Virtual Institute for Viral Immunology. MT received funding from the Deutsche Forschungsgemeinschaft (DFG) through RTG1949 (project 13), TR1208/1-1, and TR1208/2-1. MT also received support from the Kulturstiftung Essen. This work was supported by the National Natural Science Foundation of China (81861138044 and 91742114), the National Scientific and Technological Major Project of China (2017ZX10202203), and the Sino-German Virtual Institute for Viral Immunology. MT received funding from the Deutsche Forschungsgemeinschaft (DFG) through RTG1949 (project 13), TR1208/1-1, and TR1208/2-1. MT also received support from the Kulturstiftung Essen. Open access funding enabled and organized by Projekt DEAL.

Peer review: The peer review history for this article is available at https://publons.com/publon/10.1002/eji.202048780.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

Authors contributions: Conceived and designed the experiments: L VTK, UD, ML, MT, and JL. Performed the experiments: HH, MR, XY, XF, DZ, SY, WZ, L VTK, and JL. Analyzed and interpreted the data: HH, MR, L VTK, MT, and JL. Contributed reagents/materials/analysis tools: DY, YL, and JL. Drafted the manuscript: HH, MR, MT, and JL.

References

1 Schweitzer, A., Horn, J., Mikolajczyk, R. T., Krause, G. and Ott, J. J., Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet 2015.

2 Dienstag, J. L., Hepatitis B virus infection. N. Engl. J. Med. 2008. 359: 1486–1500.

3 European Association For The Study Of, L., EASL clinical practice guidelines: management of chronic hepatitis B virus infection. J. Hepatol. 2012. 57: 167–185.

4 Liu, J., Kosinska, A., Lu, M. and Roggendorf, M., New therapeutic vaccination strategies for the treatment of chronic hepatitis B. Virol. Sinus 2014. 29: 10–16.

5 Thimme, R., Wieland, S., Steiger, C., Ghayeb, J., Reimann, K. A., Purcell, R. H. and Chisari, F. V., CD8 (+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J. Virol. 2003. 77: 68–76.

6 Jung, M. C., Spengler, U., Schraut, W., Hoffmann, R., Zachoval, R., Eisenburg, J., Eichtenaub, D. et al., Hepatitis B virus antigen-specific T-cell activation in patients with acute and chronic hepatitis B. J. Hepatol. 1991. 13: 310–317.

7 Rehermann, B. and Nascimbeni, M., Immunology of hepatitis B virus and hepatitis C virus infection. Nat. Rev. Immunol. 2005. 5: 215–229.

8 Dembek, C., Protzer, U. and Roggendorf, M., Overcoming immune tolerance in chronic hepatitis B by therapeutic vaccination. Curr. Opin. Virol. 2018. 30: 58–67.

9 Pol, S., Naipal, B., Driss, F., Michel, M. L., Tiollais, P., Denis, J., Brecho, C. et al., Efficacy and limitations of a specific immunotherapy in chronic hepatitis B. J. Hepatol. 2001. 34: 917–921.

10 Vandevelipeleire, P., Lau, K. G., Leroux-Roels, G., Horsmans, Y., Gane, E., Tawande, T., Merican, M. I. 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: 8585–8597.

11 Xu, D. Z., Zhao, K., Guo, L. M., Li, L. J., Xie, Q., Ren, H., Zhang, J. M. et al., A randomized controlled phase IIb trial of antigen-antibody immunogenic complex therapeutic vaccine in chronic hepatitis B patients. PLoS One 2008. 3: e2565.

12 Yao, X., Zheng, B., Zhou, J., Xu, D. Z., Zhao, K., Sun, S. H., Yuan, Z. H. et al., Therapeutic effect of hepatitis B surface antigen-antibody complex is associated with cytolytic and non-cytolytic immune responses in hepatitis B patients. Vaccine 2007. 25: 1771–1779.

13 Xu, D. Z., Wang, X. Y., Shen, X. L., Gong, G. Z., Ren, H., Guo, L. M., Sun, A. M. et al., Results of a phase III clinical trial with an HBsAg-AS02 candidate vaccine in lamivudine-resistant chronic hepatitis B patients: experiences and findings. J. Hepatol. 2013. 59: 450–456.

14 Kosinska, A. D., Bauer, T. and Protzer, U., Therapeutic vaccination for chronic hepatitis B. Curr. Opin. Virol. 2017. 23: 75–81.

15 Martin, P., Dubois, C., Jacquier, E., Dion, S., Mancini-Bourgine, M., Godon, O., Kratzer, R. et al., TG1050, an immunotherapeutic to treat chronic hepatitis B, induces robust T cell and exerts an antiviral effect in HBV-persistent mice. Gut 2015. 64: 1961–1971.

16 Backes, S., Jager, C., Dembek, C. J., Kosinska, A. D., Bauer, T., Stephan, A. S., Dislers, A. et al., Protein-prime/modified vaccinia virus Ankara vector-boost vaccination overcomes tolerance in high-antigenemic HBV-transgenic mice. Vaccine 2016. 34: 923–932.

17 Moshkani, S., Chiale, C., Lang, S. M., Rose, J. K. and Robek, M. D., A highly attenuated vesicular stomatitis virus-based vaccine platform controls hepatitis B virus replication in mouse models of hepatitis B. J. Virol. 2019. 93. doi: 10.1128/JVI.01586-18

18 Wilski, N. A. and Snyder, C. M., From vaccine vector to oncomodulation: understanding the complex interplay between CMV and cancer. Vaccines (Basel) 2019. 7: 62.
of the carbohydrate moiety. J. Exp. Med. 2001. 195: 1697–1707.

20 Abenes, G., Lee, M., Haghjoo, E., Tong, T., Zhan, X. and Liu, F., Murine cytomegalovirus open reading frame M27 plays an important role in growth and virulence in mice. J. Virol. 2001. 75: 1697–1707.

21 Peterson, D. L., Nath, N. and Gavilanes, F., Structure of hepatitis B surface antigen. Correlation of subtype with amino acid sequence and location of the carbohydrate moiety. J. Biol. Chem. 1982. 257: 10414–10420.

22 Wang, C. J., Sung, S. Y., Chen, D. S. and Chen, P. J., N-linked glycosylation of hepatitis B surface antigen is involved but not essential in the assembly of hepatitis delta virus. Virology. 1996. 220: 28–36.

23 Beverley, P. C., Ruzsics, Z., Hey, A., Hutchings, C., Boos, S., Bolinger, B., Marchi, E. et al., A novel murine cytomegalovirus vaccine vector protects against Mycobacterium tuberculosis. J. Immunol. 2014. 193: 2306–2316.

24 Cavanaugh, V. J., Guidotti, L. G., Chisari, F. V., Inhibition of hepatitis B virus replication during adenosine and cytomegalovirus infections in transgenic mice. J. Virol. 1998. 72: 2630–2637.

25 Zelinsky, G., Myers, L., Dietze, K. K., Gibbert, K., Roggendorf, M., Liu, J., Lu, M. et al., Virus-specific CD8+ T cells upregulate programmed death-1 expression during acute friend retrovirus infection but are highly cytotoxic and control virus replication. J. Immunol. 2011. 187: 3700–3707.

26 Caposio, F., van den Worm, S., Crawford, L., Perez, W., Kreklywich, C., Gilbride, R. M., Hughes, C. M. et al., Characterization of a live-attenuated HCMV-based vaccine platform. Sci. Rep. 2019. 9: 19236.

27 Wang, C. J., Chen, P. J. and Chen, D. S., An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. Proc. Natl. Acad. Sci. USA 2006. 103: 17862–17867.

28 Brune, W., Hasan, M., Krych, M., Bubic, I., Jonjic, S. and Koszinowski, U. H., Secreted virus-encoded proteins reflect murine cytomegalovirus productivity in organs. J. Infect. Dis. 2001. 184: 1320–1324.

29 Yang, P. L., Albhage, A., Chung, J., Maier, H., Wieland, S., Isogawa, M. and Chisari, F. V., Immune effectors required for hepatitis B virus clearance. Proc. Natl. Acad. Sci. USA 2010. 107: 798–802.

30 Sylwestra, A. W., Mitchell, B. L., Edgar, J. B., Taormina, C., Pelte, C., Ruchti, F., Sleath, P. R. et al., Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J. Exp. Med. 2005. 202: 673–685.

31 Langa, K. S., Moris, A., Gouttefangeas, C., Walter, S., Teichgräber, V., Müller, M., Wernet, D. et al., High frequency of human cytomegalovirus (HCMV)-specific CD8+ T cells detected in a healthy CMV-seropositive donor. Cell. Mol. Life Sci. 2002. 59: 1076–1080.

32 Karrer, U., Sierra, S., Wagner, M., Oxenius, A., Hengel, H., Koszinowski, U. H., Phillips, R. E. et al., Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. J. Immunol. 2003. 170: 2022–2029.

33 Karrer, U., Wagner, M., Sierra, S., Oxenius, A., Hengel, H., Dumreise, T., Freitag, S. et al., Expansion of protective CD8+ T-cell responses driven by recombinant cytomegaloviruses. J. Virol. 2004. 78: 2255–2264.

34 Snyder, C. M., Cho, K. S., Bonnett, E. L., van Dommelen, S., Shellham, G. R. and Hill, A. B., Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. Immunity. 2008. 29: 650–659.

35 Hertoghs, K. M., Moerland, P. D., van Stijn, A., Remmerswaal, E. B., Yong, S. L., van de Berg, P. J., van Ham, S. M. et al., Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. J. Clin. Invest. 2010. 120: 4077–4090.

36 Schupp, A. K., Trilling, M., Rattay, S., Le-Trilling, V. T. K., Haselow, K., Jonjic, S., Koszinowski, U. et al., Secreted virus–encoded proteins reflect murine cytomegalovirus pro-
52 Petrovas, C., Price, D. A., Mattapallil, J., Ambrozak, D. R., Geldmacher, C., Cecchinato, V., Vaccari, M. et al., SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection. Blood 2007. 110: 928–936.

53 Urbani, S., Amadei, B., Tola, D., Massari, M., Schivazappa, S., Missale, G. and Ferrari, C., PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8+ exhaustion. J. Virol. 2006. 80: 11398–11403.

54 Rutigliano, J. A., Sharma, S., Morris, M. Y., Oguin, T. H., 3rd, McClaren, J. L., Doherty, P. C. and Thomas, P. G., Highly pathologcal influenza A virus infection is associated with augmented expression of PD-1 by functionally compromised virus-specific CD8+ T cells. J. Virol. 2014. 88: 1636–1651.

55 Kasprwicz, V., Schulze Zur Wiesch, J., Kuntze, T., Nolan, B. E., Longworth, S., Berical, A., Blum, J. et al., High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during acute HCV infection, irrespective of clinical outcome. J. Virol. 2008. 82: 3154–3160.

56 Greenough, T. C., Campellone, S. C., Brody, R., Jain, S., Sanchez-Merino, V., Somasundaran, M. and Luzuriaga, K., Programmed death-1 expression on Epstein Barr virus specific CD8+ T cells varies by stage of infection, epitope specificity, and T cell receptor usage. PLoS One 2010. 5: e12926.

57 David, P., Megger, D. A., Kaiser, T., Werner, T., Liu, J., Chen, L., Sitek, B. et al., The PD-1/PD-L1 pathway affects the expansion and function of cytotoxic CD8+ T cells during an acute retroviral infection. Front. Immunol. 2019. 10: 54.

58 Rafailidis, P. I., Mourtzoukou, E. G., Varbovitis, I. C. and Falagas, M. E., Severe cytomegalovirus infection in apparently immunocompetent patients: a systematic review. Virol. J. 2008. 5: 47.

59 Megger, D. A., Philipp, J., Le-Trilling, V. T. K., Sitek, B. and Trilling, M., Deciphering of the human interferon-regulated proteome by mass spectrometry-based quantitative analysis reveals extent and dynamics of protein induction and repression. Front. Immunol. 2017. 8: 1139.

60 Curtsinger, J. M., Valenzuela, J. O., Agarwal, P., Lins, D. and Mescher, M. F., Type I IFN provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J. Immunol. 2005. 174: 4465–4469.

61 Tischer, B. K., von Einem, J., Kaufer, B. and Osterrieder, N., Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. BioTechniques 2006. 40: 191–197.

62 Tischer, B. K., Smith, G. A. and Osterrieder, N., En passant mutagenesis: a two step markerless red recombination system. Methods Mol. Biol. 2010. 634: 421–430.

63 Jordan, S., Krause, J., Prager, A., Mitrovic, M., Jonjic, S., Koszinowski, U. H. and Adler, B., Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show reduced growth in salivary Glands due to a fixed mutation of MCK-2. J. Virol. 2011. 85: 10346–10353.

64 Le-Trilling, V. T. K. and Trilling, M., Mouse newborn cells allow highly productive mouse cytomegalovirus replication, constituting a novel convenient primary cell culture system. PLoS One 2012. 12: e0174695.

65 Trilling, M., Le, V. T., Zimmermann, A., Ludwig, H., Pfeffer, K., Sutter, G., Smith, G. L. et al., Gamma interferon-induced interferon regulatory factor 1-dependent antiviral response inhibits vaccinia virus replication in mouse but not human fibroblasts. J. Virol. 2009. 83: 3684–3695.

66 Brizic, I., Lisnic, B., Brune, W., Hengel, H. and Jonjic, S., Cytomegalovirus infection: mouse model. Curr. Protoc. Immunol. 2018: e51.

67 Wang, Q., Pan, W., Liu, Y., Luo, J., Zhu, D., Lu, Y., Feng, X. et al., Hepatitis B virus-specific CD8+ T cells maintain functional exhaustion after antigen reexposure in an acute activation immune environment. Front. Immunol. 2018. 9: 219.

68 Wu, J., Huang, S., Zhao, X., Chen, M., Lin, Y., Xia, Y., Sun, C. et al., Poly(I:C) treatment leads to interferon-dependent clearance of hepatitis B virus in a hydrodynamic injection mouse model. J. Virol. 2014. 88: 10421–10431.

69 Huang, L. R., Wu, H. L., Chen, P. J. and Chen, D. S., An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. Proc. Natl. Acad. Sci. U. S. A. 2006. 103: 17862–17867.

70 Zhang, X., Zhang, E., Ma, Z., Pei, R., Jiang, M., Schlaak, J. F., Roggendorf, M. et al., Modulation of hepatitis B virus replication and hepatocyte differentiation by MicroRNA-1. Hepatology 2011. 53: 1476–1485.

71 Galibert, F., Mandart, E., Fitoussi, F., Tiollais, P., Charnay, P., Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature 1979. 281: 646–650.

72 Huang, L. R., Gabel, Y. A., Graf, S., Arzberger, S., Kurts, C., Heikenwalder, M., Knolle, P. A. et al., Transfer of HBV genomes using low doses of adenovirus vectors leads to persistent infection in immune competent mice. Gastroenterology 2012. 142: 1447–1450 e1443.

73 Dietze, K. K., Schimmer, S., Kretzmer, F., Wang, J., Lin, Y., Huang, X., Wu, W. et al., Characterization of the Treg response in the hepatitis B virus hydrodynamic injection mouse model. PLoS One 2016. 11: e0151717.

74 Cripsi, I. N., Isolation of mouse intrahepatic lymphocytes. Curr. Protoc. Immunol. 2001. doi: 10.1002/0471142735.im0321s22

75 Cossarizza, A., Chang, H.-D., Radbruch, A., Acis, A., Adam, D., Adam-Klages, S., Agace, W. W. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur. J. Immunol. 2019. 49: 1457–1973.

Abbreviations: BAC: bacterial artificial chromosome · CHB: chronic hepatitis B · HBV: hepatitis B virus · HDI: hydrodynamic injection · MCMV: mouse CMV · MNC: mouse newborn cells · RhCMV: Rhesus CMV

Full correspondence: Prof. Jia Liu, Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China e-mail: jialiu77@hust.edu.cn

Additional correspondence: Prof. Mirko Trilling, Institute for Virology, University Hospital of Essen, University of Duisburg-Essen, Essen 45147, Germany e-mail: mirko.trilling@uk-essen.de

Received: 29/5/2020
Revised: 14/8/2020
Accepted: 5/10/2020
Accepted article online: 7/10/2020