Adeno-Associated Virus-Mediated Gene Transfer Leads to Persistent Hepatitis B Virus Replication in Mice Expressing HLA-A2 and HLA-DR1 Molecules

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Hepatitis B virus (HBV) persistence may be due to impaired HBV-specific immune responses being unable to eliminate efficiently or cure infected hepatocytes. The immune mechanisms that lead to HBV persistence have not been completely identified, and no appropriate animal model is available for such studies. Therefore, we established a chronic HBV infection model in a mouse strain with human leukocyte antigen A2/DR1 (HLA-A2/DR1) transgenes and an H-2 class I/class II knockout. The liver of these mice was transduced with adeno-associated virus serotype 2/8 (AAV2/8) carrying a replication-competent HBV DNA genome. In all AAV2/8-transduced mice, hepatitis B virus surface antigen, hepatitis B virus e antigen, and HBV DNA persisted in serum for at least 1 year. Viral replication intermediates and transcripts were detected in the livers of the AAV-injected mice. The hepatitis B core antigen was expressed in 60% of hepatocytes. No significant inflammation was observed in the liver. This was linked to a higher number of regulatory T cells in liver than in controls and a defect in HBV-specific functional T-cell responses. Despite the substantial tolerance resulting from expression of HBV antigens in hepatocytes, we succeeded in priming functional HBV-specific T-cell responses in peripheral tissues, which subsequently reached the liver. This AAV2/8-HBV-transduced HLA-A2/DR1 murine model recapitulates virological and immunological characteristics of chronic HBV infection, and it could be useful for the development of new treatments and immune-based therapies or therapeutic vaccines for chronic HBV infections.

Hepatitis B virus (HBV) infection is a major health problem. There are more than 350 million chronic carriers worldwide, and they are at high risk of developing liver cirrhosis and hepatocellular carcinoma (1). Chronic HBV infection is the result of impaired HBV-specific immune responses such that the infected hepatocytes cannot be eliminated or cured efficiently, but many of the associated issues remain unclear (2, 3).

Due to the paucity of in vitro and in vivo models for HBV infection, HBV-transgenic mice are the most widely used model. These mice have the viral genome integrated into the chromosome and produce infectious HBV particles or viral antigens in the liver; however, the main limitation of HBV-transgenic mouse models is that they are immunologically tolerant to viral antigens (4, 5). Various routes have been exploited to introduce the HBV genome into the hepatocytes of adult mice. One is to introduce a replication-competent HBV genome into the mouse liver by hydrodynamic injection (HDI) through the tail vein (6); although HBV replicates in the mouse liver, the virus is rapidly cleared by immune responses against HBV proteins (7). Recently, Huang and colleagues used HDI to create a nontransgenic model of persistent HBV replication (8). The virus persisted in 40% of mice or was eliminated according to the genetic background. These mice rapidly develop anti-hepatitis B virus core (Hbc) antibody, which is the first serological marker of acute HBV infection in humans. An alternative method uses adenoviral vectors to transfer 1.3 copies of the HBV genome into immunocompetent mice (9, 10), and acute or chronic HBV infection was obtained depending on the dose of adenoviral vector injected.

Here, we describe an alternative murine model for the study of HBV persistence based on the liver-targeted transduction of adeno-associated virus serotype 2/8 (AAV2/8). We produced an AAV2/8 construct carrying a replication-competent HBV DNA genome and by intravenous injection established a model of HBV persistence in humanized HLA-A2/DR1 immunocompetent mice. Hepatitis B virus surface antigen (HBsAg), hepatitis B virus e antigen (HBeAg), and HBV DNA persisted for at least 1 year in sera of all AAV2/8-injected mice, and viral replication intermediates and transcripts were detected in their livers. HbcAg was expressed in 60% of hepatocytes without significant inflammation in the liver. The persistence of infection was associated with the presence of regulatory T cells (Tregs) in the liver. This mouse model of HBV persistence recapitulates viral and histological characteristics of human chronic HBV infection in the immunotolerant stage of the disease (11, 12).

In HLA-A2/DR1 mice, cellular immune responses were completely restricted to HLA molecules. Antibody, T-helper, and cytotoxic-T-lymphocyte responses to vaccination with recombinant HBsAg or HbsAg-expressing DNA were similar to those in vaccinated humans (13, 14) or in HBV-infected individuals (15). Therefore, this AAV2/8-HBV-transduced HLA-A2/DR1 murine model may be useful for the development of therapeutic strategies that require cooperation between HLA-restricted CD4+ T-helper cells and cytolytic CD8+ T cells to clear HBV during viral persistence.

To our knowledge, this is the first mouse model with a cellular immune response restricted to human major histocompatibility complex (MHC) molecules in the context of chronic HBV infec-
tion that allows the evaluation of new treatments and immune-based therapies or therapeutic vaccines.

**MATERIALS AND METHODS**

**AAV2/8-HBV vector.** A recombinant AAV2/8 vector carrying 1.2 copies of the HBV genome (genotype D) was constructed. Virus stocks were produced and titrated by the Plateforme de Thérapie Génique in Nantes, France (INSERM U1089), with a protocol derived from one described previously by Ayuso et al. (16). Briefly, a 1.2 copy of the HBV genome was excised by PvuII digestion from the pAAY 1.2 plasmid (kindly provided by M. Melegari [17]) and ligated between the inverted terminal repeats (ITRs) of AAV2, resulting in pAAV-HBV. The 1.2-full-length HBV genome flanked by AAV2 ITRs was packaged into capsids from AAV8 after cotransfection of HEK 293 cells with pAAV-HBV and an auxiliary plasmid, leading to the AAV2/8-HBV viral vector. A stock of AAV2/8 without any transgene, referred to as AAV2/8-empty, was also produced and purified under the same conditions. The vector stocks were titrated as virus genomes (vg) per milliliter and stored at −80°C until use.

DNA vectors and peptides. Plasmid DNA was endotoxin-free and manufactured by PlasmidFactory (Germany). pCMV-S2.S awy carries the gene encoding the pre-S2 and S domains of HBsAg (genotype D), and their expression is controlled by the cytomegalovirus immediately early gene promoter (18). pCMV-HBc encodes the HBV capsid carrying HBcAg.

A set of synthetic HLA-A2- and HLA-DR1-restricted peptides derived from HBs and HBc antigens was purchased from Polypeptide Group (France) and used at a final concentration of 1 μg/ml. Peptide purity was >80%.

**Animal procedures.** Six- to eight-week-old HLA-A*0201/DRB1*0101-transgenic, H-2 class I/class II knockout (KO) mice (here referred to as HLA-A2/DR1) were used in this study and were previously described (13). All animals were housed in a specific-pathogen-free environment in the animal facilities of the Institut Pasteur, Paris, France. All protocols have been reviewed and approved by the institutional animal care committee of the Institut Pasteur for compliance with French and European regulations on animal welfare and with Public Health Service recommendations. Mice received a single tail vein injection of 5 × 10^10 vg of the AAV2/8-HBV vector or AAV2/8-empty vector or an equivalent volume of phosphate-buffered saline (PBS). Serum samples were obtained from mice by retro-orbital puncture at various times after injection. Groups (4 to 6 mice/group) were immunized once by bilateral intramuscular (i.m.) injection of 100 μg of DNA into cardiotoxin-treated tibialis anterior muscles, as previously described (18), under anesthesia (100 μl of 12.5 mg/ml ketamine and 1.25 mg/ml xylazine).

**Histological assays and immunohistochemical staining.** Liver tissues were collected from mice and fixed in 3.7% formaldehyde. Each liver was divided into six parts that were individually embedded in paraffin and sectioned. Liver sections (4 μm thick) were stained with hematoxylin-eosin or immunostained with a rabbit anti-HBcAg primary antibody (Dako, France). HBcAg-positive hepatocytes were analyzed by using ImageJ software (version 1.43; W. S. Rasband, U.S. NIH, Bethesda, MD). The percentage of HBcAg-positive hepatocytes was calculated as (HBcAg-positive hepatocytes/total hepatocytes) × 100.

**Detection of HBV antigens, antibodies, and serum aminotransferases.** HBsAg and HBcAg in mouse sera were assayed with commercial enzyme-linked immunosorbent assay (ELISA) kits (Bio-Rad, France). The HBsAg concentration was calculated in μg/ml by reference to a standard curve established with known concentrations of HBsAg. Serum HBcAg levels were determined in 10-fold-diluted sera. The threshold value for HBcAg positivity was defined as an optical density of the sample/cutoff (S/CO) ratio of ≥ 1. Antibodies were quantified by ELISA as previously described (19). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in sera of AAV2/8-HBV- and PBS-injected mice were determined by the Laboratoire de Biologie Vétérinaire Vebiotel (France). Results are expressed as U/liter.

**Quantification of HBV viremia.** Virions from mouse sera were immunoprecipitated with a monoclonal anti-HBV pre-S1 antibody (MA18/7; kindly provided by W. H. Gerlich, Giessen, Germany) and quantified by quantitative PCR as previously described (20). Results are expressed as copies/ml. The threshold of detection was 10^2 copies/ml.

**Northern and Southern blot analyses.** Northern and Southern blot analysis of total liver RNA and total genomic liver DNA were used to detect HBV RNAs and replicative HBV DNA intermediates, respectively, as previously described (21).
**Gamma interferon ELISPOT assay.** Gamma interferon (IFN-γ)-producing T cells among the splenocytes were quantified by an enzyme-linked immunosorbent spot (ELISPOT) assay after peptide stimulation for 24 h, as previously described (15). Wells containing cells (1 × 10^6) in culture medium without peptide stimulation were used as negative controls to determine background levels. A Bioreader 4000 counter (BioSys, Germany) was used to score the number of spots. Each cell population was titrated in triplicate. The response was considered positive if the median number of spot-forming cells in triplicate wells was at least twice that observed in control wells and at least 10 spot-forming cells per million splenocytes were detected after subtraction of the background.

**Flow cytometry.** Isolated cells (1 × 10^6) from spleen and liver were incubated for 1 h either with medium alone or with peptide pools. Brefeldin A (Sigma-Aldrich, France) was added to a final concentration of 2 μg/ml, and the cultures were incubated overnight at 37°C. Cells were harvested, washed, and surface stained with the following monoclonal antibodies: anti-CD3 (145-2C11), anti-CD4 (GK1.5), and anti-CD8 (53-6.7). A viability marker was also used (Live/Dead Fixable Dead Cell Yellow kit; Life Technologies, Frederick, MD). Surface-stained cells were fixed and permeabilized (BD Cytofix/Cytoperm Fixation/Permeabilization kit; BD Biosciences, France) and probed for intracellular cytokines with monoclonal anti-IFN-γ (XMG1.2), anti-interleukin-2 (IL-2) (JES6-5H4), or anti-tumor necrosis factor alpha (TNF-α) (MP6-XT22) antibodies. The Treg population was evaluated by surface staining with monoclonal anti-CD4 (L3T4) and anti-CD25 (PC61) antibodies and intracellular staining with anti-Foxp3 (MF23) antibodies. CD4^-^CD25^-^IL-10^-^ Treg cells in liver leukocyte or splenocyte preparations (2 × 10^5 cells/well) were enumerated after a 96-h culture in 96-well plates pre-coated with anti-CD3 antibody (145-2C11) in the presence of 1 μg/ml soluble anti-CD28 antibody (37-51) and 100 IU/ml human IL-2 (Roche, France). Cultured cells were stained with antibodies to CD4, CD25, and IL-10 (JES5-16E3). All antibodies were purchased from BD Biosciences (France). Stained cells were analyzed on a CyAn ADP analyzer (Beckman Coulter, Miami, FL). Data were analyzed by using FlowJo software (TreeStar, San Carlos, CA).

**Statistical analysis.** Data are expressed as means ± standard errors of the means (SEM). The Mann-Whitney U test was used for nonparametric unpaired comparisons. Statistical analysis was carried out by using Graphpad Prism 5 software (Graphpad, San Diego, CA). P values of <0.05 were considered significant.

**RESULTS**

Liver-targeted expression of HBV and persistence of HBV DNA and antigens in sera of AAV2/8-HBV-injected mice. To ensure the targeting of the HBV genome to the liver, we used an AAV serotype 8 vector that displays liver-specific transduction (22). We cloned 1.2 copies of the HBV genome between the two ITRs of AAV serotype 2 in a carrier plasmid, which was then used to produce the hybrid AAV2/8-HBV vector. Six- to eight-week-old
HLA-A2/DR1 female or male mice were given a single tail vein injection of $5 \times 10^{10}$ vg of the AAV2/8-HBV or the AAV2/8-empty (negative control) vector. Mice were regularly bled, and serum levels of HBsAg, HBeAg, and HBV DNA were determined (Fig. 1). All AAV2/8-empty-injected mice were negative for HBV antigens (data not shown), whereas all AAV2/8-HBV-injected mice were HBsAg and HBeAg positive on day 3 postinjection. In female mice, HBsAg (Fig. 1A) and HBeAg (Fig. 1B) levels increased to a mean concentration of $32.10 \pm 5.64$ ng/ml and a mean S/CO ratio of $198 \pm 22.81$, respectively, after 6 weeks. The HBsAg and HBeAg levels declined slowly thereafter, but all injected mice remained positive for 56 weeks. HBV antigen titers were higher in sera from male than in sera from female HLA-A2/DR1 mice (Fig. 1A, inset).

To exclude detection of the AAV2/8-HBV inoculum, pre-S1-carrying HBV particles were immunoprecipitated from serum samples and assayed for the presence of HBV DNA by quantitative real-time PCR. HBV particles became detectable 2 weeks after AAV2/8-HBV injection. The virus titers then remained stable until at least 1 year postinjection (mean titers were $2.48 \times 10^5$ and $1.50 \times 10^6$ copies/ml of HBV DNA in sera from females and males, respectively) (Fig. 1C). Thus, in this immunocompetent HLA-A2/DR1 mouse model, administration of AAV2/8-HBV results in persistent high levels of HBsAg and HBeAg in the sera of all injected mice and is associated with viremia. This pattern is thus similar to what is observed during persistent HBV infection in humans (12).

**HBV replication and gene expression in the liver of AAV2/8-HBV-injected mice.** To analyze the kinetics of HBV replication and transcription, liver samples from AAV2/8-HBV-injected mice were collected at various time points. At 6 weeks, 12 weeks, and 1 year postinjection, HBV DNA replicative intermediates were detected by Southern blot analysis (Fig. 2A), and pregenomic (3.5-kb) and subgenomic (2.4- and 2.1-kb) HBV RNA transcripts were detected by Northern blot analysis (Fig. 2B) of liver samples. HBV DNA replicative intermediates and HBV RNA transcripts were still detectable at 1 year but at lower levels. As in HBV-transgenic mice, HBV covalently closed circular DNA (cccDNA) was not detected (data not shown).

Liver tissues were also tested by immunostaining for HBCAg expression (Fig. 3) at 6 weeks, 12 weeks, and 1 year postinjection. The liver of each mouse was divided into six parts to evaluate the presence of HBV DNA by quantitative real-time PCR. HBV particles became detectable 2 weeks after AAV2/8-HBV injection. The virus titers then remained stable until at least 1 year postinjection (mean titers were $2.48 \times 10^5$ and $1.50 \times 10^6$ copies/ml of HBV DNA in sera from females and males, respectively) (Fig. 1C). Thus, in this immunocompetent HLA-A2/DR1 mouse model, administration of AAV2/8-HBV results in persistent high levels of HBsAg and HBeAg in the sera of all injected mice and is associated with viremia. This pattern is thus similar to what is observed during persistent HBV infection in humans (12).

Liver tissues were also tested by immunostaining for HBCAg expression (Fig. 3) at 6 weeks, 12 weeks, and 1 year postinjection. The liver of each mouse was divided into six parts to evaluate the HBcAg expression pattern (Fig. 3A to F). HBcAg was detected in both cytoplasm and nuclei, but mostly in nuclei, of hepatocytes (Fig. 3Aa, white and black arrows, respectively) in various parts of the liver. At each time point, the pattern of HBcAg expression was similar in the various lobes of the liver considered. HBcAg was expressed in about 60% of the hepatocytes at 6 weeks (Fig. 3A) and 12 weeks (Fig. 3B) post-AAV2/8-HBV injection. HBcAg expression persisted for at least 1 year (Fig. 3C) but was detectable in only around 15% of the hepatocytes at this time.

We further examined whether the HBV persistence in the AAV2/8-HBV-injected mice was associated with an inflammatory response. Livers from HBV carrier and control mice were stained with hematoxylin and eosin at 6 weeks and 1 year postinjection (Fig. 4A), and mononuclear cell infiltrates were compared. Livers from AAV2/8-HBV-injected mice presented normal architecture, with no obvious inflammatory infiltrates (Fig. 4A). Also, ASAT and ALAT levels were not higher in sera from AAV2/8-HBV-injected mice than in sera from PBS- or AAV2/8-empty-injected mice during the 1-year follow-up (Fig. 4B and C and data not shown). Thus, in our model, long-term expression of HBV antigens and HBV replication in liver following administration of AAV2/8-HBV did not cause apparent liver damage.
Defect in the HBcAg- and HBsAg-specific immune response in HBV carrier mice. Both HBc- and HBs-specific T-cell responses may contribute to viral clearance (23). Therefore, we tested whether the HBV persistence observed after injection of AAV2/8-HBV into HLA-A2/DR1 mice was due to impaired HBc- or HBs-specific immunity. HLA-A2- or -DR1-restricted peptides derived from HBV core and envelope proteins were used to detect IFN-γ-producing T cells from spleen and liver by an ELISPOT assay (Fig. 5A) and intracellular cytokine staining for IFN-γ (Fig. 5B). AAV2/8-empty-injected mice were used as negative controls and pCMV-S2.S and pCMV-HBc DNA immunization of HLA-A2/DR1 mice used as positive controls for IFN-γ-producing T cells. Results are representative of three independent experiments.

FIG 5 HBV-specific T-cell responses of AAV2/8-HBV-injected mice. IFN-γ-secreting T cells detected by ELISPOT assay of splenocytes (A) and intracellular cytokine staining for IFN-γ of intrahepatic lymphocytes (B) were obtained from 5 to 10 HLA-A2/DR1 mice injected 6 weeks previously with AAV2/8-HBV or AAV2/8-empty or immunized 2 weeks previously with HBc- and HBs-expressing DNA vectors as positive controls. (A) HLA-A2- and HLA-DR1-restricted peptides derived from either HBcAg (18-27) or HBsAg (111-125, 179-194, 183-191, 200-214, and 348-357) were used to stimulate splenocytes ex vivo. (B) Intrahepatic lymphocytes were analyzed after incubation with a pool of HLA-A2-restricted peptides (183-191, 204-212, and 348-357) or in medium alone (unstimulated). Representative fluorescence-activated cell sorter plots for IFN-γ-secreting T cells in the liver of DNA- or AAV2/8-HBV–injected mice are shown. Values in the upper right square of each panel represent the percentage of CD8+ T cells that are also IFN-γ-positive cells. Results are representative of three independent experiments.
The development of a novel animal model for HBV infection provides insights into the immune responses to HBV antigens. HBV-specific IFN-γ-producing CD8+ T cells were detected in the liver of AAV2/8-HBV-injected mice (8.56% ± 4.20%) after DNA immunization, whereas these cells were not found in liver of mice receiving AAV2/8-HBV and PBS only. The mean frequency of CD8+ T cells in liver of control mice receiving DNA vaccines only was 4.67% ± 0.99%. However, the proportion of mice with HBV-specific CD8+ T cells in liver was lower for HBV carrier mice (3/10 mice) than for control mice (7/7) (Fig. 6B).

To determine whether the decreased responses observed in the periphery and liver were due to the tolerogenic effect of the AAV2/8 vector per se, mice were injected with either the AAV-empty vector or PBS, and 32 days later, they were immunized with pCMV-S2.S and pCMV-HBc DNAs. ELISPOT assays showed that mice receiving the AAV-empty vector before DNA immunization developed comparable IFN-γ-secreting T-cell responses in the spleen (Fig. 7A). HBV-specific IFN-γ-producing CD8+ T cells were detected at a similar level in the liver of AAV2/8-empty-injected (20.42% ± 3.12%) or PBS-injected (15.41% ± 6.12%) mice after DNA immunization (Fig. 7B). This suggests that the AAV2/8 vector had no tolerogenic effect on vaccine-induced immune responses and that the observed tolerance in AAV-HBV-transduced mice is due to transgene expression.

Although HBV-specific T-cell responses were detected in spleen and liver of HBV carrier mice after immunization, these responses had apparently no effect on HBcAg or HBsAg levels in sera (Fig. 6C and D), on HBcAg intrahepatic expression, or on HBV DNA replicative intermediates when tested at 2 weeks postimmunization (data not shown). Furthermore, this immune response was not associated with an increased level of transaminase activity (data not shown).

**DISCUSSION**

We report the development of a novel animal model for HBV persistence in an immunocompetent mouse strain expressing HLA-A2/DR1 molecules (13). This involved the use of the hepatotropic hybrid serotype 2 and 8 AAV vector to deliver the entire HBV genome into mouse hepatocytes. AAV2/8 efficiently transduces hepatocytes (22). Mouse liver is permissive for HBV replication (24), so the use of AAV2/6 vectors allows the entry step of HBV infection to be bypassed. We demonstrate that a single tail

### TABLE 1 Frequencies of hepatic and splenic T-cell populations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean frequency (%) ± SEM of T-cell population</th>
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<tr>
<td></td>
<td>CD4+</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
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<tr>
<td>AAV2/8-HBV</td>
<td>21.36 ± 2.37</td>
</tr>
<tr>
<td>AAV2/8-empty</td>
<td>18.86 ± 0.87</td>
</tr>
<tr>
<td>PBS</td>
<td>17.60 ± 1.33</td>
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<tr>
<td>Spleen</td>
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<tr>
<td>AAV2/8-HBV</td>
<td>25.46 ± 1.17</td>
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<tr>
<td>AAV2/8-empty</td>
<td>23.36 ± 2.82</td>
</tr>
<tr>
<td>PBS</td>
<td>22.18 ± 1.60</td>
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* The percentage of Tregs in AAV2/8-HBV-injected mice is significantly different from that in AAV2/8-empty- or PBS-injected mice (P < 0.05).

* The percentages of the CD4+ CD25+ IL-10+ subset in AAV2/8-HBV- and in AAV2/8-empty-injected mice are significantly different from those in PBS-injected mice (P < 0.05).

* Values represent the mean percentages ± SEM of each subset of T cells in the living lymphocyte population.
vene injection of AAV2/8-HBV led to a persistent infection in all injected mice. This infection was characterized by the presence of HBsAg and HBeAg in serum for at least 1 year and the absence of seroconversion to antibodies to HBsAg or HBcAg. Viral particles containing HBV DNA were found in sera, and abundant HBV DNA replicative intermediates and HBV transcripts were found in liver both early (6 to 12 weeks) and late (1 year) after injection. The antigenemia rate and replication levels were higher in males than in females. Thus, our mouse model reproduced the epidemiological findings that serum HBsAg and HBV DNA levels are always higher in male than in female carriers (25, 26). Our protocol results in all injected mice becoming HBV carriers, for at least 1 year, after a single AAV2/8-HBV injection. In a mouse model of HBV persistence developed previously by Huang et al., only 40% of C57BL/6 mice showed the desired characteristics (8). Our model therefore allows the use of a relatively small number of animals to establish HBV carrier status for future studies. Importantly, HBeAg expression was detected in 60% of hepatocytes in our model, contrasting with the 5 to 10% of hepatocytes expressing HBeAg observed previously after HDI with the HBV genome. In addition, AAV allows homogeneous transduction of the liver, whereas not all parts of the liver are reached after HDI (7). HDI clearly induces liver inflammation, possibly leading to the clearance of HBV hepatocytes observed in most mouse strains, including ours (7, 21). Our mouse model is more similar to the recently described model of HBV persistence based on a low-dose adenovirus vector carrying the HBV genome in immunocompetent C57BL/6 mice (10). However, the kinetics of early HBV antigen expression in sera and liver differ between the two

FIG 6 HBV-specific T-cell responses of HBV carrier mice immunized with HBc- and HBs-expressing DNA vectors. (A and B) ELISPOT assays performed on splenocytes (A) and intracellular cytokine staining (B) of intrahepatic lymphocytes from 5 to 10 HLA-A2/DR1 mice immunized with HBc- and HBs-expressing DNA vectors and controls (PBS). Mice were previously injected either with AAV2/8-HBV or with PBS 32 days earlier. (A) Peptides derived from HBeAg (18-27) and HBsAg (111-125, 179-194, 183-191, 200-214, and 348-357) were used to stimulate splenocytes ex vivo. (B) Intrahepatic lymphocytes were analyzed for CD8+ T cells producing intracellular IFN-γ after incubation with a pool of HLA-A2-restricted peptides (183-191, 204-212, and 348-357) or with medium alone (unstimulated). Representative fluorescence-activated cell sorter plots used to determine the percentages of IFN-γ-secreting T cells present in the liver of mice are shown. Values in the upper right squares are the percentages of CD8+ T cells that are IFN-γ-positive cells. (C and D) HBeAg (C) and HBsAg (D) titers in sera of mice that were first injected with AAV2/8-HBV and then immunized 32 days later (arrow) with DNA or received PBS. The values reported are means ± SEM, and results are representative of three independent experiments.
models, and HBV antigens seem to persist longer at high levels in the AAV-HBV-injected mice.

Persistence of HBV in our mouse model was not deleterious for the liver and was linked to impaired HBV-specific immunity. Indeed, HBV antigenemia in AAV2/8-HBV-injected mice was high, mimicking the initial stage of natural HBV infection evolving to chronicity, in which significant levels of circulating HBV antigens but no virus-specific immunity is detected (27). The natural history of chronic HBV infection in humans has been divided into four different phases defined by virological, immunological, and clinical parameters (28). In our mice, the viral antigen levels in sera could reach approximately 2,500 IU/ml (32 μg/ml) for HBsAg and 44 Paul-Ehrlich Institute units/ml (S/CO = 200) for HBeAg in females and were even higher in males. This corresponds to values found in sera of patients in the “immune-tolerant” phase of the disease (12). In these patients, normal or low levels of ALT and mild or no liver necroinflammation are observed, which is also a characteristic of our mouse model. Another feature of these patients is the apparent state of immune tolerance toward the virus, although this was revisited in a recent study (29). In our mice, neither HBc- nor HBs-specific T cells were detected in the periphery or the liver at either early or late time points after AAV2/8-HBV injection. Given the crucial role of HBsAg- and HBeAg-specific immunity in clearance of HBV (2, 23), this lack of antigen-specific immunity is in agreement with the chronic status and may result from the continuous exposure to high levels of HBV antigens in these mice or from priming of T cells in liver. Antigen presentation in liver can skew immune responses toward tolerance and limit the effector functions of T cells (30) or promote T-cell death via the proapoptotic molecule Bim (31).

Liver-directed transgene delivery by AAV vectors in mouse models results in tolerance to a number of the proteins produced, both secreted (32, 33) and cytosolic (34). In our model, we observed Treg populations larger than those in controls, and these cells may actively suppress the functions of HBV-specific T cells and maintain strong transgene expression. Several studies have addressed the role of Tregs in HBV infection and have shown a correlation of Treg frequencies in peripheral blood and liver with different disease stages (35, 36). Depletion in vitro of CD4+ CD25+ Tregs from peripheral blood mononuclear cell samples taken from HBV-infected patients led to an increase of IFN-γ production following HBV antigen stimulation (37). Moreover,

FIG 7 HBV-specific T-cell responses of mice immunized with HBc- and HBs-expressing DNA vectors. (A) ELISPOT assays performed on splenocytes from 8 HLA-A2/DR1 mice immunized with HBc- and HBs-expressing DNA vectors. Mice were previously (−32 days) injected either with AAV2/8-empty or with PBS. The peptides derived from HBcAg (18-27) and HBsAg (111-125, 179-194, 183-191, 200-214, and 348-357) were then used to stimulate splenocytes ex vivo. The values reported are means ± SEM. (B) Intrahepatic lymphocytes were analyzed for CD8+ T cells producing intracellular IFN-γ after incubation with a pool of HLA-A2-restricted peptides (183-191, 204-212, and 348-357) or with medium alone (unstimulated). Representative fluorescence-activated cell sorter plots used to determine the percentages of IFN-γ-secreting T cells present in the liver of mice are shown. Values in the upper right squares are the percentages of CD8+ T cells that are IFN-γ-positive cells.
inhibition of viral replication reduces Treg counts and enhances the antiviral immune response in chronic hepatitis B patients (38). Tregs may also limit liver injury by controlling inflammation. We also found an increased number of T cells producing IL-10 in the liver following AAV transduction. IL-10 is a potent anti-inflammatory cytokine that inhibits Th1-, Th17-, and Th2-mediated immune responses. Production of IL-10 is also associated with regulatory responses such as Tr1 (39). Therefore, both increased numbers of Tregs and IL-10 production in liver may account for the absence of liver inflammation and the inhibition of HBV-specific immune responses in our mouse model. However, using either in vivo Treg depletion or a blockade of the IL-10 receptor, we did not find any improvement in HBV-specific immune responses. This was consistent with previous studies that failed to rescue the AAV transgene-induced CD8 T-cell ability to proliferate or to produce cytokines (40). In addition to the suppressive cytokine IL-10, a sustained high viral load (41) and the PD1–PDL-1 and Tim3–galnectin-9 inhibitory pathways are known to actively suppress T-cell responses (42). In our model, a complex interplay between the different pathways of T-cell inhibition may cooperate following AAV-mediated HBV gene expression in the liver, leading to the establishment of a chronic viral infection.

The AAV-HBV-induced persistence model that we have established is validated in mice transgenic for HLA molecules. Because of its similarities with persistent HBV infection in humans, these mice, in which mouse MHC genes have been replaced by human HLA genes, should be useful as a powerful preclinical model to evaluate antiviral and immunotherapeutic strategies. One key objective of immune therapy of chronic HBV infection is to break the immune tolerance to HBV antigens and to induce functional HBV-specific T-cell responses that are able to clear the virus. In our model, we succeeded in breaking the observed tolerance by inducing HBV-specific T cells, which were found in the periphery and liver. We were able to prime HBV-specific T cells in the periphery by i.m. injection of DNA vaccines encoding HBV antigens; this shows that the persistence of HBV antigens did not lead to the complete inhibition of HBV-specific T cells in our model. Clearance of HBV DNA in liver is mediated by at least two mechanisms involving noncytolytic inhibition of viral replication and cytolysis of HBV-infected hepatocytes (43). However, despite the production of IFN-γ by DNA-primed T cells and their localization in the liver, the responses were not potent enough to decrease the HBsAg and HBeAg levels in sera. Stronger vaccine protocols may be needed to eliminate the virus. The increased number of regulatory T cells combined with an insufficient number of HBV functional T cells or the absence of liver inflammation in our model might be responsible for HBV persistence.

We describe here a model of persistent HBV infection in immunocompetent mice that reproduces many of the immunological features of patients with chronic HBV infection, notably defective HBV-specific B-cell/T-cell responses and abnormally high numbers of Tregs and T cells producing IL-10. Despite the induction of IFN-γ-secreting T cells following DNA-based vaccination, these mice failed to respond clinically to vaccination, as the vaccine-activated T cells had no impact on HBV antigen levels, as was observed for patients enrolled in many clinical trials of therapeutic vaccination (44). Nevertheless, our mouse model of HBV persistence could be used to investigate the mechanisms underlying persistent infection following viral replication and antigen presentation in liver cells and to test potential therapeutic approaches to counteract them. Finally, the viral vector AAV2/8 used to establish our HBV persistence mouse model could be easily manipulated to change the HBV genotype to allow analysis of, for example, the cross-reactivity of T cells induced by candidate therapeutic vaccines.

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REFERENCES


