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# Optimization of immune responses induced by therapeutic vaccination with cross-reactive antigens in a humanized hepatitis B surface antigen transgenic mouse model

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ABSTRACT

The absence of relevant animal models of chronic hepatitis B virus (HBV) infection has hampered the evaluation and development of therapeutic HBV vaccines. In this study, we generated a novel transgenic mouse lineage that expresses human class I and II HLA molecules and the hepatitis B surface antigen (HBsAg). HBsAg and hepatitis B core antigen (HBcAg) administered as plasmid DNAs and recombinant proteins, either alone or in combination, were evaluated as therapeutic vaccine candidates in this mouse model. Our results emphasize the importance of the route of administration in breaking HBsAg tolerance. Although immunizing the transgenic mice with DNA encoding homologous HBsAg was sufficient to induce CD8+ T-cell responses, HBsAg from a heterologous subtype was required to induce a CD4+ T-cell response. Importantly, only prime-boost immunization protocols that combined plasmid DNA injection followed by protein injection induced the production of antibodies against the HBsAg expressed by the transgenic mice.



## Introduction

Currently, more than 400 million individuals suffer from chronic hepatitis B virus (HBV) infection worldwide. Sophisticated antiviral treatments frequently confer substantial histological improvement, but these lifelong treatments often elicit drug resistance. Consequently, alternative approaches are needed to complement or replace these antiviral treatments.

The humoral response to HBV is critical for protection from infection and for long-term clearance of HBV. During acute hepatitis and exacerbations of chronic hepatitis B, the strong responses against HBV core antigen (HBcAg) in the form of anti-HBcAg antibodies (anti-HBc) suggest important roles for these responses in controlling HBV infection and achieving recovery (Ferrari et al., 1990). The induction of neutralizing antibodies against HBV surface antigen (HBsAg), reflects the clearance of the infection and provides protective immunity against subsequent HBV infection (Chisari, 1995; Huang et al., 2006). Following activation of innate immunity, the cellular response initiated by the T-cell response to viral antigens is thought to play a key role in HBV clearance. HBV can be cleared by cytolytic mechanisms (Maini et al., 2000; Moriyama et al., 1990) and by non-cytolytic, cytokine-mediated mechanisms (Guidotti et al., 1999). Patients resolving acute hepatitis display strong polyclonal and multispecific CD4+ and CD8+ T-cell responses against nucleocapsid, polymerase and envelope HBV proteins. In contrast, T-cell responses to HBV proteins are rarely detected in chronic HBV carriers and this is thought to be due to T-cell exhaustion, to anergy or to cytokine imbalance (Chisari, 1995; Ferrari et al., 1990; Rehermann et al., 1995). An HBsAg-specific CD8+ T-cell response is undetectable in patients chronically infected with HBV and suggests that excessive serum HBsAg may function as a tolerogen during chronic infection. Although very effective in preventive vaccination, HBV envelope proteins generally do not induce a strong CD4+ T-cell response during HBV infection (Bocher et al., 1999; Ferrari et al., 1990). Nevertheless, the induction of HBV-specific CD4+ T-cells is regarded to be an important component of any immunomodulatory therapy. CD4+ T-cells contribute to the induction (Ridge et al., 1998) and maintenance of antigen-specific CD8+ T-cells, they allow dendritic cells to activate CD8+ effector T-cells (Ridge et al., 1998; Sigal et al., 1999), and they provide help for activation and differentiation of B-cells. Therapeutic vaccination has been proposed to overcome the functional defects observed in the T-cell responses present in chronically infected patients. The challenge of this strategy is to

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enhance or broaden the HBV-specific immune response in these patients. Numerous clinical trials of therapeutic immunization using conventional, prophylactic HBsAg-based vaccines have achieved limited success and have underscored the need for further improvements in the treatment of chronic HBV infection (reviewed in (Michel et al., 2011)).

Due to the lack of *in vitro* models for chronic HBV infection, HBV transgenic (Tg) mice are the most widely used models for evaluating therapeutic strategies. Despite some limitations, Tg mice expressing human HLA molecules are superior to wild-type mice for testing vaccine candidates and for developing therapeutic strategies based on the induction of T-cell responses relevant to humans (Pajot et al., 2004; Rohrlich et al., 2003). HLA-A2 and HLA-DR1 Tg mice may facilitate improvements in therapeutic strategies that require collaboration between HLA-restricted CD4+ T-helper cells and HLA-restricted cytolytic CD8+ T-cells. In this study, we generated a novel humanized HBsAg Tg mouse model as a tool to assess different HBV antigens and different routes of administration for their ability to influence T-cell responses and to achieve successful therapeutic vaccination. We compared intradermal, intranasal and intramuscular immunizations of DNA and protein based on identical antigens.

## Results

### *"Humanized" Tg mice expressing HBsAg*

To evaluate the efficacy of potential human therapeutic strategies, we generated a novel Tg mouse lineage by crossing mice that lack murine class I and II molecules but express transgenic human HLA-A2 class I and HLA-DR1 class II molecules with an HBsAg-C57BL/6 Tg mouse lineage (see Materials and Methods). The HBV transgene consisted of a copy of the HBV genome (ayw subtype) with the core gene deleted (Babinet et al., 1985). Due to the heterozygous status of the HBV transgene, only 50% of the offspring express the HBV transgene and are therefore referred to as either HBs-A2-DR1 or A2-DR1 mice, depending on whether or not they produce HBsAg. As in the parental HBsAg-C57BL/6 lineage, liver-specific synthesis of the HBV envelope proteins in the HBs-A2-DR1 mice began before birth. Quantitative measurement of liver HBV mRNA revealed similar levels of expression in the two lineages (Fig. 1A). The HBV proteins synthesized by the hepatocytes self-assembled into HBsAg particles that were secreted into the sera of mice (Fig. 1B). Western blot analysis and staining with an anti-HBs antibody showed that HBsAg particles contain the small (S), middle (preS2+S) and large (preS1+preS2+S) envelope proteins in both glycosylated and non glycosylated forms (Heermann et al., 1984) (Fig. 1C). To discriminate between dimers of small proteins and large envelope proteins, an anti-preS1 antibody specific for the large protein was used in western blot analysis (Fig. 1C, right panel). Neither differences in the concentration of the particles (Fig. 1B) nor in the ratio of the different proteins were observed in sera from HBs-A2-DR1 and parental HBsAg-C57BL/6 mice (Fig. 1C). Despite the absence of transgene expression in the thymus (data not shown), HBs-A2-DR1 mice were tolerant to HBsAg because no antibodies or T-cell responses directed to HBsAg could be detected, even after non-specific stimulation (Fig. 1B and data not shown). Nevertheless, serum HBsAg levels in the mice decreased slightly over a six-month period (Fig. 1B). This phenomenon was also observed in the parental lineage and has been attributed to hormonal regulation (Farza et al., 1987). Using flow cytometry, we determined that the HLA-A2 and HLA-DR1 molecules were expressed by spleen cells from the humanized Tg mice but not by cells from the parental HBsAg-C57BL/6 mice (Fig. 1D). In addition, the mean fluorescence intensities of the A2-DR1 and HBs-A2-DR1

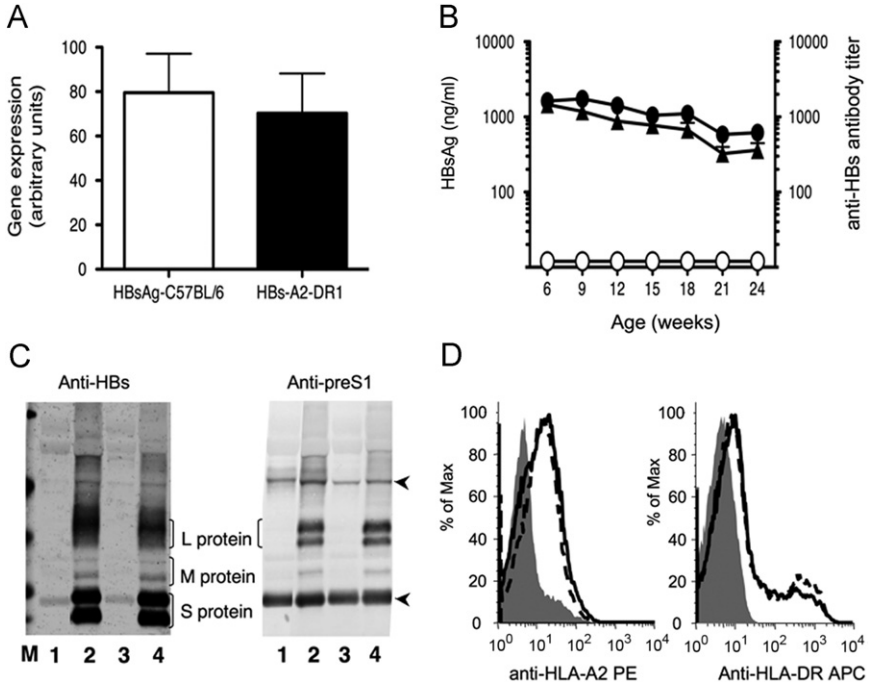


**Fig. 1.** Characterization of HBs-A2-DR1 mice. Relative expression of HBV genes was analyzed in the liver from six week-old HBsAg-C57/BL6 and HBs-A2-DR1 mice by RT quantitative PCR (A). Results from 4 mice are expressed as mean value ± SEM. (B) The HBsAg concentration (in ng/ml) was evaluated in the sera of 12 HBsAg-C57/BL6 (black triangles) and 12 HBs-A2-DR1 mice (black circles) at different time points. Particles of HBsAg ayw subtype were used to detect anti-HBs antibodies (empty circles) and antibody titers were determined by serial end-point dilutions. (C) Western Blot analysis of HBsAg particles present in mouse sera. HBV envelope proteins from C57/BL6 (1), HBsAg-C57BL/6 (2), A2-DR1 (3) and HBs-A2-DR1 (4) were detected either by rabbit anti-HBs (left) or mouse anti-preS1 (right) antibodies. Bands corresponding to the small (S), middle (M) and large (L) envelope proteins of HBV are indicated. M coresponds to protein size markers (75, 50, 37, 25, 20 kDa from the top). Arrows indicate the heavy and light chains of the mouse immunoglobulins. (D) Representative histograms for total splenocytes from A2-DR1 (black lines), HBs-A2-DR1 (dotted lines) and C57BL/6 (gray fill) mice stained for surface molecules with PE-conjugated anti-HLA-A2 (left) and APC-conjugated anti-HLA-DR (right) monoclonal antibodies.

spleen cells were similar, indicating that the presence of the HBV transgene did not hamper the expression of the HLA molecules.

*Immune response to homologous ayw HBsAg subtype after DNA-based immunization*

To ensure that the substitution of the murine MHC molecules by their human counterparts did not prevent an immune response to HBsAg, we evaluated the immune response induced by i.m. injections of the pCMV-S2.S ayw plasmid DNA in A2-DR1 mice (Fig. 2). The pCMV-S2.S expresses the small and the middle HBV envelope proteins. The resulting particles contain about 30% of middle proteins ((Michel et al., 1995) and data not shown). In *ex vivo* stimulation assays, splenocytes from immunized A2-DR1 mice secreted IFN-γ following stimulation with all HBsAg ayw-derived peptides tested (Table 1 and Fig. 2A). Depletion of specific T-cells subsets (Fig. 2B) revealed that some peptides (pep) were able to activate both CD4+ and CD8+ T-cells (pep 200–214, pep 337–357 and pep 348–357) whereas others activated only CD4+ T-cells (pep 114–128) or only CD8+ T cells (pep 183–191). IFN-γ production by CD4+ T cells after stimulation with pep 337–357 ayw was confirmed by intracellular staining (data not shown). IFN-γ-producing T-cells were also detected following plasmid DNA immunization of HBs-A2-DR1 mice (Fig. 2A). HLA-A2 restricted peptides (see Table) were able to trigger similar levels of IFN-γ secretion by HBs-A2-DR1 CD8+ T-cells and by A2-DR1 CD8+ T-cells. In contrast, following stimulation with HLA-DR1 restricted peptides 114–128 and 200–214, the number of IFN-γ-producing T-cells from immunized HBs-A2-DR1 mice was significantly lower than the number found in immunized A2-DR1 mice. Further analysis revealed that following stimulation with HLA-DR1-restricted peptides, no IFN-γ-secreting CD4+ T-cells could be detected in HBs-A2-DR1 mice by intracellular staining (data not shown). These results indicated that DNA immunization with a plasmid encoding the HBsAg ayw subtype in HBs-A2-DR1 mice expressing the same HBsAg subtype was able to generate a CD8+ T-cell response identical to the one obtained in immunized A2-DR1 mice. However, the HBs-specific CD4+ T-cell response was undetectable in immunized HBs-A2-DR1 mice.

*Cellular response after immunization with a heterologous adw HBsAg subtype combined with HBcAg*

Results obtained in HBs-A2-DR1 mice underlined a defect in the CD4+ T-cell response toward the ayw HBsAg subtype expressed by these mice. We therefore investigated whether DNA immunization of HBs-A2-DR1 mice with a plasmid encoding the heterologous adw HBsAg subtype could elicit a cross-reactive CD4+ T-cell immune response against the transgenic ayw HBsAg produced by the mice. To potentiate the immunization and because HBcAg may act as a T-cell-dependent and -independent antigen (Milich and McLachlan, 1986), HBs-A2-DR1 and A2-DR1 mice were immunized twice with a combination of pCMV-S2.S adw encoding the adw variant of HBsAg and pMasCore encoding the capsid protein of HBV. Two weeks after the final immunization, splenocytes were harvested and stimulated *ex vivo* with libraries of HBV core and envelope peptides or with the HBcAg. High frequencies of HBcAg- and HBsAg-specific IFN-γ-producing cells were detected in the spleens of immunized mice, which indicated that the DNA immunization was successful (Fig. 3A). The frequency and specificity of HBcAg-specific IFN-γ-producing cells were comparable in the two lineages. Stimulation with HBcAg particles or the HBcAg peptide pool C1–55 elicited similar numbers of IFN-γ-producing cells in each lineage suggesting that the T-cell response was focused on the N-terminal part of the capsid protein. The pCMV-S2.S adw plasmid also primed HBsAg-specific responses that were mainly directed

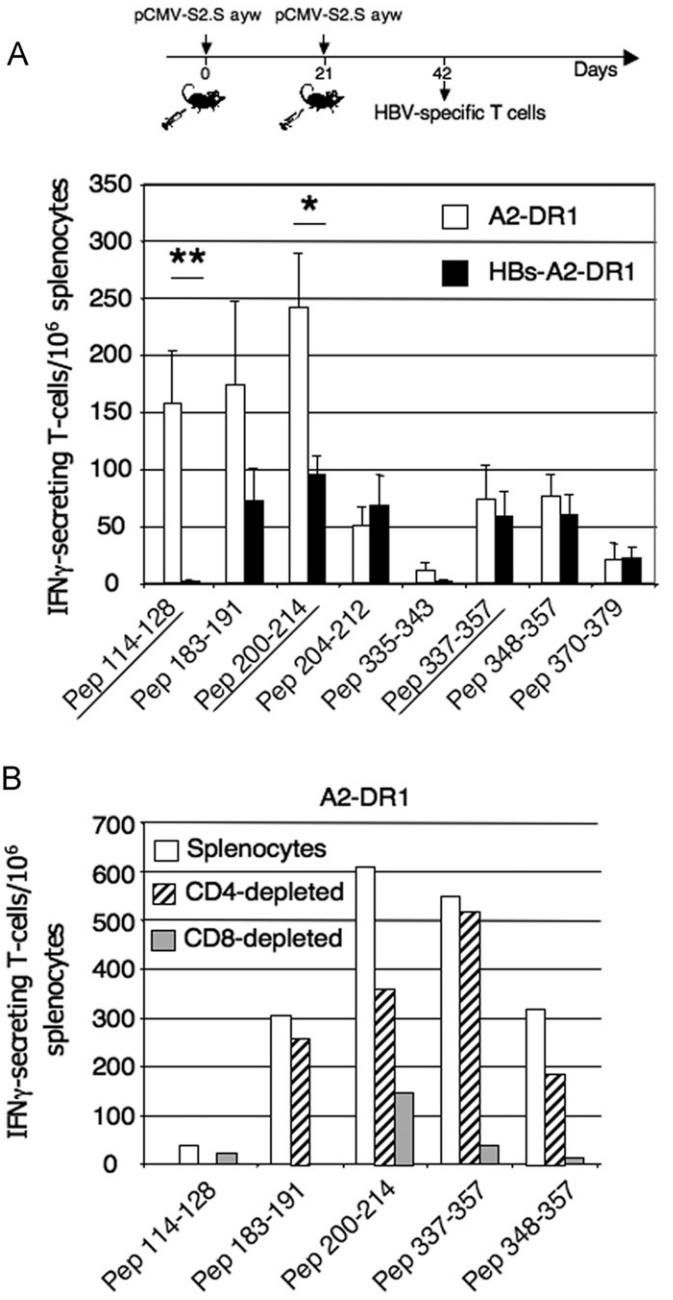


**Fig. 2.** Induction of T-cell responses in mice after pCMV-S2.S ayw immunization. Time schedule of immunization and analysis is presented on the top of the figure. Groups of A2-DR1 (white bars) and HBs-A2-DR1 (black bars) mice were immunized by i.m. injection of pCMV-S2.S ayw into cardiotoxin-treated tibialis anterior muscles on days 0 and 21. Splenocytes were collected three weeks after the second immunization and HLA-A2- or HLA-DR1-restricted (underlined) HBs-derived peptides from the ayw subtype were used to stimulate the splenocytes *ex vivo*. (A) Quantification of IFN-γ-secreting T-cells by ELISPOT assay. Each bar represents the mean value ± SEM of IFN-γ-secreting T-cells per million splenocytes ($n=3$ to 13 mice). Identical numbers of mice were used to determine the statistical significance of differences between groups by the Mann-Whitney *U* test. *, $p<0.05$; **, $p<0.01$. (B) Characterization of the IFN-γ-producing T-cell subsets by depletion experiments in A2-DR1 mice. Representative ELISPOT assays performed on CD4-depleted T-cells (striped bar), CD8-depleted T-cells (gray bars), or undepleted splenocytes (empty bars).

to pools S164–218 and S338–389 in both lineages. Conversely, no IFN-γ secretion was observed after stimulation with the preS2 ayw set of peptides indicating that either the preS2 adw region was not immunogenic or the induced T-cell response was not detectable with the preS2 ayw peptides used to stimulate the cells.

**Table 1**
HLA-restriction and specificity of HBV envelope-derived epitopes.

| Residue[a] | Amino acid sequence[b] | HLA-restriction | Subtype[c] | References |
|---|---|---|---|---|
| 114–128 | TTFHQTLQDPRVRGL | DR1 | ayw | Pajot et al., 2006 |
| 183–191 | FLLTRILTI | A2 | adw/ayw | Chisari, 1995 |
| 200–214 | TSLNFLGGTTVCLGQ | DR1 | ayw | Pajot et al., 2006 |
| | TSLNFLGGSPVCLGQ | DR1 | adw | Pajot et al., 2006 |
| 204–212 | FLGGTTVCL | A2 | ayw | Chisari, 1995 |
| | FLGGTPVCL | A2 | adw | Chisari, 1995 |
| 335–343 | WLSLLVPFV | A2 | adw/ayw | Nayersina et al., 1993 |
| 337–357 | SLLVPFVQWFVGLSPTVWLSV | DR1 | ayw | Pajot et al., 2006 |
| 348–357 | GLSPTVWLS | A2 | adw/ayw | Nayersina et al., 1993 |
| 370–379 | SILSPFLPLL | A2 | ayw | Chisari, 1995 |

[a] Positions of peptides on the HBV envelope protein are indicated starting from the first methionine of the PreS1 region in the HBV ayw sequence.
[b] Amino acid sequence of the peptides.
[c] Subtype of HBsAg.

To further characterize the T-cells involved in the secretion of IFN-γ, we stained the cells for intracellular IFN-γ. CD8+ T-cell responses were detected following stimulation with all reactive peptide pools and no significant differences were observed between the two lineages (Fig. 3B, left). The most remarkable T-cell responses were observed after stimulation with peptide pool S164–218, which contained the S183–191 and S204–212 CD8+ T-cell epitopes and the S200–214 CD4+ T-cell epitope.

Using individual peptides, we found that immunization of HBs-A2-DR1 mice with the pCMV-S2.S adw induced a CD8+ T-cell response to peptide S200–214 adw and, more precisely, to the A2-restricted epitope S204–212 adw (Fig. 3C, upper panel). We next examined whether the pCMV-S2.S adw-primed CD8+ T-cells were able to cross-react with the ayw epitope of the HBsAg that was constitutively produced by the HBs-A2-DR1 mice. The primed CD8+ T-cells showed no cross-reactive recognition of the S204–212 ayw epitope (Fig. 3C, upper panel). We detected IFN-γ-producing CD4+ T-cells in A2-DR1 mouse splenocytes stimulated with pools of HBcAg- or HBsAg-derived peptides (Fig. 3B, right). In HBs-A2-DR1 mice, and in contrast to what we observed for the CD8+ T-cell response, a CD4+ T-cell response was detectable only after stimulation with pool S164–218 (Fig. 3B, right). Stimulation with individual peptides indicated that this response was directed to the HLA-DR1-derived peptide S200–214 from the adw subtype (Fig. 3C, lower panel). These results indicate that DNA immunization with an HBsAg-encoding plasmid from a heterologous subtype is able to generate a CD4+ T-cell response in HBs-A2-DR1 mice. Adding core antigen to the immunization did not improve the response to HBsAg as no cross-reactive responses to ayw peptides could be detected.

### *Humoral response after immunization with a heterologous adw HBsAg subtype combined with HBcAg*

Since HBcAg is also a potent B-cell activator, even in the absence of adjuvant (Milich and McLachlan, 1986), we investigated the antibody responses induced in HBs-A2-DR1 and A2-DR1 mice after two i.m. injections of the pCMV-S2.S adw/pMasCore DNA combination. The immunizations achieved identical anti-HBc antibodies titers in both lineages (Fig. 4A). This result was expected since none of the humanized Tg mice expressed HBcAg. To define precisely the specificity of the anti-HBs response produced by the immunization, we used HBsAg adw particles and HBsAg ayw particles to detect the responses to the different HBsAg determinants (*i.e.*, the anti-'a', 'd' and 'w' responses). Whereas ayw particles could only detect responses to the 'a' and 'w' determinants, the adw particles could detect responses to the 'a', 'd', and 'w' determinants in the serum of mice immunized with pCMV-S2.S adw. We detected low levels of anti-HBs antibodies in each lineage that were primarily directed against the 'd' determinant since the HBsAg ayw particles coated on the solid phase detected only very low anti-HBs levels in the serum of mice (Fig. 4A). Of note, the anti-HBs titers in the A2-DR1 mice were significantly higher than those in the HBs-A2-DR1 mice.

As DNA immunization induces a weaker humoral response than protein immunization, and in order to increase anti-HBs antibody production, we immunized the two mouse lineages with protein antigens using Nasvac, a formulation containing HBcAg and HBsAg adw proteins without adjuvant (Aguilar et al., 2004). We administered the proteins by the i.d. and i.n. routes. It has been reported that immunization by the i.d. route can achieve higher antibody titers than those produced by the i.m. route (Endmann et al., 2010) and that the use of nasal immunization can also improve antigen uptake due to the numerous antigen-sampling dendritic cells located in the nasal mucosa (Kallenius et al., 2007). Three injections with the formulation were highly immunogenic in A2-DR1 and HBs-A2-DR1 mice especially with respect to anti-HBc antibodies (Fig. 4B). Nasvac immunization generated tenfold more anti-HBc antibodies than the DNA injections ($p=0.011$ for A2-DR1 mice; $p=0.002$ for HBs-A2-DR1 mice). In contrast, mice immunized by either method produced similar anti-HBs antibody levels, whether the antigens were administered as DNA or as purified proteins without adjuvant. Once again, anti-HBs antibody titers were significantly higher in A2-DR1 mice than in HBs-A2-DR1 mice ($p=0.0009$). These antibodies were mainly directed against the 'd' determinant in A2-DR1 mice and were not detectable in HBs-A2-DR1 mice. This result suggested that the anti-HBs antibodies in the HBs-A2-DR1 mice were complexed *in vivo* with the circulating HBsAg particles produced by the mice. To test this hypothesis, we determined if the induced humoral response in the immunized mice impacted the serum HBsAg concentration in HBs-A2-DR1 mice (Fig. 4C). We detected a significant decrease in HBsAg levels in sera collected three weeks after the last i.d and i.n. protein injections but not after the i.m. DNA injections. These results indicated that immunization strategies that combine core antigen with a heterologous HBsAg subtype are able to induce anti-HBs antibodies that recognize the HBsAg particles present in HBs-A2-DR1 mice.

### *Prime-boost strategy*

To optimize the immunogenic potential of the HBV DNAs and proteins, we induced cellular and humoral responses by

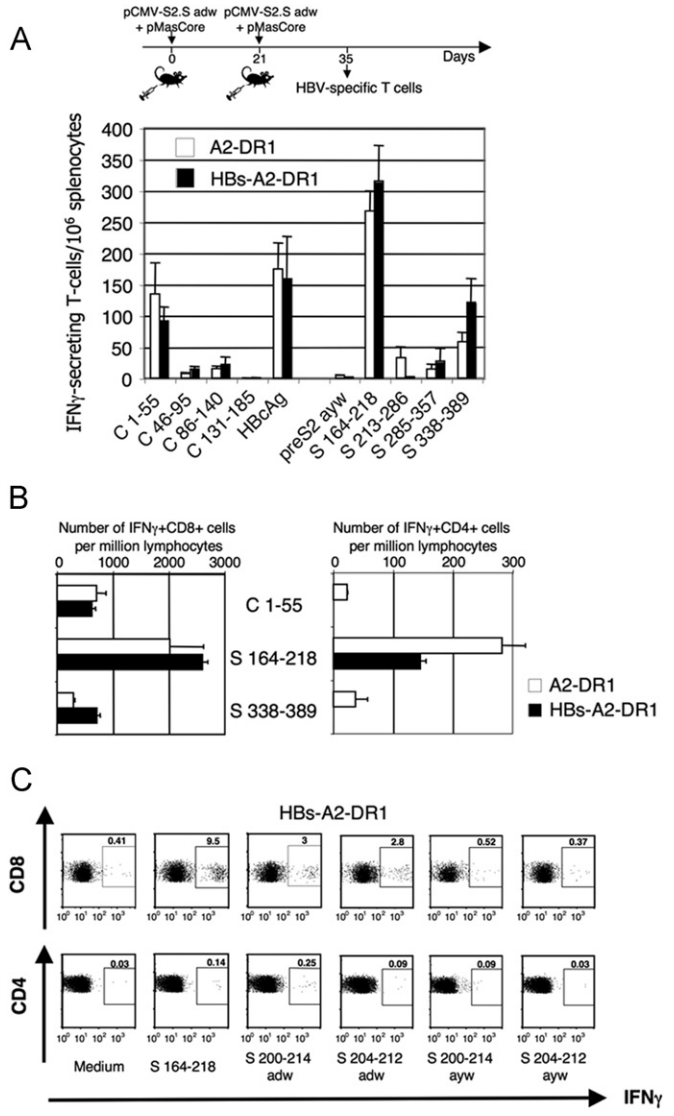


**Fig. 3.** Specificity of T-cell responses following immunization with DNAs encoding a heterologous HBsAg adw subtype and HBcAg. *Ex vivo* ELISPOT assay (A) or intracellular staining (B and C) performed on A2-DR1 (white bars) and HBs-A2-DR1 (black bars) splenocytes from mice immunized by i.m. injections with pCMV-S2.S adw and pMasCore on days 0 and 21. Two weeks after the second injection, pools of 15-mer peptides covering either the entire HBcAg (C1–55; C46–95; C86–140 and C131–185), the preS2 domain, HBsAg (S164–218; S213–286; S285–357 and S338–389) or the HBcAg protein were used to stimulate splenocytes. (B) Spleen cells were analyzed for CD8+ T-cells (left panel) or CD4+ T-cells (right panel) producing intracellular IFN-γ following stimulation with peptide pools C1–55, S164–218 and S338–389. Data are the mean ± SEM for 8 mice per group. (C) Representative FACS plots of intracellular staining used to define the percentages of the IFN-γ-secreting T-cell subsets present in the spleens of immunized HBs-A2-DR1 mice. Peptides used to stimulate the splenocytes are indicated. Values in the squares are the percentages of IFN-γ+ cells among the CD8+ (upper panels) or CD4+ (lower panels) T-cells.

combining both strategies. We immunized HBs-A2-DR1 mice with two injections of pCMV-S2.S adw/pMasCore DNAs (i.m., or i.d and i.n.) followed by three i.d and i.n. doses of Nasvac (Fig. 5A and C) or with three treatments of the Nasvac formulation (i.d and i.n.) followed by two i.m. injections of the HBV DNAs (Fig. 5B). As controls, we injected mice with PBS by the same routes. We then determined the HBsAg levels in sera collected three weeks after each immunization. After the second intramuscular injection of the DNAs or PBS, the HBsAg levels were not significantly different from preimmune levels (Fig. 5A, compare weeks 0 and 6), which confirmed the results shown in Fig. 4C. In contrast, the HBsAg levels had decreased significantly after three i.d. and i.n. injections of proteins (Fig. 5B, weeks 9 and 12). However, a similar decline was also observed in the PBS-injected group. This phenomenon was observed in all vaccination strategies only after i.d. and i.n. injections of PBS, suggesting that repeated i.d. and i.n. injections exerted a non-specific effect on HBsAg levels. Nevertheless, when the DNAs were injected i.m. prior to protein immunization, serum HBsAg levels decreased markedly and specifically (73% decrease) after the first boost of protein (Fig. 5A week 9, $p < 0.001$) and this decrease persisted up to week 15. In contrast, DNA boosts did not enhance the decrease in HBsAg levels in the sera of mice that were first immunized with proteins (Fig. 5B).

We therefore evaluated the DNA prime/protein boost strategy using DNAs and recombinant proteins that were given only by the i.d and i.n. route (Fig. 5C). Due to high variability in the groups at week 0, no significant decreases in HBsAg levels compared to preimmune levels were detected. Nevertheless, intergroup comparisons revealed that the HBsAg titers in mice from the DNA-primed group were significantly lower than those from the PBS-injected control group, starting at three weeks after the second DNA immunization. This effect was still boosted after two Nasvac injections and corresponded at week 12 to a 62% decrease in the HBsAg levels compared to preimmune levels. These results indicate that i.d and i.n injections of the DNAs were also able to decrease serum HBsAg levels in HBs-A2-DR1 mice. To assess the impact of DNA prime-protein boost strategies on intrahepatic HBV mRNA levels, livers from immunized mice were taken at week 12. Quantitative RT-PCR showed that the level of HBV mRNAs was proportional to the HBsAg concentration in sera (r value determined by the Spearman correlation test, 0.83; $P < 0.0001$) indicating that immunizations had modulated the HBV mRNA expression.

At the end of the immunization protocols, we also determined the anti-HBs humoral responses induced by the three prime-boost strategies (Fig. 5D). Despite HBsAg titers in the PBS-injected groups that were lower at week 15 than the preimmune levels, no anti-HBs antibodies were detected in any of the PBS-injected groups. In contrast, anti-HBs antibodies were detected in all three immunized groups. After the DNA prime/protein boost strategy, anti-HBs antibody titers at week 15 were between 10 and 100 times higher than after the primes only (compare Figs. 5D and 4A). The most effective protocol consisted of intramuscular DNA injections followed by i.d and i.n Nasvac immunizations. This was the only protocol in which anti-HBs antibodies were detected using the ayw particles as the capture antigen. Although our assay was unable to distinguish between an anti-'a' or an anti-'y' response, this result demonstrated the presence of antibodies able to recognize HBsAg ayw particles similar to those produced by the HBs-A2-DR1 mice. This was not the case with the protocol that used only the i.d and i.n. routes, which induced only anti-'d' antibodies. Altogether, these results show that priming with DNA immunizations followed by boosts with protein antigens were more efficient in reducing HBsAg levels in HBs-A2-DR1 mice than immunization protocols that primed with protein and boosted with DNA. Furthermore, only i.m. DNA injections were able to elicit antibodies specific for the HBsAg expressed by the HBs-A2-DR1 mice.

## Discussion

Few animal models for chronic HBV infection exist currently. Although chimpanzees can be infected and may develop chronic disease, these animals are not readily available or accessible. To evaluate the potential benefits of therapeutic HBV vaccines, surrogate models that effectively reproduce the characteristics of the chronic HBV infection are needed. Most existing models rely on Tg mice that replicate the virus or express HBV proteins. However, since

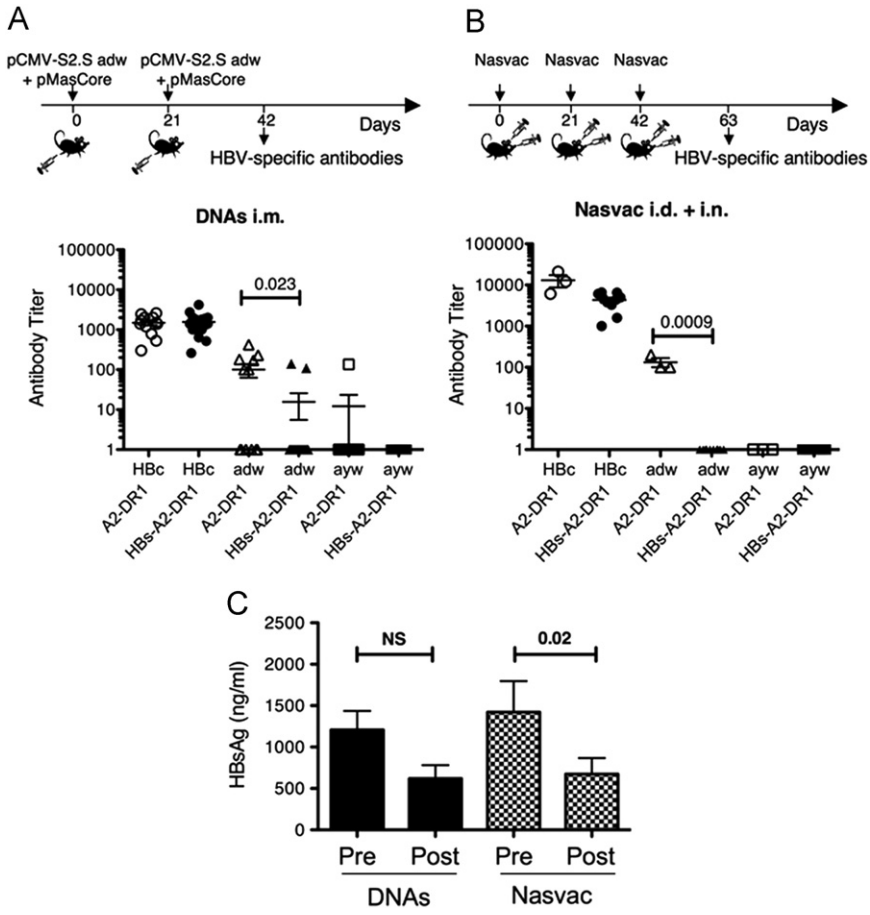


**Fig. 4.** Antibody response in immunized mice. Groups of A2-DR1 (empty symbols) and HBs-A2-DR1 (black symbols) mice were immunized twice by i.m. injection of pCMV-S2.S and pMasCore DNAs (A) or three times by i.d. and i.n. injection of Nasvac proteins (B). Three weeks after the last injection, antibodies in sera were quantified by ELISA. HBV core particles (HBc) were used to detect anti-capsid antibodies and particles of HBsAg adw (identical to the injected antigens) and HBsAg ayw (similar to particles expressed by the HBs-A2-DR1 mice) were used to detect anti-envelope antibodies. The antigens used for detection are indicated on the x axis above the name of the mouse lineage. Antibody titers were determined by serial end-point dilutions. The titer was defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum. (C) HBsAg concentration (ng/ml) in the sera of HBs-A2-DR1 mice was determined before (Pre) and three weeks after the last injection (Post) of DNAs or proteins (Nasvac). Data show the mean values ± SEM. Significant p values are indicated. NS: not significant.

therapeutic vaccination aims at inducing multispecific B- and T-cell responses, optimal models must also allow these responses to be considered in humans. In this study, we have generated a novel Tg mouse lineage that meets these criteria. As in chronic HBV carriers, these mice express large amounts of HBsAg, which is known to induce tolerance. They also express the human MHC class I and II molecules in the absence of murine MHC molecules. This allows the detection of immune responses that are potentially identical to those observed in HLA-A2 and HLA-DR1 individuals. Indeed, we showed that immunizing A2-DR1 mice with a plasmid encoding HBsAg induced a high frequency of IFN-$\gamma$-secreting T-cells in the periphery. These cells had specificity for endogenously processed HBV peptides and they exhibited recognition specificities similar to those that arise during self-limited infection or vaccination in humans (Chisari, 1995; Pajot et al., 2006). By contrast, no CD4+ T-cell responses could be detected in the HBs-A2-DR1 mice, which correlated with the situation in human chronic infection where a CD8+ T-cell response is found in the absence of a detectable CD4+ T-cell response (Urbani et al., 2005). Immune responses induced in this well-defined model system can provide useful insight into the CD4+ T-cell reactivities required to resolve chronic HBV infection. So far, therapeutic HBsAg-based vaccination of chronically infected patients has been unable to entirely control the infection (Michel et al., 2011). Therefore, our Tg mice represent a valuable model for evaluating the efficacy of prospective immunogens for therapeutic vaccination. HBcAg-specific CD4+ T-cell responses are detectable in patients during acute infection and are probably essential for controlling viremia. Moreover, HBcAg can function as both a T-cell-independent and a T-cell-dependent antigen (Milich and McLachlan, 1986) and HBcAg-specific CD4+ T-cells can sustain anti-core and anti-envelope antibody production (Milich et al., 1987). Similarly, the generation of CD4+ T-cells specific for heterologous HBsAg adw epitopes, which is induced by the endogenous (DNA immunization) and exogenous (protein immunization) processing of antigens, may facilitate the priming of CD8+ T-cells and anti-HBsAg antibody responses in HBs-A2-DR1 mice. This has been shown to be important for the CD8+ T-cell response in a therapeutic approach that used variants expressing cross-reactive CTL epitopes (Schirmbeck et al., 2003).

Anti-HBs antibodies are considered to be the predominant factor in preventing HBV infection due to their capacity to eliminate circulating viral particles. Thus, the induction of a humoral response in chronic HBV infection could eliminate the virus and reduce the established tolerance to HBsAg. The use of a single injection route induced different anti-HBs antibody responses in our two mouse lineages. The anti-HBs antibody response in HBs-A2-DR1 mice, which was characterized with adw particles to allow detection of the response to the heterologous 'd' epitope, was less intense than the one achieved in A2-DR1 mice. This weaker reactivity could be ascribed to lower antibody production by anergic B-cells. However, the humoral response to HBcAg was comparable in both lineages,

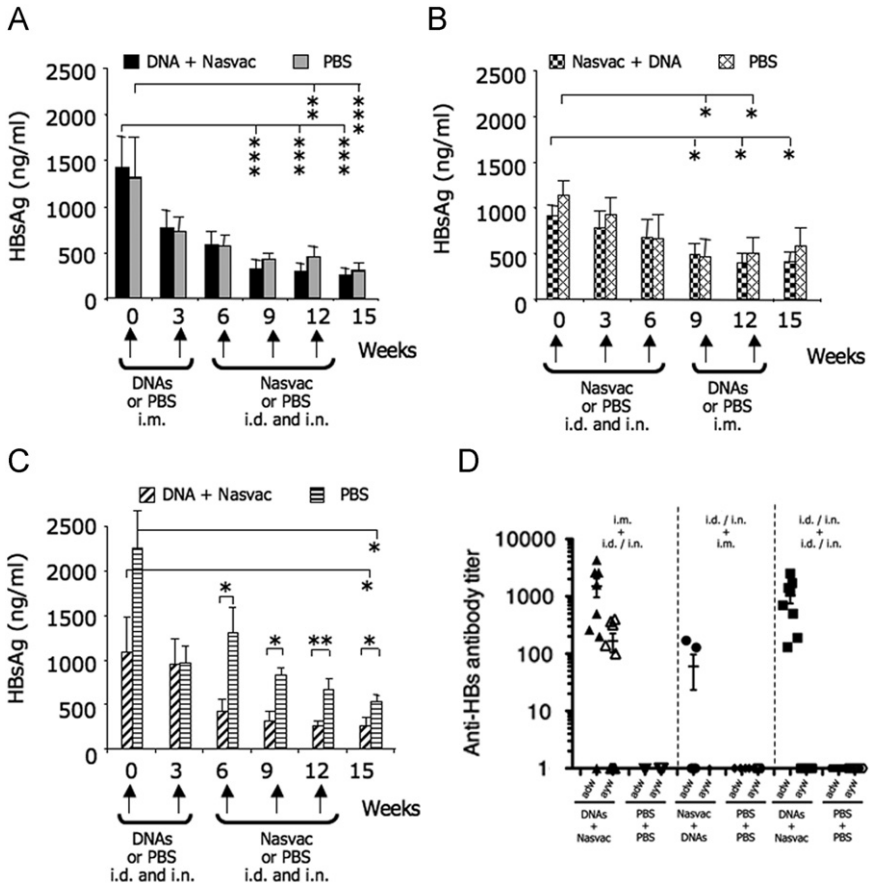


**Fig. 5.** Titration of HBsAg and anti-HBs antibodies in the sera of HBs-A2-DR1 mice following prime/boost immunization. Groups of 8 (A, C) and 5 (B) HBs-A2-DR1 mice were immunized either twice by i.m. injections of the pCMV-S2.S adw/pMasCore DNAs and three times by i.d and i.n. injections of Nasvac proteins (A, B) or five times by i.d and i.n injection of DNAs and Nasvac proteins (C) as indicated under each panel. PBS-injected HBs-A2-DR1 mice were used as controls. Sera were collected every three weeks and evaluated for HBsAg concentration (ng/ml). (D) Three weeks after the last injection, antibodies in the sera of mice immunized by the protocols described in A, B, C were quantified by ELISA as in Fig. 4. Results are given as mean values ± SEM. For multiple comparisons, an ANOVA followed by the Friedman test and Dunn's Post-test were used to determine significant differences. The Mann-Whitney *U* test was used for intergroup comparisons and stars indicate a significant difference between compared groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

demonstrating the capacity of HBs-A2-DR1 mice to produce antibodies against foreign antigens at levels identical to those produced by A2-DR1 mice. Nevertheless, we cannot exclude the possibility that some of the antibodies produced by the immunized HBs-A2-DR1 mice were directed against additional adw antigenic determinants that were recognizable on the circulating ayw particles but not the ayw antigen coated on the solid phase.

Since the HBsAg produced *in vivo* by the HBs-A2-DR1 mice can adsorb a fraction of the antibodies generated by immunization, fine analysis of the specificities of the antibody response remains difficult. However, the decrease in HBsAg titers that occurred in response to the prime-boost protocol suggests that the mice produced cross-reactive anti-HBs antibodies able to complex with circulating HBsAg. Of note, anti-subtype 'y' antibodies were produced only after i.m. DNA injection, revealing that this route of immunization was important for subverting B-cell tolerance.

Creating a successful therapeutic vaccine requires that vaccine formulation and delivery routes be optimized. Recent studies have suggested that a heterologous prime-boost vaccination approach, in which the same antigen is delivered sequentially by different types of vaccines, was very effective at eliciting enhanced and broad humoral and cellular immune responses (Lu, 2009). Such heterologous regimens can be effective when clearances of viral particles and of virus-infected cells are both required to resolve infection. Our evaluation of these DNA and protein immunogens in a prime-boost strategy revealed differences in the resulting immune response that should be taken into consideration to when optimizing therapeutic vaccination strategies. We showed that the routes of DNA immunization generated different humoral responses, probably due to the presence of different types of antigen presenting cells in skin, mucosa and muscle. Further studies should be conducted to evaluate whether prime-boost regimens generate broader T-cell responses than those produced by prime-only procedures. Of note, a decrease in HBsAg levels was observed following i.d and i.n. injections of PBS. Despite the injection of small volumes, the i.d and i.n. routes of immunizations may generate tissue damage. We speculate that following tissue damage, damage-associated molecular patterns activated by endogenous intracellular molecules released by necrotic cells and/or extracellular matrix molecules could lead to Toll-like receptors (TLR) activation that, in turn, induces inflammatory gene expression (Piccinini and Midwood, 2010). Necrotic cells have been also found to cause dendritic cell maturation in a TLR2 dependent manner. Recruitment of antigen-nonspecific inflammatory cells and production of cytokines could therefore modulate the HBV gene expression as it has been already shown in an HBV transgenic mouse model (Chisari, 1995).

Immunization with proteins usually requires that potentially toxic adjuvants be delivered with the immunizing component of the vaccine. In this study, we showed that low doses of protein antigens delivered without adjuvant induced potent polyclonal and multispecific cellular and humoral responses and that they also reduced the levels of circulating HBsAg when combined with

intramuscular injections of DNAs. The Nasvac and plasmid DNA used in this study are currently being manufactured and have demonstrated their safety in the development of vaccines (Betancourt et al., 2007; Mancini-Bourgine et al., 2004). In the setting of therapeutic vaccination, where multiple vaccine doses are often required, an effective adjuvant-free vaccine is preferred. The capacity of a candidate vaccine to generate potent polyclonal and multispecific cellular and humoral responses is an important feature for controlling chronic hepatitis B infection.

Taken together, our data show that immunization with HBV antigens can generate B- and T-cell responses even in a tolerogenic context and demonstrate that HBs-A2-DR1 mice represent a relevant model for preclinical evaluations of therapeutic vaccines.

## Materials and methods

### *Transgenic mice*

A novel Tg mouse lineage was obtained by intercrossing HLA-A*0201$^{+/+}$/HLA-DR1$^{+/+}$ double-transgenic H-2 class I ($\beta 2m^{0/0}$)-/class II ($IA\beta^{0/0}$)–KO mice (Pajot et al., 2004) and HBsAg$^{+/0}$/C57BL/6 mice (Babinet et al., 1985). Progeny were screened by PCR for the presence of the HLA-A*0201 and HLA-DR1 transgenes until HBsAg$^{+/0}$/HLA- A*0201$^{+/+}$/HLA-DR1$^{+/+}$ transgenic H-2 class I ($\beta 2m^{0/0}$)-/class II ($IA\beta^{0/0}$)–KO animals were obtained. The homozygous status of HLA-A*0201 and HLA-DR1 transgenes was confirmed by the presence of both transgenes in the progeny obtained by crossing the novel Tg mice with C57BL/6 mice. The HBsAg/HLA-A2/HLA-DR1 Tg mice express chimeric HLA-A2 MHC class I, HLA-DR1 MHC class II molecules and HBV envelope proteins from genotype D carrying the HBsAg of subtype ayw in the liver, which are secreted and present in serum throughout the animal's lifetime (see Fig. 1). Due to the heterozygous status of the HBV transgene, only 50% of the offspring express the HBV transgene and are therefore referred to as either HBs-A2-DR1 or A2-DR1 mice, depending on whether or not they produce HBsAg.

All mice were six to nine weeks of age at the first administration and were maintained at the Institut Pasteur animal facility under specific-pathogen-free conditions. All protocols were reviewed by the Institut Pasteur competent authority for compliance with the French and European regulations on Animal Welfare and with Public Health Service recommendations.

### *DNA vectors and recombinant antigens*

Plasmid DNAs were endotoxin-free and manufactured by PlasmidFactory (Germany). The pCMV-S2.S ayw and adw plasmids encode the preS2 and S domains of the ayw or adw subtype of HBsAg, respectively, and their expression is controlled by the cytomegalovirus immediate early gene promoter (Michel et al., 1995). The pMasCore plasmid (a kind gift of H. L. Davis, Coley Pharmaceutical Group) encodes the HBV capsid carrying the hepatitis B core antigen (HBcAg) of the adw subtype.

The recombinant HBsAg subtype adw was expressed by and purified from *Pichia pastoris* yeast cells and was produced at over 95% purity at the production facilities of the Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba), as a component of the commercial HBV prophylactic vaccine Heberbiovac-HB. The entire recombinant HBcAg particle of 183 amino acids was expressed in *Escherichia coli* and obtained with a purity of $>95\%$. The Nasvac formulation, containing both antigens at a concentration of 100 μg/ml, was obtained by simple mixture in phosphate-buffered saline (PBS) (Aguilar et al., 2004).

### *Protein analysis and immune staining of HBsAg particles*

Pooled mouse sera were clarified and subjected to ultracentrifugation on a 30% sucrose cushion in PBS for 4 h at 40,000 rpm. Pellets were resuspended in an appropriate volume of Laemmli buffer containing 2% β-mercaptoethanol, and subjected to protein electrophoresis on NuPAGE 4–12% Bis TRIS gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes (Biorad), and probed with specific anti-S and anti-preS1 antibodies. Membranes were then blocked with 3% nonfat milk dissolved in PBS-0.05% Tween 20 for 1 h and incubated overnight with a rabbit anti-S (R247; 1:500 dilution (Jenna and Sureau, 1999)) or a mouse anti-preS1 (Mab 18/7; 4 μg/ml kindly provided by W.H. Gerlich, Institut für Med. Virologie, Giessen, Germany) antibodies. After four washes in PBS-0.05% Tween 20 for 15 min, membranes were incubated for 35 min with appropriate secondary antibodies labeled with IRDye (Rockland Immunochemicals), with gentle shaking and protection from light. Fluorescent immunoblot images were then acquired and quantified by using an Odyssey scanner and the Odyssey 1.2 software (Li-Cor Biosciences).

### *RNA isolation and quantification of liver HBV mRNAs*

Total RNA was prepared from liver samples with TRIzol reagent and TURBO DNA-free reagent (Life Technologies). RNA (5 μg) was retrotranscribed using oligo(dT)18 primers (Fermentas) and M-MLV Reverse Transcriptase (Life Technologies). For quantitative PCR, HBV (forward 5′–GCTTTCACTTTCTCGCCAAC-3′, reverse 5′–GAGTTCCG-CAGTATGGATCG-3′) and HPRT (forward 5′–ATGCCGAGGATTTG-GAAAAA-3′, reverse 3′–ACAATGTGATGGCCTCCCA-5′) primers were used. Samples were prepared in triplicates by addition of 5 ng of cDNA to HBV and HPRT primers and SYBR Green PCR Master Mix (Applied Biosystems). Quantitative PCR was carried out on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using a standard PCR protocol (denaturation at 95 °C, annealing and extension at 60 °C). For data analysis, gene expression values were determined using the $2^{\Delta CT}$ method (Schmittgen and Livak, 2008), where $\Delta CT = CT$ (HBV primers) − CT (HPRT primers), and were reported as arbitrary units.

### *Immunization protocols*

Groups (five to ten female mice/group) were immunized by different routes, including bilateral intramuscular (i.m.) injection into cardiotoxin-treated tibialis anterior muscles as previously described (Michel et al., 1995) or intranasal (i.n.) and intradermal (i.d.) inoculations. All injections were carried out under anesthesia attained by administering 100 μL ketamine (12.5 mg/mL) and xylazine (1.25 mg/mL). Mice were immunized two times with DNAs and three times with recombinant antigens and the immunizations were given three weeks apart. Products were either dispensed slowly into both nostrils (50 μl) using a pipette tip for i.n. administration or injected into both ear pinnae (50 μl) for i.d. injection. A total of 100 μg of DNAs or 10 μg of proteins in a total volume of 100 μl was used for each injection.

### *Synthetic peptides*

Four distinct sets of synthetic peptides were used in this study: a pool of peptides containing the known HLA-A2- and HLA-DR1-restricted peptides within the preS2 and S domains of the HBV envelope proteins (see Table 1); a pool of ten 15-mer peptides spanning the entire preS2 region of HBV envelope subtype ayw; a pool of 9- to 15-mer peptides spanning the entire region of the HBV envelope subtypes ayw and adw were divided into four sets of eleven peptides (S164–218, S213–286, S285–357 and S338–389); and a pool

of 36 overlapping 15-mer HBV core peptides comprising the whole HBcAg of the adw subtype. This HBcAg pool was divided into four sets of nine peptides (C1–55, C46–95, C86–140, and C131–185).

Synthetic peptides were purchased from Polypeptide Group (Strasbourg) and were used at a final concentration of 1 μM. Peptide purity was > 80%. Peptides were dissolved in RPMI or DMSO at a concentration of 10 mg/ml and diluted to 1–2 μg/ml with culture medium before use.

*Detection of serological parameters by ELISA*

Serum samples were repeatedly obtained from vaccine-immunized mice or placebo-inoculated control mice by retro-orbital puncture using heparinized glass pipettes at different time points post-injection. ELISA was used to quantify the antibodies in the sera before and after immunization. For antibody determination, serial dilutions of sera were added to antigen-coated wells (1 μg/ml) and bound antibodies were detected by staining with anti-mouse horseradish peroxidase (Life Sciences). Purified HBcAg was used to detect anti-capsid antibodies. Purified HBsAg adw particles (identical to the injected products) and HBsAg ayw particles (identical to the antigen produced by the Tg mice) were used to detect anti-envelope antibodies (Mancini et al., 1993). Antibody titers were determined by serial end-point dilutions and were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune serum.

Detection and quantification of HBsAg were performed by using a commercial ELISA kit (Monolisa AgHBs Ultra; Bio-Rad, Marnes la Coquette, France).

*ELISPOT assay for determination of T-cell responses*

Splenocytes ($0.5 \times 10^6$ cells/well) were isolated from immunized mice and incubated with peptide pools (2 μg/ml of each peptide) in complete α-MEM medium supplemented with 10% FCS in sterile nitrocellulose MSIP 96-well plates (Millipore, Bedford, MA) coated with capture antibodies against mouse gamma interferon (IFN-γ). In some experiments, splenocytes were previously depleted of either CD4+ or CD8+ T-cells by using magnetic beads (Dynal) according to the manufacturer's instructions. The IFN-γ-coated plates were incubated at 37 °C for 18 h before scoring the number of spots with a Bioreader 4000 counter (Biosys). Splenocytes from non-immunized mice and cells in culture medium alone were used as negative controls to determine background levels. Each cell population was titrated in triplicate. The response was considered positive if the median number of spots in triplicate wells was at least twice that observed in control wells containing medium, and if at least ten IFN-γ-secreting cells per million splenocytes were detected.

*Flow cytometry*

The cell surface expression of HLA-A2 and HLA-DR1 molecules was measured on splenocytes or B220+-enriched B-cells using PE-conjugated anti-HLA-A2 and APC-conjugated anti-HLA-DR monoclonal antibodies, respectively. For intracellular cytokine detection, freshly isolated splenocytes ($1 \times 10^6$ cells) were incubated for 1 h either with medium alone or with peptide pools. Thereafter, brefeldin A (Sigma-Aldrich) 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 anti-CD3-, anti-CD4- and anti-CD8-conjugated monoclonal antibodies. Surface-stained cells were fixed with 2% paraformaldehyde in PBS. Fixed cells were resuspended in permeabilization buffer (PBS, 1% BSA, 0.05% saponin, and 0.01% sodium azide) and incubated with PE-conjugated anti-IFN-γ mAb for 30 min at 4 °C and washed twice in permeabilization buffer. Stained cells were resuspended in PBS/1% w/v BSA supplemented with 0.01% w/v sodium azide. All antibodies were purchased from BD Biosciences. Cells were acquired on FACSCanto (BD Biosciences) and frequencies were determined by flow cytometry analyses using Flowjo software (Tree Star).

*Statistical analysis*

Data were expressed as means ± SEM. Nonparametric unpaired comparisons were performed using the Mann-Whitney *U* test. For multiple comparisons of HBsAg concentrations before and after immunization, the Friedman test followed by Dunn's post-test were used. The significance level α was set 5%. Statistical analysis was carried out using Graphpad Prism 5 software. Values of $p < 0.05$ were considered significant.

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