Improving on the Ability of Endogenous Hepatitis B Core Antigen to Prime Cytotoxic T Lymphocytes

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Hepatitis B virus core antigen (HBcAg) is thought to be a major target for specific cytotoxic T cells (CTLs) in hepatitis B virus infections. A single dose of hepatitis C virus nonstructural 3/4A DNA ($<$5 μg) effectively primes functional specific CTLs, independently of CD4$^+$ T helper cells and by different routes of immunization. In contrast, HBcAg-specific CTL priming was T helper cell dependent and highly sensitive to the dose and route of delivery. Although CTL priming was improved 10-fold by codon optimization and in vivo electroporation, low levels of DNA still failed to prime CTLs effectively. Only high doses ($\geqslant$5 μg) of codon-optimized HBcAg delivered by in vivo electroporation primed in vivo lytic and polyfunctional CTLs. The ability of endogenous HBcAg to prime CTLs is surprisingly inefficient and differs from that of nonstructural 3/4A. This has important implications for the design of HBcAg-based therapeutic vaccines in humans.

Hepatitis B virus core antigen (HBcAg) is thought to be a key target for the host immune response in controlling the infection [1]. In particular, the presence of HBcAg-specific T cells has been associated with clearance of acute and chronic hepatitis B virus infections [1–3]. Subsequently, prophylactic and therapeutic vaccines that induce HBcAg-specific T cells have been developed and have shown some efficiency in infectious models [4–7]. However, despite the high immunogenicity of exogenous HBcAg, findings have been disappointing in many of the studies using endogenous HBcAg as a vaccine.

HBcAg spontaneously assembles into viruslike particles (VLPs) that are highly immunogenic in vivo [8–10]. These particles use B cells as the primary antigen-presenting cell (APC) [11] by an unusual interaction with the B cell receptor [12, 13]. HBcAg effectively primes specific T helper cells and, much less effectively, cytotoxic T cells (CTLs) as an exogenous antigen when high antigen doses in adjuvant are used [14]. Both DNA- and retrovirus-based immunizations using HBcAg have been reported to induce detectable HBcAg-specific CTLs in mice [14–18].

DNA vaccines can be used as a model to study the endogenous immunogenicity of antigens. However, direct intramuscular injections of DNA vaccines fail to prime robust immune responses in humans [19]. Different modes of DNA delivery that can be used in humans have now become available, such as transdermal delivery of DNA-coated gold beads using a gene gun [20] or treatment of the injection site by in vivo electroporation (EP) [21]. However, gene gun–based DNA immunization may preferentially prime a T helper 2–like immune response [22, 23], although this seems to depend largely on the antigen used. In vivo EP-assisted intramuscular injection has been found to effectively enhance plasmid DNA uptake and antigen expression

and to promote a local inflammatory response at the injection site [24]. We report herein the use of different antigens and different routes of DNA delivery to estimate the potency by which HBcAg induces specific CTLs. We found that HBcAg is a surprisingly poor CTL inducer in its native form.

MATERIALS AND METHODS

Overall experimental design. The studies were generally designed with a first immunization at week 0 and booster doses given at 4-week intervals. Samples were obtained and/or mice were killed at ∼2 weeks (12–17 days) after immunization. Any challenge was always given 2 weeks after the last immunization. There were generally ≥5 mice per test group.

Mice. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from B&K Universal, Charles River Laboratories, and Taconic, and CD4$^{-/-}$ knockout mice were provided by the Animal Facility at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet. All mice were 6–8 weeks old at the start of experiments, and the local committee on animal ethics approved all experiments. Mice of a C57BL/6 background were used unless otherwise stated.

Recombinant proteins, peptides, oligonucleotides, and plasmid DNA vectors. Recombinant particulate HBcAg encompassing residues 1–183 was produced in *Escherichia coli* and purified as described elsewhere [8, 25]. Ovalbumin was purchased from Sigma-Aldrich. A CpG-containing oligonucleotide (TCC ATG ACG TTC CTG ACG TT; CyberGene) was used for in vivo administration in combination with plasmid DNA.

An HBcAg-derived major histocompatibility complex (MHC) class II binding peptide composed of residues 120–140 (SFGVWIRTPPAYRPPNAPIL [26]) and 3 MHC class I binding peptides (HBcAg, MGLKFRQL and SYVNTNMGL [15, 27]; hepatitis C virus [HCV] nonstructural 3 protein (NS3), GAVQNEVTL [28]) was synthesized by means of standard techniques [29] with a multiple peptide synthesizer using 9-fluorenylmethoxycarbonyl chemistry (Syro; Multi-SynTech, Biochem).

Construction of a eukaryotic vector expressing HBcAg (designated HBcAg DNA) was described elsewhere [14]. A codon-optimized version of the same gene was made synthetically (Retrogen). A hepatitis B virus e antigen (HBeAg) gene fragment (639 nucleotides) was amplified by polymerase chain reaction from tail DNA extracted from an HBeAg-transgenic mouse [30]. The amplified gene fragment was ligated into a *Hind*III- and *Apa*I-digested pVAX1 vector. The HBeAg expression plasmid was designated HBeAg DNA and was confirmed by sequencing. The plasmid DNA used for in vivo injections was purified using Qiagen DNA purification columns according to the manufacturer's instructions.

Immunization protocols. For DNA immunization, C57BL/6 (wild type), BALB/c, and CD4$^{-/-}$ knockout mice with a C57BL/6 background were immunized either by intramuscular needle injection in the tibialis anterior muscle with 0.5–100 μg of plasmid DNA encoding wild-type HBcAg (wtHBcAg), codon-optimized HBcAg (coHBcAg), or HCV NS3/4A, with or without in vivo EP [24], or transdermally by a gene gun (Bio-Rad) with 2–18 μg of DNA [28]. Mice received booster doses every 4 weeks and were bled every second week. For detection of specific cellular immune responses, mice were killed 14 days after the last immunization [12, 28, 31].

Hydrodynamic injection and Western blot analysis. C57BL/6 mice were immunized as described above. Two weeks after the last immunization, a total volume of 1.5–1.8 mL of Ringer's solution containing 100 μg of HBcAg DNA was injected intravenously in the tail vein within <10 s [32, 33]. After 24 and 72 h, mice were killed and livers harvested for analysis of HBcAg protein by immunoprecipitation and Western blot analysis, as described elsewhere [24, 34]. HBcAg was detected using a rabbit anti-mouse HBcAg monoclonal antibody (Dakopatts) by chemiluminescence (Western Breeze; Invitrogen). The expression levels in tibialis anterior muscles were analyzed in the same way.

Detection of HBcAg-specific immunoglobulin (Ig) G and subclasses. Detection of specific HBcAg antibodies and IgG isotype distribution was determined by solid-phase enzyme immunoassay, as described elsewhere [31, 35].

Detection of HBcAg-specific CTLs. Lytic HBcAg-specific CTLs were detected in spleen cells restimulated in vitro for 5 days by a standard 4-h chromium 51 (^{51}Cr) release assay, as described elsewhere [14, 36]. HBcAg$_{93-100}$ or HCV NS3 MHC class I peptide–loaded RMA-S cells were used as target cells [37]. The percentage of specific ^{51}C release was calculated as [(experimental release − spontaneous release)/(maximum release-spontaneous release)] × 100. Results are shown as the mean percentage of specific lysis for triplicate values.

The frequency of HBcAg-specific CD8$^+$ T cells was analyzed by ex vivo staining of spleen cells with the HBcAg$_{93-100}$ MHC class I H-2K^b (MGLKFRQL) pentamer (ProImmune). In brief, spleen cells (1 × 10^6) were washed and resuspended in phosphate-buffered saline/1% fetal bovine serum (fluorescence-activated cell sorter buffer) and incubated with R-phycoerythrin (PE)–labeled H-2K^b (MGLKFRQL) pentamer for 15 min in the dark at room temperature (22°C). To block Fc binding, cells were washed and incubated with anti-mouse CD16/32 antibodies (BD Biosciences). Cells were then incubated with anti-mouse CD8–fluorescein isothiocyanate (clone KT15) and anti-mouse CD19–PE–cyanine (Cy) 5 (clone 6D5) for 20 min. The cells were fixed in 2% paraformaldehyde in phosphate-buffered saline for analysis, and 100,000 total events from each sample were acquired on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software, version 6.0. From a live lymphocyte gate, CD19-positive events were excluded, and the re-

maining cells were gated for CD8 expression. The frequency of HBcAg H-2K^b (MGLKFRQL)–positive events within this population was determined.

Enzyme-linked immunospot assays. Enzyme-linked immunospot assays were performed as described elsewhere [24, 38]. The number of spots was scored using the AID ELISPOT reader system (version 2.6; Autoimmun Diagnostika).

Fluorescence-activated cell sorter staining. A total of 5×10^5 spleen cells were restimulated for 12 h in the presence of GolgiPlug (BD Biosciences) and pools of overlapping peptides (20 amino acids) corresponding to HBcAg. The 2 pools contained either 5 peptides (0.3 μg/mL per peptide) covering amino acids 51–110, spanning the H-2^b–restricted CTL epitope [15], or 5 peptides covering amino acids 101–160, spanning the H-2^b–restricted T helper epitope [26]. After restimulation, the cells were stained for different surface markers and intracellular cytokines, using the following antibodies: Pacific Blue anti-mouse CD3 (BioLegend) allophycocyanin–cyanine 7 rat anti-mouse CD8a (BD Biosciences), PE rat anti-mouse CD107a (BD Biosciences), allophycocyanin anti-mouse interleukin-2 (BioLegend), and fluorescein isothiocyanate anti-mouse interferon (IFN)–γ (BioLegend). Cells were analyzed with the CyAn ADP flow cytometer (Dako) and FlowJo software (version 8.8.6; Tree Star).

Statistical analysis. Statistical comparisons were performed using StatView (version 5.0, 3/20/98, Power PC version; SAS Institute) and Excel for Mac (version 11.3.7; Microsoft) software packages for Macintosh. Frequencies were compared using the Fisher exact test (StatView).

RESULTS

Endogenous HBcAg priming of antibodies to HBcAg. Endogenously produced HBcAg is quite effective in priming a rapid production of antibodies to HBcAg (anti-HBc) (Figure 1A). A single intramuscular injection of 100 μg of wtHBcAg DNA primed anti-HBc titers of >10,000 at 4 weeks after the injection (Figure 1A). In contrast, transdermal delivery of 50-fold lower doses required ≥2 injections to reach titers of >1000 (Figure 1B). The addition of CpG oligonucleotides improved the antibody titers after transdermal delivery by ∼10-fold, without altering the IgG subclass profile (Figure 1B). The IgG2b-dominated subclass distribution of anti-HBc was maintained regardless of the delivery route or adjuvants, mimicking the IgG subclass distribution seen after immunization with exogenous HBcAg [39, 40], suggesting that this is an intrinsic property of HBcAg [39, 40].

Endogenous HBcAg priming of lytic CTLs at high DNA doses. To characterize the ability of wtHBcAg DNA to prime lytic CTLs at high and low doses we used intramuscular and transdermal delivery with the gene gun. The standard ^{51}C release assay includes a 5-day restimulation and should therefore be

considered a highly sensitive assay for CTL detection. Two intramuscular 100-μg injections of wtHBcAg or wtHBeAg DNA primed detectable CTLs, but the same plasmids delivered transdermally in a low dose did not (Figure 2A–2D). Three immunizations with the 100-μg dose given intramuscularly primed CTLs (Figure 2E, 2G, and 2I), but 3 2-μg doses given transdermally (total dose, 6 μg) did not (Figure 2F). Three 6-μg transdermal doses (total dose, 18 μg) induced detectable CTLs in C57BL/6 and BALB/c mice (Figure 2H and 2J). A single transdermal or intramuscular immunization with wtNS3/4A or coNS3/4A genes induced detectable CTLs (Figure 2K–2N). Thus, these findings suggest that native HBcAg is a comparatively poor inducer of specific CTLs.

HBcAg-specific CTL priming and CD4$^+$ T helper cell dependence. The priming of HCV NS3/4A-specific CTLs is independent of the presence of CD4$^+$ T cells [33, 36]. We therefore examined the role of CD4$^+$ T cells in the priming of HBcAg-specific CTLs. Wild-type and CD4$^{-/-}$ mice were immunized once with 100 μg of wtHBcAg DNA, and the appearance of HBcAg-specific IFN-γ–producing CTLs was determined 4 days and 2 weeks (17 days) after immunization. These data show that HBcAg-specific IFN-γ–producing CTLs were not detectable ex vivo at day 4 but became detectable after 2 weeks (Figure 3A and 3B). The corresponding CTL responses failed to appear in the CD4$^{-/-}$ mice (Figure 3C and 3D). Thus, the priming of HBcAg-specific CTLs is more dependent on CD4$^+$ T helper cells than the priming of NS3/4A-specific CTLs [33, 36].

The importance of CD4$^+$ T helper cells for the priming of CTLs suggested that this dependence might be corrected by a prime-boost strategy; that is, a preexisting pool of HBcAg-specific T helper cells might improve CTL priming by DNA. To test this, hypothesis H-2^b mice were immunized with a peptide representing the dominant CD4$^+$ T helper epitope at residues 120–140 of HBcAg 9 days before DNA immunization. This approach was found elsewhere to greatly improve humoral responses to genetic immunization with a retroviral vector [16]. Again, one or two 100-μg doses of HBcAg DNA delivered intramuscularly primed HBcAg-specific IFN-γ–producing T helper cells and CTLs (Figure 3E and 3I), whereas low doses failed to do so (Figure 3F and 3J). Priming with the HBcAg-derived T helper peptide effectively induced HBcAg-specific IFN-γ–producing T helper cells that were recalled in vitro by both the peptide itself and recombinant HBcAg (Figure 3G and 3K). However, the presence of a preexisting pool of HBcAg-specific IFN-γ–producing T helper cells did not improve the ability of low doses of HBcAg DNA to prime CTLs (Figure 3H and 3L).

Functional HBcAg-specific CTLs in vivo. Using transiently transgenic mice, we tested whether the CTLs primed by high doses of HBcAg DNA given intramuscularly were functional
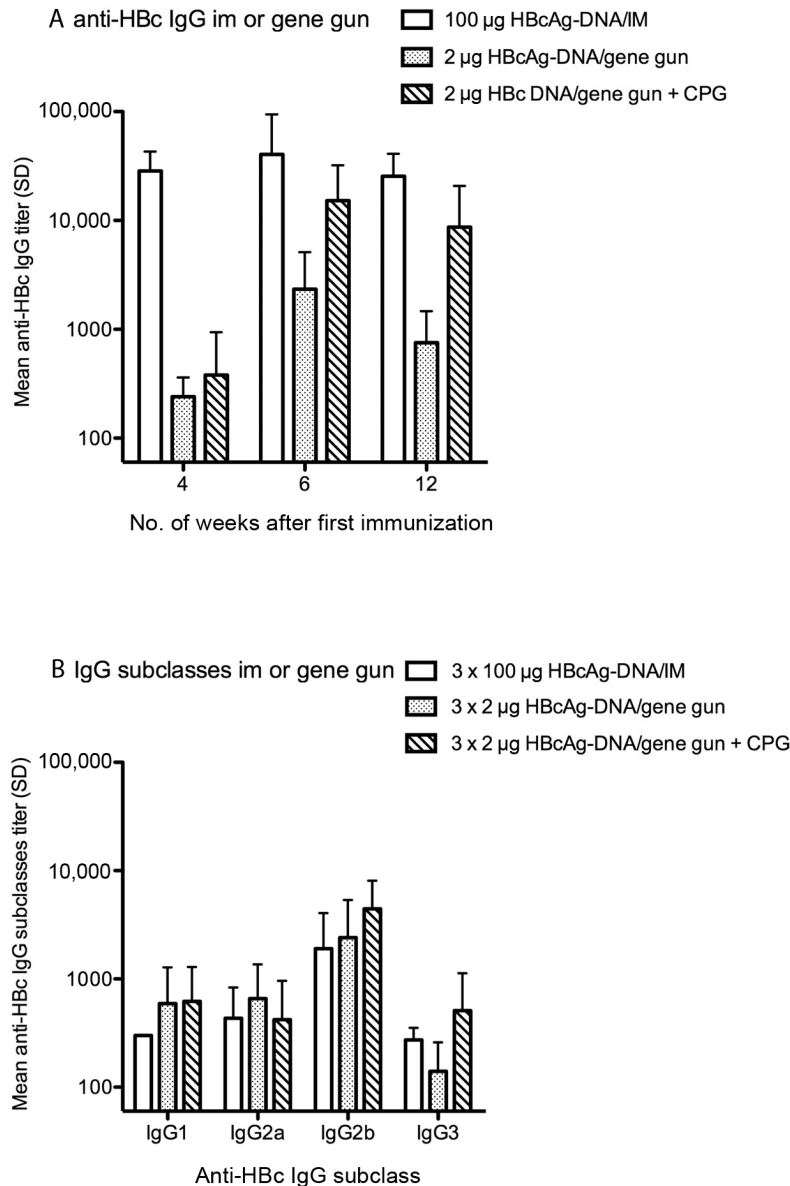
Figure 1. *A,* Titers for antibody to hepatitis B virus core antigen (HBcAg) (anti-HBc) immunoglobulin (Ig) G primed by 3 monthly injections with 100 μg of HBcAg DNA given intramuscularly (IM) or 2 μg of HBcAg DNA given transdermally by gene gun, with or without CpG as an adjuvant. Samples were obtained 4, 6, and 12 weeks after the first vaccination. *B,* Anti-HBc IgG subclass distribution 1 month after the third immunization in the same groups of mice. All data are given as mean end-point titers (+ standard deviations [SDs]).

in vivo [32]. In this model the clearance of antigen-expressing hepatocytes is dependent on CD8+ CTLs [33]. The transient expression of HBcAg in hepatocytes is generated by a hydrodynamic injection of HBcAg DNA and was monitored at 24 and 72 h by Western blot analysis (Figure 4) [32]. At 24 h after hydrodynamic injection of HBcAg DNA, all mice had detectable hepatic expression of HBcAg, regardless of whether or not CTLs had been primed (Figure 4). Within 72 h after hydrodynamic injection, HBcAg expression was cleared in all vaccinated mice (Figure 4) (0 of 4 vs 4 of 4; $P < .05$, by the Fisher exact test). In the unvaccinated mice, no statistical evidence of clearance

was found when HBcAg expression at 24 was compared with that at 72 h (Figure 4) (0 of 4 vs 1 of 4; $P =$ not significant, by the Fisher exact test). Thus, functional CTLs are primed in vivo by vaccinations with HBcAg DNA, delivered intramuscularly with or without in vivo EP (data not shown). These CTLs enter the liver and eliminate HBcAg-expressing hepatocytes, which is a desired feature of a therapeutic vaccine. This finding confirms that the CTLs primed by the high-dose DNA vaccination recognized endogenously produced hepatic HBcAg presented within the context of hepatic MHC class I molecules in vivo.

intramuscular

transdermal

Effector:Target ratio
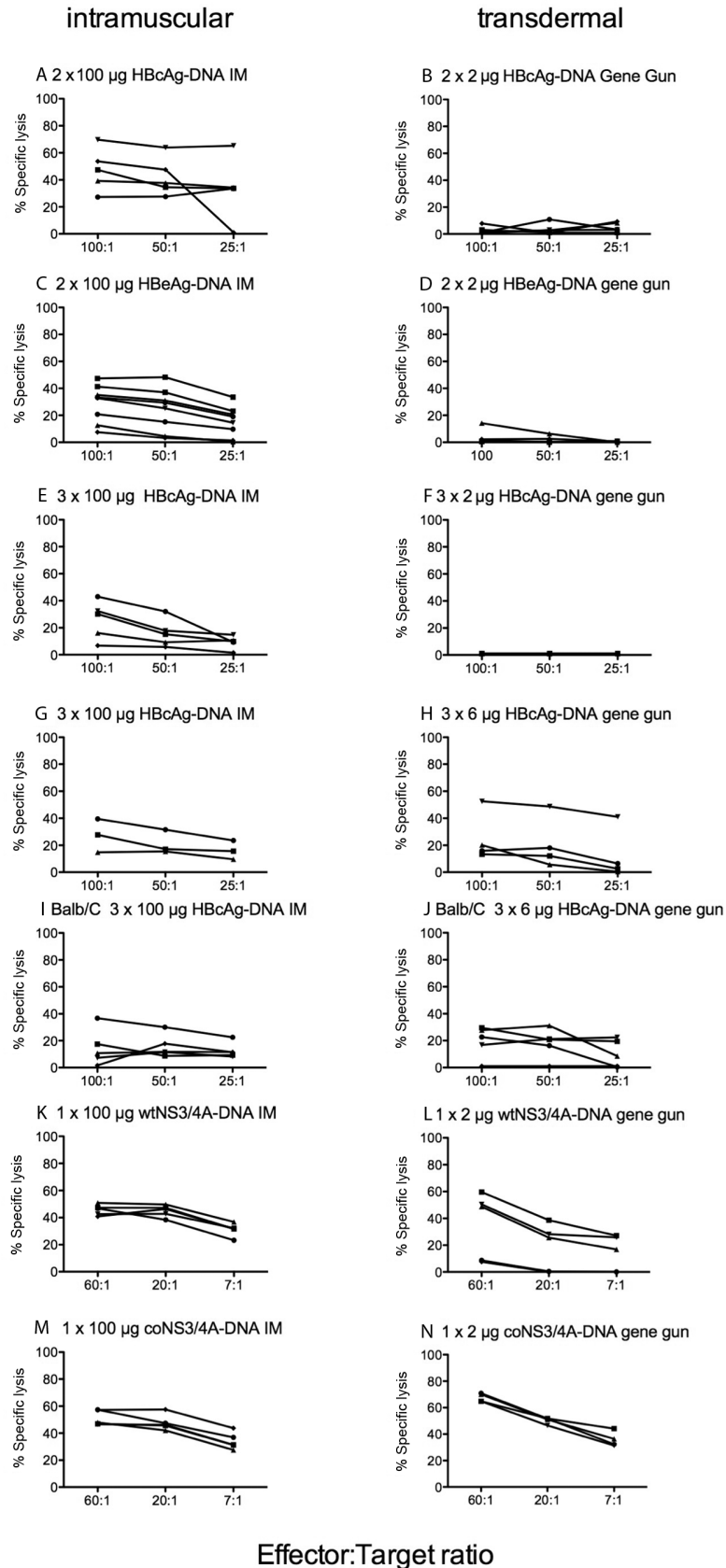
Figure 2. Efficiency of cytotoxic T lymphocyte priming by intramuscular (IM) or transdermal immunization and with different doses of wild-type (wt) hepatitis B virus core antigen (HBcAg) DNA or wtHBeAg DNA in C57BL/6 mice (BALB/c in *I* and *J*), shown as the percentage of specific lysis for HBcAg or nonstructural (NS) 3 peptide–loaded RMA-S target cells. Mice were immunized as indicated, and lytic activity was determined by a standard 4-h chromium 51 release assay; co, codon-optimized.
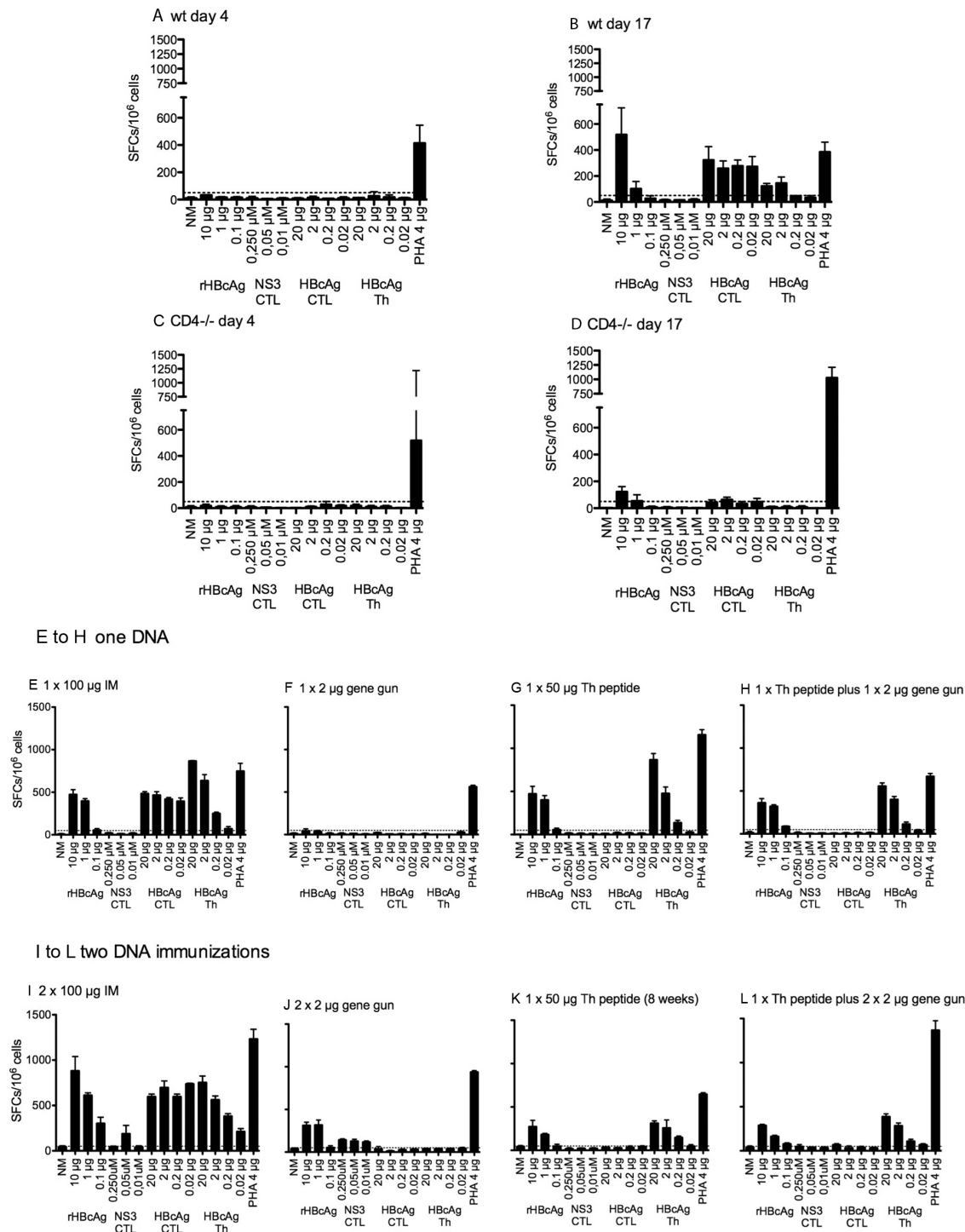
Figure 3. *A–D*. Kinetics of the priming of interferon (IFN) γ–producing hepatitis B virus core antigen (HBcAg)–specific CD8^{+} T cells in H-2[b] C57BL/6 (*A* and *B*) or CD4^{−/−} knockout (*C* and *D*) mice, as determined by enzyme-linked immunospot (ELISPOT) assay. Groups of 5 mice were immunized intramuscularly (IM) with 100 μg of wild-type (wt) HBcAg DNA, and IFN-γ production was detected 4 and 17 days after immunization. Values represent IFN-γ spot-forming cells (SFCs)/10[6] cells. CTL, cytotoxic T lymphocyte; NM, normal medium; NS3, nonstructural 3 protein; PHA, phytohemagglutinin; rHBcAg, recombinant HBcAg; Th, T helper cells. *E–L*, Pretreatment with T helper peptide could not restore priming of HBcAg-specific CD8^{+} T cells in mice immunized by gene gun. To determine whether the priming of HBcAg-specific CD4^{+} T cells was a limiting factor for the priming of specific CTLs, mice were preimmunized with 100 μg of a T helper peptide in incomplete Freund's adjuvant, given subcutaneously 9–11 days before DNA immunization. HBcAg-specific IFN-γ–producing CD8^{+} and CD4^{+} cells were determined by ELISPOT assay. Mice were divided into groups of 5 and received either 100 μg of wtHBcAg DNA intramuscularly or 2 μg of wtHBcAg DNA transdermally by gene gun. Mice received 1 (*E–H*) or 2 (*I–L*) immunizations at weeks 0 and 4. Values are presented as SFCs/10[6] cells (+SD); dotted line indicates cutoff of 50 SFCs/10[6] splenocytes.
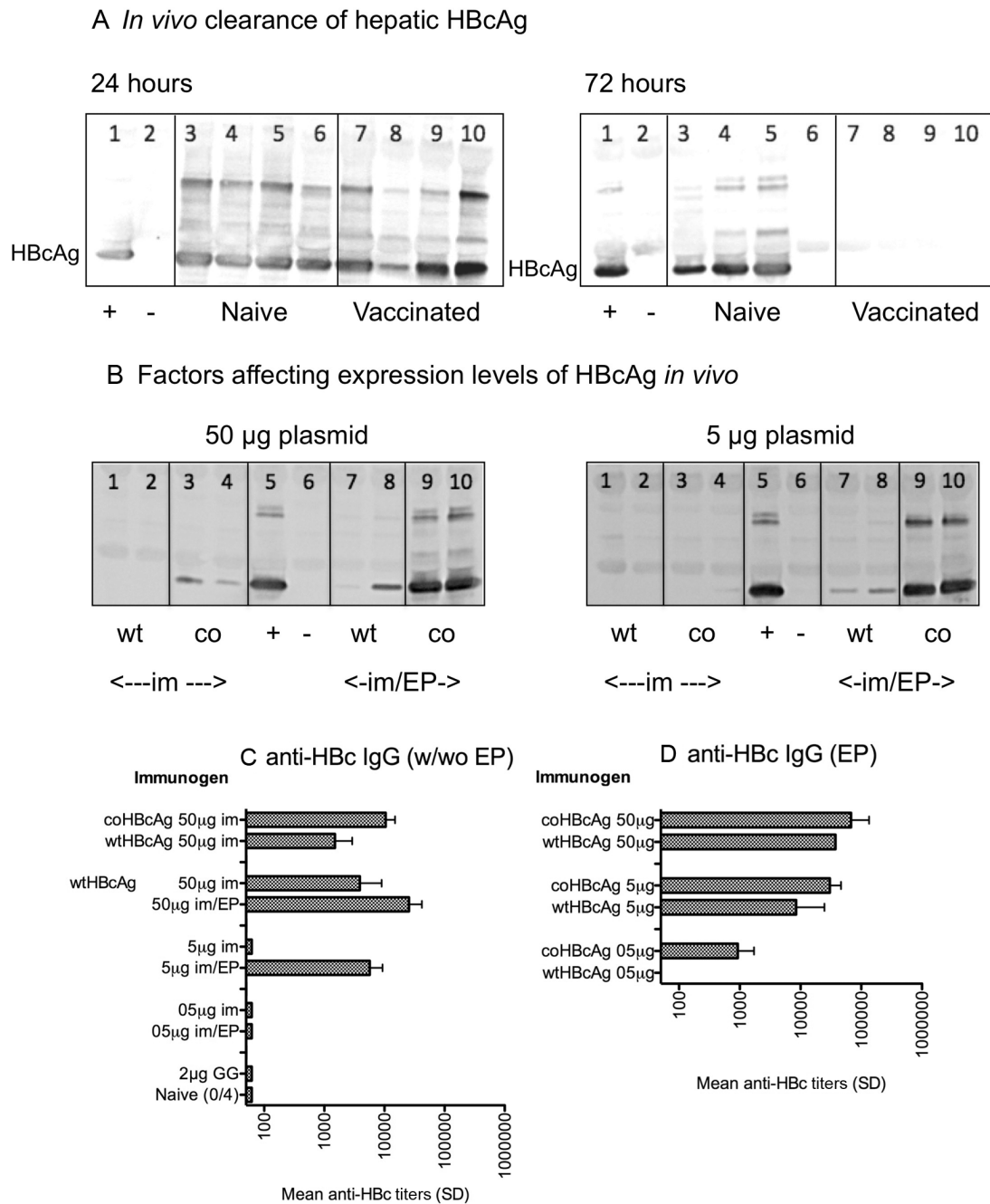
Figure 4. *A*, Hepatitis B virus core antigen (HBcAg)–specific cytotoxic T lymphocytes (CTLs), primed by a single 50-μg dose of DNA given intramuscularly (im) with in vivo electroporation (EP), are functional in vivo as determined by clearance of HBcAg-expressing hepatocytes in transiently transgenic mice. Mice were given a hydrodynamic injection of wild-type (wt) HBcAg DNA; 24 and 72 h later, livers were analyzed for the presence of HBcAg by immunoprecipitation followed by Western blot analysis. Lanes 3–10 indicate individual mice; controls are codon-optimized (co) HBcAg DNA–transfected HepG2 cells (*plus sign*) and a liver lysate from a naive mouse (*minus sign*). *B*, In vivo expression of HBcAg, as determined by immunoprecipitation and Western blot analysis, at the site of injection 72 h after vaccination with 50 (*left*) or 5 (*right*) μg of DNA with wtHBcAg or coHBcAg DNA. DNA was administered intramuscularly, with or without in vivo EP. Each lane indicates an individual mouse except for lane 5, corresponding to coHBcAg DNA–transfected HepG2 cells (*plus sign*), and lane 6, corresponding to a muscle lysate from a naive mouse (*minus sign*). *C* and *D*, Codon optimization and in vivo electroporation improves the responses of antibody to HBcAg (anti-HBc) immunoglobulin (Ig) G responses determined 2 weeks after intramuscular injection of 0.5, 5, or 50 μg of HBcAg DNA. Values represent mean antibody titers + standard deviations (SDs); GG, gene gun.

A wtHBcAg   B wtHBcAg/EP   C coHBcAg/EP

Effector:target ratio

D wtHBcAg   E wtHBcAg/EP
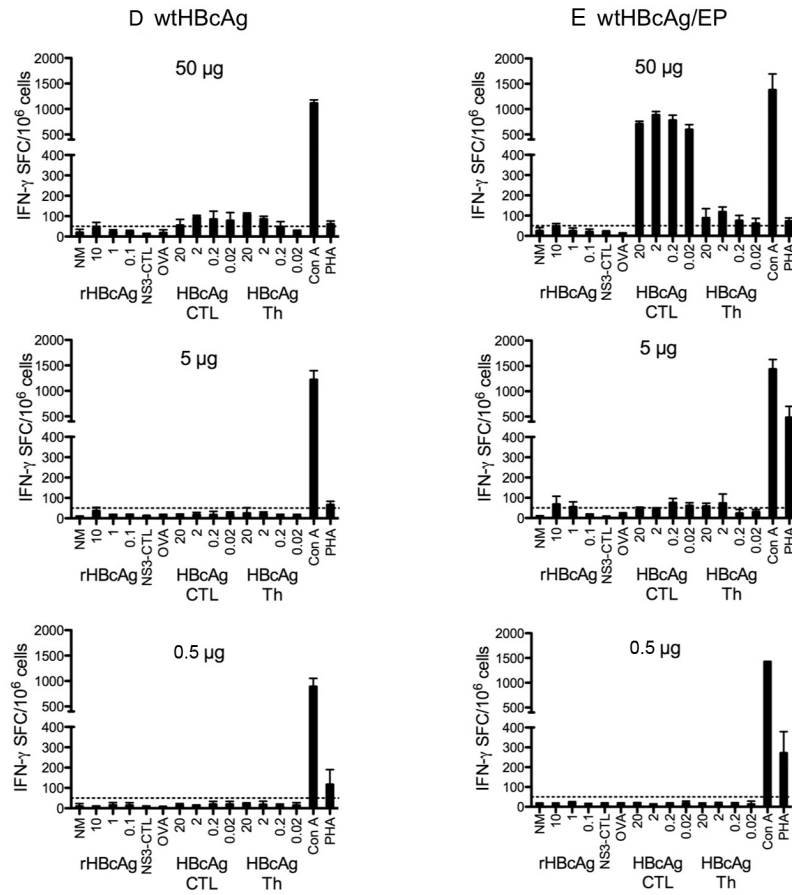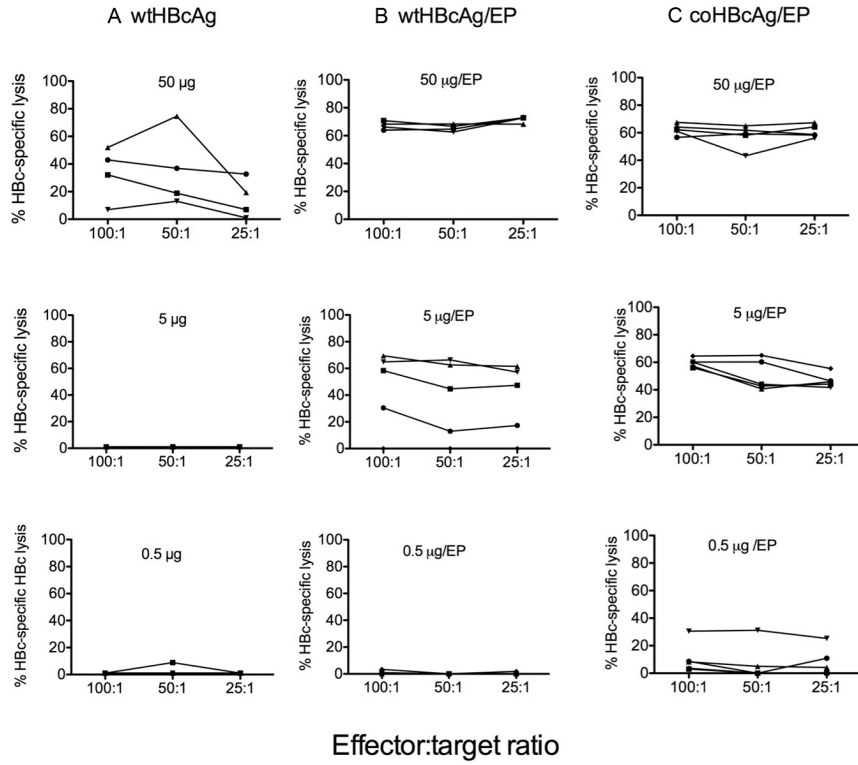
In vitro recall antigen

HBcAg expression in vivo and B cell immunogenicity. Co-don optimization and in vivo EP can be combined, resulting in improved expression levels of a desired gene. Using immunoprecipitation and Western blot analysis, we analyzed the effects of these 2 factors on in vivo expression levels of HBcAg at the site of injection (tibialis anterior) at 72 h after immunization (Figure 4). Both approaches clearly improved expression levels of HBcAg in vivo, and the combined effect represents a 10–100-fold improvement. Specifically, 50 μg of wtHBcAg DNA given without EP is barely detectable by immunoprecipitation and Western blot analysis, whereas 5 μg of coHBcAg given with EP is clearly detectable (Figure 4).

The priming of anti-HBc IgG was also improved by increasing in vivo expression levels of HBcAg (Figure 4). Vaccination with coHBcAg DNA using in vivo EP raised detectable anti-HBc after a single 0.5-μg dose (Figure 4). In contrast, wtHBcAg given intramuscularly at doses <50 μg without in vivo EP, or given with the gene gun, failed to induce detectable anti-HBc (Figure 4).

HBcAg expression and T cell immunogenicity. We next determined the DNA doses needed for an effective priming of HBcAg-specific T cell responses by the intramuscular route. Numerous reports have shown that HBcAg DNA induces CTLs at high doses, usually 50–100 μg per injection in mice [14, 15, 18, 27]. We found that endogenously produced HBcAg demonstrated a surprisingly rapid loss in its ability to prime lytic CTLs (Figures 5–7). HBcAg-specific lytic CTLs, but not IFN-γ–producing CTLs, were detected at the 5-μg dose only when the intramuscular injection of wtHBcAg was accompanied by adjuvant in vivo EP (Figures 5 and 6). Single doses of <5 μg— delivered intramuscularly, with or without in vivo EP, or delivered by the gene gun (Figures 2, 3, 6, and 7)—failed to effectively prime detectable CTLs. This is distinct from HCV NS3/4A protein, which effectively induces CTLs by many delivery routes and at low doses [24, 28, 36, 41–44]. These data were further confirmed by quantifying the number of HBcAg-specific CTLs ex vivo with pentamer staining (Figure 7). A potent expansion of CTLs was noted only after priming with the coHBcAg delivered by in vivo EP gene at doses of $\geqslant$5 μg (Figure 7). Additional analysis was performed using flow cytometry of T cells primed by 5-μg immunization with coHBcAg DNA and in vivo EP. This showed that the CTL-containing

peptide pool (amino acids 51–110) primed mainly IFN-γ–producing T cells and, to a minor extent, polyfunctional T cells producing both IFN-γ and interleukin-2 (Figure 7).

DISCUSSION

HBcAg is an unusual antigen. When expressed in vitro, it spontaneously forms capsidlike particles [45]. These capsids can bind to the naive B cell receptor through a noncanonical binding and use naive B cells as the primary APC. The efficiency of the B cell as the primary APC for exogenous HBcAg is most likely explained by the cross-linking surface-bound immunoglobulin, whereby the B cells matures into an activated APC [11–13]. Exogenous HBcAg has been found to be highly immunogenic on a B- and T helper cell level and can transfer this immunogenicity to foreign sequences inserted into the tip of the protruding spikes [8, 10]. Although it has been shown that genetic immunization with HBcAg (ie, endogenously produced HBcAg) clearly induces immune responses, including antibodies, T helper cells, and CTLs [14–18, 27], little has been done to quantify its intrinsic immunogenicity. We have now found that HBcAg as an endogenously produced antigen is a comparatively poor CTL inducer.

We immediately found that although HBcAg seems quite immunogenic at first glance, it certainly is not when the levels of DNA are reduced. This finding is in contrast to our previous experience with the HCV NS3/4A gene, which is highly immunogenic in its native or codon-optimized form [24, 28, 36]. In particular, NS3/4A DNA can prime CTLs at single doses of <1 μg [24], whereas HBcAg is unable to do so regardless of adjuvants or increased antigen expression.

The evidence that endogenous HBcAg is a poor immunogen is as follows: First, the immunogenicity of HBcAg DNA drops off rapidly between 50- and 5-μg doses when delivered intramuscularly, even if in vivo EP is used as an adjuvant. HCV NS3/4A DNA effectively primes specific CTLs at a single 0.5-μg dose when delivered by in vivo EP [24]. Second, HBcAg DNA fails to activate IFN-γ–producing T helper cells and CTLs at intramuscular doses <5 μg, even with in vivo EP and a codon-optimized gene. HCV NS3/4A DNA again effectively primes these responses down to 0.5 μg [24]. Thus, when delivered intramuscularly, with or without in vivo EP, HBcAg DNA is at

Figure 5. Dose dependency of the priming of lytic cytotoxic T lymphocytes (CTLs) by hepatitis B virus core antigen (HBcAg) DNA. C57BL/6 mice (*n* = 5 per group) were primed once with 50, 5, or 0.5 μg of wild-type HBcAg (wtHBcAg) or codon-optimized HBcAg (coHBcAg) DNA, with or without in vivo electroporation (EP). *A–C*, Lytic CTL responses were determined 14 days later as the lysis of radio-labeled peptide-loaded RMA-S target cells; values are shown as the percentage of specific lysis. wtHBcAg also effectively induces specific interferon (IFN) γ–producing HBcAg-specific T cells at high DNA doses when delivered by in vivo EP. *D* and *E*, C57BL/6 mice were primed once with 50, 5, or 0.5 μg of wtHBcAg DNA, and HBcAg-specific IFN-γ–producing responses were determined 14 days later with enzyme-linked immunospot assay. Values represent IFN-γ spot-forming cells (SFCs)/10^6 cells; dotted line, cutoff of 50 SFCs/10^6 splenocytes. Con A, concanavalin A; NM, normal medium; NS3, nonstructural 3; OVA, ovalbumin; PHA, phytohemagglutinin; rHBcAg, recombinant HBcAg; Th, T helper cells.
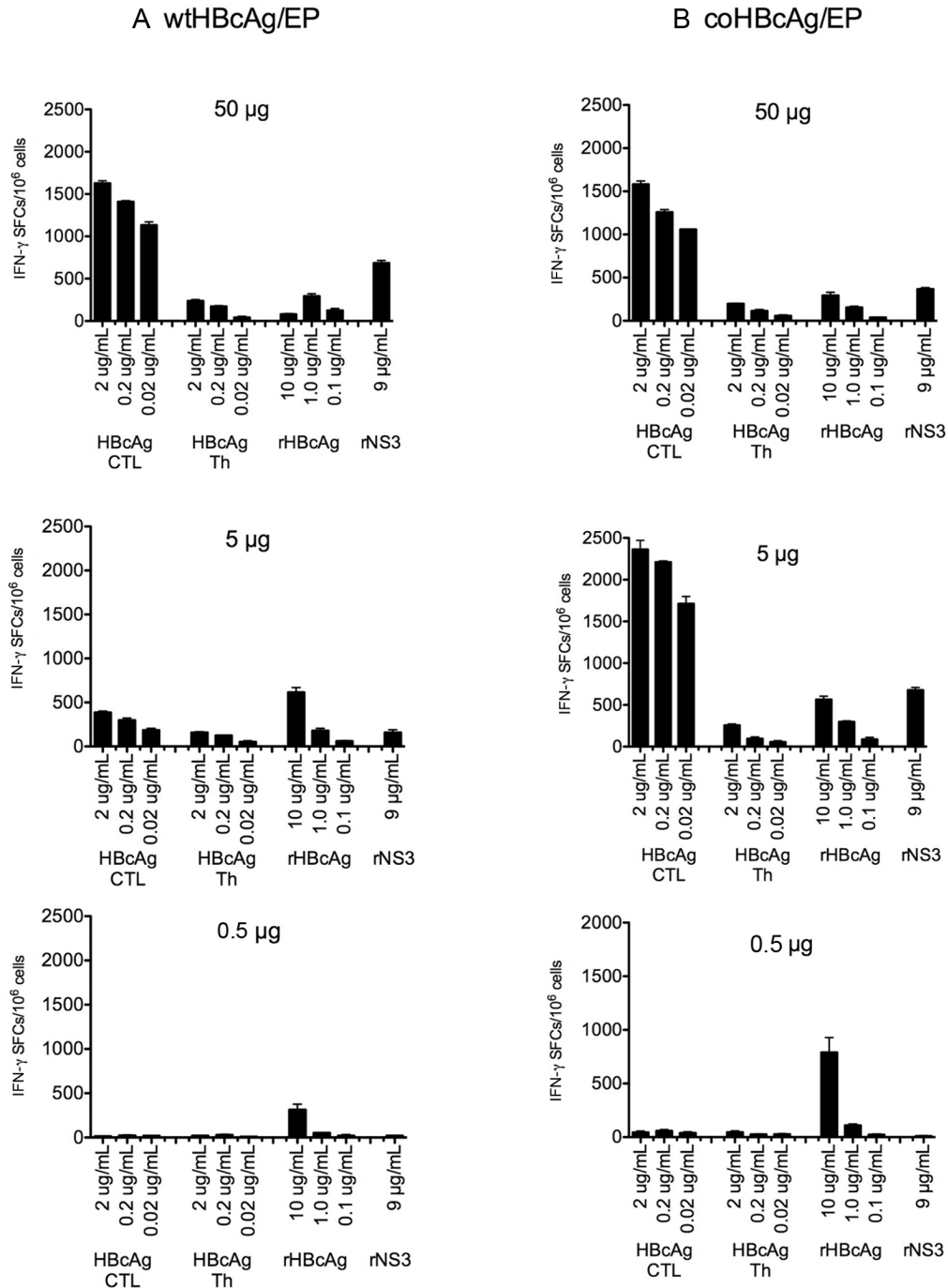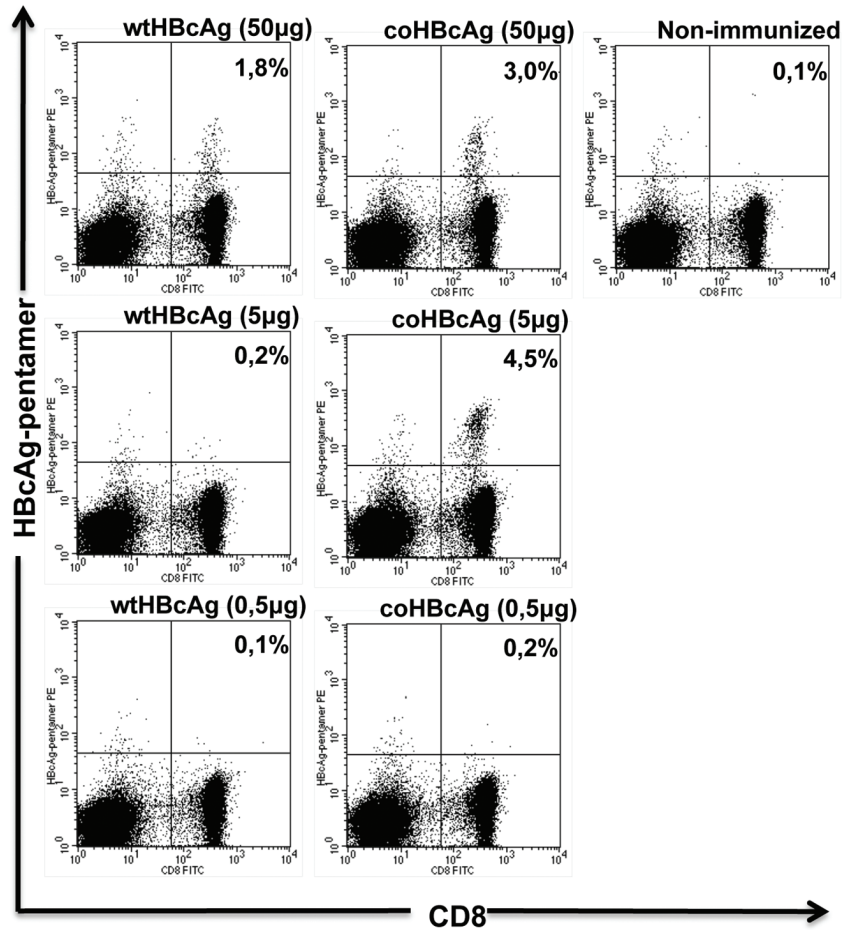
Figure 6. Codon optimization improves the ability of hepatitis B virus core antigen (HBcAg) DNA to induce specific interferon (IFN) γ–producing HBcAg-specific T cells at high doses when delivered by in vivo electroporation. *A* and *B*, C57BL/6 mice ($n = 5$ per group) were primed once with 50, 5, or 0.5 μg of wild-type HBcAg or codon-optimized DNA, and HBcAg-specific IFN-γ–producing responses were determined 14 days later using enzyme-linked immunospot assay. Values represent IFN-γ spot-forming cells (SFCs)/10^6 cells (means + standard deviations); dotted line, cutoff of 50 SFCs/10^6 splenocytes.

least 10–100-fold less effective in T cell priming than HCV NS3/4A DNA. With respect to other routes of administration, HBcAg DNA was unable to induce CTLs when administered transdermally in 3 doses of 2 or 4 μg (total dose, 6 or 12 μg; data not shown), whereas the NS3/4A gene primes in vivo functional CTLs transdermally after a single dose of 2 μg [32, 33]. HBcAg DNA eventually induces HBcAg-specific CTLs when administered transdermally in 3 6-μg doses (total dose,

Figure 7. Codon optimization improves the ability of hepatitis B virus core antigen (HBcAg) DNA to prime high frequencies of HBcAg-specific cytotoxic T lymphocytes (CTLs), when delivered at high doses by in vivo electroporation, as determined by pentamer staining. C57BL/6 mice (*n* = 5 per group) were primed once with 50, 5, or 0.5 μg of codon-optimized HBcAg (coHBcAg) or wild-type HBcAg (wtHBcAg) DNA, and HBcAg-specific CTLs were determined 14 days later using pentamer staining. FITC, fluorescein isothiocyanate. Values are given as percentages of HBcAg-specific CD8^{+} T cells. *B*, Scattergrams from analysis of cytokine production by multicolor flow cytometry to determine polyfunctionality of HBcAg-specific T cells. Splenocytes were restimulated for 12 h with the indicated peptide pools. Pie chart shows percentages of cytokine-positive HBcAg-specific T cells that produce interleukin (IL)–2 and/or interferon (IFN)–γ. SFCs, spot-forming cells.

18 μg) and is thus, $\geq$9-fold less efficient than NS3/4A DNA delivered transdermally.

To better understand the inability of low doses of HBcAg DNA to prime specific CTLs, we added CpG oligonucleotides and preprimed HBcAg-specific T helper cells. Unlike our own experience [28], others have suggested that gene gun immunization may favor a T helper 2–like response [22, 23]. CpG oligonucleotides have been shown to sometimes, but not always, shift a gene gun–mediated DNA immune response from T helper 2 toward T helper 1 cells [46, 47]. Thus, in an attempt to override the potential Th2 bias of transdermal delivery, we coated gold beads with a mix of plasmid DNA and immune-stimulating CpG oligonucleotides. Although the presence of CpG oligonucleotides improved immunogenicity, with a 10-fold increase in anti-HBc titers, it could not correct the inability of low doses of HBcAg DNA to prime HBcAg-specific CTLs.

We found that the priming of HBcAg-specific CTLs was dependent on CD4$^+$ T helper cells. This prompted us to investigate whether the presence of a preexisting pool of HBcAg-specific T helper cells could correct the inability of low doses of HBcAg DNA to prime CTLs, but this was not the case. Thus, HBcAg seems to have an intrinsically poor ability to prime CTLs, which is quite unexpected. We did find that increasing expression levels a step at a time, by codon optimizing the HBcAg gene and by introducing in vivo EP-assisted delivery, improved the CTL priming by HBcAg. Again, surprisingly, despite all of these measures HBcAg was unable to prime CTLs effectively at low DNA doses.

The high immunogenicity exerted by exogenous HBcAg is probably explained by a number of factors. First, HBcAg can effectively use B cells as a primary APC [11–14]. Second, multimeric capsid antigens often have a high intrinsic immunogenicity toward a humoral immunity. Third, HBcAg can bind RNA, which can act as an adjuvant [48, 49]. The most immunogenic property of endogenous HBcAg seems to be its ability to rapidly prime anti-HBc, although this also drops off quite rapidly at lower DNA doses. The least immunogenic property of endogenous HBcAg is its ability to prime CTLs, which requires high expression levels of HBcAg mediated by high levels of HBcAg DNA and/or improved uptake and expression. One could speculate that the poor priming of CTLs results from intracellular processing of HBcAg that does not favor class I presentation—for example, poor proteosomal degradation and/or improper ubiquitination [50]. This will be an interesting question to resolve.

In conclusion, we herein describe strategies to improve the immunogenicity of endogenous produced HBcAg, which is highly important in designing vaccine strategies for human use.

References

1. Bertoletti A, Ferrari C, Fiaccadori F, et al. HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. Proc Natl Acad Sci USA 1991; 88:10445–10449.
2. Ferrari C, Penna A, Bertoletti A, et al. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. J Immunol 1990; 145:3442–3449.
3. Jung MC, Diepolder HM, Spengler U, et al. Activation of a heterogeneous hepatitis B (HB) core and e antigen-specific CD4+ T cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection. J Virol 1995; 69:3358–3368.
4. Iwarson S, Tabor E, Thomas HC, Snoy P, Gerety RJ. Protection against hepatitis B virus infection by immunization with hepatitis B core antigen. Gastroenterology 1985; 88:763–767.
5. Menne S, Maschke J, Tolle TK, Lu M, Roggendorf M. Characterization of T-cell response to woodchuck hepatitis virus core protein and protection of woodchucks from infection by immunization with peptides containing a T-cell epitope. J Virol 1997; 71:65–74.
6. Menne S, Tennant BC, Gerin JL, Cote PJ. Chemoimmunotherapy of chronic hepatitis B virus infection in the woodchuck model overcomes immunologic tolerance and restores T-cell responses to pre-S and S regions of the viral envelope protein. J Virol 2007; 81:10614–10624.
7. Sallberg M, Hughes J, Javadian A, et al. Genetic immunization of chimpanzees chronically infected with the hepatitis B virus, using a recombinant retroviral vector encoding the hepatitis B virus core antigen. Hum Gene Ther 1998; 9:1719–1729.
8. Billaud JN, Peterson D, Barr M, et al. Combinatorial approach to hepadnavirus-like particle vaccine design. J Virol 2005; 79:13656–13666.
9. Billaud JN, Peterson D, Lee BO, et al. Advantages to the use of rodent hepadnavirus core proteins as vaccine platforms. Vaccine 2007; 25:1593–1606.
10. Sallberg M, Hughes J, Jones J, Phillips TR, Milich DR. A malaria vaccine candidate based on a hepatitis B virus core platform. Intervirology 2002; 45:350–361.
11. Milich DR, Chen M, Schodel F, Peterson DL, Jones JE, Hughes JL. Role of B cells in antigen presentation of the hepatitis B core. Proc Natl Acad Sci USA 1997; 94:14648–14653.
12. Lazdina U, Cao T, Steinbergs J, et al. Molecular basis for the interaction of the hepatitis B virus core antigen with the surface immunoglobulin receptor on naive B cells. J Virol 2001; 75:6367–6374.
13. Watts NR, Cardone G, Vethanayagam JG, et al. Non-canonical binding of an antibody resembling a naive B cell receptor immunoglobulin to hepatitis B virus capsids. J Mol Biol 2008; 379:1119–1129.
14. Lazdina U, Alheim M, Nystrom J, et al. Priming of cytotoxic T cell responses to exogenous hepatitis B virus core antigen is B cell dependent. J Gen Virol 2003; 84:139–146.
15. Kuhrober A, Pudollek HP, Reifenberg K, et al. DNA immunization induces antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2b mice. J Immunol 1996; 156:3687–3695.
16. Sallberg M, Townsend K, Chen M, et al. Characterization of humoral and CD4+ cellular responses after genetic immunization with retroviral vectors expressing different forms of the hepatitis B virus core and e antigens. J Virol 1997; 71:5295–5303.
17. Townsend K, Sallberg M, O'Dea J, et al. Characterization of CD8+ cytotoxic T-lymphocyte responses after genetic immunization with retrovirus vectors expressing different forms of the hepatitis B virus core and e antigens. J Virol 1997; 71:3365–3374.
18. Frelin L, Wahlstrom T, Tucker AE, et al. A mechanism to explain the

selection of the hepatitis e antigen-negative mutant during chronic hepatitis B virus infection. J Virol **2009**; 83:1379–1392.

19. Laddy DJ, Weiner DB. From plasmids to protection: a review of DNA vaccines against infectious diseases. Int Rev Immunol **2006**; 25:99–123.

20. Roy MJ, Wu MS, Barr LJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine **2000**; 19:764–778.

21. Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. Gene Ther **1999**; 6:508–514.

22. Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J Immunol **1997**; 158:2278–2284.

23. McCluskie MJ, Brazolot Millan CL, Gramzinski RA, et al. Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. Mol Med **1999**; 5:287–300.

24. Ahlen G, Soderholm J, Tjelle TE, et al. In vivo electroporation enhances the immunogenicity of hepatitis C Virus nonstructural3/4A DNA by increased local DNA uptake, protein expression, inflammation, and infiltration of CD3+ cells. J Immunol **2007**; 179:4741–4753.

25. Billaud JN, Peterson D, Schodel F, et al. Comparative antigenicity and immunogenicity of hepadnavirus core proteins. J Virol **2005**; 79:13641–13655.

26. Milich DR, McLachlan A, Moriarty A, Thornton GB. Immune response to hepatitis B virus core antigen (HBcAg): localization of T cell recognition sites within HBcAg/HBeAg. J Immunol **1987**; 139:1223–1231.

27. Kuhrober A, Wild J, Pudollek HP, Chisari FV, Reimann J. DNA vaccination with plasmids encoding the intracellular (HBcAg) or secreted (HBeAg) form of the core protein of hepatitis B virus primes T cell responses to two overlapping Kb- and Kd-restricted epitopes. Int Immunol **1997**; 9:1203–1212.

28. Frelin L, Alheim M, Chen A, et al. Low dose and gene gun immunization with a hepatitis C virus nonstructural (NS) 3 DNA-based vaccine containing NS4A inhibit NS3/4A-expressing tumors in vivo. Gene Ther **2003**; 10:686–699.

29. Sallberg M, Ruden U, Magnius LO, Norrby E, Wahren B. Rapid "tea-bag" peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids applied for antigenic mapping of viral proteins. Immunol Lett **1991**; 30:59–68.

30. Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proc Natl Acad Sci USA **1990**; 87:6599–6603.

31. Hultgren C, Milich DR, Weiland O, Sallberg M. The antiviral compound ribavirin modulates the T helper (Th) 1/Th2 subset balance in hepatitis B and C virus-specific immune responses. J Gen Virol **1998**; 79(pt 10):2381–2391.

32. Ahlen G, Nystrom J, Pult I, Frelin L, Hultgren C, Sallberg M. In vivo clearance of hepatitis C virus nonstructural 3/4A-expressing hepatocytes by DNA vaccine-primed cytotoxic T lymphocytes. J Infect Dis **2005**; 192:2112–2116.

33. Ahlen G, Derk E, Weiland M, et al. Cleavage of the IPS-1/Cardif/MAVS/VISA does not inhibit T cell-mediated elimination of hepatitis C virus non-structural 3/4A-expressing hepatocytes. Gut **2009**; 58:560–569.

34. Frelin L, Brenndorfer ED, Ahlen G, et al. The hepatitis C virus and immune evasion: non-structural 3/4A transgenic mice are resistant to

35. Hultgren C, Desombere I, Leroux-Roels G, et al. Evidence for a relation between the viral load and genotype and hepatitis C virus-specific T cell responses. J Hepatol **2004**; 40:971–978.

36. Frelin L, Ahlen G, Alheim M, et al. Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus nonstructural 3/4A gene. Gene Ther **2004**; 11:522–533.

37. Ljunggren HG, Stam NJ, Ohlen C, et al. Empty MHC class I molecules come out in the cold. Nature **1990**; 346:476–480.

38. Soderholm J, Ahlen G, Kaul A, et al. Relation between viral fitness and immune escape within the hepatitis C virus protease. Gut **2006**; 55:266–274.

39. Milich DR, Schodel F, Hughes JL, Jones JE, Peterson DL. The hepatitis B virus core and e antigens elicit different Th cell subsets: antigen structure can affect Th cell phenotype. J Virol **1997**; 71:2192–2201.

40. Milich DR, Peterson DL, Schodel F, Jones JE, Hughes JL. Preferential recognition of hepatitis B nucleocapsid antigens by Th1 or Th2 cells is epitope and major histocompatibility complex dependent. J Virol **1995**; 69:2776–2785.

41. Brinster C, Chen M, Boucreux D, et al. Hepatitis C virus non-structural protein 3-specific cellular immune responses following single or combined immunization with DNA or recombinant Semliki Forest virus particles. J Gen Virol **2002**; 83:369–381.

42. Brinster C, Muguet S, Lone YC, et al. Different hepatitis C virus non-structural protein 3 (Ns3)-DNA-expressing vaccines induce in HLA-A2.1 transgenic mice stable cytotoxic T lymphocytes that target one major epitope. Hepatology **2001**; 34:1206–1217.

43. Lang KA, Yan J, Draghia-Akli R, Khan A, Weiner DB. Strong HCV NS3- and NS4A-specific cellular immune responses induced in mice and Rhesus macaques by a novel HCV genotype 1a/1b consensus DNA vaccine. Vaccine **2008**; 26:6225–6231.

44. Luckay A, Sidhu MK, Kjeken R, et al. Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. J Virol **2007**; 81:5257–5269.

45. Crowther RA, Kiselev NA, Bottcher B, et al. Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. Cell **1994**; 77:943–950.

46. Schirmbeck R, Reimann J. Modulation of gene-gun-mediated Th2 immunity to hepatitis B surface antigen by bacterial CpG motifs or IL-12. Intervirology **2001**; 44:115–123.

47. Weiss R, Scheiblhofer S, Freund J, Ferreira F, Livey I, Thalhamer J. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that over-rules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. Vaccine **2002**; 20:3148–3154.

48. Riedl P, Stober D, Oehninger C, Melber K, Reimann J, Schirmbeck R. Priming Th1 immunity to viral core particles is facilitated by trace amounts of RNA bound to its arginine-rich domain. J Immunol **2002**; 168:4951–4959.

49. Lee BL, Tucker A, Frelin L, et al. Interaction of the hepatitis B core antigen and the innate immune system. J Immunol **2009**; 182:6670–6681.

50. Rodriguez F, Zhang J, Whitton JL. DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. J Virol **1997**; 71:8497–8503.