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Interaction of the Hepatitis B Core Antigen and the Innate Immune System

Byung O. Lee,* Amy Tucker,* Lars Frelin,* Matti Sallberg,† Joyce Jones,* Cory Peters,* Janice Hughes,* David Whitacre,‡ Bryan Darsow,** Darrell L. Peterson,§ and David R. Milich*‡

Previous studies demonstrated that the primary APCs for the hepatitis B core Ag (HBcAg) were B cells and not dendritic cells (DC). We now report that splenic B1a and B1b cells more efficiently present soluble HBcAg to naive CD4+ T cells than splenic B2 cells. This was demonstrated by direct HBcAg-biotin-binding studies and by HBcAg-specific T cell activation in vitro in cultures of naive HBcAg-specific T cells and resting B cell subpopulations. The inability of DC to function as APCs for exogenous HBcAg relates to lack of uptake of HBcAg, not to processing or presentation, because HBcAg/anti-HBc immune complexes can be efficiently presented by DC. Furthermore, HBcAg-specific CD4+ and CD8+ T cell priming with DNA encoding HBcAg does not require B cell APCs. TLR activation, another innate immune response, was also examined. Full-length (HBcAg183), truncated (HBcAg149), and the nonparticulate HBeAg were screened for TLR stimulation via NF-κB activation in HEK293 cells expressing human TLRs. None of the HBe/HBcAgs activated human TLRs. Therefore, the HBe/HBcAgs proteins are not ligands for human TLRs. However, the ssRNA contained within HBcAg183 does function as a TLR-7 ligand, as demonstrated at the T and B cell levels in TLR-7 knockout mice. Bacterial, yeast, and mammalian ssRNA encapsidated within HBcAg183 all function as TLR-7 ligands. These studies indicate that innate immune mechanisms bridge to and enhance the adaptive immune response to HBcAg and have important implications for the use of hepadnavirus core proteins as vaccine carrier platforms. The Journal of Immunology, 2009, 182: 6670–6681.

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These studies reveal the involvement of the innate immune system in a rather unique APC pathway for exogenous HBeAg particles, which is dependent on the mode of immunization.

Materials and Methods

Mice

C57BL/10 (B10), B10.S, and 7/16-5 TCR-transgenic (Tg) mice were obtained from the breeding colony of the Vaccine Research Institute of San Diego. The B cell knockout (KO; μMT) mice originally produced by K. Rajewsky (14) were backcrossed onto B10, B10.S, and 7/16-5 TCR-Tg backgrounds. The TLR-7KO mice were obtained from R. Flavell (Yale University, New Haven, CT) and bred at the Vaccine Research Institute of San Diego. The C3H. HeJ and C3H. HeSm mice and the CBA/J and CBA/N (sid) mice were obtained from The Jackson Laboratory. All animal care was performed according to National Institutes of Health standards as set forth in the Guide for the Care and Use of Laboratory Animals (1996).

Recombinant proteins and synthetic peptide

Full-length rHBcAg183 of the ayw subtype was produced in E. coli, as described previously (5). Yeast-derived HBcAg183 (ayw) was obtained commercially from Meridian Life. A truncated version of HBcAg (residues 1–149) was produced in E. coli. This material is particulate, but expresses both HBeAg and HBeAg antigenicity at the level of Ab recognition and is designated HBcAg149 or HBeAg149 (additional C-terminal cysteine for biotinylation). A rHBcAg corresponding in sequence to serum-derived HBeAg encompassing the 10 precore amino acids remaining after cleavage of the precursor and residues 1–149 of HBcAg was produced, as described previously (15). The presence of the 10 precore amino acids prevents particle assembly, and HBeAg is recognized efficiently by an HBeAg-specific mAb, but displays little HBc antigenicity. Recombinant hybrid woodchuck hepatitis core Ag (WHcAg) particles containing a Plasmodium falciparum malaria circumsporozoite repeat sequence (NANPNVDPNANP3) inserted in the external loop were produced, as previously described (16). Anti-HBc mAbs 3105 and 3120 were purchased from the Immunology Institute.

Purification of core Ag

The core proteins were precipitated from the bacterial lysate by the addition of solid ammonium sulfate to 45% saturation (277 g/liter). The precipitates were collected by centrifugation, redissolved in a minimum of buffer (10 mM sodium phosphate buffer (pH 6.8)), and dialyzed extensively against the same buffer. The protein solutions were then applied to a Bio-Rad BioGel HTP hydroxypatite column (5 cm × 5–10 cm, depending on amount of protein) and eluted with 50 mM sodium phosphate buffer (pH 6.8). The core Ag pass through unretained. The proteins were then applied to a Sepharose CL-4B column (5 × 100 cm).

Endotoxin was removed from the core preparations by a modification of a phase separation with Triton X-114 (17). A solution of the protein at a concentration of 5 mg/ml was made with 1% Triton X-114 and incubated at 4°C for 30 min with constant stirring. The solution was then incubated at 37°C for 10 min and then centrifuged at 20,000 × g for 10 min. The protein solution was recovered from above the detergent. This procedure was repeated four times. Finally, the protein was precipitated by lowering the pH to 5. Residual detergent remained in solution. The protein was recovered by centrifugation and dissolved in endotoxin-free buffer. Before Triton X-114 treatment, the core preparations contained ~10 ng of endotoxin/μg HBeAg; after phase separation with Triton X-114, the endotoxin content was between 0.01 ng/μg HBcAg to an undetectable amount as determined by the QCL-1000 chromogenic Limulus amebocyte lysate endo- toxin assay (Cambrex). Yeast-derived HBcAg183 contained no measurable endotoxin. Unless otherwise specified, only endotoxin-free HBeAg preparations were used.

Synthetic peptides derived from the HBcAg or WHcAg sequences or the P. falciparum repeat sequence were synthesized by the simultaneous pep- tide synthesis method, as previously described (18).

Plasmid DNA and tumor cell line expressing HBeAg

A HBeAg gene fragment (552 nt) of the ayw subtype was amplified and cloned into the eukaryotic expression vector pVAX1 (Invitrogen), as previously described (19). The HBeAg expression plasmid was designated HBeAg-pVAX1. Plasmid DNA was grown and purified, as described (20). The purified plasmid DNA was dissolved and diluted in sterile PBS to a concentration of 1 mg/ml. A Rauscher virus-induced T cell lymphoma (RBL-5) cell line (H-2b) stably expressing HBcAg (RBL-5/C) (21) was provided by F. Chisari (The Scripps Research Institute, La Jolla, CA).

Biotinylation of HBeAg150C

rHBcAg150C (2 mg/ml) in 1 ml of PBS containing 1 mM EDTA was mixed with 100 μl of a biotin-N-(6-(biotinamido)hexyl)-3-[(2-pyridyl- dithio)-propionamido] stock solution (4 mM; Pierce) and vortexed, and the reaction mixture was incubated for 2 h at room temperature. Excess reagents were removed by dialysis against PBS. Based on the absorbance change at 343 nm, the biotin reacted with between 0.5 and 1 cysteine/monomer (240 monomers/particle).

Immunization protocols

For protein immunization with HBeAg/WHcAg, groups of three to five female mice were injected i.p. with 10–20 μg of protein either in saline or emulsified in CFA or IFA for both Ab production and T cell experiments. For DNA immunization, groups of 5–10 female mice were injected with 100 μg of plasmid DNA in PBS, and then saline against PBS. Based on the absorbance change at 343 nm, the biotin reacted with between 0.5 and 1 cysteine/monomer (240 monomers/particle).

ELISA for detection of murine Abs

Sera were collected from mice by retro-orbital bleeding of isofluorane-anesthetized mice and pooled. Abs were detected by an indirect solid-phase ELISA with rHBcAg or rHBeAg or P. falciparum repeat peptides as the solid-phase ligands, as described previously (2). Serial dilutions of both test and preimmunization sera were made, and the data were expressed as Ab endpoint titers, in which the highest dilution of sera required to yield an OD (492 nm) three times the preimmunization sera was considered positive. IgG-, IgG1-, IgG2a-, and IgG3-specific secondary Abs were purchased from Southern Biotechnology Associates.

Determination of CD4+ cytokine production

Spleen cells from either primed wild-type or B cell KO mice were cultured (5 × 106 cells/ml) with various concentrations of a panel of Ag. Culture supernatants were harvested at 48 h for IL-2 determination and at 96 h for IFN-γ determination. Cytokines were measured using commercial ELISA kits, according to the manufacturer’s protocol (eBiosciences).

Detection of HBcAg-specific lytic CTLs

Spleen cells derived from DNA plus tumor cell-immunized B10 mice were resuspended in complete RPMI 1640 medium, and in vitro stimulation was conducted for 5 days in the presence of 0.05 μM H-2Kk-restricted HBcAg93–100 peptide (sequence MGLKFRQL) (22). After 5 days of in vitro stimulation, effector cells were harvested and a 4-hour 51Cr assay was performed in 96-well V-bottom plates in a total volume of 200 μl. A total of 1 × 106 target cells (e.g., RMA-S) was pulsed with 50 μM H-2Kk-restricted HBeAg93–100 peptide for 90 min, and then subsequently labeled for 1 h with 25 μl of 51Cr (5 mCi/ml; MP Biomedicals) and washed three times in PBS. Different numbers of effectors and 5 × 104 labeled target cells (5 × 105 cells/well) were added to wells at E/T ratios of 100:1, 50:1, and 25:1. The level of cytolytic activity was determined after incubation of effectors and targets for 4 h.

Fractionation of cell populations

Five- to 10-wk-old 7/16-5 TCR-Tg mice were used as a CD4+ T cell source. Single-cell suspensions of splenocytes were labeled with anti-CD4 Microbeads (Miltenyi Biotec) and passed through a LS column (Miltenyi Biotec); the cells retained in the column were collected as CD4+ T cells. For fractionation of B cells, splenocytes from B10 mice were labeled with anti-CD90.2 Microbeads to deplete T cells and then passed through the LS column. Cells contained in the flow-through was termed enriched B line- age cells. Enriched B cells were then labeled with anti-CD5 Microbeads for positive selection of B1a cells. Similarly, CD5+ B1a cells were collected from peritoneal exudate cells. Purity of B1a cells was subsequently analyzed by FACS. For isolation of the splenic B1b population (B220+ CD21low CD11b+), flow through from the B1a cells was further labeled with CD11b Microbeads and positively selected as an enriched B1b cell population. FACS analysis was performed to determine the purity and showed >75% were B220+ CD11b+. Flow through from this procedure was collected and used as B2 cells. Bone marrow-derived DC (BMDC) were prepared by an established protocol. Briefly, bone marrow progenitor cells were harvested from femur and tibia bones of B10 mice and cultured in PBS, 10% FCS with 20 ng/ml mouse rGM-CSF. On day 2, supernatant and nonadherent cells were aspirated and replaced with fresh medium containing mouse rGM-CSF. Adherent immature DC were harvested by gentle pipetting on day 7.

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FIGURE 1. Naive B cells function as APCs for naive, HBcAg-specific CD4\(^+\) T cells in vitro. A, Fractionated, HBcAg-specific CD4\(^+\) T cells (1 \(\times\) 10\(^6\)) derived from three unprimed, TCR-Tg mice (7/16-5) were cocultured for 4 days with either purified, resting splenic B cells (1 \(\times\) 10\(^6\)) or splenic DC/M\(\phi\) (1 \(\times\) 10\(^6\)) derived from three wild-type mice, plus the indicated concentrations of HBcAg\(_{183}\) or the HBcAg-derived peptide (p120–140). CD4\(^+\) T cell activation was measured by CD25 expression, as determined by FACS analysis. B, Unprimed spleen cells pooled from either three wild-type (WT) 7/16-5 TCR-Tg mice or three 7/16-5 TCR-Tg mice bred on a B cell KO (BKO) background were cultured (5 \(\times\) 10\(^5\)) with a B cell KO background (BKO) with the indicated concentrations of HBcAg\(_{183}\) and IL-2 and IFN-\(\gamma\) production was determined by ELISA at day 2 or 4, respectively. Values are given as pg/ml. This experiment was performed on two occasions, and the results are representative.

Flow cytometry analysis

To analyze T cell activation, Ag-specific T cells from 7/16-5 TCR-Tg mice (V\(\gamma\)B11\(^+\) CD4\(^+\) T cell) were cultured with HBcAg or p120–140 peptide for 4 days. At day 4, cultured cells were stained for CD25. For surface staining, cells were incubated in 3% FBS in PBS containing 10 ng/ml 2.4G2 to block FcR binding, followed by the addition of fluorochrome-conjugated Abs. All fluorochrome-conjugated Abs were obtained from eBioscience. Flow cytometry was performed on a LSR flow cytometer (BD Biosciences) at the Salk Institute. Data were analyzed using FlowJo software (Tree Star).

TLR ligand screening

TLR stimulation was tested by assessing NF-\(\kappa\)B activation in HEK293 cells ectopically expressing seven different human TLRs, including the following: TLR 2, 3, 4, 5, 7, 8, and 9, and a reporter plasmid, pNiFty, carrying the human secreted embryonic alkaline phosphatase gene controlled by an NF-\(\kappa\)B-inducible ELAM-1 composite promoter (InvivoGen). Four HBc/HBeAg preparations were incubated with the TLR-expressing HEK293 cells at a concentration of 2 \(\mu\)g/ml. The four HBc/HBeAg preparations varied in the amounts of contaminating LPS ranging from 10 ng/\(\mu\)g to undetectable.

Core protein-binding assay

B1a and B2 cells were prepared as described above. Biotinylated HBc\(_{150c}\) was added to 1 \(\times\) 10\(^6\) B cells and incubated at 4°C for 1 h. Cells were collected and stained for B220 and streptavidin-PE for further FACS analysis.

Statistical analysis

Data were analyzed using the Student’s \(t\) test. SPSS software was used for all statistical analysis.

Results

Resting B cells, but not DC/M\(\phi\), can efficiently present HBcAg and prime naive T cells

Fractionated B cells (B220\(^+\)) or DC/M\(\phi\) cells representing the APC source were cocultured with naive HBcAg-specific, splenic CD4\(^+\) T cells derived from 7/16-5 TCR-Tg mice (Fig. 1A). Both APC populations are capable of presenting the HBcAg-derived peptide p120–140 to naive 7/16-5 CD4\(^+\) T cells, resulting in T cell activation, as demonstrated by up-regulation of IL-2 and IFN-\(\gamma\) cytokine production. HBcAg-specific IL-2 and IFN-\(\gamma\) production was determined by ELISA at day 2 or 4, respectively. Values are given as pg/ml. This experiment was performed on two occasions, and the results are representative.

To determine whether the preferential B cell presentation of HBcAg was unique to 7/16-5 CD4\(^+\) T cells and/or relevant in vivo, B10.S/\(\text{+/+}\) mice or B10.S/BKO mice were immunized with...
HBcAg₁₈₃ either emulsified in CFA, IFA, or saline (only the results of HBcAg/CFA immunization are shown in Fig. 2). In B10.S/+/+ mice, HBcAg efficiently primed T cells measured by IL-2 and IFN-γ production in response to a panel of recall Ag (HBcAg, nonparticulate HBeAg, and the dominant peptidic T cell site, p120–140). The HBcAg Ag was the superior recall Ag in vitro. In contrast, HBcAg-specific T cells were not efficiently primed in B10.S/BKO mice (Fig. 2). This defect was specific for the HBcAg

FIGURE 2. Efficient CD4⁺ T cell priming in vivo with soluble HBcAg requires B cells. Groups of three B10.S/+/+ or B10.S/BKO mice were immunized (i.p.) with HBcAg₁₈₃ (10 μg) emulsified in CFA. Ten days after immunization, spleen cells were harvested, pooled, and cultured with the indicated concentrations of a panel of recall Ag (HBcAg₁₈₃, HBeAg, and p120–140). Culture supernatants were collected at days 2 and 4 for the determination of IL-2 and IFN-γ, respectively, by ELISA. Groups of three mice each were also immunized with the HBsAg (20 μg, CFA), and spleen cells were cultured with varying concentrations of HBsAg in vitro (inset). This experiment was performed on at least three occasions, and the results are representative.

FIGURE 3. Preferential Ag presentation of HBcAg by B1a and B1b cells. A, Fractionated, splenic B cells (4 × 10⁵) or BMDC (4 × 10⁵) were cocultured with unprimed, CFSE-labeled 7/16-5 TCR-Tg CD4⁺ T cells (1 × 10⁵) in the presence of 2 μg/ml HBcAg₁₄₉ or p120–140 peptide. After 4 days, dilution of CFSE as a measure of CD4⁺ T cell proliferation was determined by FACS analysis. B, The indicated fractionated cell populations (4 × 10⁵) (BMDC, splenic B1a, splenic B1b, and splenic B2 cells) derived from unprimed B10 mice were cultured with HBcAg₁₄₉ (2.0 μg/ml) and CD4⁺ T cells (1 × 10⁵) derived from 7/16-5 TCR-Tg mice. After 4 days of in vitro culture, CD4⁺ T cell activation was measured by CD25 expression determined by FACS analysis. C, Unprimed, B10, total spleen cells, fractionated splenic B2 cells, or fractionated, splenic B1a cells (4 × 10⁵) were cocultured with unprimed, 7/16-5 TCR-Tg CD4⁺ T cells (1 × 10⁵) and HBcAg₁₄₉ (0.04–5.0 μg/ml) and CD4⁺ T cell activation was measured by IL-2 (day 2) and IFN-γ (day 4) production, as determined by ELISA. The results are representative of experiments performed on two separate occasions.
because the particulate envelope protein of HBV, HBsAg, primed HBsAg-specific T cells equally in H11001/H11001 and BKO mice (Fig. 2, inset). It was also notable that the low number of T cells primed in B10.S/BKO mice by HBcAg was more efficiently activated in vitro by HBeAg and p120–140 rather than the HBcAg, demonstrating that HBeAg and p120–140 are not as dependent on B cells for APC function as the particulate HBcAg.

**B cell subsets preferentially present HBcAg to naive CD4+ T cells**

We next examined whether a specific B cell subset was responsible for the APC function. Fractionated, naive HBcAg-specific CD4+ T cells (from 7/16-5 TCR-Tg mice) were labeled with CFSE and cocultured with fractionated splenic B cells or BMDC as APCs (Fig. 3A). Only CD4+ T cells cocultured with splenic B cells proliferated efficiently in the presence of HBcAg149. Note that both B cells and BMDC were efficient APCs for the HBcAg-derived peptide, p120–140. Importantly, splenic B1a cells and splenic B1b cells were superior APCs as compared with splenic B2 cells, and BMDC were unable to present HBcAg149 efficiently to naive HBcAg-specific CD4+ T cells, as measured by CD25 up-regulation on CD4+ T cells (Fig. 3B) and IL-2 and IFN-γ cytokine production in vitro (Fig. 3C).

Furthermore, direct binding studies of biotinylated-HBcAg150C particles to separated BMDC, splenic B2 cells, or splenic B1a cells were performed. Fig. 4 demonstrates that biotinylated HBcAg150C binds preferentially to an enriched B1a cell population in a dose-dependent manner and not to a B2 cell population. BMDC do not bind biotinylated HBcAg150C (data not shown). Furthermore, to illustrate the consequences of B1a cell APC function in vivo, we immunized CBA/N (xid) mice, which lack B1a cells, with HBcAg149 in saline and compared the response with CBA/J control mice (Fig. 5). Significantly lower and slower developing anti-HBc Ab responses were apparent in CBA/N (xid) mice. Note, however, that CBA/N (xid) mice did demonstrate a moderate secondary anti-HBc Ab response. Therefore, it appears that B1a cells may be responsible for the early and/or initial anti-HBc Ab response. As a further control, these two strains were also immunized with the HBsAg, and no significant differences were observed in the anti-HBs Ab responses. We also measured IgM and the various anti-HBc IgG isotype responses in CBA vs CBA/N mice (Fig. 5B). Note that CBA/J mice possess IgM anti-HBc Abs before immunization (pre-M), whereas CBA/N mice do not. All murine strains

**FIGURE 4.** Splenic B1a cells preferentially bind biotinylated HBcAg150C. Enriched B2 cells or B1a cells (1 x 106) were incubated at 4°C for 1 h with the indicated concentrations of biotinylated HBcAg150C. Cells were collected and stained for the B cell marker, B220, and for streptavidin binding, and analyzed by FACS.

**FIGURE 5.** Anti-HBc Ab production in vivo is influenced by B1a cells. A, Groups of five wild-type CBA/J or CBA/N (xid) mice were immunized (1°) with HBcAg149 (20 μg in saline) and boosted (2°) (i.p.) with 10 μg of HBcAg149 in saline, and serum anti-HBc Ab levels were determined by ELISA at the indicated times (○-○, ●-●). Serum anti-HBc IgG Ab was quantitated by endpoint dilution of sera (titer). Groups of three mice each were also immunized (1°) with HBsAg (20 μg; IFA), and anti-HBs Ab was determined by ELISA (□-□, ■-■). B, Groups of three CBA/J or CBA/N (xid) mice were immunized (i.p.) with HBcAg149 (20 μg of insaline), and 4 wk later serum IgM and IgG isotype-specific anti-HBc Abs were determined by ELISA. Pre-M, pre-existing IgM anti-HBc (before immunization). Results represent the mean values (±SD) of individual mouse sera (*, p = 0.02; **, p = 0.005; *** p = 0.002).
previously tested possess IgM anti-HBc Abs spontaneously, which most likely represents natural Ab produced by the innate immune system; in mice at least, this appears to be produced by B1a cells because it is absent in CBA/N mice. However, upon immunization, CBA/N mice did produce IgM anti-HBc Abs in levels equal to CBA/J mice (Fig. 5B). Therefore, pre-existing IgM anti-HBc is produced by B1a cells, and IgM anti-HBc produced in response to immunization is produced by another B cell subset, most likely B1b cells or marginal zone B2 cells. CBA/J mice produced significantly more IgG anti-HBc Abs of all IgG isotypes than CBA/N mice.

The requirement for B cell APCs does not apply to immune-complexed HBcAg or HBcAg expressed intracellularly

The preceding experiments were performed using protein rHBcAg as Ag and immunogen and demonstrated that DC/Mφ cells do not serve as efficient APCs for exogenous HBcAg. However, a single immunization with a DNA plasmid encoding the HBcAg (HBcAg-pVAX1) did not require B cells as APCs for efficient CD4+ T cell priming (Fig. 6A). Both wild-type B10 mice and B10/BKO mice immunized with HBcAg-pVAX1 demonstrated efficient HBcAg-, HBcAg-, and p120–140-specific T cell cytokine responses in splenic in vitro Ag recall cultures (Fig. 6A). We have previously shown that a prime/boost immunization strategy using HBcAg-pVAX1 and a boost with a HBcAg-expressing tumor cell line (RBL-5/C) elicits an efficient CD8+ CTL response to the HBcAg (23). The induction of an efficient CD8+ CTL response to intracellular HBcAg also does not require B cells as APCs (Fig. 6B). It appears that DC/Mφ APCs can cross-present intracellular HBcAg derived from transfected or tumor cells. Furthermore, non-B cell APCs can process and present HBcAg when it is complexed to anti-HBc mAbs (mAbs 3105 + 3120), but not free HBcAg. In contrast, B cell APCs can present free HBcAg or immune-complexed HBcAg, as shown in Fig. 7. Therefore, it appears that DC/Mφ can process and present HBcAg to naive CD4+ and CD8+ T cells, but do not efficiently bind and internalize free exogenous HBcAg.
HBcAg even though it is a particulate Ag. This most likely reflects the absence of any ligands for DC/Mφ endocytic receptors on HBcAg (i.e., no carbohydrate or lipid).

Potential role of TLRs in Ag presentation of the HBcAg

Various preparations of HBcAg and the nonparticulate HBeAg were examined for the ability to signal through a number of human TLRs (Fig. 8). Only a truncated HBcAg149 preparation, which contained E. coli-derived LPS as a contaminant (10 ng/µg HBcAg), signaled through TLR-2 and TLR-4, whereas the clearest HBcAg183 preparation, which was produced in yeast, demonstrated zero TLR signaling. Therefore, it appears that the HBcAg/HBeAg proteins do not possess TLR-binding capability, but the LPS that can copurify with rHBcAg produced in E. coli can bind and signal through TLRs 2 and 4. Recently, a number of publications have suggested that this contaminating LPS may explain the enhanced immunogenicity of HBcAg in mice and that researchers have been unaware of this problem (11, 12). Although we disagree that researchers have not been aware of this issue and, in fact, have rigorously controlled for the presence of LPS, it was important to address the possible effects that contaminating LPS may have on HBcAg immunogenicity studies in mice. Therefore, LPS-sensitive (C3H. HeSn) and congenic LPS-insensitive (C3H. HeJ) mice, which possess a mutation preventing TLR-4 signaling, were immunized with either truncated HBcAg149 or full-length HBcAg183, and anti-HBc Ab production was then determined. As shown in FIGURE 9, in LPS-sensitive C3H. HeSn mice, the LPS-negative HBcAg149 preparation (10 ng/ml) or a HBcAg149 preparation free of LPS contamination (LPS−) were immunized i.p. with 10 µg of HBcAg149 in saline, and 5 wk later serum anti-HBc IgG isotypes were measured by ELISA.

Role of TLR-7

Full-length HBcAg183, which contains a protamine-like C terminus, encapsidates pregenomic RNA during natural infection, and rHBcAg encapsidates ssRNA when produced in prokaryotic or eukaryotic expression systems. Furthermore, the enhanced immunogenicity of full-length vs truncated HBcAg is well known and has been attributed to the encapsidated ssRNA (13). However, formal proof that the encapsidated ssRNA acts as a TLR-7 ligand is lacking. Therefore, B10/f/+ and B10/TLR-7KO mice were immunized with either truncated HBcAg149 or full-length HBcAg183, and anti-HBc Ab production was then determined. As shown in FIGURE 10, enhanced immunogenicity of full-length HBcAg183 is mediated by TLR-7 signaling. Groups of three wild-type B10/f/+ or B10/TLR-7KO mice were immunized i.p. with 10 µg in saline of either full-length HBcAg183 (ssRNA+) or HBcAg149 (ssRNA−). At 1, 2, 4, and 6 wk after immunization, IgG anti-HBc Abs in pooled sera were measured by ELISA. Groups of three wild-type B10/f/+ mice or B10/TLR-7KO mice were immunized i.p. with 10 µg in saline of full-length HBcAg183 (ssRNA+). Two weeks after immunization, anti-HBc Abs of the indicated IgG isotypes were measured in pooled sera by ELISA. The results depicted represent mean values (±SD) of experiments performed using pooled serum samples on two separate occasions (⁎, p = 0.05; **, p = 0.005).
Fig. 10, immunization (10 μg in saline) with full-length HBcAg183 in B10/+/+ mice elicited significantly higher titer (~25-fold) anti-HBc Ab at every time point compared with truncated HBcAg149. Full-length HBcAg183 also elicited significantly higher titer anti-HBc Ab in B10/+/+ mice as compared with B10/TLR-7KO mice. Truncated HBcAg149 was equally immunogenic in B10/+/+ and B10/TLR-7KO mice. Also note that the humoral response to full-length HBcAg183 in B10/TLR-7KO mice was identical with the response to the truncated HBcAg149 in either B10/+/+ mice or B10/TLR-7KO mice. Furthermore, the anti-HBc IgG isotype distribution was dramatically influenced by the absence of TLR-7 signaling (Fig. 10). Anti-HBc IgG1 was enhanced in TLR-7KO mice, IgG2a anti-HBc Abs were totally absent in TLR-7KO mice, and IgG2b anti-HBc Abs were significantly reduced in TLR-7KO mice as compared with wild-type mice. Anti-HBc IgG3 Abs were not significantly different in wild-type vs TLR-7KO mice. These results indicate that the ssRNA of bacterial origin that is encapsidated within full-length HBcAg183 acts as a TLR-7 ligand and enhances anti-HBc Ab production while shifting IgG isotype distribution via signaling through endosomal TLR-7 within B cells or other APCs. These results also indicate that the difference in immunogenicity between full-length HBcAg183 and truncated HBcAg149 resides solely in ssRNA signaling through TLR-7 because HBcAg183 and HBcAg149 are equivalently immunogenic in

FIGURE 11. HBcAg-specific T cell cytokine production is regulated by TLR-7 signaling. Groups of three B10/+/+ and B10/TLR-7KO mice were immunized i.p. with 10 μg of full-length HBcAg183 (ssRNA+) in saline, and 4 wk later in vitro splenic recall cultures were performed in the presence of the indicated concentration of HBcAg183. HBcAg-specific IL-2 (day 2) and IFN-γ (day 4) production was measured by ELISA and expressed as pg/ml. The results are representative of two separate experiments.

FIGURE 12. Ab production to hybrid-WHcAg particles is influenced by TLR-7 signaling. A, Groups of three B10/+/+ or B10/TLR-7KO mice were immunized i.p. with 20 μg of either truncated hybrid-WHcAg (149-Mal-78-UTC) (ssRNA−) or full-length hybrid-WHcAg (FLW-Mal-78-UTC) (ssRNA+) particles emulsified in IFA. Six weeks after immunization, anti-WHc (carrier), anti-NANP (insert), and anti-NVDP (insert) Abs were measured in pooled sera by ELISA. B, Groups of three B10 wild-type mice were immunized i.p. with 20 μg of either truncated hybrid-WHcAg (149-Mal-78-UTC) (ssRNA−) or full-length hybrid-WHcAg (FLW-Mal-78-UTC) (ssRNA+) particles emulsified in IFA. Six-week sera were collected and pooled, and IgG1 and IgG2a anti-WHc, anti-NANP, and anti-NVDP Abs were measured by ELISA. G1/G2a ratios are also shown. The results represent mean values (±SD) of experiments performed using pooled serum samples on two separate occasions (*, p = 0.05; **, p = 0.005).
TLR-7KO mice. rHBcAg183 expressed in E. coli or yeast behaves similarly; therefore, both prokaryotic and eukaryotic ssRNA within HBcAg can function as TLR-7 ligands. Immunization with a pVAX1 DNA vector encoding full-length HBcAg183 elicits greater anti-HBc Ab production than a pVAX1 vector encoding truncated HBcAg149, suggesting that mammalian ssRNA can also function as a TLR-7 ligand (data not shown). The ssRNA-TLR-7 pathway predictably affected HBcAg-specific CD4+ T cell priming as well as in vivo anti-HBc Ab production, as shown in Fig. 11. Splenic T cells derived from B10/TLR-7KO mice immunized with HBcAg183 (i.e., ssRNA−) produced significantly less IL-2 and IFN-γ than T cells derived from B10/+/+ -immunized mice in HBcAg recall spleen cultures.

Because the woodchuck hepatitis virus core protein (WHcAg) has been developed as a vaccine carrier platform (16), it was of interest to determine whether TLR-7 signaling was also relevant to immunization with hybrid-WHCag particles. For this purpose, full-length WHcAg187 (ssRNA+) carrying two P. falciparum malaria-neutralizing B cell epitopes (i.e., NANP and NVDP) in the loop region at position 78 and a truncated WHcAg149 (ssRNA−) particle carrying the identical malaria B cell inserts were used to immunize B10/+/+ and B10/TLR-7KO mice; anti-WHC and anti-ssRNA− hybrid-WHCag particles elicited IgG2a-predominant Ab responses (Fig. 12A). The anti-WHC, anti-NANP, and anti-NVDP Ab responses were significantly higher (13- to 25-fold) in wild-type (+/+ ) mice immunized with the ssRNA− hybrid-WHCag particle as compared with the ssRNA+ particle. Furthermore, the ssRNA+ hybrid-WHCag particle elicited 25-fold higher anti-ssRNA− Ab responses in B10/+/+ mice as compared with B10/TLR-7KO mice, demonstrating the positive effect of signaling through TLR-7. Note also that the immunogenicity of ssRNA+ and ssRNA− hybrid-WHCag particles was virtually identical in TLR-7KO mice, again illustrating that only TLR-7 signaling distinguished between the full-length and truncated hybrid-WHCag particles. Furthermore, immunization of B10/+/+ mice with ssRNA− hybrid-WHCag particles elicited IgG2a-predominant anti-WHC, anti-NANP, and anti-NVDP Abs, whereas ssRNA− hybrid-WHCag particles elicited IgG1-predominant Ab responses (Fig. 12B).

Evidence that early TLR-7 signaling occurs in B cells

The preceding experiments indicate that B cells, and preferentially B1a and B1b cells, act as the primary APCs for exogenous HBcAg, and that TLR-7 signaling enhances anti-HBc Ab production and HBcAg-specific T cell cytokine production. This suggests, but does not prove that TLR-7 signaling occurs, at least initially, in B cells. To determine whether TLR-7 signaling occurs in HBcAg-specific B cells and is relevant to in vivo anti-HBc Ab production, a B cell transfer experiment was used. Recipient B cell KO mice were adoptively transferred with purified, splenic B cells (30 × 106) derived either from wild-type (+/+ ) mice or from TLR-7KO mice, in which case only B cells lacked TLR-7 expression and non-B cell APC populations were wild type with respect to TLR-7. Three days after the B cell transfer, mice were immunized (20 μg) and boosted (10 μg) with HBcAg183 (ssRNA−) in saline, and IgM anti-HBc Abs were measured by ELISA (using this protocol IgG anti-HBc is not efficiently produced). As shown in Fig. 13, TLR-7 expression in B cells was required for an efficient primary IgM anti-HBc response in vivo, indicating the relevance of TLR-7 signaling to this response. After the boost, IgM anti-HBc Ab production was less dependent on B cell TLR-7 expression. An in vitro correlate assay also indicated the importance of B cell TLR signaling for IgM anti-HBc Ab production. As noted in Fig. 1, unprimed B cells present HBcAg to and activate naive, 7/16-5

![FIGURE 13. TLR-7 signaling occurs in B cells. Two groups of six B10/B1KO mice served as recipients for the adoptive transfer of 30 × 106 fractionated splenic B cells derived from either B10/+/+ mice or B10/TLR-7KO mice. Successful B cell grafting was confirmed by the presence of serum IgG 3 days after adoptive transfer. At 7 days after adoptive transfer, mice were immunized i.p. with HBcAg183 (ssRNA−) (20 μg in saline) and boosted with 10 μg of HBcAg183 in saline. Sera were collected, pooled, and assayed for IgM anti-HBc Abs at the indicated time points by ELISA.

![FIGURE 14. In vitro IgM anti-HBc Ab production requires three signals. TCR-Tg CD4+ T cells in culture, and HBcAg149 as well as HBcAg183 can activate T cells. However, for production of IgM anti-HBc Ab to occur in this primary in vitro culture system, the HBcAg must be associated with a TLR signal (Fig. 14). Naive, HBcAg-specific 7/16-5 spleen cells cultured with HBcAg in vitro for 4–5 days produced IgM anti-HBc Abs in the supernatant only if the HBcAg possessed ssRNA as a TLR-7 ligand or if HBcAg was associated with LPS as a contaminant, which is a TLR-4 ligand (Fig. 14). The LPS does not have to be physically associated with the HBcAg because HBcAg plus soluble LPS will elicit IgM anti-HBc Ab production as well (data not shown). It appears that primary in vitro anti-HBc Ab production requires three signals, including the following: BCR cross-linking, cognate T cell help, and a TLR signal.](http://www.jimmunol.org/)

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**Discussion**

Although previous studies indicated that HBcAg-specific B cells were the primary APCs for HBcAg, the readout system consisted of presentation to HBcAg-specific T cell hybridomas, which do not require costimulatory signals for T cell activation (6, 24). In the current study, it was demonstrated that naive HBcAg-specific CD4+ T cells, derived from unprimed 7/16-5 TCR-Tg mice, were activated by HBcAg presented by resting, naive B cells in vitro, and furthermore, that DC/Mφ APCs could not serve as efficient APCs for exogenous HBcAg. The ability of resting B cells to prime naive CD4+ T cells remains a controversial subject, with opinions ranging from B cells being essential as APCs (25–27); B cells being unable to function as APCs (28, 29); and B cells inducing anergy in naive T cells (30). This lack of consensus may relate to the nature of the Ag under study. For example, the HBcAg is a particulate, multivalent protein Ag capable of cross-linking BCRs sufficiently to induce B7.2 and B7.1 costimulatory molecules on resting, HBcAg-specific B cells (6). Furthermore, in the present study, it was demonstrated that B1a cells are superior APCs for the HBcAg as compared with B2 cells and a relatively high frequency of B1a cells bind biotinylated HBcAg150C. Furthermore, in mice lacking B1a cells, the in vivo anti-HBc response was delayed as compared with wild-type mice. B1a cells are members of the innate immune system that produce IgM natural Abs and play a role in defense against bacterial infection (31). The specificities characteristic of B1a cells are encoded by distinctive germline genes, possibly with preferential H chain-L chain combinations, resulting in a restricted or hard-wired Ab repertoire (32).

In this regard, it is interesting that a linear motif that binds HBcAg has been identified in the framework region 1-CDR1 junction of an IgM anti-HBc hybridoma (9C8). This motif is present in H chains of the mouse V\_\_H1 family and human V\_\_H1 and V\_\_H7 families (33). A cryo-electron microscope reconstruction of an immune complex between HBcAg and a Fab of 9C8 indicates binding via the V\_\_H chain rather than the V\_\_L chain; however, the V\_\_H chain framework region 1-CDR1 junction region contains a motif with 73% similarity to the V\_\_H1 consensus motif (34). These studies suggest interaction between HBcAg and Igs derived from restricted V region germline genes, which is consistent with preferential recognition of HBcAg by B1 cells. Because WHcAg as well as HBcAg is preferentially presented by B cell APCs (data not shown) and these hepatavirus core proteins are not cross-reactive at the Ab level, we suggest that structural features of the spikes on the capsids surface rather than specific amino acids per se may serve as recognition sites for B1 cell Ig receptors. Although not extensively studied, B1a cells have been reported to be very efficient APCs (35).

As interesting as the HBcAg-B1 cell APC pathway may be, the more surprising observation is the inability of non-B cell professional APCs (i.e., DC/Mφ) to efficiently present exogenous HBcAg to CD4+ T cells in vitro or in vivo. DC/Mφ cells are known to be highly efficient APCs due to constitutive expression of costimulatory molecules, and are also known to be able to cross-present virus-like particles and other large particulate Ag (36–38). Nevertheless, immunization of B cell KO mice even with HBcAg emulsified in CFA, a very strong APC activator, was unable to efficiently prime CD4+ T cells (Fig. 2). The fact that immunization in CFA was unable to prime HBcAg-specific CD4+ T cells in B cell KO mice indicated that the defect in APC function was not at the level of DC/Mφ activation, and suggested that the uptake of free HBcAg particles by DC/Mφ was the limiting factor. Because the HBcAg is nonglycosylated and contains no lipid and apparently no other ligands recognized by DC/Mφ endocytic receptors, it appears that HBcAg particles are ignored by DC/Mφ APCs at least at concentrations of 1 μg/ml or less. The finding that DC/Mφ cells were efficient APCs for HBcAg immune complexed to HBcAg-specific mAbs established that once the HBcAg is internalized, presumably through FcR-mediated uptake, processing of HBcAg by DC/Mφ APCs and subsequent presentation to naive CD4+ T cells occur efficiently. Furthermore, immunization with a DNA plasmid encoding HBcAg elicited very efficient CD4+ T cell and CD8+ CTL responses in B cell KO mice. This indicates that B cells are not required as APCs for intracellular HBcAg, which can apparently be cross-presented by DC/Mφ to both HBcAg-specific CD4+ and CD8+ T cells. Because HBcAg exists as an exogenous Ag as well as an intracellular Ag within hepatocytes during a natural infection, B cell presentation of exogenous HBcAg to CD4+ T cells and DC/Mφ cross-presentation of intracellular HBcAg to CD4+ and CD8+ T cells are both relevant APC pathways during HBV infection. Predictably, B cell presentation of exogenous HBcAg leads to significantly greater anti-HBc Ab production as compared with DC/Mφ presentation of intracellular HBcAg; and, reciprocally, HBcAg-specific CD8+ T cells and possibly CD4+ T cells are more efficiently primed by DC/Mφ presentation of intracellular HBcAg.

The HBcAg and WHcAg have been proposed as vaccine carrier platforms for heterologous T and B cell epitopes (16, 39). Vaccination with hybrid-HBcAg/WHcAg particles would at least initially rely on B cells for primary APC function; however, once anti-HBc and anti-insert Abs are produced, immune-complexed hybrid-HBcAg/WHcAg particles would be recognized by DC/Mφ APCs as well.

Historically, naive B cell activation, maturation, and Ab production have been assumed to be dependent on the sequential integration of two signals, as follows: BCR cross-linking by Ag, followed by cognate interaction with Th cells through an immunological synapse involving CD40/CD40L interaction and cytokines (40, 41). More recent studies suggest a three-signal pathway for naive B cell activation that includes TLR ligands acting directly on B cells, resulting in coligation of the BCR and TLRs (42, 43). Although the absolute requirement for TLR signaling in naive B cell activation has been questioned (44), there is no disagreement that coligation of BCRs and TLRs augments Ab responses. There has been some debate as to whether HBcAg can function directly as a TLR ligand or whether LPS or some other contaminant indirectly activates TLR-2/4 (45). The current study did not find any evidence that the HBcAg protein possessed TLR-activating activity, which was restricted to HBcAg particles that were contaminated with LPS (i.e., TLR-2/4 activation). However, the ssRNA encapsidated in either E. coli-derived or yeast-derived full-length HBcAg stimulated significant TLR-7 activation. For example, full-length HBcAg,\_\_B (ssRNA+) elicited 25-fold higher anti-HBc responses in wild-type mice as compared with TLR-7 KO mice. Furthermore, in the absence of TLR-7 (TLR-7 KO mice), full-length HBcAg,\_\_B (RNA-) and truncated HBcAg,\_\_B (ssRNA-) were equally immunogenic. Therefore, the significantly greater immunogenicity of HBcAg,\_\_B as compared with HBcAg,\_\_B in wild-type mice is mediated entirely through ssRNA-TLR-7 signaling within B cell APCs, as demonstrated by adoptive transfer of TLR-7-negative B cells into B cell KO mice immunized with HBcAg,\_\_B (Fig. 13). Predictably, the ssRNA encapsidated within HBcAg,\_\_B also enhanced HBcAg-specific T cell cytokine production. Furthermore, ssRNA-TLR-7 signaling skewed the anti-HBc Ab isotype distribution toward IgG2a, IgG2b and away from IgG1 indicative of a Th1-type response.

Because of our interest in using the WHcAg as a vaccine carrier platform for heterologous B and T cell epitopes, hybrid-WHcAg
carrying *P. falciparum* malaria-protective B cell epitopes (NANP, NVDP) were used to immunize wild-type and TLR-7−/− mice. Hybrid-WHcAg particles containing ssRNA were significantly more immunogenic and elicited IgG2a/2b-predominant anti-WHc and anti-NANP and anti-NVDP Ab responses as compared with ssRNA-negative hybrid-WHcAg particles in wild-type mice and equivalent Ab responses in TLR-7−/− mice. The finding that anti-ssRNA is capable of enhancing innate immune responses to the HBcAg during a natural infection (50, 51), suggesting that the innate immune system is able to sense HBV through TLR-7 signaling. It appears that hybrid-WHcAg particles are excellent delivery vehicles for nucleic acid TLR ligands because the ssRNA is protected from nucleases within the WHcAg capsid and is delivered intracellularly to the APCs, in this case, Ag-specific B cells. The ssRNA present in hybrid-WHcAg can also be removed by RNase treatment and replaced with other TLR ligands, such as CpG, a TLR-9 ligand (46). The adverse systemic effects reported for TLR ligands such as CpG mixed with Ag as an adjuvant should not be problematic because TLR ligands encapsidated within hybrid-WHcAg particles are present in relatively low amounts and are delivered intracellularly to Ag-specific B cells or other APCs.

Given the spatial segregation between the BCR on the plasma membrane and both TLR-7 and TLR-9, which are endosomal receptors, it has not been clear whether enhanced B cell responses to ssRNA- or dsDNA-containing Ag are achieved by synergistic BCR-TLR coligation and/or whether coligation occurred in the same cellular compartment. However, a recent study clarified this point by demonstrating that as internalized BCR-Ag traffics to autophagosomes, it continues to signal through a phospholipase D-dependent pathway to recruit TLR-7-containing endosomes to autophagosomes, where they colocalize with BCR-Ag complexes (47). The recruitment of TLR-9 to BCR-containing compartments permits TLR-9 to sample BCR-bound Ag for the presence of its ligand (47). Although not experimentally demonstrated, it is likely that the synergistic interaction between BCRs and TLR-9 also occurs between BCRs and TLR-7 because coligation of BCR and TLR-7 within B cells has been described (48) and TLR-7 and TLR-9 are both localized in endosomes.

Although the relevance of TLR ligands encapsidated within hybrid-HBcAg/WHcAg particles used for vaccine purposes is clear, the relevance of the viral RNA/DNA incorporated in HBcAg to TLR signaling in a natural HBV infection is less obvious. The HBV has been characterized as a stealth virus in terms of innate immunity because in the liver of acutely HBV-infected chimpanzees, HBV does not induce an intrahepatic innate immune response during the lag phase of infection or the log phase of viral spread (49). It was suggested that HBV RNA/DNA species may be shielded from recognition by TLR or other nucleic acid sensors because HBV replication occurs within HBcAg particles (49). However, this would not preclude TLR signaling in APCs during processing of HBcAg, which may enhance both innate and adaptive immune responses to the HBcAg during a natural infection. Furthermore, recent studies of very early acute HBV infections in humans indicate early development of NK and NT cell responses, suggesting that the innate immune system is able to sense HBV infection (50, 51).

The ability of the HBcAg to interact with the innate immune system was demonstrated by the preferential binding and presentation of HBcAg to CD4+ T cells by B1a and B1b APCs and TLR-7 signaling within B cell APCs. These interactions serve to bridge the innate and adaptive immune responses to the HBcAg. However, it is important to emphasize that these innate immune interactions enhance, but are not necessary for an adaptive immune response to the HBcAg. For example, in CBA/N (xid) mice, which lack efficient B1a cell function, pre-existing IgM anti-HBc Abs are not present in serum, and the induced IgG anti-HBc Ab response is lower and delayed as compared with wild-type CBA mice. Nevertheless, a secondary IgG anti-HBc Ab response occurs after boosting in CBA/N (xid) mice, although less efficient than the wild-type response (Fig. 5). Furthermore, truncated HBcAg_{149}, which lacks encapsidated ssRNA, is still a relatively strong immunogen in the absence of TLR-7 signaling, and TLR-7−/− mice are still capable of producing anti-HBc Abs (Fig. 10).

The suggestions that HBcAg protein binding to TLR-2 (9) or full-length HBcAg_{183} binding to cell membrane HS (9, 10), or the activity of contaminating LPS may contribute to the enhanced immunogenicity of the HBcAg in vivo (11, 12), could not be substantiated in this study (see Figs. 8 and 9). These previous suggestions were based solely on in vitro systems, whereas the present study relied heavily on evaluation of in vivo immune responses to the HBcAg. For example, in vitro IgM, anti-HBc production requires three signals, as follows: BCR, cognate Th, and TLR-7; and LPS signaling through TLR-4 can substitute for the TLR-7 signal. However, the amount of LPS present even in a highly contaminated HBcAg preparation had no significant effect on anti-HBc Ab production in vivo (Fig. 9). Similarly, we observed nonspecific activation of DC/Mφ in the presence of full-length HBcAg_{183} in vitro at relatively high concentrations (≥1.0 μg/ml), which may be mediated by HBcAg C-terminal binding to cell membranes (9, 52) (data not shown). However, the in vivo relevance is not apparent because DC/Mφ do not efficiently present exogenous HBcAg_{183} to resting or primed CD4+ T cells; and, in the absence of TLR-7 signaling, full-length HBcAg_{183} and truncated HBcAg_{149} are equally immunogenic. Therefore, the particulate structure of the HBcAg characterized by the optimal spacing of the antigenic loops at the tip of the capsid spikes, which are capable of cross-linking BCRs, together with efficient CD4+ T cell recognition, explains the efficient adaptive humoral response to HBcAg, which can certainly be enhanced by the innate immune mechanisms described in this study.

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**Disclosures**

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