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Cytokine Induction by the Hepatitis B Virus Capsid in Macrophages Is Facilitated by Membrane Heparan Sulfate and Involves TLR2

Arik Cooper,* Guy Tal,† Ofer Lider,1† and Yosef Shaul2*

The hepatitis B virus (HBV) core Ag (HBcAg) is a 21-kDa protein that serves as the structural subunit of the highly immunogenic, 30-nm viral capsid (1). HBcAg possesses a N-terminal domain (residues 1–144) that directs capsid assembly and a C-terminal, arginine-rich domain (residues 150–183) that binds nucleic acids in a sequence nonspecific manner (2–4). Native capsids encapsulate the pregenomic RNA, whereas capsids assembled in bacteria incorporate bacterial RNA (4). The particle lattice is fenestrated by 1.5-nm pores that allow portions of the arginine-rich domain of HBcAg to become accessible on the shell exterior (5).

Alternative translational start codons from the precore/core gene of HBV direct the synthesis of either HBcAg or a core-related protein termed the HBV e Ag (HBeAg). HBeAg is a nonparticulate, secreted protein that is synthesized as a 25-kDa precursor harboring the entire primary sequence of HBcAg and additional 29 N-terminal residues. The 25-kDa precursor is processed en route the secretory pathway, resulting in removal of a 19-aa signal sequence from its N terminus and 34 residues corresponding to the arginine-rich domain of HBcAg from its C terminus, yielding the mature 17-kDa HBeAg. Although HBcAg and HBeAg share identical T cell epitopes (6), studies have shown that when injected into mice, HBcAg elicits a vigorous Th1 response while HBeAg triggers Th2 immunity (7, 8). Furthermore, HBeAg can skew immunity to HBcAg toward a Th2 phenotype by various mechanisms, including depletion of HBcAg-specific Th1 cells and induction of clonal-specific anergy (9–11). This interplay may have a profound clinical significance because HBc/HBeAg-specific Th1 immunity is associated with serological clearance of the virus while a Th2 response triggered by HBeAg promotes viral persistence (9).

Proinflammatory and regulatory cytokines produced by APCs play important roles in promoting Th1 immunity and controlling HBV infection (12). Using HBV transgenic animals, it was shown that TNF-α produced by resident liver macrophages enforces noncytopathic viral clearance from hepatocytes (13, 14). IL-12 and IL-18 released by macrophages were shown to contribute to the noncytopathic antiviral effect by inducing IFN-γ in other immune cells (14, 15). Clinical data suggest that capsids released from damaged hepatocytes during infection trigger the release of regulatory cytokines by APCs (16). Consistently, HBcAg induced the transcription and secretion of IL-18 in PBMCs (17). Also, Th1 immunity induced by HBcAg in mice involved the release of IL-12 from dendritic cells (8). Importantly, HBeAg and capsids lacking the arginine-rich domain of HBcAg did not induce substantial production of IL-12 and IL-18 in APCs (8, 17). RNA bound to the arginine-rich domain of HBcAg was suggested to have an adjuvant effect in facilitating Th1 immunity in the murine system (8). However, the molecular mechanism by which the capsid triggers activation of the innate immune response has remained elusive.

Macrophages represent a major source for inflammatory and regulatory cytokines during HBV infection (12). In the present study, we investigated the capacity of the HBV capsid to induce proinflammatory and regulatory cytokines in human macrophages and the possible underlying mechanisms. We found that the viral capsid is a potent inducer of TNF-α, IL-12p40, and IL-6 in human THP-1 macrophages. This induction involves capsid attachment to

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3 Abbreviations used in this paper: HBV, hepatitis B virus; HBcAg, HBV core Ag; HBeAg, HBV e Ag; PKA, protein kinase A; HEK, human embryonic kidney; F/DTC, pyrrolidine dithiocarbamate; CHO, Chinese hamster ovary; GAG, glycosaminoglycan; HCC, hepatocellular carcinoma.
cells that is largely promoted by interaction of the arginine-rich domain of HBcAg with heparan sulfate proteoglycans on the macrophage surface. Furthermore, we found that serine phosphorylation in the arginine-rich region can modulate the interaction of HBcAg with macrophages and the cytokine response. Finally, we show that cytokine induction by HBcAg involves TLR2-mediated NF-kB activation and ERK-1/2 and p38 MAPK signaling.

Materials and Methods

Escherichia coli-derived HBcAg capsids

Recombinant HBV capsids produced and purified from E. coli were kindly provided by Prof. P. Pumpens (Biomedical Research and Study Center, University of Latvia, Riga, Latvia) and were described previously (2, 18, 19). The recombinant HBc capsid consists of monomers encompassing the full-length open reading frame of HBcAg (aa 1–183; subtype ayw). The recombinant HBc-144 capsid assembles from monomers encompassing aa 1–144 and therefore lacks the arginine-rich domain of HBcAg. Protein analysis of the capsids by Coomassie brilliant blue staining confirmed a high purity level for both preparations (data not shown). The preparations contained up to 5.6 pg of LPS/µg protein as determined by the Limulus amebocyte lysate test (Milouda Biological Industries Services).

Removal of the bacterial RNA from the HBc capsid interior was performed by capsid permeabilization and nucleic digestion essentially as described previously (20). Briefly, capsids were diluted in water to reduce NaCl concentration to <20 mM, and RNAse A (Sigma-Aldrich) was added to a final concentration of 150 µg/ml. Capsids were incubated at 40°C for 10 min followed by incubation at room temperature for 30 min to allow capsid permeabilization and nucleic digestion, respectively. Removal of the RNA from the capsid interior using this protocol does not affect its particulate morphology (20). For analysis of the products, RNAse A-treated or untreated capsids were applied to a native agarose gel followed by Coomassie brilliant blue or ethidium bromide staining (20).

Phosphorylation of HBc capsids was performed as described previously (20). Briefly, permeabilized, RNA-free HBc capsids (100 µg/ml) were incubated with 150 U of protein kinase A (PKA) catalytic subunit (Sigma-Aldrich) or the phosphorylation buffer containing 200 HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, and 100 µM ATP. For radioactive labeling, phosphorylation was performed in the presence of 100 µCi of [γ-³²P] ATP (specific activity, 5000 Ci/mmol/ml). Radioactively phosphorylated core proteins were visualized by autoradiography after separation of the samples by SDS-PAGE with a 12.5% gel.

Labeling of capsids with FITC was performed according to the manufacturer’s manual (Molecular Probes). Briefly, capsids (2.5 mg/ml) were incubated with FITC (0.2 mg/ml) for 0.1 M NaHCO₃ (pH 9.0) for 2 h at room temperature with shaking. To remove nonconjugated FITC molecules, the reaction was layered on top of a 30% (w/v) sucrose cushion and spun in a TLA.120.1 rotor (Beckman Coulter) at 100,000 × g protein as determined by the Bradford method (3) at 280 nm. The pellet containing the capsids was resuspended in PBS and frozen at −80°C until use.

HBcAg capsids from mammalian cells

Human embryonic kidney (HEK) 293 cells were plated in 10-cm plates and transfected with 10 μg of mammalian expression vectors encoding either a full-length core open reading frame, a full-length core open reading frame harboring serine to alanine substitutions at all potential phosphorylation sites, a C-terminally deleted core open reading frame (aa 1–144), or with an empty vector using the calcium phosphate method. Forty-eight hours after transfection, cells were harvested and lysed by sonication and three cycles of freezing and thawing. Cell debris was removed by high-speed centrifugation. Nucleic acids, a potential source for macrophage activation (21–23), were removed by treatment with 100 µg/ml DNase I for 30 min at 37°C. The lysates were layered on top of a 30% (w/v) sucrose cushion and spun in a TLA.120.1 rotor (Beckman Coulter) at 100,000 × g for 1 h. Pellets containing the capsids were resuspended in PBS and frozen at −80°C until use.

Plasmids, Abs, and reagents

Expression vectors for human TLR2, TLR4, TLR9, and CD14 were kindly provided by Prof. R. J. Ulevitch (The Scripps Research Institute, La Jolla, CA) and were described previously (24–26). For construction of mammalian expression vectors encoding the full-length and C-terminally truncated (aa 1–144) core proteins, the respective sequences were PCR amplified from the HBV genomic DNA using the appropriate primers containing an EcoRI restriction site. The PCR products were digested with EcoRI restriction enzyme and cloned into pCDNA3 (Invitrogen Life Technologies) digested with the same enzyme. Plasmid pCMV-CM1 (kindly provided by Prof. J.-H. Ou, University of Southern California, Los Angeles, CA) encodes HBcAg (subtype adw2) in which residues 157, 164, and 172 were mutated from serines into alanines. Serine residues at positions 178 and 180 encoded by this mutant were changed into alanines by site directed mutagenesis.

Polyclonal rabbit Abs against HBcAg were generated in our laboratory by repeated injections with recombinantly expressed, denatured HBcAg. The monoclonal mouse anti-HBcAg Ab was generated by injection of recombinant HBcAg capsids, followed by selection of hybridoma-secreting anti-HBcAg Abs. Rhodamine red X-conjugated anti-rabbit Abs was purchased from Jackson ImmunoResearch Laboratories, and anti-IκBα was purchased from Santa Cruz Biotechnology. PMA, LPS, from E. coli 055:B5, peptide glycophosphatidylcholine from Staphylococcus aureus, chloroquine, 2-deoxy-o-glucose, and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich. U0126 and SB203580 were obtained from Calbiochem. Ham’s F-12 medium, DMEM, and FCS were purchased from Invitrogen Life Technologies. Ham’s F-12 medium was obtained from Biological Industries.

Polysaccharides and size defined heparin

Heparin (bovine lung) was purchased from Calbiochem. Heparan sulfate (bovine intestinal mucosa), chondroitin sulfate A (bovine trachea), chondroitin sulfate B (dermatan sulfate; porcine intestinal mucosa), and chondroitin sulfate C were purchased from Sigma-Aldrith. Size-defined heparin fragments were from Enzyme Research Laboratories.

Cell culture

Human monocytic THP-1 cells were grown in RPMI 1640 medium containing 10% heat-inactivated FCS, 2 mM glutamine, 25 µM 2-ME, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Differentiation into macrophages was attained by growing the cells in the presence of PMA (100 ng/ml) for 60 h. Differentiated THP-1 macrophages in 6-well plates (Nalge Nunc International; 2 × 10⁶ cells/well) or in 96-well plates (Nalge Nunc International; 140,000 cells/well) were washed once with PBS and incubated with HBV capsids or LPS in growth medium for the indicated times. Blood-derived macrophages were obtained from the peripheral blood of healthy human donors (Blood Bank) and purified as described previously (27). Blood-derived macrophages in 96-well plates (10⁶ cells/ml) were incubated with the indicated concentrations of HBc and HBc-144 capsids for 12 h.

Chinese hamster ovary (CHO)-pgsA745 and CHO-pgsD677 mutant cell lines were kindly provided by Prof. J. D. Esko (University of California, San Diego, La Jolla, CA). The CHO-pgsA745 line is deficient in xylosyltransferase activity, hence lacking expression of all glycosaminoglycan (GAG) species (28). Line CHO-pgsD677 is defective in the activity of both N-acetylgalactosaminyltransferase and glucuronosyltransferase specifically responsible for heparan sulfate polymerization. This line lacks cell surface heparan sulfate and possesses three times more chondroitin sulfate relative to the wild-type CHO-K1 strain (29). The parental CHO-K1 cell line and the mutant cell lines were grown in Ham’s F-12 medium supplemented with 10% FCS and antibiotics. HEK 293 cells were maintained in DMEM supplemented with 10% FCS and antibiotics. All cell lines were cultured at 37°C in a 5.6% CO₂ humidified incubator.

Immunostaining

Nuclei were stained with Hoechst 33342 (Molecular Probes) by brief incubation before fixation. Cells were extensively washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 25 min at room temperature, washed with PBS containing 0.2% Tween 20 (PBS-T), and blocked with FCS containing 10% (v/v) skim milk and 0.2% (v/v) Tween 20 for 45 min. Cells were then incubated with polyclonal rabbit anti-HBcAg Abs (diluted 1/175) in PBS-T containing 10% skim milk for 1 h, washed six times for 5 min with PBS-T, and incubated with rhodamine red X-conjugated anti-rabbit Abs for 40 min. Finally, the cells were washed six times as above, and the coverslips were mounted in Aqua PolyMount (Polysciences). Microscopic images were obtained using a Bio-Rad MRC-1024 confocal system, using an argon-krypton mixed gas laser and mounted on a Zeiss Axiovert microscope.

Flow cytometry

Differentiated THP-1 macrophages seeded 3 days before the experiment in 12-well plates (Nalge Nunc International; 10⁶ cells/well) were incubated with FITC-labeled capsids (1 nM) for the indicated times. Subsequently, the cells were washed, scraped by a rubber policeman in ice-cold PBS, extensively washed with the same buffer, and analyzed with a FACScan flow cytometer.
**RT-PCR**

Total RNA was isolated by using TRI Reagent (Molecular Research Center) and was reverse transcribed using Superscript II reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s recommendations. PCR amplification was performed with the ReadyMix master mix (Abgene) using the following primers: 3′-ATGAAATGACCCAG-3′ and reverse, 5′-CATCTCGTTCTCGAAGTCCA-3′; TNF-α, forward, 5′-CAGAGGAAGAGTCCCCCAAGC-3′ and reverse, 5′-CGTCTGAGTCTGGTCTGGTAGGAGACG-3′; IL-6, forward, 5′-ATGAACTCTCCCTCCACACAGG-3′ and reverse, 5′-GAAGGACCCCTCAAGGTCGAACG-3′; and IL-12p40, forward, 5′-GGACCAGACAGTGAGGTCCT-3′ and reverse, 5′-CTCCTTGTGTGCCTCCCTCTGA-3′. The RT PCR products were analyzed by electrophoresis through 2% agarose gels containing ethidium bromide.

**Cytokine measurement**

Concentration of human TNF-α in the culture supernatants was determined by ELISA according to the manufacturer’s instructions (BioSource International).

**EMS**

THP-1 cells were incubated with HBV capsids for the indicated times. Nuclear extracts were mixed with a ⅢP-labeled probe containing a consensus NF-κB binding site as previously described (30), electrophoresed through a 5% polyacrylamide gel, and visualized by autoradiography.

**Transfection and NF-κB reporter assay**

HEK 293 cells in 12-well plates were transiently transfected with the indicated amounts of TLR expression vectors, CD14, and reporter plasmids using the calcium phosphate method. Twenty-four hours after transfection, the cells were stimulated with either recombinant HBV capsids or S. aureus peptidoglycan for 12 h. The cells were subsequently harvested, lysed and luciferase activity was measured using lucy3 (Anthos).

**Results**

**The arginine-rich domain of HBcAg promotes cytokine induction in THP-1 macrophages**

To study innate immune responses toward HBcAg particles, we used the human monocytic cell line THP-1. These cells differentiate into macrophages upon treatment with PMA (31). Recombinant full-length (HBc) capsids induced the production of TNF-α in the culture supernatant of differentiated THP-1 cells in a dose- and time-dependent manner (Fig. 1, A and B). Significant TNF-α induction was detected already at the earliest time point (2.35 ng/ml after 3 h of incubation with 1 nM HBc capsids) and at the lowest dose of HBc capsids (1.22 ng/ml with 0.25 nM HBc capsids at 4 h). In contrast, C-terminally deleted capsids (HBc-144) were inefficient in TNF-α induction (~25% of the level induced by the HBc capsids at all time points and doses). Similarly, HBc but not HBc-144 capsids induced strong TNF-α production in human blood-derived macrophages (Fig. 1C). Neither HBc nor HBc-144 capsids induced TNF-α production in undifferentiated THP-1 monocytes (data not shown). Therefore, all additional experiments were conducted with differentiated cells.

To determine whether TNF-α induction occurred at the transcriptional level, TNF-α mRNA levels were evaluated by semiquantitative RT-PCR. Also, the levels of the transcripts encoding IL-6 and the IL-12p40 subunit were tested. HBc capsids strongly induced the transcripts of all these cytokines, whereas induction by HBc-144 capsids was substantially lower (Fig. 1D). These results demonstrate that the recombinant HBc capsid is a highly potent inducer of proinflammatory and regulatory cytokines in THP-1 macrophages and that this potential is largely dependent on its arginine-rich domain.

The kinetics of the transcriptional induction of the cytokines was next examined. TNF-α mRNA levels were induced to a maximal level already at 2 h after HBc capsid administration, decreased at 4 h, and was undetectable by 8 h, demonstrating a highly transient nature for TNF-α induction (Fig. 1E). Induction of IL-6 transcripts was also detectable at 2 h but was sustained throughout the experiment (up to 16 h). In contrast, slower induction was observed for the IL-12p40 transcript with a peak at 8 h and a decrease at 16 h. Thus, induction of the various cytokines by the HBc capsid occurs with different kinetics.

The recombinant *E. coli*-derived capsid preparations contained minute amounts of bacterial LPS (1.6 pg of LPS/μg protein as determined by the Limulus amebocyte lysate test). To exclude the possibility that residual LPS contamination contributed to cytokine induction, HBc and HBc-144 capsids were either untreated or preboiled before their appliance to the culture medium. As a control, *E. coli* LPS, either untreated or preboiled, was separately added to THP-1 macrophages. Analysis by ELISA and RT-PCR revealed that preboiling of the capsids but not of LPS completely abolished cytokine induction (Fig. 1, F and G). This result rules out the possibility that LPS contamination is responsible for cytokine induction by *E. coli*-derived capsids.

However, a possibility remained that another, heat-sensitive bacterial contamination (e.g., lipopeptides) in the *E. coli*-derived capsid preparation was involved in the induction of cytokines. To address this possibility, capsids from a mammalian source were used. HEK 293 cells were transfected with expression vectors encoding either HBc or HBc-144. Two days after transfection, the cells were lysed, and the capsids were concentrated by ultracentrifugation (see Materials and Methods for details). In addition, HEK 293 cells were transfected with an empty vector and extracted in a similar manner. Samples from the protein preparations were subjected to SDS-PAGE followed by Western blotting with a monoclonal anti-core Ab. Responsive bands at the expected size of 16 and 21 kDa were detected for the HEK 293-derived HBc-144 and HBc proteins, respectively (293-HBc-144 and 293-HBc, respectively; Fig. 1H, left panel). No signal was detected with the protein extract made from HEK 293 cells transfected with an empty expression vector (293-mock), demonstrating the specificity of the anti-core Ab. The 293-HBc and 293-HBc-144 proteins migrated similarly to *E. coli*-derived HBc capsids on a native agarose gel, demonstrating their particulate morphology (Fig. 1H, right panel).

Capsids derived from HEK 293 cells were applied to THP-1 macrophages, and transcriptional induction of TNF-α and IL-6 was tested by semiquantitative RT-PCR. 293-HBc capsids triggered a strong transcriptional induction of both cytokines, whereas 293-HBc-144 capsids induced a detectable but largely compromised response (Fig. 1I). Analysis by ELISA showed that 293-HBc capsids induced TNF-α protein production to a level similar to that triggered by *E. coli*-derived HBc capsids (Fig. 1J). An extract prepared from mock-transfected cells (293-mock) failed to induce TNF-α and IL-6 transcription and TNF-α protein secretion (Fig. 1, I and J). This indicates that the responses observed with human cell-derived capsids do not involve cellular components or other potential contaminants. Collectively, the results obtained with HEK 293-derived capsids were highly consistent with the activation observed with *E. coli*-derived capsids, ruling out contamination with *E. coli* lipopeptides as being involved in the cytokine induction.

The RNA associated with the arginine-rich domain of HBcAg is dispensable for cytokine induction in THP-1 macrophages

The arginine-rich domain of HBcAg mediates encapsidation of bacterial RNA in a sequence nonspecific manner during capsid assembly in *E. coli* (4, 8). Native agarose gel analysis revealed nucleic acids in association with *E. coli*-derived HBc but not HBc-144 capsids (Fig. 2A, lanes 2 and 1, respectively). The associated...
macrophages were incubated with HBc capsids (1 nM) for the indicated times, and the mRNA levels of TNF-α were determined by ELISA. B. THP-1 macrophages were incubated with 1 nM HBc capsids (1 nM) or HBc-144 capsids (1 nM) for 4 h, and TNF-α production was determined by ELISA. C. Human blood-derived macrophages were incubated with the indicated concentrations of HBc capsids (1 nM) or HBc-144 capsids (1 nM) for 12 h, and TNF-α production was determined by ELISA. D. THP-1 macrophages were either untreated or incubated for 4 h with HBc capsids or HBc-144 capsids (1 nM each) and TNF-α, IL-6, and IL-12p40 mRNA levels were determined by semiquantitative RT-PCR. E. Kinetics of cytokine mRNA induction by HBc capsids. THP-1 macrophages were incubated with HBc capsids (1 nM) for the indicated times, and the mRNA levels of TNF-α, IL-6, and IL-12p40 were tested by RT-PCR. F. Effect of preboiling on TNF-α production. HBc and HBc-144 capsids (left panel; final concentration 1 nM) and E. coli LPS (right panel; final concentration 1 μg/ml) were either untreated or boiled for 1 h before their appliance to the culture medium of THP-1 macrophages for 12 h. TNF-α production was determined by ELISA. G. HBc capsids, HBc-144 capsids, and E. coli LPS were either untreated or preboiled for 1 h before their appliance to the culture medium of THP-1 macrophages for 4 h. Total RNA was extracted from the cells, and TNF-α, IL-6, and IL-12p40 mRNA levels were determined by semiquantitative RT-PCR. UV exposure of products from LPS-treated cells was lowered to monitor changes in the intensity of the bands. H. Preparation of HBV capsids in human cells. HEK 293 cells were transfected with mammalian expression vectors encoding either a full-length core open reading frame (293-HBc), a C-terminally deleted core open reading frame (293-HBc-144), or with an empty vector (293-mock). Proteins were extracted 48 h later, and particles were enriched as described in Materials and Methods. Samples from the preparations were analyzed by SDS-PAGE and Western blotting with a monoclonal anti-core Ab (left panel) or by electrophoresis through a nondenaturing agarose gel followed by protein staining with Coomassie brilliant blue (right panel). HBc, 5 μg of E. coli-derived HBc capsids. I. THP-1 macrophages were either untreated or incubated for 4 h with 293-HBc capsids (1 nM), 293-HBc-144 capsids (1 nM), or an extract prepared from mock-transfected cells (293-mock). Total RNA was extracted from the cells, and the mRNA levels of TNF-α and IL-6 were determined by semiquantitative RT-PCR. J. THP-1 macrophages were either untreated or incubated for 4 h with 293-HBc capsids (1 nM), E. coli-derived HBc capsids (HBo; 1 nM), or an extract from mock-transfected cells (293-mock). TNF-α protein levels in the culture supernatants were determined by ELISA. Each value in A, B, C, F, and J represents the mean of triplicate samples ± SD. Results are representative of three independent experiments.

nucleic acids were RNase but not DNase sensitive (data not shown), in agreement with previous reports (4, 20). To test whether the RNA within capsids plays a role in cytokine induction in THP-1 macrophages, HBc capsids were permeabilized to allow effective accessibility of nucleases to the capsid interior, and the RNA was removed by RNase A treatment, as described previously (20). Treatment with RNase A resulted in removal of the RNA from the capsid interior and loss of capsid migration through the gel (Fig. 2A, lane 4), in consistence with a previous report (20). The process of capsid permeabilization affected neither the migration of the capsids through the gel nor the RNA content of the capsids (Fig. 2A, lane 3). RNase A-treated capsids, as well as permeabilized or untreated capsids, were applied to the medium of THP-1 macrophages, and TNF-α production was determined by ELISA. The level of TNF-α production induced by RNase-treated capsids was enhanced by 30% relative to untreated capsids (Fig. 2B). RNase A alone did not trigger TNF-α production, excluding a role for the endonuclease in cytokine induction. Also, capsid permeabilization per se did not change its capacity to induce TNF-α production. Finally, RNA-free HBc capsids were highly effective in inducing TNF-α, IL-6, and IL-12p40 transcripts (Fig. 2C). Hence, the RNA associated with the arginine-rich domain of HBcAg is dispensable for cytokine induction in THP-1 macrophages.

The arginine-rich domain of HBcAg promotes capsid attachment to macrophages

We next sought other possible features of the arginine-rich domain that may underlie its contribution to the response in THP-1 macrophages. In recent years, arginine-rich proteins and peptides were
shown to mediate cellular uptake of various macromolecules (32, 33). Consistently, arginine-rich peptides derived from the C terminus of HBcAg were shown to enhance the adjuvant effect of synthetic immunostimulatory oligodeoxynucleotides (34). To test whether the arginine-rich domain of HBcAg promotes capsid binding to macrophages, THP-1 cells were either untreated or incubated with HBc or HBc-144 capsids and immunostained with a polyclonal anti-HBcAg Ab. Although a faint signal was observed for HBc-144 capsids, strong staining for HBcAg, predominantly at the cell boundaries, was detected following incubation with HBc capsids (Fig. 3A). No signal was obtained with untreated cells, demonstrating the specificity of the anti-HBcAg Ab.

Capsid association with THP-1 macrophages was also visualized by flow cytometry using FITC-labeled capsids. A strong shift in cell fluorescence was observed following incubation with FITC-labeled HBc capsids (bold blue line) for 4 h in the continuous presence of 30 mM 2-deoxy-d-glucose and 0.1% sodium azide. Cells incubated with capsids were either washed with PBS (bold red line) or trypsinized to remove cell surface proteins (bold blue line). D, FACS analysis of THP-1 macrophages that were either untreated (thin green line) or incubated with 1 nM FITC-labeled HBc capsids for 4 h with the addition of 30 mM 2-deoxy-D-glucose and 0.1% sodium azide. Cells incubated with capsids were either washed with PBS (bold red line) or trypsinized (bold blue line) before analysis. E, FACS analysis of THP-1 macrophages incubated for 4 h with FITC-labeled HBc capsids that were either pretreated with RNase A (bold blue line) or untreated (bold red line). Control cells are represented by a thin green line.
The arginine-rich domain of HBcAg directs capsid binding to cells through interaction with membrane heparan sulfate proteoglycans.

The mechanism of HBc capsid attachment to cells was next investigated. The arginine-rich domain of HBcAg is highly positively charged, prompting the possibility that negatively charged moieties on the cell membrane are involved in HBc capsid attachment to cells. Proteoglycans have been frequently implicated in electrostatic interactions with positively charged ligands through their GAG chains (35). To examine the possible involvement of these molecules in capsid attachment, soluble GAGs were tested as competitive antagonists of capsid binding to THP-1 macrophages. Chondroitin sulfate A, chondroitin sulfate B (dermatan sulfate), and chondroitin sulfate C had a limited effect on HBc capsid attachment and uptake (Fig. 4A; 10–30% inhibition). In contrast, heparan sulfate and its highly sulfated structural homologue heparin markedly blocked capsid association with cells (70 and 75% inhibition, respectively).

To test whether the inhibitory effect of heparin was through direct sequestration of capsids, an in vitro binding assay to immobilized heparin was performed. Heparin-agarose beads were incubated with either HBc or HBc-144 capsids, and bound proteins were eluted with increasing NaCl concentrations. HBc capsids avidly bound heparin, with the peak of particles eluted with 1 M NaCl (Fig. 4B). In contrast, HBc-144 capsids did not bind heparin, in correlation with their inefficient binding to cells. These results imply that soluble heparin directly targets arginine-rich sequences exposed on the particle exterior, further supporting an active role for these motifs in capsid attachment to cells.

Heparin consists of repeating disaccharide units (36). To determine the minimal size of heparin required to occupy the binding site on the capsid, THP-1 cells were incubated with FITC-labeled HBc capsids in the presence of homogeneous heparin-derived fragments ranging in size from 2- to 16-saccharide units. Quantitative analysis by flow cytometry showed that an oligosaccharide consisting of 14 sugar monomers was the smallest fragment that efficiently inhibited capsid attachment (Fig. 4C). This indicates that the strong inhibitory effect of heparin on capsid cell attachment is not a consequence of nonspecific charge effects but a result of a defined heparin-ligand interaction.

Capsid attachment was observed with other cell types, including CHO, and in all cases, it was strongly inhibited by heparan sulfate and heparin but not by chondroitin sulfates (data not shown). Heparan sulfate but not heparin is a common GAG on the cell exterior (36). To directly address the possibility that heparan sulfate on the cell membrane participates in capsid binding to cells, CHO mutant cell lines with defects in GAG biosynthesis were used. Line pgs-A745 is deficient in xylosyltransferase activity, hence lacking expression of all GAG species (28). Line pgs-D677 is defective in the activity of both N-acetylgalactosaminyltransferase and glucuronyltransferase specifically responsible for heparan sulfate polymerization. This line lacks cell surface heparan sulfate and possesses three times more chondroitin sulfate relative to the wild-type CHO-K1 strain (29). The wild-type and mutant cell lines were incubated with FITC-labeled HBc capsids, and cellular fluorescence was analyzed by flow cytometry. Although the capsids effectively associated with the wild-type cell line, no binding and uptake were detected with the mutant cell lines (Fig. 4D). These results imply that the mechanism of HBc capsid binding to cells involves a specific recognition of the arginine-rich domain by cell surface heparan sulfate proteoglycans.

![FIGURE 4](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)

**A.** Effect of soluble GAGs on capsid binding and uptake. THP-1 macrophages were coincubated with FITC-labeled HBc capsids (1 nM) and different soluble GAGs (15 μg/ml) for 4 h and analyzed by FACS. Each value represents the mean fluorescence intensity of cells calculated relative to cells incubated with labeled capsids in the absence of antagonists. Results are representative of three experiments. Hep, heparin; HS, heparan sulfate; CS-A, -B, -C, chondroitin sulfate A, B, and C, respectively. **B.** Capsid binding to heparin. HBc or HBc-144 capsids were incubated with heparin immobilized on agarose beads. The beads were thoroughly washed and proteins were eluted by a step-gradient of increasing concentrations of NaCl. A sample from each fraction was analyzed by Western blot analysis using monoclonal anti-HBcAg Ab. Lane 1, one-tenth of inputs; lanes 2–5, fractions eluted with increasing NaCl concentrations as pointed at the top of the panels. Upper panel, HBc capsids; lower panel, HBc-144 capsids. **C.** Effect of homogeneous, size-defined heparin oligosaccharides on capsid binding and uptake. THP-1 macrophages were coincubated with FITC-labeled HBc capsids (1 nM) and heparin oligosaccharides of increasing length (all 0.66 μM) for 4 h and analyzed by FACS. Each value represents the mean fluorescence intensity of cells calculated relative to cells incubated with labeled capsules in the absence of antagonists. Data represents the mean ± SD of two independent experiments performed in duplicates. FL, full-length heparin. **D.** HBc capsids do not bind cells defective in GAG biosynthesis. CHO-K1 (parental source of mutant lines), pgs-A745 (no GAGs), and pgs-D677 (no heparan sulfate, three times more chondroitin sulfate) cells were incubated with 1 nM FITC-labeled HBc capsids (bold lines) or left untreated (thin lines). After 4 h, the cells were extensively washed and analyzed by FACS.
results demonstrate that GAGs are necessary for capsid attachment to the cells. Furthermore, heparan sulfate is specifically required, and an excess of chondroitin sulfate does not compensate for loss of heparan sulfate chains.

**Capsid association with heparan sulfate on macrophages facilitates cytokine induction**

Identification of heparan sulfate involvement in capsid binding to cells allows the relation between capsid attachment and cytokine induction to be directly investigated. To this end, HBc capsids were added to the culture medium of THP-1 macrophages in the absence or presence of various soluble GAGs, and TNF-α production was determined by ELISA. Only soluble heparan sulfate and heparin strongly inhibited TNF-α production (Fig. 5A; 70 and 75% inhibition, respectively). The inhibition levels are in good correlation with the inhibition degree the same soluble GAGs had on capsid binding to the cells (Fig. 4A). Heparan sulfate and heparin had a strong inhibitory effect on the mRNA levels of TNF-α, IL-6, and IL-12p40 induced by the capsid, whereas chondroitin sulfates had only a limited effect (Fig. 5B). Soluble GAGs alone had no effect on the basal level of the cytokines (data not shown). Also, soluble GAGs had no effect on the cytokine mRNA levels and TNF-α protein production induced by LPS (Fig. 5, C and D). Hence, soluble GAGs have a specific effect on cytokine production induced by the HBc capsid. This result further rules out the possibility that LPS contamination is responsible for cytokine induction by the recombinant capsid.

To further demonstrate the link between heparan sulfate-facilitated capsid association with macrophages and cytokine induction, HBc capsids were added to the culture medium of THP-1 macrophages in the absence or presence of homogeneous, size-defined heparin fragments. Heparin oligosaccharides consisting of 14-mer subunits were the smallest fragments that efficiently reduced cytokine induction by the capsid (Fig. 6, A and B). Again, these effects highly correlate with the inhibition enforced by the heparin fragments with respect to capsid attachment to cells (Fig. 4C). Collectively, these results indicate that interaction of the arginine-rich domain of HBcAg with membrane heparan sulfate, leading to capsid attachment and uptake, greatly facilitates cytokine induction in THP-1 macrophages.

**Serine phosphorylation within the arginine-rich domain of HBcAg modulates capsid binding and cytokine induction in macrophages**

The arginine-rich domain of HBcAg contains several serine residues shown to be phosphorylated by various kinases in vitro and in vivo (20, 37). To further substantiate our hypothesis that the arginine-rich region is important for response induction, we examined the effect of serine phosphorylation within this domain on HBc capsid binding to macrophages and cytokine induction. It was shown previously by Kann et al. (20) that PKA specifically phosphorylates serine residues within the arginine-rich domain of HBcAg in vitro. To generate phosphorylated cores, permeabilized HBc capsids were incubated with PKA and [γ-32P]ATP as described previously (20). Samples containing equal amounts of core proteins were separated by SDS-PAGE and either stained with Coomassie brilliant blue or visualized by autoradiography. A strong radioactive signal at the expected size for HBc monomers was detected following incubation of HBc capsids with PKA in the presence of [γ-32P]ATP (Fig. 7A, lower panel, lane 2). No radioactive signal was visualized for HBc capsids incubated either PKA or [γ-32P] ATP alone (Fig. 7A, lower panel, lanes 3 and 4, respectively), confirming the specificity of the signal. No signal was detected with HBc-144 capsids incubated with PKA and [γ-32P] ATP (Fig. 7A, lower panel, lane 5). Taken together, these results demonstrate that serine residues within the arginine-rich region of HBcAg are phosphorylated by PKA in vitro, in agreement with the study by Kann et al. (20).
THP-1 macrophages were incubated with either phosphorylated or unphosphorylated HBc capsids, and binding activity was examined by Western blotting of total cell extracts using a monoclonal anti-core Ab. Also, cytokine induction was tested by semiquantitative RT-PCR. Unphosphorylated HBc capsids strongly bound the cells and induced TNF-α and IL-6 mRNA synthesis (Fig. 7, B and C, lane 2). HBc phosphorylation markedly inhibited capsid association with cells and cytokine induction (Fig. 7, B and C, lane 3). No changes in macrophage binding and cytokine induction were noted with HBc capsids incubated with either the kinase or ATP alone, ruling out their involvement in the responses (Figs. 7, B and C, lanes 4 and 5). These results demonstrate that phosphorylation of serine residues embedded in the arginine-rich domain of HBc modulates capsid binding and cytokine induction in THP-1 macrophages. These data further provide direct biochemical evidence for the role of the arginine-rich region in capsid binding and cytokine induction in macrophages.

Next, we tested whether phosphorylation of serines within the arginine-rich region of HBcAg modulates the responses induced by HBV capsids expressed in mammalian cells. Previous studies identified serine residues located at aa 157, 164, 172, 178, and 180 as the in vivo phosphorylation sites in HBcAg (37, 38). A plasmid encoding HBcAg in which all these serines were mutated into alanines was expressed in HEK 293 cells. Importantly, it was shown that HBcAg phosphorylation in human cells is completely abolished for this mutant (37). A sample from the mutant protein preparation (293-HBc-SA), as well as a sample from wild-type 293-HBc capsids, were subjected to SDS-PAGE followed by Western blotting with a monoclonal anti-core Ab. Responsive bands at the expected size of 21 kDa were detected for the 293-HBc-SA and 293-HBc proteins (Fig. 7D, left panel). HBc293-HBc-SA cores migrated similarly to 293-HBc capsids on an agarose gel followed by protein staining with Coomassie brilliant blue (right panel). E, THP-1 macrophages were incubated for 4 h with an extract prepared from mock-transfected cells (293-mock), 293-HBc capsids (1 nM), or 293-HBc-SA capsids (1 nM). Total RNA was extracted from the cells, and the mRNA levels of TNF-α were determined by semiquantitative RT-PCR. F, THP-1 macrophages were incubated for 6 h with an extract prepared from mock-transfected cells (293-mock), 293-HBc capsids (1 nM), or 293-HBc-SA capsids (1 nM). TNF-α protein levels in the culture supernatants were determined by ELISA.
rose gel, demonstrating their particulate structure (Fig. 7D, right panel).

THP-1 cells were incubated with equal amounts of either 293-HBc or 293-HBc-SA capsids. Semiquantitative RT-PCR revealed enhanced levels of TNF-α mRNA in cells incubated with 293-HBc-SA capsids relative to cells incubated with 293-HBc capsids (Fig. 7E). Consistently, the level of TNF-α protein was enhanced by ~50% in the supernatant from cells incubated with 293-HBc-SA capsids compared with cells incubated with 293-HBc (Fig. 7F). These results are in accord with the data obtained with in vitro-phosphorylated HBc capsids, demonstrating that in vivo phosphorylation of serine residues within the arginine-rich region modulates the cytokine response induced by HBV capsids in macrophages.

Cytokine induction by recombinant HBV capsids involves signaling through NF-κB and MAPKs

To investigate the nature of the signaling events that lead to cytokine induction by the HBc capsid, the effect of various inhibitors was tested. PDTC, a blocker of NF-κB activation, completely abolished the TNF-α transcript and protein production but did not affect IL-6 and IL-12p40 transcript levels induced by the HBc capsid (Fig. 8, A and B). UO126, a specific inhibitor of ERK-1/2 MAPKs, strongly impeded mRNA induction of all cytokines, as well as protein production of TNF-α. SB203580, a specific inhibitor of the p38 MAPK, blocked mRNA production of IL-6 but had no adversary effect on the mRNA levels of TNF-α and IL-12p40 and on TNF-α protein production. Taken together, these results demonstrate that NF-κB and the MAPKs ERK-1/2 and p38 are differentially involved in induction of cytokines by the capsid.

In agreement with the possibility that NF-κB is involved in TNF-α induction by the capsid, EMSA analysis revealed strong NF-κB activation in THP-1 macrophages incubated with HBc but not HBc-144 capsids (Fig. 8C). The signal induced by HBc capsids was detectable already at 1 h following particle addition to the culture medium, peaked at 4 h, and leveled off at 6 h. Consistently, a substantial decrease in the protein level of the NF-κB inhibitor IκBα was observed only with the appliance of HBc capsids (Fig. 8D). HBcAg-induced IκBα degradation was markedly blocked in the presence of heparin, in agreement with our finding that capsid association with membrane heparan sulfate greatly contributes to cytokine induction.

Finally, chloroquine, known to inhibit endosomal acidification, did not impair cytokine induction by the HBc capsid (Fig. 8, A and B). Therefore, endosomal uptake of capsids may be nonessential for generation of the signal in THP-1 macrophages.

**NF-κB activation by HBcAg involves TLR2**

The signal transmitted following capsid attachment to macrophages involves NF-κB and MAPKs, which become activated in signaling transduced by members of the TLR family (39). These receptors are responsible for activation of innate immunity in response to various pathogens, including certain viruses (40). To test the possible involvement of TLRs in the signal transduced by HBcAg, HEK 293 cells were cotransfected with a NF-κB-luciferase reporter plasmid together with either an empty vector or an expression plasmid encoding TLR2 or TLR9. Transfectants were either untreated or incubated with 293-HBc, HBc, or HBc-144 capsids. Also, transfectants were incubated with S. aureus peptidoglycan as a positive control for TLR2. Cells transfected with an empty vector, as well as cells expressing TLR9, were unresponsive to HBcAg capsids (Fig. 9). Expression of TLR2 rendered the cells responsive to HBc and 293-HBc capsids. Coexpression of TLR2 and CD14, an accessory molecules that facilitates signaling by TLR2 ligands (26, 41), further enhanced NF-κB activation by HBc and 293-HBc capsids. No response was observed in 293 cells expressing CD14 alone and incubated with the capsids. A visible yet highly compromised response was detected in cells expressing either TLR2 alone or together with CD14 and incubated with HBc-144 capsids. Also, heparin markedly reduced NF-κB activation by HBc in cells expressing TLR2 alone and in combination with CD14. Finally, HBc and HBc-144 did not induce NF-κB activation in 293 cells coexpressing CD14 and the LPS-responsive TLR4 (data not shown). Collectively, NF-κB activation by the capsids specifically involves TLR2 and is facilitated by CD14 and membrane heparan sulfate.

**Discussion**

Activation of innate immunity plays a central role in virus-host relations, greatly influencing the development of adaptive immunity and the clinical outcome of infection. In this study, we analyzed the mechanism of induction of proinflammatory and regulatory cytokines by HBcAg in human macrophages. We show that the HBc capsid is a potent inducer of cytokines in THP-1 macrophages and in human blood-derived macrophages and that its arginine-rich domain is required for a vigorous response. Activation was abolished following boiling of E. coli-derived capsids and was...
recapitulated using capsids produced in a mammalian system, ruling out the possibility that bacterial contaminants are involved in the response. Hence, our results demonstrate that the HBc capsid is a bona fide activator of macrophages, relaxing concerns raised regarding authenticity of the immune activation by recombinant HBV capsids (42). We further demonstrate that the capsid binds macrophages in a manner largely dependent on its arginine-rich domain and membrane heparan sulfate. Using competitive heparan sulfate antagonists, a direct correlation between capsid binding to macrophages and the level of cytokine induction is demonstrated. Hence, cytokine induction by the capsid is strongly potentiated by capsid binding to membrane heparan sulfate. Furthermore, cell activation by the capsid involves signaling through TLR2 and is executed by NF-κB, ERK-1/2, and p38 MAPK.

C-terminally deleted capsids were markedly compromised in their capacity to bind macrophages in culture and did not interact with immobilized heparin in vitro. Full-length HBc capsids bound normal CHO cells in culture but failed to bind cells specifically defective in heparan sulfate biosynthesis. Combined with the avid binding of HBc capsids to immobilized heparin, these findings show that capsid attachment to cells is facilitated by direct, electrostatic interactions between the arginine-rich domain of HBcAg and sulfated heparan sulfate. Consistently, this domain harbors contiguous clusters of arginines (43), a motif implicated in binding to heparin by facilitating electrostatic and hydrogen bonding to sulfate ions presented on the sugar moiety (35). Notably, direct interaction with membrane heparan sulfate suggests that this domain is at least partially accessible on the capsid exterior. Consistently, foreign sequences inserted at the C terminus of the core protein, as well as portions of the arginine-rich domain itself, were shown to be partially accessible on the particle exterior (44–46). A flexible hinge links the assembly domain and the arginine-rich C terminus of HBcAg (46). This linker was suggested to confer accessibility of portions of the arginine-rich domain on the particle exterior by allowing these regions to protrude via 1.5-nm pores that traverse the shell lattice (5). Hence, the protruded arginine-rich sequences mediate capsid attachment to heparan sulfate. The enhanced association of RNase-treated capsids with cells is consistent with this model, as removal of the RNA from the capsid interior expectedly freed arginine-rich, heparan sulfate binding motifs to become further externally exposed.

We found that phosphorylation of serine residues located in the arginine-rich domain of HBcAg negatively affected cytokine induction through decreased capsid binding to THP-1 macrophages. Decreased capsid binding likely stems from the addition of negatively charged phosphates to the positively charged region, leading to a reduced binding affinity to negatively charged heparan sulfate chains. This notion is consistent with studies showing compromised affinity of phosphorylated HBcAg to nucleic acids (20, 43, 47). In addition, conformational changes in HBcAg brought about by serine phosphorylation in this domain (48, 49) may affect capsid recognition by macrophages. HBcAg phosphorylation is regulated throughout the viral life cycle, and HBV nucleocapsids contain a cellular born kinase (20, 50). HBcAg is underphosphorylated in resting cells but becomes hyperphosphorylated when cells enter the cell cycle (38, 51). Hepatocytes are normally quiescent but enter cycles during liver injury and regeneration (51, 52). Thus, capsids are expected to become hyperphosphorylated during ongoing hepatitis. Consistently, upon partial hepatectomy and liver regeneration in mice, HBc translocates from the nucleus to the cytoplasm (52), a shift correlating with HBcAg hyperphosphorylation (38, 51). The finding that phosphorylated capsids induce a largely compromised response in macrophages suggests that during active hepatitis, HBcAg undergoes hyperphosphorylation as part of a viral strategy to evade innate immunity.

Heparan sulfate proteoglycans are either GPI-anchored (termed glypicans) or transmembrane proteins with short intracellular domains (termed syndecans) that have not been directly implicated in cytokine induction. A requirement for NF-κB, ERK-1/2, and p38 MAPK for cytokine induction by the capsid prompted the conjecture that a TLR family member is involved in transmission of the signal intracellularly. Consistently, we found that NF-κB activation by the capsid in HEK 293 cells specifically required expression of TLR2. TLR2-dependent NF-κB activation by HBV capsids was enhanced by CD14, supporting the notion that the latter facilitates the recognition of a broad variety of ligands by TLR2 (26, 41). Also, in agreement with the role played by heparan sulfate in promoting cytokine induction by the capsid in THP-1 macrophages, soluble heparin significantly reduced NF-κB activation by the capsid in HEK 293 cells expressing TLR2. This implies that heparan sulfate is involved in capsid presentation to TLR2. Remarkably, heparan sulfate is commonly used for anchorage of viruses on the cell surface (53). Hence, cell attachment involving heparan sulfate may promote TLR activation by other viruses. The possibility that heparan sulfate binding by respiratory syncytial virus F and G proteins is related to their ability to activate TLR4 was proposed recently (54).

Particulate HBcAg was shown to induce Th1 immunity in mice, whereas HBeAg, as well as C-terminally deleted capsids, both of which lack the arginine-rich domain of HBcAg, primed a Th2-biased response (7, 8). Potent synthesis of IL-12 in murine dendritic cells induced by full-length but not C-terminally truncated capsids is suggestively involved in priming Th1 immunity to HBcAg in vivo (8). Our study showing that HBc but not HBe-144 capsids are capable of robustly inducing proinflammatory and regulatory cytokines in macrophages is consistent with these findings. RNA bound to the arginine-rich domain was suggested to function as an adjuvant in promoting cytokine induction and Th1 immunity in the murine system (8). However, in our study, removal of the RNA from the capsid interior did not hamper its capacity to induce...
cytokines in THP-1 macrophages. Conversely, the level of TNF-α induced by RNase-treated capsids was relatively increased and was accompanied by a proportional enhancement in capsid association with macrophages. A role for TLR2 in NF-κB activation by the capsid is consistent with this finding because TLR2 is not involved in viral RNA recognition (23). Also, chloroquine that specifically inhibits recognition of RNA and unmethylated CpG DNA via endosomal TLRs (55) did not impair cytokine induction by the capsid. Hence, our findings suggest that activation of human macrophages by the capsid is core protein-intrinsic. The different experimental settings used in the studies may underlie the above discrepancy. A contribution for the encapsulated RNA in inducing cytokines in THP-1 macrophages may thus contribute to oncogenesis associated with chronic HBV infection. It was shown recently that TNF-α specifically inhibits recognition of RNA and unmethylated CpG DNA via Toll-like receptor 7 and 8. Interleukin-18 inhibits hepatitis B virus replication in the livers of transgenic mice. J. Virol. 76: 10702–10707. Cavanaugh, V. J., G. L. Guidotti, and F. V. Chisari. 1997. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. J. Virol. 71: 3236–3245.

Identification of heparan sulfate and TLR2 as components potentially involved in the inflammatory response provoked by HBcAg may have future clinical implications. In recent years, chronic inflammation caused by infectious agents has become increasingly recognized as an important factor in development of pathogen-associated cancers (56). Consistently, chronic hepatitis B is associated with additional factors in the in vivo system.

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References


