Role of Immunoproteasome Catalytic Subunits in the Immune Response to Hepatitis B Virus[▽]

Michael D. Robek,^{1*} Mayra L. Garcia,¹ Bryan S. Boyd,² and Francis V. Chisari²

Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510,¹ and Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037²

Received 16 August 2006/Accepted 20 October 2006

Inhibition of hepatitis B virus (HBV) replication and viral clearance from an infected host requires both the innate and adaptive immune responses. Expression of interferon (IFN)-inducible proteasome catalytic and regulatory subunits correlates with the IFN- α/β - and IFN- γ -mediated noncytopathic inhibition of HBV in transgenic mice and hepatocytes, as well as with clearance of the virus in acutely infected chimpanzees. The immunoproteasome catalytic subunits LMP2 and LMP7 alter proteasome specificity and influence the pool of peptides available for presentation by major histocompatibility complex class I molecules. We found that these subunits influenced both the magnitude and specificity of the CD8 T-cell response to the HBV polymerase and envelope proteins in immunized HLA-A2-transgenic mice. We also examined the role of LMP2 and LMP7 in the IFN- α/β - and IFN- γ -mediated inhibition of virus replication using HBV transgenic mice and found that they do not play a direct role in this process. These results demonstrate the ability of the IFN-induced proteasome catalytic subunits to shape the HBV-specific CD8 T-cell response and thus potentially influence the progression of infection to acute or chronic disease. In addition, these studies identify a potential key role for IFN in regulating the adaptive immune response to HBV through alterations in viral antigen processing.

Hepatitis B virus (HBV) can cause either acute or chronic hepatitis in infected individuals (13). Progression of a primary HBV infection to either acute or chronic disease depends in large part on the efficacy of the host immune response to the virus (6). Acute HBV infection is associated with a vigorous, multispecific T-cell response to the virus, while chronic infection is associated with a weaker, more narrowly focused T-cell response (6, 31). Control of HBV replication by the host immune response involves both the gamma interferon (IFN- γ)-mediated noncytopathic clearance of HBV from infected cells and the killing of infected hepatocytes by virus-specific cytotoxic T lymphocytes (47). Thus, both innate and adaptive immune responses appear to be critical for the clearance of HBV from an infected host.

HBV replication is blocked noncytolytically by both IFN- α/β and IFN- γ , as well as by the IFN-related protein IL-28/IFN- λ (14). Although HBV infection does not induce a strong IFN- α/β response during acute infection of chimpanzees, IFN- γ produced by intrahepatic antigen specific or nonspecific immune cells plays a central role in controlling virus replication (20, 43, 44). Cell culture and transgenic mouse models of HBV replication have been used to demonstrate that the cellular IFN response inhibits HBV DNA replication in hepatocytes without affecting expression of viral mRNA by inhibiting the assembly of viral pregenomic RNA-containing capsids (45, 46). Gene expression analyses of IFN-induced genes in transgenic mouse livers and hepatocytes indicated that a number of proteins potentially contribute to this process, including the IFN-inducible immunoproteasome catalytic (LMP2, LMP7,

MECL-1) and regulatory (PA28 α/β) subunits (48). Utilizing this information, we previously determined that the IFN-mediated inhibition of HBV requires cellular gene expression and proteasome activity (32, 46). However, the precise proteins and molecular events that mediate this inhibition have not yet been defined.

A subset of the IFN-inducible proteasome subunits was also identified as being associated with HBV clearance in acutely infected chimpanzees, consistent with a potential role for these proteins in the adaptive immune response to HBV (43, 44). The immunoproteasome subunits are not normally expressed in the liver but are highly induced during an intrahepatic inflammatory immune response (22). The proteasome is responsible for generating the pool of peptides that are presented on the cell surface by major histocompatibility complex class I (MHC-I) molecules (33). The IFN-inducible catalytic subunits LMP2 and LMP7 alter the pool of peptides available for class I antigen presentation through enhanced substrate cleavage after basic and hydrophobic amino acid residues compared to the constitutive proteasome catalytic subunits (10, 12). This process has the potential to shape the CD8 T-cell response to viral antigens both by increasing the diversity of peptides produced and by favoring the production of peptides with carboxyl-terminal amino acid residues that more tightly bind MHC-I molecules. However, although immunoproteasomes produce some peptide epitopes more efficiently than constitutive proteasomes, the processing of other peptides is not enhanced by these subunits and in some cases is even suppressed (39).

The goal of this study was to determine whether the IFN-induced proteasome catalytic subunits LMP2 and LMP7 influence the innate and adaptive immune responses to HBV. We found that the CD8 T-cell response to the HBV envelope (ENV) and polymerase (POL) proteins is altered in mice that lack LMP7. We also determined that the antiviral effect of IFN

* Corresponding author. Mailing address: Department of Pathology, Yale University School of Medicine, P.O. Box 208023, 310 Cedar Street LH315A, New Haven, CT 06520-8023. Phone: (203) 785-6174. Fax: (203) 785-6127. E-mail: michael.robek@yale.edu.

[▽] Published ahead of print on 1 November 2006.

against HBV does not require expression of LMP2 or LMP7. These results indicate that in addition to its role in the noncytopathic inhibition of HBV replication, the cellular IFN- γ response may also influence the CD8 T-cell response to HBV by inducing expression of immunoproteasome subunits in hepatocytes.

MATERIALS AND METHODS

Animals and reagents. HLA-A2/K^b (A2/K^b) transgenic mice were provided by Epimmune Corporation (La Jolla, CA). To produce A2/K^b transgenic mice genetically deficient for LMP2 or LMP7, homozygous A2/K^b transgenic mice were mated with either LMP2 or LMP7 knockout mice (provided by M. Gaczynska, University of Texas, courtesy of L. van Kaer, Vanderbilt University, Nashville, TN, and H. Fehling, University of Ulm, Ulm, Germany) (9, 40). The resulting A2/K^b $\times$ LMP2^{+/-} and A2/K^b $\times$ LMP7^{+/-} mice were backcrossed to the respective LMP2^{-/-} or LMP7^{-/-} strain to produce mice heterozygous for the A2/K^b transgene and deficient for LMP2 or LMP7. These mice were further mated to LMP2^{-/-} or LMP7^{-/-} mice to produce additional -/- mice and were mated to C57BL/6 mice to produce control (+/-) mice. HLA-A2 transgene expression was analyzed by flow cytometric analysis of peripheral blood mononuclear cells, and LMP2 and LMP7 genotypes were assessed by PCR analysis for the presence of the deleted LMP2 and LMP7 exons. Groups of mice in all experiments were matched for age (8 to 12 weeks) and sex before use. All animals were housed in specific-pathogen-free rooms under strict barrier conditions. All animal procedures were performed in accordance with the Animal Care and Use Guidelines of The Scripps Research Institute and Yale University.

HBV transgenic mice (strain 1.3.32, C57BL/6 background) have been previously described (16, 17). To produce HBV transgenic mice genetically deficient for LMP2 or LMP7, homozygous HBV transgenic mice were backcrossed with either LMP2 or LMP7 knockout mice as described above (9, 40). Groups of mice in all experiments were matched for age (8 to 12 weeks), sex, and HBeAg expression levels (International Immuno-Diagnostics, Foster City, CA) before use.

Murine IFN- γ was provided by Yoshiaki Yanai (Hayashibara Co. Ltd.) and was also purchased from Calbiochem (La Jolla, CA). The anti-CD40 monoclonal antibody (MAb) was purified from a hybridoma (FGK45) provided by A. Rolink (Basel Institute for Immunology, Basel, Switzerland) (23). Poly(I)-poly(C) [poly(IC)] and MG-132 were purchased from Sigma (St. Louis, MO). The HBV POL plasmid vector expresses the POL protein under control of the cytomegalovirus (CMV) immediate-early promoter and has been previously described (21). The HBV ENV plasmid vector (generously provided by R. Whalen and H. Davis) expresses the middle and major ENV proteins under control of the CMV promoter (8). Recombinant vaccinia viruses expressing the POL and major ENV proteins have also been previously described (21).

Mouse immunizations. Mice were immunized by a DNA prime, recombinant vaccinia virus boost procedure to induce a virus-specific CD8 T-cell response, as previously described (21). Briefly, 50 μ g of the HBV ENV or HBV POL expression vector was injected intramuscularly (100 μ g/mouse) into regenerating tibialis anterior muscle 5 days after injection of cardiotoxin. Mice were injected 3 weeks later intravenously or intraperitoneally with 2×10^7 PFU of recombinant vaccinia virus expressing the corresponding antigen.

Intracellular IFN- γ staining. CD8 T-cell responses were analyzed ex vivo 2 weeks after the vaccinia virus boost by intracellular cytokine staining (ICCS) for IFN- γ expression or IFN- γ enzyme-linked immunospot (ELISPOT) analysis using splenocytes from immunized mice. For the ICCS analysis, splenocytes were stimulated with 10 μ g of ENV- or POL-derived peptides per ml for 5 h in the presence of 50 U recombinant mouse interleukin 2 (IL-2)/ml and 1 μ g brefeldin A/ml. After stimulation, the cells were harvested, washed in phosphate-buffered saline (PBS) containing 1% fetal bovine serum, and incubated for 20 min on ice with culture supernatant from the hybridoma cell line 2.4G2 (ATCC) to block nonspecific binding to the Fc receptor. The cells were then stained for surface CD8 α expression with a fluorescein isothiocyanate-conjugated antibody (Pharmingen, La Jolla, CA), followed by fixation and permeabilization (Cytofix/Cytoperm, Pharmingen) according to the manufacturer's instructions. Cells were then stained for IFN- γ (Pharmingen), fixed in 2% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer. Data were analyzed with CellQuest software (Becton Dickinson, San Jose, CA).

Splenocytes were stimulated as follows in the experiment in which cells were expanded in vitro prior to analysis. Cells were either seeded in a 2:1 ratio with irradiated HLA-A2-positive splenocytes that were previously incubated for 90 min at 37°C with 10 μ g of each individual peptide per ml (mice 1, 2, 5, 6, and 7

in Fig. 3) or were directly stimulated with 10 μ g of each peptide per ml (mice 3, 4, 8, and 9 in Fig. 3). Cells were harvested 7 days later and were restimulated with the corresponding peptide prior to ICCS staining.

ELISPOT analysis. Two hundred thousand splenocytes were seeded in duplicate in 96-well ELISPOT plates coated with IFN- γ capture antibody (BD Biosciences, San Diego, CA). Cells were stimulated with ENV₁₈₃₋₁₉₁ or ENV₃₇₀₋₃₇₉ peptide (10 μ g/ml) or overlapping peptide pools (8 to 10 peptides/pool; 2.5 μ g of each peptide/ml) overnight at 37°C. Splenocytes from two unimmunized mice were also stimulated with peptide, and splenocytes from immunized mice were incubated without peptide as negative controls. The remainder of the assay was performed exactly as described by the manufacturer's instructions (BD Biosciences). Results were analyzed with an ImmunoSpot ELISPOT analyzer and software (Cellular Technology Ltd., Cleveland, OH).

Proteasome purification. HeLa S3 cells were untreated or treated with 100 U IFN- γ /ml for 3 days. Constitutive proteasomes and immunoproteasomes were purified by modification of a previously described protocol (12). Approximately 5×10^7 cells were lysed by three freeze-thaw cycles in 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM ATP, and 250 mM sucrose. Nuclei and cellular debris were removed from the lysates by centrifugation at $10,000 \times g$ for 20 min. Supernatants were then centrifuged at $100,000 \times g$ for 1 h, followed by a second $100,000 \times g$ spin for 5 h. Pellets were resolubilized in 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM ATP, and 20% (vol/vol) glycerol. High-molecular-weight proteins were further enriched by centrifugation through a Centricon filter unit with a molecular mass cutoff of 100 kDa (Millipore, Billerica, MA). Increased incorporation of LMP2 and LMP7 in proteasomes purified from IFN- γ -treated cells was confirmed by Western blot analysis with LMP2- or LMP7-specific antibodies (BIOMOL, Plymouth Meeting, PA).

Proteasome activity assays. Synthetic fluorogenic peptides corresponding to the carboxy-terminal 4 amino acids of the ENV₁₈₃₋₁₉₁ and POL₈₀₃₋₈₁₁ epitopes [benzyloxycarbonyl(Cbz)-Ile-Leu-Thr-Ile-7-amino-4-methylcoumarin (AMC) and Cbz-Ser-Pro-Ser-Val-AMC, respectively] were synthesized by Genscript (Piscataway, NJ). Ten micrograms of proteasomes were incubated for 3 h with 25 μ M (each) peptide substrate in reaction buffer (25 mM HEPES, pH 7.6–0.5 mM EDTA–5 U apyrase/ml) at 37°C, and fluorescence emission at 480 nm was measured at 30-min intervals with a Perkin-Elmer Victor V3 instrument after excitation at 355 nm. Similar reactions were also performed without addition of proteasomes or with the known proteasome substrate *N*-succinyl-Leu-Leu-Val-Tyr-AMC as negative and positive controls, respectively.

Statistical data analysis. A two-tailed Student's *t* test was used to determine significant differences in the CD8 T-cell responses to POL and ENV. *P* values of <0.05 were considered statistically significant.

RESULTS

HLA-A2-restricted CD8 T-cell response to HBV ENV and POL. We used HLA-A2 transgenic mice to analyze the influence of LMP2 and LMP7 on the CD8 T-cell response to HBV, as these mice allowed examination of the contribution of these subunits to the generation of HLA-restricted epitopes with a mouse model. These HLA-A2/K^b mice express an MHC molecule derived from the HLA-A2 α 1 and α 2 domains and the mouse K^b α 3 region for efficient binding to mouse CD8 (41). Because the HBV core protein contains relatively few known HLA-A2-restricted epitopes and the POL and ENV proteins contain multiple epitopes (Table 1), we focused our analysis on POL and ENV (6, 24, 42).

Previous studies demonstrated HBV ENV- and POL-specific HLA-A2-restricted CD8 T-cell responses in HLA-A2 transgenic mouse models (24, 42). However, the immunization method we employed to induce an HBV-specific T-cell response differed from that used in previous studies. We therefore first confirmed the specificity of the CD8 T-cell response in immunized HLA-A2/K^b transgenic mice. HLA-A2/K^b transgenic mice were primed by intramuscular injection of ENV or POL plasmid expression vectors, followed by boosting with recombinant ENV- and POL-expressing vaccinia virus 3 weeks later. The CD8 T-cell response was quantified 2 weeks after

TABLE 1. HLA-A2-restricted peptide epitopes

Epitope	Sequence
HBV POL	
442–450	GLSRYVARL
491–499	HLYSHPIIL
562–570	FLLSLGIHL
642–650	ALMPYACI
803–811	SLYADSPSV
HBV ENV	
183–191	FLLTRILTI
260–269	LLDYQGMLPV
335–343	WLSLLVPFV
348–356	GLSPTVWLS
370–379	SILSPFLPLL

the boost by measuring IFN- γ production in splenocytes after a 5-h stimulation with the HLA-A2-restricted peptides (Table 1). When analyzed directly ex vivo, virtually the entire HLA-A2-restricted cytotoxic T-lymphocyte (CTL) response was limited to a single POL (POL_{803–811}) or ENV (ENV_{183–191}) epitope (Fig. 1A and C). No HLA-A2-restricted response was observed in unimmunized HLA-A2/K^b mice or in immunized C57BL/6 mice (data not shown). When splenocytes from immunized mice were expanded in vitro for 1 week by stimulation with the individual peptides, responses to other HLA-A2-restricted epitopes were detected (Fig. 1B and D). In particular,

CD8 T cells specific for the ENV_{370–379} epitope were consistently found. Thus, a POL- and ENV-specific HLA-A2-restricted CD8 T-cell response with a clear immunological hierarchy is generated in HLA-A2/K^b mice by using this immunization protocol.

Altered HLA-A2-restricted T-cell response in the absence of LMP7. We next determined whether the magnitude or specificity of the CD8 T-cell response to POL or ENV was altered in the absence of LMP2 or LMP7 expression. HLA-A2/K^b transgenic mice were crossed with LMP2^{−/−} or LMP7^{−/−} mice to produce A2/K^b × LMP2^{−/−}, A2/K^b × LMP7^{−/−} mice and corresponding control A2/K^b × LMP2^{+/-} and A2/K^b × LMP7^{+/-} mice. The CD8 T-cell response to the known HLA-A2-restricted peptides in POL- and ENV-immunized mice was again examined ex vivo by intracellular IFN- γ staining 2 weeks after vaccinia virus boost. IFN- γ staining of POL_{803–811}⁺, ENV_{183–191}⁺, and ENV_{370–379}-specific CD8 T cells in immunized LMP7^{+/-} and LMP7^{−/−} mice is shown in Fig. 2A. The magnitude and specificity of the CD8 T-cell response were not altered in POL-immunized LMP2^{−/−} mice compared to control heterozygous mice, as a strong response was generated to the immunodominant POL_{803–811} epitope in both strains (Fig. 2B). While the specificity of the response to POL in LMP7^{−/−} mice was also unchanged, the magnitude of the response to the POL_{803–811} epitope was decreased (mean specific CD8 T cells, 2.5%) compared to control LMP7^{+/-} mice (12.8%), LMP2^{+/-}

A. HBV POL Ex Vivo

B. HBV POL In Vitro

C. HBV ENV Ex Vivo

D. HBV ENV In Vitro

FIG. 1. HLA-A2-restricted CD8 T-cell response in HLA-A2/K^b transgenic mice. HBV POL (A and B)- or ENV (C and D)-specific CD8 T-cell responses were generated in HLA-A2/K^b transgenic mice by using a DNA prime, vaccinia virus boost immunization procedure. The HLA-A2-specific CTL response was measured by intracellular cytokine staining of splenocytes for IFN- γ expression after a 5-h stimulation with 10 μ g of each indicated peptide per ml either directly ex vivo (A and C) or after a 1-week in vitro expansion with the same peptide (B and D). N.D., none detected.

FIG. 2. Altered HLA-A2-restricted CD8 T-cell response in LMP7 knockout mice. (A) Example of intracellular IFN- γ staining of POL₈₀₃₋₈₁₁, ENV₁₈₃₋₁₉₁, ENV₃₇₀₋₃₇₉-specific CD8 T cells in immunized LMP7^{+/−} and LMP7^{−/−} mice. (B) Three to six mice of the indicated genotype were immunized to generate a POL-specific CD8 T-cell response. The magnitude of the response to the POL₈₀₃₋₈₁₁ epitope was then analyzed ex vivo by intracellular IFN- γ staining of POL₈₀₃₋₈₁₁-stimulated splenocytes. Each data point represents the percentage of CD8⁺ cells specific for this epitope in an individual mouse. (C) Four LMP7^{+/−} control and six LMP7^{−/−} mice were immunized to generate an ENV-specific T-cell response. The magnitude of the CD8 T-cell response to the immunodominant ENV₁₈₃₋₁₉₁ epitope and the subdominant ENV₃₇₀₋₃₇₉ epitope was analyzed ex vivo by intracellular IFN- γ staining of peptide-stimulated splenocytes. Each data point represents the percent of CD8⁺ T cells specific for each epitope in an individual mouse.

mice (10.4%), and LMP2^{−/−} mice (12.0%) (Fig. 2B). This decrease in the CD8 T-cell response was statistically significant ($P = 0.007$) when the response in the LMP7^{−/−} mice was compared to that in the other three strains, which all express LMP7. Therefore, incorporation of LMP7 into proteasomes may enhance the production of the immunodominant POL₈₀₃₋₈₁₁ epitope.

We also examined the ability of LMP2 and LMP7 to alter the magnitude and specificity of the CD8 T-cell response to HBV ENV. As with POL, no consistent change was observed in the CD8 T-cell response to ENV in LMP2^{−/−} mice compared to heterozygous controls (data not shown). However, the CD8 T-cell response to the immunodominant ENV₁₈₃₋₁₉₁ epitope was reduced in the absence of LMP7 (mean specific CD8 T cells, 0.18%) compared to the control heterozygous strain (1.6%) (Fig. 2C). The decrease in the CD8 T-cell response to ENV₁₈₃₋₁₉₁ in the LMP7^{−/−} mice was again significant ($P = 0.03$) compared to the response observed in the LMP2^{+/−}, LMP2^{−/−}, and LMP7^{+/−} mice. Furthermore, the response generated to the subdominant ENV₃₇₀₋₃₇₉ epitope was increased in the absence of LMP7 expression (specific cells, 0.96% and 0.06% in LMP7^{−/−} and LMP7^{+/−} mice, respectively). Once again, this change was significant when the response is compared to that observed in all LMP7-positive strains ($P = 0.008$). Therefore, LMP7 alters both the magnitude and specificity of the CD8 T-cell response to HBV ENV in this model system.

We were unable to detect ENV₃₇₀₋₃₇₉-specific T-cell responses in the majority (three of four) of control mice or ENV₁₈₃₋₁₉₁-specific responses in the majority (four of six) of LMP7-deficient mice by intracellular IFN- γ staining of peptide-stimulated cells (Fig. 2C). However, the possibility existed that weak CD8 T-cell responses were generated below the limit of detection (approximately 0.1% of CD8⁺ cells) of this assay. Therefore, we immunized five additional control heterozygous and LMP7-deficient mice and analyzed the T-cell response to these epitopes by the more sensitive IFN- γ ELISPOT assay. Responses to both ENV₁₈₃₋₁₉₁ and ENV₃₇₀₋₃₇₉ were consistently detected in both control and LMP7-deficient mice by this assay, although the absolute magnitude of the response varied and was occasionally weak (data not shown). However, the number of ENV₁₈₃₋₁₉₁-specific cells in control LMP7^{+/−} mice was consistently greater than the number of cells specific for ENV₃₇₀₋₃₇₉ (mean, 5.2-fold greater) (Fig. 3). In contrast, the number of cells specific for ENV₁₈₃₋₁₉₁ was lower than the number of cells specific for ENV₃₇₀₋₃₇₉ in the LMP7 KO mice (mean, 1.5-fold lower). The difference in the response between the two groups was again statistically significant ($P = 0.028$). These results again confirm that the CD8 T-cell response to ENV₁₈₃₋₁₉₁ is reduced in the absence of LMP7, while the response to ENV₃₇₀₋₃₇₉ is increased in the absence of this subunit.

One limitation to this analysis is that we chose to focus on the CD8 T-cell response generated to known HLA-A2-restricted CD8 T-cell epitopes. These studies therefore did not address the possibility that the results may be influenced by the CTL response to either uncharacterized HLA-A2-restricted epitopes or murine *H-2^b*-restricted peptides. Therefore, we also used ELISPOT analysis to examine the CD8 T-cell response to the entire HBV ENV protein by using an overlap-

FIG. 3. ELISPOT analysis of the ENV₁₈₃₋₁₉₁- and ENV₃₇₀₋₃₇₉-specific T-cell response in HBV ENV-immunized mice. Five LMP7^{+/+} control and five LMP7^{-/-} mice were immunized to generate an ENV-specific T-cell response. The magnitude of the T-cell response to the immunodominant ENV₁₈₃₋₁₉₁ epitope and the subdominant ENV₃₇₀₋₃₇₉ epitope was analyzed ex vivo by IFN- γ ELISPOT analysis with peptide-stimulated splenocytes. Data are presented as the ratio of the number of spots observed after ENV₁₈₃₋₁₉₁ stimulation compared to ENV₃₇₀₋₃₇₉ stimulation in an individual mouse.

ping peptide library. This library consisted of 76 15-mer peptides that overlap by 10 amino acids and span the entire ENV amino acid sequence. Splenocytes from five ENV-immunized control and LMP7-deficient mice were stimulated with pools of overlapping peptides (8 or 10 per pool), followed by IFN- γ ELISPOT analysis of peptide-specific cells. Control cultures (nonimmunized with peptide stimulation and immunized without peptide stimulation) and the majority of peptide pool-stimulated cultures did not show a significant T-cell response, as evidenced by fewer than 10 spots per well (data not shown). However, T-cell responses specific for a subset of the peptide pools were observed in both the control and LMP7-deficient mice (Fig. 4). Interestingly, weak responses generated to certain peptide pools were generally unaffected by the absence of LMP7 expression, while strong responses in control mice were often weaker in the LMP7-deficient mice. Therefore, changes in the overall CD8 T-cell response to HBV ENV occur in the absence of LMP7 but appear to be limited to stronger immunodominant specificities.

Cleavage of ENV₁₈₃₋₁₉₁ and POL₈₀₃₋₈₁₁ C termini by constitutive proteasomes and immunoproteasomes. Because the altered CD8 T-cell response may be due to factors other than cleavage efficiency of the protein substrate by the proteasome, we also examined the ability of constitutive proteasomes and immunoproteasomes to cleave model peptide substrates derived from the HBV ENV and POL proteins. Proteasomes were isolated from HeLa S3 cells that were untreated or treated with 100 U of human IFN- γ /ml for 3 days. Increased incorporation of LMP2 and LMP7 in the proteasomes purified from IFN- γ -treated cells was confirmed by Western blot analysis (Fig. 5A). Proteasomes from IFN- γ -treated cells also cleaved a known proteasome substrate (succinyl-LLVY-AMC)

FIG. 4. ELISPOT analysis of HBV ENV-immunized mice, using an overlapping peptide library. Five LMP7^{+/+} control mice (A) and five LMP7^{-/-} mice (B) were immunized to generate an ENV-specific T-cell response. The magnitude of the T-cell response to pools of 8 or 10 overlapping 15-mer peptides spanning the entire ENV protein was analyzed ex vivo by IFN- γ ELISPOT analysis with peptide-stimulated splenocytes. Data are presented as the average number of spots in duplicate assays for peptide pools in which a response above background was detected. Each bar represents the response to a particular pool in an individual mouse.

more efficiently than proteasomes from untreated cells (data not shown). Furthermore, the proteasome inhibitor MG-132 inhibited protease activity in the preparations with a 50% inhibitory concentration of approximately 10 nM, indicating that the purified protease activity was proteasome-specific (Fig. 5B).

Degradation of proteins by the proteasome generates peptides that are further trimmed by N-terminal peptidases but retain their C-terminal end generated by proteasome cleavage (3, 7, 26, 29, 38). The C-terminal amino acid is often a critical residue for determining binding specificity to MHC-I molecules. Synthetic peptides were generated that were carboxy-

FIG. 5. ENV peptide cleavage by constitutive proteasomes and immunoproteasomes. (A) Western blot analysis of LMP2 and LMP7 in proteasomes purified from IFN- γ -treated or untreated HeLa S3 cells. Expression of the noninducible α -catalytic subunits is shown as a protein loading control. (B) Inhibition of protease activity in the purified preparations by the proteasome inhibitor MG-132. Error bars represent standard deviations from triplicate experiments. (C and D) Proteasomes purified from untreated and IFN- γ -treated HeLa S3 cells were used in proteolytic assays to determine the cleavage rate of ENV₁₈₃₋₁₉₁- and POL₈₀₃₋₈₁₁-derived peptide substrates (Cbz-ILTI-AMC and Cbz-SPSV-AMC, respectively). Proteasomes purified from IFN- γ -treated cells cleaved each peptide more efficiently than proteasomes from untreated cells. Error bars represent standard deviations from four replicate experiments. The difference in fluorescence at each time point was statistically significant ($P < 0.001$).

terminally labeled with the fluorescent molecule AMC. Cleavage after the C-terminal amino acid releases AMC, allowing for fluorescent emission after excitation. Peptides consisting of the C-terminal 4 amino acids of the ENV₁₈₃₋₁₉₁ and POL₈₀₃₋₈₁₁

epitopes were cleaved more efficiently by proteasomes purified from IFN- γ -treated HeLa S3 cells than by proteasomes isolated from untreated cells (Fig. 5C and D). Therefore, the increased CD8 T-cell response to these epitopes in mice expressing LMP7 correlates with increased cleavage of the peptides by proteasomes containing LMP2 and LMP7.

The antiviral effect of IFN does not require LMP2 or LMP7.

We also examined whether the noncytopathic inhibition of HBV replication by IFN- α/β or IFN- γ requires the activity of the IFN-inducible proteasome catalytic subunits LMP2 or LMP7. HBV 1.3.32 transgenic mice were mated with mice genetically deficient for LMP2 or LMP7 to produce HBV $\times$ LMP2^{-/-} and HBV $\times$ LMP7^{-/-} mice and the corresponding control HBV $\times$ LMP2^{+/-} and HBV $\times$ LMP7^{+/-} mice. An intrahepatic IFN response was induced in the mice by intravenous injection of poly(IC) (doses of 200 μ g or 10 μ g per mouse), IFN- γ (2×10^5 units/mouse), or anti-CD40 MAb (100 μ g/mouse). These stimuli have all been demonstrated to inhibit HBV replication by inducing IFN- α/β , IFN- γ , or both (19, 23, 25). With all stimuli, the antiviral effect of IFN in the control LMP2 or LMP7 heterozygous mice was similar to that observed in the LMP2- or LMP7-deficient mice, as determined by densitometry quantification of HBV total and single-stranded DNA replication intermediates after Southern blot analysis (Fig. 6). Thus, although the immunoproteasome catalytic subunits may influence the specificity of the HBV-specific T-cell response, they do not appear to play a role in the IFN-mediated noncytopathic inhibition of virus replication.

DISCUSSION

Intrahepatic gene expression analysis of acutely infected chimpanzees has shown that the immunoproteasome subunits are among the genes that are induced in the liver in correlation with HBV clearance (43). One mechanism by which these subunits could contribute to viral clearance might be by enhancing the generation of HBV immunogenic peptide epitopes. This hypothesis can be correct, however, only if LMP2 or LMP7 has the capacity to alter the processing and presentation of HBV antigenic peptides. A single HLA-Aw68-restricted HBV core protein epitope has previously been shown to require the immunoproteasome subunits for efficient production (36). We have expanded these observations by showing that LMP7 may contribute to the production of the HLA-A2-restricted POL₈₀₃₋₈₁₁ and ENV₁₈₃₋₁₉₁ peptides by using an HLA-A2 transgenic mouse model. CD8 T cells specific for these two epitopes are often found in HLA-A2-positive infected individuals, and the ENV₁₈₃₋₁₉₁ peptide is recognized in the majority of HLA-A2-positive infected individuals (6, 28). Of course, further studies with human systems may be necessary to rule out species-specific differences in antigen processing and presentation in this phenomenon.

In addition to observing a reduced response to the immunodominant ENV₁₈₃₋₁₉₁ epitope in the absence of LMP7 expression, we also found an increase in the CD8 T-cell response to a second ENV epitope (ENV₃₇₀₋₃₇₉). CD8 T cells specific for this peptide were rarely observed ex vivo in mice with a functional LMP7 gene, and when they were detected, the response was generally very weak (Fig. 1, 2, 3, and data not shown). However, T cells specific for this epitope could be

FIG. 6. The antiviral effect of IFN does not require LMP2 or LMP7. Groups of age-, sex-, and serum HBeAg-matched HBV $\times$ LMP2 $^{+/-}$ and HBV $\times$ LMP2 $^{-/-}$ (A) or HBV $\times$ LMP7 $^{+/-}$ and HBV $\times$ LMP7 $^{-/-}$ mice (B) (four or five mice per group) were injected intravenously with a single dose of either 0.9% NaCl (saline), 200 μ g poly(IC) (200), 10 μ g poly(IC) (10), 2×10^5 U murine IFN- γ , or 100 μ g anti-CD40 MAb. HBV replication was examined 24 h after injection by Southern blot analysis of relaxed-circle and single-stranded DNA (ssDNA) replication forms and compared to control saline-injected mice. The relative levels of HBV ssDNA or total DNA were quantified by phosphorimager analysis. Data are expressed as HBV DNA levels normalized to the transgene relative to the control saline-injected mice of each genotype. Error bars represent standard deviations.

readily detected after in vitro expansion of cells from wild-type immunized mice, indicating that it is in fact a subdominant epitope (Fig. 1D). Thus, proteasomes that contain LMP7 may produce this peptide less efficiently than proteasomes that lack LMP7. Although immunoproteasomes can enhance the production of certain epitopes through greater cleavage efficiency after certain amino acids, they may also suppress epitope production through increased cleavage at a location within the

peptide (4). The general pattern of a reduced T-cell response to the immunodominant epitope and an enhanced response to a subdominant epitope in the absence of IFN-induced subunits has been described for other viral proteins. LMP2-deficient mice display reduced CTL responses to immunodominant influenza virus epitopes (5, 40) and enhanced responses to a subdominant epitope (5). Likewise, Schwartz et al. demonstrated that expression of the IFN-induced proteasome subunits enhances the presentation of an immunodominant LCMV epitope (35), while Basler et al. have shown that immunoproteasomes down-regulate presentation of a subdominant LCMV epitope (2). Thus, our results are consistent with the notion that immunoproteasomes can influence the specificity and hierarchy of the host T-cell response to a viral antigen.

While we found consistent effects on the HLA-A2-restricted T-cell response in LMP7-deficient mice, we did not observe any changes in the response in LMP2-deficient mice. There are a number of possible reasons for this finding. Perhaps the most likely explanation is the fact that LMP7 specifically enhances the cleavage of peptides after hydrophobic amino acid residues, while LMP2 primarily enhances the cleavage after basic residues (10, 12). As the canonical HLA-A2-binding motif includes a C-terminal hydrophobic amino acid, we would predict that production of these epitopes might be influenced more by LMP7 than by LMP2. However, Sijts et al. have also shown that LMP7 may alter the structure of the proteasome in a way that alters protein cleavage specificity beyond that attributable to the LMP7 catalytic active site alone (36). Thus, the LMP7-deficient proteasomes may have greater alterations in cleavage specificity than LMP2-deficient proteasomes.

There are a few potential caveats to the present study. As described previously, other groups have also noted changes in the CTL response to viral antigen in the absence of LMP2 or LMP7 expression. However, some others have not found such changes, notably a recent study by Nussbaum et al. that clearly showed a normal immune response to LCMV in infected LMP2- and LMP7-deficient mice (30). While the precise nature of these differences is unclear, they may reflect differences in the method of generating an immune response. Thus, we cannot rule out the possibility that our DNA-prime/vaccinia virus boost approach for inducing a CTL response is unusually sensitive to immunoproteasome expression. Furthermore, Chen et al. have shown that LMP2 can influence the CD8 T-cell response to influenza virus not only through the processing of the viral antigens but also by an altered CD8 T-cell repertoire in the LMP2-deficient mice (5).

Despite the fact that much is known about the requirement for IFN in the cytokine-mediated noncytopathic inhibition of HBV, relatively little is known about the intracellular molecular mechanism that ultimately inhibits replication. The antiviral effect of IFN- α/β and IFN- γ against HBV was recently shown to occur through a disruption in assembly of viral pre-genomic RNA-containing capsids (45). In addition, the inhibition of HBV replication by IFN- γ is dependent upon inducible nitric oxide synthase expression, while the IFN- α/β -dependent antiviral effect is not (18). Furthermore, the IFN- α/β - and IFN- γ -induced antiviral effects do not require the IFN-inducible antiviral proteins IRF1, PKR, RNase L, or Mx1 (19). To identify the cellular factors that inhibit HBV replication, Wieland et al. performed a gene expression analysis utilizing

HBV transgenic mice and an immortalized hepatocyte cell line derived from these mice (48). Among the various classes of genes that were regulated in a manner that correlated with the antiviral effect of IFN- α/β and IFN- γ , a number of proteins involved in protein degradation were induced, including the IFN-inducible proteasome catalytic subunits LMP2 and LMP7. Using this information, we previously demonstrated that proteasome activity is required for the antiviral effect of IFN against HBV in vitro using small-molecule pharmacological inhibitors (32).

The IFN-inducible LMP2 and LMP7 catalytic subunits are known to alter proteasome specificity in a way that enhances cleavage after hydrophobic or basic amino acid residues (1, 10, 12). However, these subunits generally do not change the overall rate of degradation of whole proteins (11). Furthermore, incorporation of these subunits into active proteasomes generally requires multiple days (22), and these kinetics are seemingly incompatible with those of the IFN-mediated antiviral effect against HBV, which occurs over 12 to 24 h (16). Nevertheless, we examined the influence of these subunits on the antiviral effect using HBV transgenic mice genetically deficient for LMP2 or LMP7. Numerous stimuli that induce an intrahepatic IFN- α/β or IFN- γ response efficiently blocked HBV replication even in the absence of LMP2 or LMP7. Thus, although the antiviral effect of IFN requires proteasome activity, this inhibition occurs independently of the activity of the IFN-inducible proteasome catalytic subunits.

Although perhaps unlikely for the reasons cited above, we cannot rule out the formal possibility that LMP2 or LMP7 are each sufficient to inhibit HBV replication, and thus, simultaneous deletion would be necessary to observe changes in the IFN antiviral effect. However, the genes for these subunits are encoded in close proximity to one another in the MHC locus. Because they are closely genetically linked, a double-deficient mouse cannot be created by crossing the two individual knockout strains. We have also not yet examined the possibility that other IFN-inducible proteasome subunits (PA28, MECL-1) have a function in mediating the inhibition of HBV replication, but these experiments are outside of the scope of the present study.

The immunoproteasome subunits may play an important role in the host immune response to HBV for a number of reasons. Although hepatocytes express only low levels of these subunits in the absence of IFN- γ , their expression is rapidly induced during an intrahepatic inflammatory immune response in virus- and bacterium-infected mice (22). The expression of these subunits is also seen during the elimination of the virus from the liver of acutely infected chimpanzees (43). Because the priming (and hence the specificity) of the T-cell response to HBV presumably occurs in professional antigen presenting cells which constitutively express immunoproteasomes, the IFN-induced expression of LMP2 and LMP7 in hepatocytes may facilitate recognition of infected hepatocytes by primed virus-specific CTL (15, 27, 34, 37). This hypothesis predicts that antigen processing may be differentially regulated in acute versus chronic infection, in which different levels of inflammatory cytokines (including IFN- γ) may be expressed in the liver. In fact, the T-cell response to acute HBV infection has been shown to be both vigorous and multispecific, while it is weaker and more narrowly restricted in chronically infected

patients (6, 31). A better understanding of the cellular factors that contribute to a successful immune response to HBV may provide a basis for future immunomodulation-based therapies for chronic infections.

ACKNOWLEDGMENTS

This work was supported by grant CA40489 (F.V.C.), Ruth L. Kirschstein National Research Service Award AI49665 (M.D.R.), and Research Scholar Development Award K22 AI64757 (M.D.R.) from the NIH.

We thank Yoshiaki Yanai (Hayashibara Co. Ltd.) for the gift of murine IFN- γ , Epimmune for the gift of HLA-A2/K^b transgenic mice, and Maria Gaczynska, Luc Van Kaer, and Hans Fehling for providing LMP2- and LMP7-deficient mice. Finally, we thank Stefan Wieland, Masanori Isogawa, and Luca Guidotti for helpful discussions.

REFERENCES

1. Akiyama, K., S. Kagawa, T. Tamura, N. Shimbara, M. Takashina, P. Kristensen, K. B. Hendil, K. Tanaka, and A. Ichihara. 1994. Replacement of proteasome subunits X and Y by LMP7 and LMP2 induced by interferon-gamma for acquirement of the functional diversity responsible for antigen processing. *FEBS Lett.* **343**:85–88.
2. Basler, M., N. Youhnovski, M. Van Den Broek, M. Przybylski, and M. Groettrup. 2004. Immunoproteasomes down-regulate presentation of a subdominant T cell epitope from lymphocytic choriomeningitis virus. *J. Immunol.* **173**:3925–3934.
3. Beninga, J., K. L. Rock, and A. L. Goldberg. 1998. Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J. Biol. Chem.* **273**:18734–18742.
4. Chapiro, J., S. Claverol, F. Piette, W. Ma, V. Stroobant, B. Guillaume, J. E. Gairin, S. Morel, O. Burlet-Schiltz, B. Monsarrat, T. Boon, and B. J. Van den Eynde. 2006. Destructive cleavage of antigenic peptides either by the immunoproteasome or by the standard proteasome results in differential antigen presentation. *J. Immunol.* **176**:1053–1061.
5. Chen, W., C. C. Norbury, Y. Cho, J. W. Yewdell, and J. R. Bennink. 2001. Immunoproteasomes shape immunodominance hierarchies of antiviral CD8⁺ T cells at the levels of T cell repertoire and presentations of viral antigens. *J. Exp. Med.* **193**:1319–1326.
6. Chisari, F. V., and C. Ferrari. 1995. Hepatitis B virus immunopathogenesis. *Ann. Rev. Immunol.* **13**:29–60.
7. Craiu, A., T. Akopian, A. Goldberg, and K. L. Rock. 1997. Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. *Proc. Natl. Acad. Sci. USA* **94**:10850–10855.
8. Davis, H. L., R. Schirmbeck, J. Reimann, and R. G. Whalen. 1995. DNA-mediated immunization in mice induces a potent MHC class I-restricted cytotoxic T lymphocyte response to the hepatitis B envelope protein. *Hum. Gene Ther.* **6**:1447–1456.
9. Fehling, H. J., W. Swat, C. Laplace, R. Kuhn, K. Rajewsky, U. Muller, and H. von Boehmer. 1994. MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science* **265**:1234–1237.
10. Gaczynska, M., A. L. Goldberg, K. Tanaka, K. B. Hendil, and K. L. Rock. 1996. Proteasome subunits X and Y alter peptidase activities in opposite ways to the interferon-gamma-induced subunits LMP2 and LMP7. *J. Biol. Chem.* **271**:17275–17280.
11. Gaczynska, M., K. L. Rock, and A. L. Goldberg. 1993. Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* **365**:264–267.
12. Gaczynska, M., K. L. Rock, T. Spies, and A. L. Goldberg. 1994. Peptidase activities of proteasomes are differentially regulated by the major histocompatibility complex-encoded genes for LMP2 and LMP7. *Proc. Natl. Acad. Sci. USA* **91**:9213–9217.
13. Ganem, D., and A. M. Prince. 2004. Hepatitis B virus infection—natural history and clinical consequences. *N. Engl. J. Med.* **350**:1118–1129.
14. Guidotti, L. G., and F. V. Chisari. 1999. Cytokine-induced viral purging—role in viral pathogenesis. *Curr. Opin. Microbiol.* **2**:388–391.
15. Guidotti, L. G., and F. V. Chisari. 2006. Immunobiology and pathogenesis of viral hepatitis. *Annu. Rev. Pathol. Mech. Dis.* **1**:23–61.
16. Guidotti, L. G., T. Ishikawa, M. V. Hobbs, B. Matzke, R. Schreiber, and F. V. Chisari. 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity* **4**:25–36.
17. Guidotti, L. G., B. Matzke, H. Schaller, and F. V. Chisari. 1995. High-level hepatitis B virus replication in transgenic mice. *J. Virol.* **69**:6158–6169.
18. Guidotti, L. G., H. McClary, J. M. Loudis, and F. V. Chisari. 2000. Nitric oxide inhibits hepatitis B virus replication in the livers of transgenic mice. *J. Exp. Med.* **191**:1247–1252.
19. Guidotti, L. G., A. Morris, H. Mendez, R. Koch, R. H. Silverman, B. R. Williams, and F. V. Chisari. 2002. Interferon-regulated pathways that control hepatitis B virus replication in transgenic mice. *J. Virol.* **76**:2617–2621.

20. Guidotti, L. G., R. Rochford, J. Chung, M. Shapiro, R. Purcell, and F. V. Chisari. 1999. Viral clearance without destruction of infected cells during acute HBV infection. *Science* **284**:825–829.
21. Kakimi, K., M. Isogawa, J. Chung, A. Sette, and F. V. Chisari. 2002. Immunogenicity and tolerogenicity of hepatitis B virus structural and nonstructural proteins: implications for immunotherapy of persistent viral infections. *J. Virol.* **76**:8609–8620.
22. Khan, S., M. van den Broek, K. Schwarz, R. de Giuli, P. A. Diener, and M. Groettrup. 2001. Immunoproteasomes largely replace constitutive proteasomes during an antiviral and antibacterial immune response in the liver. *J. Immunol.* **167**:6859–6868.
23. Kimura, K., K. Kakimi, S. Wieland, L. G. Guidotti, and F. V. Chisari. 2002. Activated intrahepatic antigen-presenting cells inhibit hepatitis B virus replication in the liver of transgenic mice. *J. Immunol.* **169**:5188–5195.
24. Loirat, D., F. A. Lemonnier, and M. L. Michel. 2000. Multiepitopic HLA-A*0201-restricted immune response against hepatitis B surface antigen after DNA-based immunization. *J. Immunol.* **165**:4748–4755.
25. McClary, H., R. Koch, F. V. Chisari, and L. G. Guidotti. 2000. Relative sensitivity of hepatitis B virus and other hepatotropic viruses to the antiviral effects of cytokines. *J. Virol.* **74**:2255–2264.
26. Mo, X. Y., P. Cascio, K. Lemerise, A. L. Goldberg, and K. Rock. 1999. Distinct proteolytic processes generate the C and N termini of MHC class I-binding peptides. *J. Immunol.* **163**:5851–5859.
27. Morel, S., F. Levy, O. Burlet-Schiltz, F. Brasseur, M. Probst-Kepper, A. L. Peitrequin, B. Monsarrat, R. Van Velthoven, J. C. Cerottini, T. Boon, J. E. Gairin, and B. J. Van den Eynde. 2000. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. *Immunity* **12**:107–117.
28. Nayersina, R., P. Fowler, S. Guilhot, G. Missale, A. Cerny, H. J. Schlicht, A. Vitiello, R. Chesnut, J. L. Person, A. G. Redeker, et al. 1993. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. *J. Immunol.* **150**:4659–4671.
29. Niedermann, G., S. Butz, H. G. Ihlenfeldt, R. Grimm, M. Lucchiari, H. Hoschutzky, G. Jung, B. Maier, and K. Eichmann. 1995. Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules. *Immunity* **2**:289–299.
30. Nussbaum, A. K., M. P. Rodriguez-Carreno, N. Benning, J. Botten, and J. L. Whitton. 2005. Immunoproteasome-deficient mice mount largely normal CD8+ T-cell responses to lymphocytic choriomeningitis virus infection and DNA vaccination. *J. Immunol.* **175**:1153–1160.
31. Rehmann, B., P. Fowler, J. Sidney, J. Person, A. Redeker, M. Brown, B. Moss, A. Sette, and F. V. Chisari. 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. *J. Exp. Med.* **181**:1047–1058.
32. Robek, M. D., S. F. Wieland, and F. V. Chisari. 2002. Inhibition of hepatitis B virus replication by interferon requires proteasome activity. *J. Virol.* **76**:3570–3574.
33. Rock, K. L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A. L. Goldberg. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**:761–771.
34. Sallusto, F., and A. Lanzavecchia. 1999. Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. *J. Exp. Med.* **189**:611–614.
35. Schwarz, K., M. van Den Broek, S. Kostka, R. Kraft, A. Soza, G. Schmidtke, P. M. Kloetzel, and M. Groettrup. 2000. Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28 alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. *J. Immunol.* **165**:768–778.
36. Sijts, A. J., T. Ruppert, B. Rehmann, M. Schmidt, U. Koszinowski, and P. M. Kloetzel. 2000. Efficient generation of a hepatitis B virus cytotoxic T lymphocyte epitope requires the structural features of immunoproteasomes. *J. Exp. Med.* **191**:503–514.
37. Steinman, R. M., K. Inaba, S. Turley, P. Pierre, and I. Mellman. 1999. Antigen capture, processing, and presentation by dendritic cells: recent cell biological studies. *Hum. Immunol.* **60**:562–567.
38. Stoltze, L., M. Schirle, G. Schwarz, C. Schroter, M. W. Thompson, L. B. Hersch, H. Kalbacher, S. Stevanovic, H. G. Rammensee, and H. Schild. 2000. Two new proteases in the MHC class I processing pathway. *Nat. Immunol.* **1**:413–418.
39. Van Den Eynde, B. J., and S. Morel. 2001. Differential processing of class-I-restricted epitopes by the standard proteasome and immunoproteasome. *Curr. Opin. Immunol.* **13**:147–153.
40. Van Kaer, L., P. G. Ashton-Rickardt, M. Eichelberger, M. Gaczynska, K. Nagashima, K. L. Rock, A. L. Goldberg, P. C. Doherty, and S. Tonegawa. 1994. Altered peptidase and viral-specific T-cell response in LMP2 mutant mice. *Immunity* **1**:533–541.
41. Vitiello, A., D. Marchesini, J. Furze, L. A. Sherman, and R. W. Chesnut. 1991. Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human-mouse class I major histocompatibility complex. *J. Exp. Med.* **173**:1007–1015.
42. Vitiello, A., A. Sette, L. Yuan, P. Farness, S. Southwood, J. Sidney, R. W. Chesnut, H. M. Grey, and B. Livingston. 1997. Comparison of cytotoxic T lymphocyte responses induced by peptide or DNA immunization: implications on immunogenicity and immunodominance. *Eur. J. Immunol.* **27**:671–678.
43. Wieland, S., R. Thimme, R. H. Purcell, and F. V. Chisari. 2004. Genomic analysis of the host response to hepatitis B virus infection. *Proc. Natl. Acad. Sci. USA* **101**:6669–6674.
44. Wieland, S. F., and F. V. Chisari. 2005. Stealth and cunning: hepatitis B and hepatitis C viruses. *J. Virol.* **79**:9369–9380.
45. Wieland, S. F., A. Eustaquio, C. Whitten-Bauer, B. Boyd, and F. V. Chisari. 2005. Interferon prevents formation of replication-competent hepatitis B virus RNA-containing nucleocapsids. *Proc. Natl. Acad. Sci. USA* **102**:9913–9917.
46. Wieland, S. F., L. G. Guidotti, and F. V. Chisari. 2000. Intrahepatic induction of alpha/beta interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. *J. Virol.* **74**:4165–4173.
47. Wieland, S. F., H. C. Spangenberg, R. Thimme, R. H. Purcell, and F. V. Chisari. 2004. Expansion and contraction of the hepatitis B virus transcriptional template in infected chimpanzees. *Proc. Natl. Acad. Sci. USA* **101**:2129–2134.
48. Wieland, S. F., R. G. Vega, R. Muller, C. F. Evans, B. Hilbush, L. G. Guidotti, J. G. Sutcliffe, P. G. Schultz, and F. V. Chisari. 2003. Searching for interferon-induced genes that inhibit hepatitis B virus replication in transgenic mouse hepatocytes. *J. Virol.* **77**:1227–1236.