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The Immunodominant, L\(^d\)-Restricted T Cell Response to Hepatitis B Surface Antigen (HBsAg) Efficiently Suppresses T Cell Priming to Multiple D\(^d\)-, K\(^d\)-, and K\(^b\)-Restricted HBsAg Epitopes

Reinhold Schirmbeck, Detlef Stober, Shereen El Kholy, Petra Riedl, and Jörg Reiman

MHC-I-restricted CTL responses of H-2\(^d\) (L\(^d\)+ or L\(^d\)-) and F\(_1\) H-2\(^d\)-k\(^b\) mice to hepatitis B surface Ag (HBsAg) are primed by either DNA vaccines or HBsAg particles. The D\(^d\)/S\(_{201-209}\) and K\(^b\)/S\(_{199-208}\) epitopes are generated by processing endogenous HBsAg; the K\(^b\)/S\(_{208-215}\) epitope is generated by processing exogenous HBsAg; and the L\(^d\)/S\(_{28-39}\) epitope is generated by exogenous as well as endogenous processing of HBsAg. DNA vaccination primed high numbers of CTL specific for the L\(^d\)/S\(_{28-39}\) HBsAg epitope, low numbers of CTL specific for the D\(^d\)/S\(_{201-209}\) or K\(^b\)/S\(_{199-208}\) HBsAg epitopes in BALB/c mice, and high numbers of D\(^d\)/S\(_{201-209}\) and K\(^b\)/S\(_{199-208}\)-specific CTL in congenic H-2\(^d\)/L\(^d\)\(_x\) dm2 mice. In F\(_1\) dm2 mice, the K\(^b\)-, D\(^d\)-, and K\(^b\)-restricted CTL responses to HBsAg were strikingly suppressed in the presence but efficiently elicited in the absence of L\(^d\)/S\(_{28-39}\)-specific CTL. Once primed, the K\(^d\)- and D\(^d\)-restricted CTL responses to HBsAg were resistant to suppression by immunodominant L\(^d\)/S\(_{28-39}\)-specific CTL. The L\(^d\)-restricted immunodominant CTL reactivity to HBsAg can thus suppress priming to multiple alternative epitopes of HBsAg, independent of the processing pathway that generates the epitope, of the background of the mouse strain used, and of the presence/absence of different allelic variants of the K and D MHC class I molecules.


Efficient priming of multispecific CTL responses is limited by the poor immunogenicity of subdominant MHC class I-binding epitopes. Although immunodominance is a central feature of multispecific CTL responses, many cellular and molecular mechanisms regulating the establishment of immunodominance are unknown. Presentation of epitopes to CTL precursors is controlled at different levels, including processing efficiency, avidity to bind MHC class I molecules, and stability of the MHC class I/peptide complex on the cell surface (reviewed in Refs. 1 and 2). In addition, suppression by CTL specific for immunodominant determinants can limit the immunogenicity of subdominant determinants.

Different MHC class I-binding epitopes of the hepatitis B surface Ag (HBsAg) are recognized by CTL from H-2\(^d\) and H-2\(^b\) mice (Table I). These include the following peptides: S\(_{28-39}\) binding to L\(^d\) that stimulates multispecific and polyclonal CTL responses (3); S\(_{201-209}\)-binding to D\(^d\) and S\(_{199-208}\)-binding to K\(^d\) (4); S\(_{208-215}\)-binding to K\(^b\) (5); and (not yet mapped) epitopes in the C-terminal region of HBsAg that bind to K\(^b\) (5). Different processing pathways generate these peptides. The K\(^b\)-binding S\(_{208-215}\) epitope is generated by processing exogenous HBsAg (i.e., in cells pulsed with HBsAg particles) (5). The D\(^d\)-binding S\(_{201-209}\) and K\(^b\)-binding S\(_{199-208}\) and the (not yet mapped) C-terminal K\(^b\)-binding epitopes are generated only by endogenous processing of HBsAg (i.e., in transfectants) (Ref. 5 and data shown in this paper), whereas the L\(^d\)-binding S\(_{28-39}\) epitope is generated by both exogenous and endogenous processing of HBsAg (5). In H-2\(^d\) mice, the L\(^d\)-restricted CTL response to HBsAg is immunodominant (3, 4). Thus, the 226-residue envelope protein of hepatitis B virus (HBV) contains multiple antigenic epitopes recognized by class I-restricted CTL that are generated in different processing pathways.

To characterize immunodominance in multispecific CTL responses, three key questions have to be considered. 1) What is the type of immunization used to prime multispecific CTL responses (or which processing pathway is accessed by the Ag delivered by the vaccine)? 2) Which technique is used to detect primed CTL in vitro? 3) Is there a difference in the biological role that dominant and subdominant CTL reactivities play in vivo?

Different vaccination strategies can be used to prime multispecific CTL responses. DNA-based vaccination is a potent technique to prime CTL responses. This novel immunization technique seems to preferentially prime CTL responses to epitopes generated in TAP- and proteasome-dependent pathways, although cross-priming is also involved in stimulating CTL immunity by this technique. In contrast, injection of low doses of HBsAg proteoliposomes without adjuvants into mice primes CTL responses to some but not all antigenic epitopes. In particular, responses to peptides generated in alternative (TAP- and proteasome-independent) pathways can be efficiently primed by HBsAg particle injections.

Functional CTL responses can be detected ex vivo either in ELISPOT- or FACS-based readout systems after specific in vitro restimulation for a few hours or in cytolytic assays after specific
clonal expansion for a few days. It is unknown which system yields a more informative, quantitative estimate of the functional CTL reactivity prevalent in vivo. A critical factor is the type of specific in vitro restimulation used to detect primed CTL. The restimulation protocols available to prepare stimulator cells include the pulse of cells with synthetic peptides, the pulse of cells with particulate Ag, or the coculture of CTL with transfected, Ag-expressing cells. While the former protocol is expected to be processing-independent, the latter protocols depend on exogenous or endogenous processing of the Ag. The latter readouts may give a more realistic picture of immunodominance hierarchies established in vivo but require some information on the processing pathway that generates the epitope.

A further question is whether dominant and subdominant CTL populations mediate in vivo different biological effector functions, or similar functions with different efficacy. It has proved difficult to correlate the strength of a CTL response apparent in in vitro readouts with its biological role in vivo.

We studied the induction of CTL responses to HBsAg in H-2d and F1 mice using different vaccination approaches, combined with different in vitro restimulation and readout protocols. We report that the immunodominant L1-restricted CTL response to HBsAg suppresses concomitant CTL responses to all other HBsAg epitopes tested, irrespective of the processing pathways involved in generating these subdominant epitopes, of BALB/c and/or C57BL/6 background genes, and of the presence of different H-2D and H-2K molecules that present the epitopes. Despite this efficient suppression, primed subdominant CTL responses are functional in vivo in a specific tumor rejection assay.

Materials and Methods

Mice

C57BL/6Bom (B6) mice (H-2b), DBA/2 mice (H-25 Ld), BALB/cBom mice (H-2d), BALB/c (dm2) mice (H-2d Ld), BALB/c × C57BL/6F1 mice, and (dm2 × C57BL/6)F1 mice were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University. C57BL/6)F1 mice were bred and kept under similar functions with different efficacy. It has proved difficult to correlate the strength of a CTL response apparent in in vitro readouts with its biological role in vivo.

Cells

The H-2d mastocytoma cell line P815 (TIB64) was obtained from the American Tissue Culture Collection (Manassas, VA). The H-2b (B6-derived) T lymphoma cell line RBL5 was obtained from Dr. H.-U. Wetzljen (University of Freiburg, Freiburg, Germany). The chicken hepatoma cell line LMH was obtained from Dr. H.-J. Schlcht (University of Ulm, Ulm, Germany).

Plasmids

Construction of the SV40 large tumor Ag (T-Ag), cyttoplasmic (c)T-Ag, and HBV S-encoding pCI vectors has been described previously (6–8).

pCI-T-FSI. The HBV sequence, encoding the HBV-S amino acid sequence from 1 to 100, was cloned in frame to the heat shock protein (hsp73)-binding cT-Ag272 fragment (9). The CCACAAGCTTATGGAGAAAGCGGCCGCTTAAACAAATGGCACTAGTAAACT (+) primers were used for PCR-based DNA amplification, digested with HindIII and NotI, and cloned into the HindIII/NotI site of the pBluescript vector, yielding the vector pBlue-SII. pBlue-SII was ligated with the c-TAg272-containing HindIII fragment to generate the pCI-cT-SII expression vector.

Expression of cT- fusion constructs by transfected cells

LMH cells were transiently transfected with pCI-based expression plasmid DNA using the CaPO4 method. Cells were metabolically labeled for 12–15 h with [35S]methionine 36 h after transfection and extracted with lysis buffer (120 mM NaCl, 1% aprotinin (Trasylol; Bayer, Leverkusen, Germany), leupeptin, 0.5% Nonidet P-40, and 50 mM Tris-hydrochloride (pH 8)) for 30 min at 4°C. Extracts were cleared by centrifugation and immunoprecipitated for T-Ag using the mAb PAB108 (directed against the extreme N terminus of the T-Ag) and protein A-Sepharose. Immunocomplexes bound to protein A-Sepharose were purified with wash buffer (300 mM LiCl, 1% Nonidet P-40, 100 mM Tris-hydrochloride (pH 8.5), followed by two washes in PBS and 0.1% PBS. Immunocomplexes were recovered from protein A-Sepharose with elution buffer (1.5% SDS, 5% 2-ME, and 7 M Tris-hydrochloride (pH 6.8)), processed for SDS-PAGE, and analyzed by fluorography.

Tumor cell transplantation

P815 tumor cells were grown in serum-free medium (BioWhittaker, Ver- viers, Belgium) and washed in PBS, and 100 μl of the cell suspension (105) was injected s.c. into the shaved right flank. Experimental groups consisted of five mice. Tumor development was followed by serial measurements of tumor size at two perpendicular diameters.

Determination of splenic CTL frequencies

Spleen cells (1 × 106/ml) were incubated for 1 h in RPMI 1640 medium with 5 μg/ml HBsAg-derived peptides, syngeneic HBsAg-expressing target cells (105/ml), or HBsAg-pulsed cells. Thereafter, 5 μg/ml brefeldin A (catalog no. 15870; Sigma-Aldrich, St. Louis, MO) was added, and the cultures were incubated for another 4 h. Cells were harvested and surface stained with PE-conjugated anti-CD8 mAb (catalog no. 01045B; BD Pharmingen, San Diego, CA). Surface-stained complexes were fixed with 2% paraformaldehyde in PBS before intracellular staining for IFN-γ. Fixed cells were resuspended in permeabilization buffer (HBSS, 0.5% BSA, 0.5% saponin, 0.05% sodium azide) and incubated with FITC-conjugated anti-IFN-γ mAb (catalog no. 55441; BD Pharmingen) for 30 min at room temperature and washed twice in permeabilization buffer. Stained cells were resuspended in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. We deter- mined the frequencies of CD8+ IFN-γ+ CTL by flow cytometry (FCM) analy- ses. The double-positive CD8+ IFN-γ+ T cells per 106 CD8+ spleen cells are determined.

CTL assays

Single cell suspensions were prepared from spleens of mice in α-MEM tissue culture medium supplemented with 10 mM HEPES buffer, 5 × 10−5 M 2-ME, antibiotics, and 10% v/v FCS (PAA Laboratories, Linz, Austria). A selected batch of Con A-stimulated rat spleen cell supernatant (2% v/v) was added to the culture medium. A total of 3 × 105 responder cells were cocultured with 1 × 106 irradiated, syngeneic transfectants or HBsAg particle-pulsed cells. Coculture was performed in 10 ml medium in upright 25-cm2 tissue culture flasks in a humidified atmosphere/5% CO2 at 37°C. After 5 days of culture, cells were harvested, washed, and assayed for specific cytolytic reactivity. Serial dilutions of effector cells were cultured with 2 × 105 51Cr-labeled targets in 200-μl round-bottom wells. Specific cytolytic activity of cells was tested in short-term 51Cr release assays against transfected, HBsAg-, or peptide-pulsed targets or nontreated targets. After a 4-h incubation at 37°C, 50 μl of supernatant was collected for gamma radiation counting. The percentage of specific release was calculated as [(experimental release − spontaneous release)/total release − spontaneous release] × 100. Total counts were measured by resuspending target cells. Spontaneously released counts were always <15% of the total count.
Recombinant HBsAg particles and antigenic HBsAg peptides

HBsAg particles were obtained from Dr. K. Melber (Rhein Biotech, Düsseldorf, Germany). HBsAg was produced in the H. polymorpha host strain RB10 (10). HBsAg particles were purified from crude yeast extracts by adsorption to silica gel, column chromatography, and isopionic ultracentrifugation. Purified HBsAg particle preparations were characterized by SDS-PAGE and electron microscopy (11). The synthetic L4-binding S28–39, IQPSLDGWWTSL peptide, D4-binding S201–209, WGPSLYSIL peptide, Kd-binding S199–208, WYGPSLYSI peptide, and the Kd-binding S208–215, ILSPFPL peptide were obtained from Jerini BioTools (Berlin, Germany). Peptides were dissolved in a DMSO solution at a concentration of 10 mg/ml and diluted with culture medium before use.

Results

Construction of plasmids for the expression of HBsAg fragments

CTL from H-2d or H-2b mice specifically recognize antigenic peptides of the small surface (S, HBsAg) protein of HBV that bind to murine Ld, Dd, Kd, or Kk MHC class I molecules (Table I and Fig. 1A). For selective induction of CTL responses to these epitopes, we subcloned sequences of the 226-residue HBsAg gene in frame behind the hsp73-binding mutant SV40 T-Ag N terminus (Fig. 1B). This system allows efficient expression of protein fragments of 20–800 aa (9). We chose three overlapping HBsAg fragments: the N-terminal 100-residue fragment SI (aa 1–100), containing the L4-binding S28–39 epitope (3, 12); the central 100-residue fragment SII (aa 80–180), containing none of the known CTL epitopes but the well-known Ab-binding “a” determinant; and the C-terminal 86-residue fragment SIII (aa 140–226), including the D4-binding S201–209, Kd-binding S199–208, and Kd-binding S208–215 epitopes (4, 5). Control constructs included the pCI/S plasmid containing the complete HBsAg-S (encoding sequence (13) and the pCI/cT-preS plasmid containing the HBV preS-encoding sequence of large surface (LS) Ag cloned behind the N-terminal cT fragment (9). Transient transfection of LMH cells with the pCI/cT-SI, pCI/cT-SII, pCI/cT-SIII, and subsequent immunoprecipitation analyses revealed the efficient expression of fusion proteins (Fig. 1C). The transfected cells expressed the cT-SI, cT-SII, or cT-SIII fusion proteins that coprecipitated the constitutively expressed, cytotoxic stress protein hsp73, indicating tight, noncovalent association between the two proteins (Fig. 1C), confirming our previous data (8, 9, 14). We have thus constructed a panel of DNA vaccines in which HBsAg fragments containing different antigenic epitopes could be tested for immunogenicity.

L4-, Kd-, and D4-restricted, HBsAg-specific CTL responses primed by DNA vaccination in Ld- and Ld- H-2d mice

CTL responses specific for the L4-, Kd-, and D4-restricted epitopes of HBsAg were primed in BALB/c mice by a single injection of pCI/S plasmid DNA (Fig. 2A, group 1), confirming and extending published data (4, 15). High frequencies were detected for CTL specifically recognizing transfected P815/S cells, but lower frequencies were found for CTL specifically recognizing P815 cells pulsed with recombinant HBsAg. High frequencies were detected for CTL specifically recognizing the L4-, and D4-binding HBsAg epitopes. A similar distribution of CTL frequencies was found in four independent experiments using BALB/c or DBA/2 (H-2b) mice in which the response was read out between days 10 and 20 postvaccination.

When mice were immunized with DNA vaccines expressing the HBsAg fragments, CTL specific for the L4-restricted S28–39 epitope were primed only by pCI/cT-SI (group 2), no CTL responses were elicited by vaccination with pCI/cT-SII (group 3), Kd- and D4-restricted CTL responses were primed by injecting pCI/cT-SIII plasmid DNA (group 4), and no CTL reactivity was detected in mice injected with (negative control) pCI/cT-preS plasmid DNA (group 5). These data confirmed the mapping of the CTL epitopes in H-2d mice. They indicate the usefulness of the expression system as a DNA vaccine for eliciting selective T cell responses against fragments of the Ag. The data revealed priming of unexpectedly strong CTL responses against the subdominant Kd- and D4-binding HBsAg peptides by in vivo processed Ag.

In parallel series of experiments, we immunized Ld- mutant BALB/c(H-2d) (dm2) mice with the five different DNA vaccines described. These animals generated no L4-restricted (Fig. 2B, groups 1 and 2) but potent Kd- and D4-restricted (Fig. 2B, groups 1 and 4) CTL responses to HBsAg. Unexpectedly, the frequencies of HBsAg-specific, Kd- and D4-restricted CTL stimulated in dm2 mice by the pCI/S and pCI/cT-SII plasmid DNA vaccines were reproducibly 5- to 15-fold higher than those stimulated in BALB/c mice (Fig. 2, compare A and B). When this CTL response was read out against the P815/S target, the measured frequency estimates were lower (Fig. 2B, groups 1 and 4). Similar data were observed when primed CTL were restimulated with other syngeneic HBsAg transfectants (data not shown).

Table I. Murine CTL-defined epitopes of HBsAg

<table>
<thead>
<tr>
<th>Group</th>
<th>Residuesa</th>
<th>Sequenceb</th>
<th>Restrictionc</th>
<th>Processingd</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28–39</td>
<td>IQPSLDGWWTSL</td>
<td>Ld</td>
<td>Exo/endo</td>
<td>3. 5</td>
</tr>
<tr>
<td>2</td>
<td>201–209</td>
<td>WGPSLYSIL</td>
<td>D4</td>
<td>Endo</td>
<td>4’</td>
</tr>
<tr>
<td>3</td>
<td>199–208</td>
<td>WYGPSLYSI</td>
<td>Kd</td>
<td>Endo</td>
<td>4’</td>
</tr>
<tr>
<td>4</td>
<td>208–215</td>
<td>ILSPFPL</td>
<td>Kd</td>
<td>Exo</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>140–226</td>
<td>Unknownf</td>
<td>Kd/Dd</td>
<td>Endo</td>
<td>5</td>
</tr>
</tbody>
</table>

a Amino acid residue of the HBsAg subtype ayw.
b Amino acid sequence.
c Mouse H-2 class I molecule binding the epitope.
d Processing pathway that can generate the antigenic peptide. Exo, Exogenous; endo, endogenous.
e Data in this paper.
f Induced in B6 mice by pCI/cT-SIII but not pCI/cT-SII or pCI/cT-SI.

Endogenous but not exogenous processing generates antigenic Kd- and D4-binding HBsAg peptides

Ld-, Kd-, and D4-binding peptides from HBsAg presented to CTL are generated in different processing pathways. Confirming our previously reported findings (5), transfected and HBsAg-pulsed P815 cells presented the L4-restricted epitope to CTL (Fig. 2A, groups 1 and 2). The L4-binding peptide is thus generated by processing endogenous and exogenous HBsAg, as described previously (reviewed in Ref. 16). In contrast, only transfected but not HBsAg-pulsed cells efficiently presented the Kd- and D4-restricted epitopes to CTL (Fig. 2, groups 1 and 4), indicating that processing...
endogenous but not exogenous HBsAg generates the K<sub>d</sub>- and D<sub>d</sub>-binding peptides. These data were generated by restimulating in vivo primed CTL (derived from BALB/c and dm2 mice) for 5–6 h in vitro with the respective peptides or APC. We asked whether similar processing requirements operate in priming CTL responses to HBsAg delivered as either an endogenous Ag (DNA vaccine) or an exogenous Ag (recombinant HBsAg proteoliposomes). These data indicate that DNA vaccination efficiently primes all CTL in BALB/c and dm2 mice that specifically recognize epitopes from endogenously processed HBsAg, while immunizations with HBsAg particles primed CTL responses to the L<sub>d</sub>-restricted HBsAg epitope (generated by exogenous processing of HBsAg) but not the K<sub>d</sub>- and D<sub>d</sub>-restricted epitopes (generated only by endogenous processing of HBsAg) (Figs. 2 and 3). Neither the codelivery of the potent CTL-stimulating (CpG-containing) adjuvant oligodeoxynucleotide (ODN) (17) nor repeated boost injections allowed priming of K<sub>d</sub>- or D<sub>d</sub>-restricted CTL to HBsAg by particle injection (Fig. 3, group 3, and data not shown). These data support the notion that endogenous processing of HBsAg is required to generate the K<sub>d</sub>- and D<sub>d</sub>-binding peptides while the L<sub>d</sub>-binding peptide of HBsAg can be generated by different processing pathways. They further indicate that the two vaccination approaches used selectively prime in vivo CTL subsets recognizing HBsAg epitopes generated by either exogenous or endogenous processing.

**K<sub>d</sub>- and D<sub>d</sub>-restricted HBsAg-specific CTL are difficult to detect in vivo**

L<sub>d</sub>-restricted HBsAg-specific CTL were detected in the spleen of pCI/S-vaccinated BALB/c or DBA/2 mice specifically restimulated for a few hours ex vivo (Figs. 2A and 3A, group 1). The K<sub>d</sub>- and D<sub>d</sub>-restricted CTL reactivities were difficult to detect in cytolytic assays after splenic T cells primed in vivo were cocultured in vitro for 5 days with HBsAg-expressing P815/S transfectants. A representative example is shown in Fig. 4A. In BALB/c mice immunized with different vaccines, L<sub>d</sub>-restricted, HBsAg-specific CTL were readily revealed in standard cytolytic assays using stimulator/target cells processing exogenous or endogenous HBsAg. In contrast, selective K<sub>d</sub>- and D<sub>d</sub>-restricted CTL from pCI/cT-SIII vaccinated BALB/c or dm2 mice showed only low specific lysis of HBsAg-expressing, syngeneic transfectants (Fig. 4A). The inefficient presentation of the D<sub>d</sub>- and K<sub>d</sub>-binding HBsAg epitopes by transfectants or a limited potential to expand in vivo may explain

**FIGURE 1.** Construction of HBV S fragments using a novel hsp73-binding, SV40 T-Ag-derived expression system. A, Schematic representation of antigenic regions of the HBV S Ag. The defined CTL and Ab epitope(s) are indicated. B, Construction of hsp73-binding cT<sub>272</sub> fusion proteins. Fusion fragments consist of the N-terminal hsp73-binding unit (cT<sub>272</sub>) and the in frame fusion sequences S<sub>1-100</sub> (cT-SI), S<sub>100-180</sub> (cT-SII), and S<sub>140-226</sub> (cT-SIII). C, Expression of HBsAg or chimeric, hsp73-bound fusion Ags. The HBsAg-encoding sequence and the respective constructs were cloned into the pCI expression vector and tested in transient transfection assays. Transiently transfected LMH cells were labeled with [<sup>35</sup>S]methionine, extracted, immunoprecipitated with anti-T-Ag mAb PAB108, and processed for SDS-PAGE, followed by fluorography of the gels. The positions of hsp73 and the respective cT-S fusion Ags are indicated. LMH cells were also transiently transfected with pCI/S DNA, and HBsAg immunoprecipitated by anti-HBsAg rabbit antiserum was analyzed by SDS-PAGE.
During the repeated in vitro restimulation of pCI/S-primed, multi-

FIGURE 2. Priming HBsAg-specific CTL responses. BALB/c mice and dm2 (Ld-) mice were vaccinated i.m. with 100 µg/mouse plasmid DNA of the vectors: pCI/S (group 1), pCI/cT-SI (group 2), pCI/cT-SII (group 3), pCI/cT-SIII (group 4), or the unrelated (control) HBV preS-encoding DNA pCI/cT-preS (group 5). Spleen cells obtained 11 days postvaccination were restimulated for 5 h with HBsAg-derived peptides S_{28-39}, S_{215-225}, S_{201-209}, or S_{199-208}-HBsAg-expressing P815/S cells, or HBsAg-pulsed P815 cells (P815 + HBsAg). T cells were surface-stained for CD8 and intracellularly stained for IFN-γ. We determined the frequencies of CD8^+ IFN-γ^+ T cells per 10^5 CD8^+ spleen cells by FCM analyses. Restimulation with nontreated/nontransfected P815 cells were used to determine nonspecific background frequencies. The mean number of IFN-γ^+CD8^+ T cells/10^5 CD8^+ spleen cells ± SD of four individual mice are shown.

these findings. As splenic T cells from pCI/cT-SIII-primed BALB/c mice induced strikingly lower specific responses of HBsAg-specific memory CTL after brief restimulation with P815/S transfectants as compared with restimulation with S_{201-209} or S_{199-208} peptide-pulsed P815/S cells, there is evidence that transfectants display a presentation defect. Despite this relative presentation defect of D^d- and K^d-binding HBsAg epitopes processed from endogenous HBsAg, CTL precursors were efficiently primed in vivo by pCI/S or pCI/cT-SIII DNA vaccines.

The K^d- and D^d-restricted CTL populations were rapidly lost during the repeated in vitro restimulation of pCI/S-primed, multispecific CTL populations by P815/S transfectants, whereas HBsAg-specific, L^d/S_{215-225}-restricted CTL populations were greatly expanded (Fig. 4B). We have described a similar in vitro selection of immunodominant CTL reactivities at the expense of subdominant CTL reactivities in the multispecific response of B6 mice to the SV40 T-Ag (18). These data show that the magnitude of K^d- and D^d-restricted, HBsAg-specific CTL reactivity detected in vitro in a conventional cytotoxic assay does not correlate well with the CTL frequencies measured directly ex vivo. To validate the efficient priming of subdominant CTL populations in vivo, we asked whether these CTL subsets could mediate effector functions in vivo.

Rejection of (nontransfected or transfected) P815 tumors growing s.c. in syngeneic DBA/2 hosts is CD8^+ CTL dependent (7). Mice primed with the pCI/cT-SIII DNA vaccine rejected s.c. growing P815/S tumors after some transient growth but did not control s.c. growth of nontransfected P815 tumors (Fig. 5). Similarly, mice immunized with the pCI/S or the pCI/cT-SI vaccine efficiently suppressed growth of a lethal P815/S (but not a P815) tumor cell challenge. Control experiments demonstrated the specificity of the rejection response (Fig. 5) (Ref. 7 and data not shown). These data indicate that, despite inefficient presentation of the K^d/D^d- epitopes by HBsAg-expressing P815/S cells, K^d- and D^d-restricted CTL reactivities to HBsAg were sufficient to reject P815/S in vivo.

The immunodominant L^d-restricted CTL response to HBsAg inhibits generation of additional CTL responses to HBsAg restricted by unrelated MHC class I molecules

The data in Fig. 2 indicate that L^d- dm2 mice generate higher K^d- and D^d-restricted CTL responses to HBsAg than congenic L^d- H-2^d strain mice. This is also evident from the data shown in Fig. 6, groups 2-5. This suggests that the immunodominant, polyclonal, and multispecific L^d-restricted CTL response to HBsAg (3) down-regulates or partially suppresses the development of concomitant CTL responses to other HBsAg epitopes restricted by other MHC class I molecules. This was confirmed in studies of K^b-restricted CTL responses (generated by exogenous or endogenous HBsAg processing) in L^d^- F_{1,ab} (BALB/c X B6) and L^d^- F_{1,ab} (dm2 X B6) mice. B6 mice immunized with the pCI/S DNA vaccine efficiently induced HBsAg-specific, K^b-restricted CTL responses to the S_{201-209} epitope and against peptides that are presented by HBsAg-expressing transfectants, the fine specificity of which is not yet mapped (5). S_{201-209}-specific CTL were detected only after restimulation with HBsAg particle-pulsed cells but do not (cross-)react to HBsAg-expressing transfectants (5). In H-2^b
mice, the generated HBsAg-specific CTL thus recognize epitopes that are generated by processing either exogenous or endogenous HBsAg (5). In pCI/S-vaccinated B6 and Ld−/F1 (B6 × dm2) mice we found similar frequencies of IFN-γ-expressing CD8+ CTL recognizing either the Kb-restricted S208–215 epitope or HBsAg-expressing transfectants (Fig. 6, groups 1 and 2). In contrast, only low numbers of Kb-restricted, S208–215-specific CTL were induced in pCI/S-vaccinated Ld−/F1 (B6 × BALB/c) mice (Fig. 6, group 4). The immunodominant Ld-restricted, S28–39-specific CTL response thus inhibits the development of concomitant Dd-, Kd-, and

**FIGURE 3.** HBsAg-specific Dd and Kd epitopes are generated in the endogenous processing pathway. BALB/c mice and dm2 (Ld−) mice were vaccinated i.m. with 100 μg/mouse HBsAg-encoding pCI/S plasmid (group 1), 5 μg/mouse HBsAg particles in PBS (group 2), 5 μg/mouse HBsAg particles mixed with 30 μg CpG-containing ODN-1826 in PBS (group 3), or 100 μg/mouse noncoding plasmid DNA (group 4). Spleen cells obtained 14 days postvaccination were restimulated with HBsAg-derived peptides S28–39, S201–209, or S199–208, HBsAg-expressing P815/S transfectants, or P815 cells pulsed with HBsAg particles (P815 + HBsAg). The mean numbers of restimulated IFN-γ+ CD8+ T cells/10^5 spleen cells ± SD of four individual mice are shown.

**FIGURE 4.** Detection of HBsAg-specific CTL responses in cytotoxic assays. A, BALB/c mice and dm2 (Ld−) mice were vaccinated i.m. with 100 μg/mouse plasmid DNA of the vectors pCI/S, pCI/cT-SI, pCI/cT-SIII, or (noncoding) pCI, or with 5 μg/mouse HBsAg particles mixed with 30 μg CpG-containing ODN-1826 in PBS (HBsAg/CpG-ODN). Spleen cells obtained from immune mice 2–4 wk postvaccination were specifically restimulated in vitro for 5 days with either HBsAg-expressing P815 transfectants (endogenous Ag processing) or HBsAg particle-pulsed P815 cells (exogenous Ag processing) and tested for specific cytotoxicity in a 4-h 3Cr release assay. HBsAg-expressing P815/S targets were used to detect endogenous CTL (left panel); HBsAg-pulsed P815 targets were used to detect exogenous CTL (right panel). Mean specific lysis values (of triplicates) at the indicated E:T ratios are shown. The nonspecific lysis of control P815 (<5%) was subtracted. B, pCI/S-primed BALB/c spleen cells were stimulated three times in vitro at weekly intervals with P815/S cells and tested for specific cytotoxic activity against (nontreated or with the anti-Ld mAb 30-5-7 pretreated) P815/S targets or P815 targets pulsed with the Ld- or Dd-binding peptides. Mean specific lysis values (of triplicates) at an E:T ratio of 20 are shown.
Kb-restricted CTL responses to HBsAg, irrespective of the processing requirements that have to be met to generate these MHC-I-binding epitopes.

Primed, subdominant, Dd- or Kd-restricted, HBsAg-specific CTL are resistant to suppression by immunodominant Ld-restricted CTL. When coprimed by HBsAg, the immunodominant, Ld-restricted CTL response down-regulates all concomitant CTL responses to other epitopes of the same Ag. We performed experiments to prime or boost CTL to dominant or subdominant epitopes of HBsAg. A single immunization of BALB/c mice with the pCI/S DNA vaccine induced similar frequencies of IFN-γ/H9253 CD8/H11001 CTL specific for the Ld-restricted S28–39 epitope, independent of additional prime or boost injections with the pCI/cT-SIII DNA vaccine (Fig. 7, groups 1, 2, 6, and 7). Mice primed and boosted with the pCI/S vaccine developed enhanced, Ld-restricted CTL responses (Fig. 7, group 5), indicating that the response can be boosted. In contrast, only low numbers of Dd-restricted, S201–209-specific CTL were induced in mice immunized with pCI/S (Fig. 7, groups 1, 2, and 5), suggesting that this CTL response is not boosted by repeated pCI/S injections. Mice immunized with pCI/cT-SIII DNA developed Dd-restricted, S201–209-specific CTL responses (Fig. 7, groups 3, 4, and 8) that were efficiently boosted in mice primed with pCI/S and boosted with pCI/cT-SIII, or vice versa (Fig. 7, groups 6 and 7). Similar data were generated for the Kd-restricted CTL response (data not shown). In mice with established Dd/Kd-restricted, HBsAg-specific CTL immunity, the priming of an immunodominant Ld-restricted, S28–39-specific CTL response did not inhibit the boost of the subdominant Dd/Kd-restricted CTL responses to HBsAg. In fact, the Kd/Dd-restricted HBsAg-specific CTL frequencies were even higher after a boost injection with pCI/S than after a boost injection with pCI/cT-SIII (Fig. 7, groups 7 and 8). Inhibition of the Dd/Kd-restricted CTL by Ld-restricted CTL thus operates in the priming stage.

Discussion
We comparatively studied two alternative vaccine delivery strategies that seem to preferentially target different processing pathways. These were DNA-based vaccination for delivering Ag to endogenous processing (reviewed in Refs. 19 and 20) and virus-like particle-based vaccination for delivering recombinant Ag to exogenous processing (reviewed in Ref. 16). Modifications of DNA vaccination were used to prime HBsAg-specific CTL only against a selected set of epitopes. Following vaccination, we comparatively evaluated methods for (semi-)quantitative, epitope-specific in vitro detection of primed CTL. A direct ex vivo enumeration of IFN-γ-producing CTL specifically restimulated for a few hours revealed efficient priming of CTL specific for HBsAg epitopes that were difficult to detect (or undetectable) in conventional cytolytic assays (after specific in vitro restimulation for a few days).

A single i.m. injection of the pCI/S DNA vaccine into BALB/c or DBA/2 mice primed Ld-, Kd-, and Dd-restricted CTL populations (Fig. 2A). The specific CTL reactivities primed by the different DNA vaccines were as expected: the pCI/S vaccine primed CTL to all three known Ld-, Kd-, and Dd-restricted HBsAg epitopes; the pCI/cT-SI vaccine primed CTL to Ld but not Kd- and Dd-restricted HBsAg epitopes; the pCI/cT-SII vaccine primed no HBsAg-specific CTL; and the pCI/cT-SIII primed CTL to Kd- and Dd, but not Ld-restricted HBsAg epitopes (Fig. 2A). Substantially more Ld- than Kd- or Dd-restricted, HBsAg-specific CTL were
detected in the spleens of primed mice when cells were restimulated for 6 h ex vivo with peptides, syngeneic transfected cells expressing HBsAg (P815/S), or syngeneic cells pulsed with HBsAg particles (P815 plus HBsAg). Ld-restricted CTL were restimulated by transfected and pulsed targets, whereas Kd- and Dd-restricted CTL were detected only by transfected but not pulsed targets (Fig. 2A). This pointed to different processing requirements that have to be met to generate the respective epitope. A different picture emerged when BALB/c-congenic Ld/H-2dm2 mice were immunized with the pCI/S or pCI/cT-SIII DNA vaccines (Fig. 2B). These animals showed no Ld-restricted CTL reactivity, as expected. Their Kd- and Dd-restricted CTL responses were strikingly enhanced. This provided the first evidence that immunodominant Ld-restricted CTL responses to HBsAg can partially suppress concomitant CTL responses to other epitopes of the same Ag.

The HBsAg-derived peptides bind to Ld, Kd, and Dd with comparable and high affinity (4). Thus, it seems unlikely that immunodominance reflects the strength of binding of the respective antigenic peptides to their MHC class I molecules. In contrast, affinities of the TCR for their peptide/MHC class I molecule that are selected in CTL responses to HBsAg may differ. CTL clones with very high TCR affinities for the Ld/S28–39 ligand have been described to be present in the polyclonal and multispecific CTL population raised against this epitope (3). This may contribute to the immunodominance we describe.

Only the immunodominant, Ld-binding HBsAg epitope is generated in the conventional endogenous (TAP- and proteasome-independent) pathway, as well as in an alternative, exogenous (TAP- and proteasome-independent) pathway (5, 21–23). The data shown in Figs. 2 and 3 indicate that exclusively endogenous processing of HBsAg generates the Kd- and Dd-binding peptides. The Kd- and Dd-restricted CTL were primed after injection of DNA vaccines (Fig. 2, groups 1 and 4, and Fig. 3, group 1) but not after the injection of HBsAg particles (Fig. 3, group 2). Because CTL precursors are cross-primed during DNA vaccination, it was unexpected that such a clear-cut picture emerged at the stage of in vivo CTL priming. The in vitro restimulation data confirmed the differential processing requirements of the three HBsAg epitopes.
tested: detection of Kα- and Dα-restricted CTL required restimulation by peptide-pulsed or transfected (but not HBsAg particle-pulsed) targets, whereas Lβ-restricted CTL were restimulated by transfected, peptide-pulsed, or HBsAg particle-pulsed targets (Figs. 2 and 3). Confirming these data, detection of the specific cytolytic reactivity of Kα- and Dα-restricted CTL primed by pCI/S or pCI/cT-III in dm2 mice required 5-day in vitro restimulation with transfecteds and testing of the cytolytic reactivity against transfectants (Fig. 4A). Endogenous processing of HBsAg is thus required for efficient Kα- and Dα-restricted epitope presentation for priming in vivo, restimulation in vitro, and specific effector function delivery in vitro. These data generated with H-2d mice are similar to our previously reported data in H-2b mice (5). In this system, the Kα-restricted S208-215 epitope is exclusively generated by processing exogenous HBsAg, whereas the (not yet mapped C-terminal) Kβ/Dβ-restricted HBsAg epitopes are generated only by endogenous processing. Despite the complex in vivo situation operating during priming of CTL responses by DNA vaccines encoding intracellular Ag, Ag delivered by this mean accesses only some but not all processing pathways, which restricts the epitope repertoire against which CTL can be primed using a single vaccination (or Ag delivery) strategy.

The pCI/S DNA vaccine primes high numbers of Lβ-restricted CTL but low numbers of Kα- and Dα-restricted CTL in BALB/c mice. Lβ- but not Kα- or Dα-restricted CTL populations were detected in 5-day cytolytic assays after specific restimulation in vitro (Fig. 4). Specific restimulation of pCI/S-primed, BALB/c-derived CTL by either P815/S transfecants or HBsAg-pulsed P815 supported expansion of Lβ- but not Kβ/Dβ-restricted CTL (Fig. 4A). Kβ/Dβ-restricted CTL from pCI/S-primed dm2 mice expanded poorly in vitro when restimulated by P815/S transfecants. Either the poor presentation of Kβ/Dβ-restricted epitopes derived from endogenous processing of HBsAg or their restricted potential for in vitro expansion makes it difficult to detect these CTL populations in conventional cytotoxicity assays in vitro. Readout systems involving in vitro restimulation followed by specific cytolytic readouts thus do not yield a reliable picture of the CTL repertoire primed in vivo.

The immunodominance in MHC class I-restricted T cell responses has multifactor causes (reviewed in Refs. 1, 2, and 24). It is evident in natural virus infections (25) but it is not stable in the course of an ongoing virus infection (26). Immunodominance can operate either at the epitope presentation level or at the T cell level. Interference between responding T cells (“T cell competition model”) (27, 28) and/or incomplete CTL differentiation stimulated by a subdominant viral Ag (29) can contribute in establishing immunodominance at the T cell level. Immunodominance is usually not an absolute feature of a given epitope, but is also defined in relation to other epitopes within the same Ag or APC. Our data indicate that the Lβ-binding HBsAg epitope generated by multiple processing pathways can down-modulate CTL responses to many other epitopes of the same viral Ag (generated in exogenous or endogenous processing pathways) in an unexpectedly efficient way, irrespective of the presence of many nonrestriciting MHC class I molecules and alternative “background” gene (BALB/c, C57BL/6) expression.

Polyepitope vaccines have been constructed using recombinant viruses (30, 31), DNA-based vaccines (32), or synthetic peptides. The presence of suppressive epitopes has raised concern about the advantage and efficacy of such vaccines. The presence of a dominant suppressive epitope in the construct was feared to limit the polyvalent efficacy of the vaccine. Our data suggest that, although immunodominance hierarchies between multiple CTL-defined epitopes are evident, the presence of immunodominant epitopes does not necessarily impair the biological efficacy of CTL specific for subdominant epitopes.

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