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HLA-C-restricted T cells have been shown to play an important role in HIV control, but their impact on protection or pathogenesis in other viral infections remains elusive. Here, we characterized the hierarchy of HLA class I-restricted hepatitis B virus (HBV) epitopes targeted by CD8 T cells in HBV-infected subjects. The frequency of CD8 T cells specific for a panel of 18 HBV epitopes (restricted by HLA-A*0201/03/07 [hereinafter HLA-A*0201/03/07], -A1101, -A2402/07, -B5801, -B4001, -B1301, and -Cw0801) was quantified in a total of 59 subjects who resolved HBV infection. We found that the HLA-Cw0801-restricted epitope comprised of Env residues 171 to 180 (Env171–180) is immunoprevalent in the Southeast Asian subjects (10/17 HLA-Cw0801-positive subjects) and immunodominant in the majority of HLA-Cw0801-positive subjects able to control HBV infection. HLA-Cw0801-restricted Env171–180-specific CD8 T cells recognized endogenously produced HBV surface antigen (HBsAg) and tolerated amino acid variations within the epitope detected in HBV genotypes B and C. In conclusion, we demonstrate that the HLA-Cw0801-restricted Env171–180 T cell response is an important component of the HBV-specific adaptive T cell immunity in Asians infected with HBV. Thus, HLA-C-restricted T cells might play an important role in various viral infections.

During viral infections, CD8 T cells clear virus-infected cells due to their ability to recognize viral proteins presented, in the form of short peptides, by different major histocompatibility complex (MHC) class I molecules on the surface of the cells. Two allelic forms of MHC class I proteins coded by three distinct genes, HLA-A, -B, and -C, are expressed in human nucleated cells. Virus-specific CD8 T cells recognizing HLA-A/B viral peptide complexes have been amply characterized in humans, with HLA-B-restricted CD8 T cells often associated with superior antiviral activity (1, 2). In contrast, since HLA-C molecules seem to be expressed at levels 10% lower than HLA-A and HLA-B molecules, CD8 T cells specific for viral peptides presented by HLA-C molecules have been thought to be rare and characterized by weak antiviral activity (3).

Seminal data obtained in HIV infection has, however, challenged this concept. The first observation was derived from a genome-wide association study that identified a strong association between a dimorphism 35 kb upstream of the HLA-C gene promoter and levels of HIV viremia (4). Such results were complemented by the finding that the HLA-C variant −35C, associated with lower viremia, was linked with higher expression of HLA-C molecules in European/American populations, showing that higher expression of HLA-C molecules confers protection from HIV (5, 6). Recently, the protective value of HLA-C-restricted T cell responses in HIV infections was extended to Asian populations, where it was shown that the high expression of HLA-C molecules results in a stronger HLA-C-restricted HIV-specific immune response and an increased frequency of viral mutations on targeted epitopes (7).

However, the protective impact of HLA-C-restricted T cells might be an exclusive feature of HIV infection since during HIV replication, the HIV negative replication factor protein ( nef) selectively downregulates HLA-A and HLA-B expression without interfering with HLA-C expression levels (8). Even though HLA-C-restricted CD8 T cells have been detected in other viral infections (human cytomegalovirus [HCMV], Epstein-Barr virus [EBV], and hepatitis C virus [HCV]) (3), the role played by HLA-C-restricted T cells in viral protection or pathogenesis remains elusive.

We recently characterized in a Han Chinese patient with acute hepatitis B virus (HBV) infection an HLA-Cw*0801 (hereinafter HLA-Cw0801)-restricted T cell response specific for the conserved region of residues 171 to 180 of the envelope protein (Env171–180) of HBV genotypes A, C, D, and F (9). Whether this epitope is frequently targeted by HBV-specific T cells in HLA-Cw08-positive patients and whether such HLA-C08-restricted T cells represent a dominant or subdominant response within an infected individual are not known.

Such information is important since knowledge of the repertoire of HBV-specific CD8 T cells is very limited in patients infected with HBV genotypes B and C (the dominant HBV strains in Northeast/Southeast Asia), and an understanding of the prevalence of the CD8 response against defined HBV epitopes in South Asian patients carrying specific HLA class I (horizontal immu-
nudominate or immunoprevalence) is needed. At the moment, HBV-specific CD8 T cell characterization in this patient population is limited to few epitopes (10, 11) or is based on theoretical epitopes predicted by the presence of mutations associated with selected HLA alleles (10, 12). Furthermore, we have demonstrated that CD8 T cell epitopes frequently recognized and immunodominant in HLA-A02 Caucasian patients infected by HBV genotypes A and D are rarely detected in HLA-A02 South Asian patients (13).

Thus, to have a better understanding of the HBV epitopes targeted by CD8 T cells in subjects infected with HBV genotypes B and C, we interrogated the HBV-specific T cell repertoire of 59 HBV-infected individual, the dominant CD8 T cell response should exert more antiviral activity than subdominant ones.

By reporting that the HLA-Cw0801-restricted Env171–180 epitope represents a horizontal and vertical immunodominant HBV epitope in South Asian populations, we provide valuable information about the important antigenic region targeted by the immune system during HBV infection and also show that the substantial role of HLA-C-restricted T cells in viral infection might not be restricted only to HIV infection.

**MATERIALS AND METHODS**

**Patient population.** HBV-infected patients of Southeast Asian origin were enrolled at the National University Hospital of Singapore or at Chulalongkorn University, Bangkok, Thailand. This study was approved by the ethics committees of both Chulalongkorn University and the National University Hospital of Singapore. Eight patients had clinical, biochemical, and virological evidence of acute HBV infection (alanine aminotransferase [ALT] levels of >10 times the upper limit of normal, detection of HBV surface antigen [HBsAg] and serum anti-HBc core protein [HBc] IgM, and HBsAg clearance within 2 months from clinical onset of hepatitis). Fifty-one patients had only serological evidence of HBV contact (HBsAg negative and IgG anti-Hbc and anti-HBc surface protein [HBs] positive) but no history of acute hepatitis. All patients were HLA typed (BG1, Hong Kong, China), gave written informed consent, and were serologically negative for HIV and HCV. We also selected three patients with chronic HBV infection (HBsAg positive for over 6 months) that were positive for the HLA-Cw0801 allele.

**Synthetic peptides.** A library of 313 synthetic 15-mer peptides overlapping by 10 amino acid (aa) residues covering the whole HBV genotype C proteome sequence were purchased from Chiron Mimotopes (Victoria, Australia). The pools of core and X peptides were made into a 9- by 8-matrix, containing eight or nine peptides/pool, respectively, whereas enveleope peptides were pooled in a 9- by 9-matrix containing nine peptides/pool and polymerase peptides formed a 14- by 12-matrix containing 12 or 14 peptides/pool, as described previously (13). The known HBV epitopes were purchased from Proimmune (Oxford, United Kingdom) and from GenScript (Piscataway, NJ).

**Stimulation of PBMCs.** Peripheral blood mononuclear cells (PBMCs) from patients were isolated by Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co., St. Louis, MO) and resuspended in AIM-V medium (Gibco-BRL Laboratories, Gaithersburg, MD) with 2% human AB serum. PBMCs were in vitro expanded with peptides for 10 days before assays were performed. For full proteome screening, 20% of PBMCs were pulsed with 10 μg/ml of each overlapping peptide for 1 h at 37°C and then washed and cocultured with the remaining PBMCs (80%) in AIM-V medium with 2% human AB serum and 20 U/ml of interleukin-2 (IL-2) (R&D Systems, Abingdon, United Kingdom). For single peptide expansion, HBV peptides were added directly at 5 μg/ml for 15-mer peptides and at 1 μg/ml for 9- to 10-mer peptides.

**Intracellular cytokine staining (ICS) and degranulation assays.** In vitro-expanded PBMCs were incubated in medium alone (control) or with viral peptides (5 μg/ml) for 5 h in the presence of brefeldin A (10 μg/ml). After being washed, the cells were stained with anti-CD8 phycoerythrin (PE)-Cy7 and anti-CD3 peridinin chlorophyll protein (PerCP)-Cy5.5 monoclonal antibodies (MABs) for 30 min at 4°C and then fixed and permeabilized using Cytofix/Cytoperm Fixation/Permeabilization solution (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. Cells were then stained with anti-gamma interferon (IFN-γ) PE for 30 min on ice, washed, and analyzed by flow cytometry. To assess degranulation activity, CD107a PE antibody (BD Pharmingen, San Di-iego) was added to all wells at the beginning of the 5 h of incubation. Following the incubation, cells were washed and labeled with CD8 PE-Cy7 and CD3 PerCP-Cy5.5 as described above.

**IFN-γ ELISPOT assay.** IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays were performed as previously described (13) using a panel of 313 overlapping peptides covering the full proteome sequence of HBV genotype C pooled in the described mixtures. HBV-specific T cell responses were analyzed in IFN-γ ELISPOT assays either ex vivo using fresh or frozen PBMCs or after short-term peptide-specific polyclonal T cell expansion (10 days). Briefly, 96-well plates (Multiscreen-HTS; Milli- pore, Billerica, MA) were coated overnight at 4°C with 5 μg/ml capture mouse anti-human IFN-γ monoclonal antibody (1DIK; Mabtech, Swe- den). Plates were then blocked with AIM-V medium supplemented with 10% heat-inactivated fetal calf serum (FCS) for 30 min at room temperature. A total of 1 × 105 PBMCs or 5 × 104 cells from short-term polyclonal T cell lines were seeded per well, in duplicates for each individual peptide mixture. Plates were incubated for 18 h at 37°C in the absence or presence of peptides (at a final concentration of 5 μg/ml). After the incubation, plates were developed using the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride (BCIP/NBT; KPL, MD) according to the recommended protocol from Mabtech. The colorimetric reaction was stopped after 10 to 15 min by washing the plates with distilled water. Plates were air dried, and spots were counted using an automated ELISPOT reader (ImmunoSpot reader; CTL Technologies, OH). The number of peptide-specific IFN-γ-secreting cells was calculated by subtracting the nonstimulated control value from that of the stimulated sample. Positive controls consisted of PBMCs stimulated with phorbol myristate acetate (10 ng/ml) and ionomycin (100 ng/ml). In the direct ex vivo assays, wells were considered positive when the number of spot-forming units (SFU) was above 5 and at least three times the mean value of the unstimulated control wells (three wells/patient). The positivity criteria for in vitro ELISPOT assays is less stringent, requiring that wells that have SFU counts above 5 and at least two times the mean value of unstimulated control wells, but the responses were reconfirmed using IFN-γ ICS.

**HLA restriction, fine specificity, and epitope avidity assay.** The HLA restriction of the T cell responses was deduced by coculturing the short-term T cell lines for 5 h with a panel of HLA class I-matched Epstein-Barr virus (EBV)-transformed B cells pulsed with the specific peptide. After T cells were cocultured with EBV B cells, IFN-γ- and CD107a-expressing CD8+ T cells were quantified by flow cytometry. To determine the minimal epitope, short peptides (8- to 10-mer) were designed based on the responding 15-mer peptide and tested using IFN-γ ICS. The functional avidity of the minimal epitopes was determined by pulsing EBV B cell lines carrying the appropriate HLA class I molecules with serially diluted concentrations of the corresponding peptides (1 μM, 100 nM, 1 nM, 100 pM, and 1 pM), followed by coculturing with the corresponding short-term T cell lines (derived from the PBMCs of HBV
peptide-responding patients) and quantification of the IFN-γ and CD107a response by flow cytometry. We then calculated the percent-transformed B cells was pulsed with 1 μM Env171–180 and subsequently acquired on a FACSCanto instrument. As a positive control, the cells were stained for CD8, IFN-γ/H9253, tumor necrosis factor alpha (TNF-α), and IL-2, according to the ICS protocol detailed above, and restimulation with different peptide pools. We have recently demonstrated, directly or indirectly, the presence of the protein transport inhibitor brefeldin A. After the culture, the horizontal immunodominance (immunoprevalence) of the HLA-Cw0801/Env171–180 response. For this purpose, we then evaluated the vertical immunodominance of the HLA-Cw0801-restricted T cell response represents a highly immunoprevalent CTL response in HBV patients, where the response against the core protein epitope consisting of residues 18 to 27 (core18 –27) was highly common, the prevalence of this response was low in our HLA-A0201-positive Southeast Asian cohort (two positive subjects out of eight tested) (Fig. 1A). Conversely, the response to the polymerase epitope consisting of residues 455 to 463 (Pol455–463) that was less frequently found in HLA-A0201-positive Caucasians was one of the most prevalent responses observed in our HLA-A0201-positive Southeast Asian subjects (four positive subjects out of seven tested) (Fig. 1A).

In our Southeast Asian cohort, four responses were the most frequently detected among various CD8 T cell epitopes tested. In descending order, the first was against the HLA-Cw0801-restricted Env171–180 epitope, which was detected in 10 of the 17 HLA-Cw0801 subjects; the second was against the Pol455–463 epitope restricted to HLA-A0201 (4 out of 7 subjects); third was the HLA-A2402-restricted core117–125 epitope found in 5 out of 9 subjects; and the fourth response was again directed at the Pol455–463 epitope but restricted to HLA-A0203 (Fig. 1A). Taking into account the greater prevalence of HBV-Cw0801 in Southeast Asia (Fig. 1B), our data show that the HLA-Cw0801-restricted Env171–180 CD8 T cell response represents a highly immunoprevalent CTL response in Southeast Asian patients with self-limiting HBV infection.

**Vertical immunodominance of the HLA-Cw0801-restricted T cell response.** We then evaluated the vertical immunodominance of the HLA-Cw0801/Env171–180 response. For this purpose, the whole HBV-specific T cell repertoire was studied in two HLA-Cw0801+ patients with acute HBV infection. PBMCs of the two patients, obtained 4 weeks after the onset of acute hepatitis symptoms (patient 1) or after resolution of disease and HBsAg seroconversion (patient 2), were stimulated with 61 mixtures of overlapping peptides covering the whole proteome of HBV genotype C. ELISPOT assays to detect IFN-γ-producing cells were performed directly ex vivo and after 10 days of in vitro expansion. In accordance with previous results, direct ex vivo analysis of IFN-γ-producing cells did not generate results that could be used with confidence to elucidate an immunodominance hierarchy (data not shown). At best, 8 to 6 spots/10^5 cells were visualized by stimulation with different peptide pools. We have recently demonstrated, directly ex vivo, that the lack of detectable functional HBV-specific T cells during the acute phase of HBV infection is the result of the combined action of T cell exhaustion and the suppressive effect of arginase (14). On the other hand, in patient 2, the few HBV-specific IFN-γ spots detected ex vivo are compatible with the low frequency of memory HBV-specific CD8 T cells present after resolution of infection.

**Results**

**Horizontal immunodominance of HLA-Cw0801-restricted T cells.** The horizontal immunodominance (immunoprevalence) of 18 distinct HBV-specific CD8 T cell epitopes restricted by HLA-A0201/03/07, HLA-A1101, HLA-A2402/07, HLA-B5801, HLA-B4001, HLA-B1301, and HLA-Cw0801 (Table 1) were tested in HBV patients of Southeast Asian ethnicities (Chinese Han, Thai, Malay, and Indonesian Javanese). The amino acid sequences of different epitopes and their HLA-class I restrictions are displayed in Table 1. These epitopes were chosen as they are restricted by HLA alleles commonly present in the Southeast Asian population. To analyze the frequency of subjects carrying the respective HLA-class I allele who are able to respond to the corresponding HBV epitopes, the HLA-class I profile (four-digit resolution) of 59 subjects (HBsAg negative and anti-HBc and anti-HBs positive) with or without a reported episode of acute hepatitis was characterized.

Selected subjects were positive for the following alleles: 24 for HLA-A02 (8 A0201, 9 A0203, and 9 A0207), 13 for HLA-A24 (9 A2402 and 4 A2407), 17 for Cw0801, 24 for A1101, 8 for B1301, 8 for B5801, and 7 for B4001. PBMCs of each subject were stimulated with the respective HLA class I-restricted epitopes in vitro for 10 days. The presence of epitope-specific CD8 T cells was determined with ICS and ELISPOT assays. Figure 1 shows a summary of the results obtained.

As described previously by Tan et al., the immunoprevalence profile of CD8 T cell responses varies between the different populations. Unlike the situation in HLA-A0201-positive Caucasian HBV patients, where the response against the core protein epitope consisting of residues 18 to 27 (core18 –27) was highly common, the prevalence of this response was low in our HLA-A0201-positive Southeast Asian cohort (two positive subjects out of eight tested) (Fig. 1A). In our Southeast Asian cohort, four responses were the most frequently detected among various CD8 T cell epitopes tested. In descending order, the first was against the HLA-Cw0801-restricted Env171–180 epitope, which was detected in 10 of the 17 HLA-Cw0801 subjects; the second was against the Pol455–463 epitope restricted to HLA-A0201 (4 out of 7 subjects); third was the HLA-A2402-restricted core117–125 epitope found in 5 out of 9 subjects; and the fourth response was again directed at the Pol455–463 epitope but restricted to HLA-A0203 (Fig. 1A). Taking into account the greater prevalence of HBV-Cw0801 in Southeast Asia (Fig. 1B), our data show that the HLA-Cw0801-restricted Env171–180 CD8 T cell response represents a highly immunoprevalent CTL response in Southeast Asian patients with self-limiting HBV infection.

**Vertical immunodominance of the HLA-Cw0801-restricted T cell response.** We then evaluated the vertical immunodominance of the HLA-Cw0801/Env171–180 response. For this purpose, the whole HBV-specific T cell repertoire was studied in two HLA-Cw0801+ patients with acute HBV infection. PBMCs of the two patients, obtained 4 weeks after the onset of acute hepatitis symptoms (patient 1) or after resolution of disease and HBsAg seroconversion (patient 2), were stimulated with 61 mixtures of overlapping peptides covering the whole proteome of HBV genotype C. ELISPOT assays to detect IFN-γ-producing cells were performed directly ex vivo and after 10 days of in vitro expansion. In accordance with previous results, direct ex vivo analysis of IFN-γ-producing cells did not generate results that could be used with confidence to elucidate an immunodominance hierarchy (data not shown). At best, 8 to 6 spots/10^5 cells were visualized by stimulation with different peptide pools. We have recently demonstrated, directly ex vivo, that the lack of detectable functional HBV-specific T cells during the acute phase of HBV infection is the result of the combined action of T cell exhaustion and the suppressive effect of arginase (14). On the other hand, in patient 2, the few HBV-specific IFN-γ spots detected ex vivo are compatible with the low frequency of memory HBV-specific CD8 T cells present after resolution of infection.

**Table 1 List of known HBV epitopes tested**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Protein and amino acid position</th>
<th>HBV genotype C</th>
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<tr>
<td>HLA-A alleles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0201/07</td>
<td>Core 8–16</td>
<td>EFGASVELL</td>
</tr>
<tr>
<td>A0201/07</td>
<td>Core 18–27</td>
<td>FLPSDFFPSI</td>
</tr>
<tr>
<td>A0201/07</td>
<td>Env 61–70</td>
<td>NLLGWSPQA</td>
</tr>
<tr>
<td>A0201/07</td>
<td>Env 183–191</td>
<td>FLTRILTI</td>
</tr>
<tr>
<td>A0201/07</td>
<td>Env 333–343</td>
<td>WSLVPVFV</td>
</tr>
<tr>
<td>A0201/07</td>
<td>Env 338–347</td>
<td>LLYPEFQWVFV</td>
</tr>
<tr>
<td>A0201/07/03</td>
<td>Env 348–357</td>
<td>GLSPTVLWSLV</td>
</tr>
<tr>
<td>A0201/07/03</td>
<td>Env 370–379</td>
<td>NILNPFPLPL</td>
</tr>
<tr>
<td>A0201/07</td>
<td>Pol 455–463</td>
<td>GLPRYVVARL</td>
</tr>
<tr>
<td>A1101</td>
<td>Core 88–96</td>
<td>YVNVNMGKL</td>
</tr>
<tr>
<td>A1101</td>
<td>Core 141–151</td>
<td>TLPETTVRR</td>
</tr>
<tr>
<td>A2402/07</td>
<td>Core 117–125</td>
<td>EYLVSGGVV</td>
</tr>
<tr>
<td>A2402/07</td>
<td>Pol 756–764</td>
<td>KYTSPWLL</td>
</tr>
<tr>
<td>HLA-B alleles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B5801</td>
<td>Env 356–364</td>
<td>SVIWMMDWYW</td>
</tr>
<tr>
<td>B5801</td>
<td>Pol 165–174</td>
<td>ASFCGPSYSW</td>
</tr>
<tr>
<td>B4001</td>
<td>Core 75–83</td>
<td>LEDPASREL</td>
</tr>
<tr>
<td>B1301</td>
<td>Pol 386–395</td>
<td>SRLVDFSQF</td>
</tr>
<tr>
<td>HLA-C allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cw0801</td>
<td>Env 171–180</td>
<td>FLGPLVLLQQA</td>
</tr>
</tbody>
</table>

a Amino acid positions with genotype-specific mutations are highlighted in bold.
b Predicted epitope (12).
In contrast to the *ex vivo* data, a clear population of HBV peptide-responsive T cells was detected with ELISPOT assays in both patients after *in vitro* expansion. Deconvolution of the single peptide responsible for the T cell activation and definition of their CD4 or CD8 phenotype was performed on the individual T cell lines with intracellular cytokine staining. Figure 2A summarizes the results of these consecutive experiments, displaying only the spot counts that were derived from mixtures containing peptides that were confirmed to activate HBV-specific CD8 T cells. CD8 T cells responding to the Env\textsubscript{171–180} HLA-Cw0801-restricted epitope clearly represent a numerically important response in both acute patients. In patient 1, even though the response to a Pol\textsubscript{71–85} epitope was slightly bigger, Env\textsubscript{171–80} was able to stimulate the strongest CD8 T cell response specific to the envelope protein. Remarkably, Env\textsubscript{171–80}-specific CD8 T cells were dominant over the response to the HLA-A201-restricted CD8 T cell epitope core\textsubscript{18-27}, one of the most studied HBV-specific CD8 T cell epitopes. In patient 2, the memory CD8 T cell response to Env\textsubscript{171–180} was dominant in comparison to responses against polymerase- and core-specific CD8 T cell responses.

The vertical immunodominance of the Cw0801-restricted CD8 T cell response detected in the two patients with acute hepatitis B was then measured in an additional 15 HLA-Cw0801/H11001 subjects with serological profiles indicative of previous HBV infection (anti-HBs and anti-HBc positive). In 10 out of these 15 HLA-Cw0801/H11001 subjects, HBV-specific T cells directed against

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**FIG 1** Immunoprevalence of HLA-Cw0801-restricted Env\textsubscript{171–180}-specific T cell response in Southeast Asian subjects who resolved HBV infection. (A) Bars show the prevalence of the HLA-Cw0801/Env\textsubscript{171–180} T cell response in Southeast Asian subjects who resolved HBV infection in comparison to previously characterized HBV epitopes restricted by HLA alleles commonly found in the Southeast Asian population. The inset shows the HLA-Cw0801/Env\textsubscript{171–180} T cell response from a representative subject. Cells were gated for CD3 positivity, and the epitope-specific CD8 T cell response is shown as the frequency in the total T cell population. The number of subjects tested and the HLA restriction for each epitope are indicated below the bars. (B) Phenotypic frequency and the geographical distribution of HLA alleles commonly found in the Southeast Asian population. Heat maps and data were obtained from NCBI MHC database (dbMHC [http://www.ncbi.nlm.nih.gov/gv/mhc]).
peptides covering the whole HBV proteome were detectable after in vitro expansion. Seven out of the 10 HLA-Cw0801+/H11001 subjects with detectable expanded HBV-specific T cells showed an Env171–180-specific CD8 T cell response. As visually displayed in Fig. 2B, the HLA-Cw0801/Env171–180-specific CD8 T cells represent the dominant response in 50% of HLA-Cw0801+/H11001 HBV-infected subjects, in addition to being the most abundant CD8 T cell response in five out of the seven Env171–180-responsive subjects.

Since the Cw0801-restricted CD8 T cell response against Env171–180 sequence is immunodominant in the majority of HLA-Cw08+/H11001 subjects able to control HBV infection, we wanted to determine whether the Env171–180 response could also be detected in patients with chronic HBV infection. Three HLA-Cw0801+/H11001 chronic HBV patients were tested. Not surprisingly, we were unable to detect the Env171–180 response even after 10 days of in vitro expansion (data not shown), consistent with the established view of dysfunctional/deleted HBV-specific T cell responses in chronic HBV patients.

Functional affinity of HBV-specific CD8 T cells. In a previous analysis of the relative magnitude of several different HIV- and EBV-specific CD8 T cell responses, the vertical and horizontal immunodominance of the epitope-specific CD8 T cell response was directly correlated with its functional affinity (2). We therefore analyzed the functional affinity of CD8 T cells specific for HLA-Cw0801/Env171–180 + A0201/core18–27 and HLA-B5801/Env356–364 that were able to recognize target cells pulsed with peptides at concentrations as low as 1 to 10 pM. Other CD8 T cell specificities displayed lower functional affinity (HLA-B1301/Pol386–395 and -A0201/Pol455–463), being activated only by targets pulsed with at least 1 to 10 nM concentrations of the different peptides and with EC50s that were 2 logs higher. HLA-Cw0801/Env171–180-specific CD8 T cells displayed a functional affinity curve that was between these two categories.
We analyzed whether HLA-Cw0801-specific CD8 T cells to recognize target cells pulsed with the peptides LLGPLLVLQA (HBV genotypes B and F) and FLGPLLVLOQA (HBV genotypes A, C, D, and E). We also tested whether the two peptides have similar in vitro abilities to expand Env171–180-specific CD8 T cells. PBMCs of HLA-Cw0801-positive subjects were stimulated with the two peptides, and the expansion of Env171–180 CD8 T cells was tested. Figure 4C shows that both peptides were able to expand in vitro HLA-Cw0801/Env171–180-restricted CD8 T cells. In different subjects the expansion of Env171–180 CD8 T cells specific for the two peptides differs, perhaps because of primary infection with different HBV genotypes. Overall, in all of the subjects the variations at aa 171 did not abolish the immunogenicity of the Env171–180 region.

**Alternative HLA restriction of the Env171–180 epitope.** Interestingly, by analyzing the T cell responses against the HBV envelope in HLA-Cw0801-negative HBV-infected patients, we detected one subject (HLA-A1101, -A3303, -B3503, -B4001, -C0403, and -C1203) that mounted a specific CD8 T cell response to the region of residues 165 to 180, containing the HLA-Cw0801/Env171–180 epitope. Env165–180-specific CD8 T cell lines were expanded, and HLA class I restriction and fine specificity were determined (Fig. 5A and B). We were able to demonstrate that this patient presented an HLA-B3503-restricted CD8 T cell response against the same Env171–180 region, which appears, therefore, to be a promiscuous epitope capable of being presented by at least two different HLA class I molecules, HLA-B3503 and HLA-Cw0801. Similar to the result with the HLA-Cw0801/Env171–180 epitope, the amino acid mutation at position 171 did not alter the immunogenicity of this HLA-B3503-restricted epitope.

Among the HLA-B35 alleles, the HLA-B3501 subtype is the most frequently detected HLA-B35 allele globally (the top three most frequent HLA-B35 alleles are shown in Fig. 5C). Also, while HLA-B3503 is rarely found in South Asian populations, the incidence of the HLA-B3501 subtype is moderately frequent in Caucasians in the United States (~10% phenotype frequency) (Fig. 5C). Hence, we tested whether HLA-B3503-restricted CD8 T cells can also recognize Env171–180 when it is presented by HLA-B3501. Using a short-term T cell line specific for Env171–180 derived from the HLA-B3503-expressing subject, we were able to show that HLA-B3501 can present the Env171–180 peptide to HLA-B3503/Env171–180-specific CD8 T cells (Fig. 5D), suggesting that the same T cell response could also be present in the Caucasian population, which frequently expresses the HLA-B3501 allele.

**Endogenous processing and presentation of Env171–180.** Finally, we sought to determine whether this epitope could be processed and presented in a physiological manner, a crucial point for T cell responses characterized using synthetic peptides. A HLA-Cw0801-expressing hepatocellular carcinoma cell line, SNU-423, was transduced with the full-length HBV genotype C envelope protein (SNU-423Env). The cell line was then used as a target to stimulate an in vitro-expanded short-term T cell line specific for Env171–180 derived from a patient with self-limiting HBV infection. As shown in Fig. 6, SNU-423Env efficiently stimulated T cells specific for Env171–180 to produce IFN-γ, TNF-α, and IL-2, with frequencies comparable to those obtained using EBV-transformed B cells pulsed with the corresponding peptides. This confirms that the Env171–180 epitope could be naturally processed, presented, and subsequently activate specific T cells in HLA-Cw0801 patients infected with HBV.

#### Table 3

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Interpolated Log10 EC50</th>
<th>% prevalence (no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cw0801 / Env171</td>
<td>3.52</td>
<td>58.8 (17)</td>
</tr>
<tr>
<td>A0201 / Core18</td>
<td>1.66</td>
<td>25 (8)</td>
</tr>
<tr>
<td>B5401 / Env356</td>
<td>2.00</td>
<td>14.3 (7)</td>
</tr>
<tr>
<td>A0201 / Env370</td>
<td>4.41</td>
<td>0 (8)</td>
</tr>
<tr>
<td>A0201 / Pol455</td>
<td>4.29</td>
<td>57.1 (7)</td>
</tr>
<tr>
<td>B1301 / Pol386</td>
<td>4.41</td>
<td>12.5 (8)</td>
</tr>
</tbody>
</table>

FIG 3 Comparative functional affinity of the HLA-Cw0801/Env171–180 epitope against known HBV epitopes. EBV-transformed B cells expressing specific HLA alleles were pulsed with different concentrations of the corresponding HBV epitopes and used as targets to stimulate in vitro expanded epitope-specific T cells. The dose-response curve was generated using IFN-γ production as a measurement of activation. The table below shows the interpolated EC50 of the various epitopes and the corresponding immunoprevalence of the response. Max, maximum.
DISCUSSION

During HBV infection, CD8 T cells classically target multiple epitopes and establish a hierarchy of dominant and subdominant HBV-specific CD8 T cell responses within the infected subject. Measurement of the presence of a defined CD8 T cell response against a single epitope in subjects sharing the same HLA class I molecule is called horizontal immunodominance and is important to understanding the prevalence of a defined T cell response in a given population. The analysis of the hierarchy of dominant and subdominant responses within a single individual, defined as vertical immunodominance, represents, in contrast, a functional parameter that helps in the definition of possible protective epitopes (15). Often, within an infected individual, the dominant CD8 T cell response suppresses viral replication more efficiently than subdominant responses, and the dominant response is also associated with better functional avidity.

By analyzing the responses against several previously characterized epitopes, we showed that the HLA-Cw0801-restricted CD8 T cell response specific for the Env171–180 epitope is both horizontally and vertically immunodominant in HBV-infected patients of South Asian origin. The HLA-Cw0801 molecule is expressed in <5% of European/American populations, but it is, in contrast, present in 18 to 20% of the Southeast Asian population, with peaks of about 35 to 40% in specific large ethnic groups (Malay and Indonesian Javanese). Based on these observations, we think that this response might play an important role in the control or pathogenesis of HBV infection in Asian patients, who are the primary world population most affected by HBV persistence. Importantly, by demonstrating that HLA-C-restricted T cells are frequently detectable in HBV infection, our data demonstrate that HLA-C-restricted T cells are not an exclusive feature of HIV infection but are a robust component of antiviral immunity in infections with viruses, like HBV, that are not known to interfere with HLA-A and HLA-B molecule expression.

The immunodominance of the HLA-Cw0801/Env171–180 T cell response in comparison to other HBV-specific CTL responses can be explained by virological and immunological features. First, the Env171–180 epitope resides in the first transmembrane region of HBV envelope that is critical for infectivity (16), suggesting that this region has a high level of conservation among various HBV
genotypes and, hence, the frequent T cell response against it. In addition, we demonstrated that the Env171–180-specific CD8 T cells were able to tolerate the genotype-specific amino acid mutation present at position 171 and be activated by both variants of the epitope. This is unlike the HLA-A0201-restricted core18–27 response (found mainly in subjects infected with HBV genotypes A and D) where amino acid mutation (isoleucine in genotypes B and C; valine in genotypes A and D) at position 27 has a clear negative impact on functional recognition by T cells (13). Therefore, the Env171–180 epitope could potentially be found in HLA-Cw0801 subjects infected by all HBV isolates. Indeed, our immunoprevalence data derived from the study of patients of South Asian origin likely infected by either HBV genotype B or C support this hypothesis.

The other important factor that likely facilitates the horizontal and vertical immunodominance of the HLA-Cw0801/Env171–180 CD8 T cell response is the expression levels of HLA-Cw08 alleles on nucleated cells. HLA-Cw alleles (both HLA-Cw0801 and -Cw0802) have been shown to be in strong linkage disequilibrium with the −35C polymorphism that was associated with high HLA-C cell surface expression (6). More importantly, the higher surface expression of HLA-C correlates with a stronger HLA-C-restricted CD8 T cell response in HIV-infected patients (7). Hence, the elevated expression of HLA-Cw08 is also likely to contribute to the immunodominance of our Env171–180 T cell response in HBV patients.

Note that our data do not directly demonstrate that Env171–180-specific CD8 T cells have a protective or pathogenic role during HBV infection. Such conclusions might be better supported by large population studies associating HLA-class I and/or class II profiles of HBV-infected patients with normal subjects. However, recently the superior antiviral activity of vertical immunodominant epitopes has been demonstrated in a work that investigated the impact of HIV-specific CTL responses on HIV control and showed that vertical immunodominant epitopes exerted more pressure on HIV variability (15). Therefore, the vertical immu-
nondominance of the HLA-Cw0801/Env\textsubscript{171–180}–specific CD8 T cells, in addition to their exclusive detection in subjects that controlled the infection, supports the important antiviral activity of such responses during HBV infection.

Other than the immunodominance of the Env\textsubscript{171–180} epitope, its location within transmembrane domain I of HBV envelope further substantiated its significant antiviral potential. Experimental analysis of the role of the transmembrane domains in the production of HBV surface protein has clearly shown that mutations introduced within the first transmembrane domain (at positions 172 and 178) completely blocked S protein synthesis and subviral particle production (17). Together with the demonstration introduced within the first transmembrane domain (at positions 172 and 178) completely blocked S protein synthesis and subviral particle production (17). Together with the demonstration that this domain is also crucial for infectivity (16), this means that it is unlikely that HBV will escape CTL pressure that targets this region due to the major inherent viral fitness cost, making the Env\textsubscript{171–180} epitope a T cell response with important antiviral potential.

On a different note, the demonstration that an important CD8 T cell epitope can target a conserved viral region also exposed the limitation of using viral mutations associated with HLA-class I profile as a method of identifying new CTL epitopes. Such a method has been used extensively in past years and is driven by a sound rationale, but this approach will never be able to pinpoint the CTL epitopes against conserved regions of the virus that might represent responses crucial for protection. This caveat will have to be properly considered in works employing such an approach to CTL epitope characterization.

Another interesting observation of the Env\textsubscript{171–180} response was also the ability of the epitope to be presented by a different HLA class I molecules, showing the strong immunogenicity of this epitope. Perhaps the conserved nature of this region might facilitate the detection of CD8 T cell epitopes since our method still relies on peptides that were synthesized based on published sequences and might not be identical to the sequence of the infecting virus. One other possibility is that the transmembrane region might actually be presented more efficiently by HLA class I molecules since their embedded nature in the endoplasmic reticulum (ER) membrane might facilitate HLA class I loading. More detailed analysis of different CD8 T cell epitopes will need to be performed to understand whether epitopes located in transmembrane regions of envelope proteins might have a selective advantage to be presented by HLA class I molecules.

Overall, in addition to showing that an HLA-Cw-restricted CD8 T cell response can be easily triggered in not only HIV but also HBV infection, we also provide information on the hierarchy of new HBV–specific CD8 T cell epitopes that will facilitate pathogenetic studies of HBV infection in patients of Asian ethnicities.

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REFERENCES


