An immunodominant HLA-A*1101-restricted CD8+ T-cell response targeting hepatitis B surface antigen in chronic hepatitis B patients

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Hepatitis B virus (HBV) infection is a worldwide public health problem. HBV-specific CD8+ CTLs are vital for viral clearance. Identification of immunodominant CTL epitopes from HBV-associated antigens is necessary for therapeutic vaccine development. We showed that the HLA-A*1101 allele is one of the most common alleles in both healthy individuals and chronic hepatitis B (CHB) patients in the Chongqing area, China. However, less than 10% of epitopes of HBV-associated antigens have been identified in an HLA-A*1101 context. Here, we describe an immunodominant CD8+ T-cell response targeting a hepatitis B surface antigen determinant (HBs295–304) restricted by HLA-A*1101 in both healthy individuals and CHB patients. Moreover, HBs295–304 is more immunogenic for CTL induction than a known naturally HLA-A*1101-processed epitope from hepatitis B core antigen (HBc88–96). Therefore, the newly identified epitope, HBs295–304, will benefit the development of immunotherapeutic approaches for HBV infection.

Chronic hepatitis B virus (HBV) infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, affecting more than 350 million people worldwide (Chisari & Ferrari, 1995; Merican et al., 2000). HBV-specific CD8+ CTLs, which recognize short viral antigenic peptides (epitopes) presenting by HLA class I molecules on the surface of the infected hepatocytes, play a fundamental role in viral clearance and liver injury. The HBV-specific CTL response is multispecific, polyclonal and vigorous during acute hepatitis B (Thimme et al., 2003), but minimal or undetectable during chronic infection, which is generally acknowledged as a major determinant of viral persistence in this disease (Löhr et al., 1993; Penna et al., 1991; Rehermann et al., 1995).

Identification of immunodominant HBV-specific CTL epitopes is critical for the development of epitope-based therapeutic vaccine for chronic HBV infection (Inchauspe & Michel, 2007). HLA-A*0201 is a common HLA class I allele in the general population, with a stronger predominance in Western Caucasian populations than in Asian populations (Zhang et al., 2003). Previous studies of CTL epitopes from HBV-associated proteins have mostly focused on the HLA-A*0201 allele (Penna et al., 1991; Bertoletti et al., 1993; Nayersina et al., 1993; Loirat et al., 2000). However, HLA-A*1101, rather than HLA-A*0201, is the most common HLA class I allele in Asian countries, and in China in particular (Lee et al., 1988; Lin et al., 2001). However, compared with a considerable number of HLA-A*0201-restricted HBV-derived epitopes, less than 10% of HBV epitopes currently described fall within the HLA-A*1101 context (Desmond et al., 2008). Therefore, identification and characterization of HBV-specific CTL epitopes restricted by HLA-A*1101 alleles is extremely important for the development of therapeutic vaccines for the Chinese population. Here, we identified an immunodominant CTL epitope HBs295–304 restricted by HLA-A*1101. The study was approved by the Ethics Committee of the Third Military Medical University and all subjects gave informed consent.

Firstly, we explored the HLA-A allele distribution in chronic hepatitis B (CHB) patients and healthy individuals in the Chongqing area. Genomic DNA was isolated from frozen peripheral blood samples of 49 CHB patients and 44 healthy controls for low-resolution HLA PCR-SSP
genotyping, according to the manufacturer’s instructions (Morga HLA SSP A Typing kit, Texas BioGene). All CHB patients fulfilled the diagnostic criteria of the Chinese National Program for Prevention and Treatment of Viral Hepatitis. Exclusion criteria included co-infection with human immunodeficiency virus or hepatitis C virus, and diagnoses of autoimmune or alcoholic hepatitis. HLA class I supertypes have been defined based upon overlapping peptide–HLA-binding specificities (Kangueane et al., 2005). The distributions of HLA-A2, HLA-A3 and HLA-A24 supertypes in healthy individuals and CHB patients from the Chongqing area were analyzed (Table S1, available in JGV Online). We found that HLA-A*1101 is the most frequent allele among carriers of the A3 supertype in healthy controls, as well as in CHB patients (Table S1). Accordingly, the HLA-A*1101 allele has been reported to be the most frequent HLA-A allele in the population from other regions in China (Yao et al., 2009; Trachtenberg et al., 2007). After comparing the allele frequency of A*1101 and A*0201 between healthy controls and CHB patients, our results suggested that A*0201, rather than A*1101, may correlate with persistent HBV infection. However, this suggestion was not supported by other data, which indicated that the HLA-A*0201 allele may be related to HBV clearance in Chinese populations in Northern China (Zhang et al., 2006).

To screen candidate HLA-A*1101-binding peptides from hepatitis B surface antigen (HBsAg; GenBank accession no. P03138) and hepatitis B core antigen (HBcAg; GenBank accession no. P03146), we used integrated methods combining several T-cell epitope prediction programs, including SYFPEITHI (http://www.syfpeithi.de/), IEDB (http://tools.immuneepitope.org/mhci/), SVMHC (http://abi.inf.uni-tuebingen.de/Services/SVMHC), NETMHIC (http://www.cbs.dtu.dk/services/NetMHC/) and EPIJEN (Doytchinova et al., 2006). Nine peptides, predicted as potential HLA-A*1101 binders by at least two methods, are listed in Table S2. Considering the advantage of the integrated prediction approach and for economic reasons, only three peptides, viz., HBs295–304, HBs324–332 and HBc88–96, were selected for further analyses of the peptide-specific CD8+ T-cell response. HBs295–304 was positively evaluated by all programs except SVMHC (MHCEPI; Brusic, 1998) and EPIJEN; HBs324–332 was predicted to be a poor HLA-A*1101 binder by SYFPEITHI and EPIJEN; HBc88–96 peptide was estimated to be a good HLA-A*1101 binder by virtually all the programs. Both HBs88–96 and HBs295–304 had also been described as HLA-A11-binding peptides in two patents (PCT/US1998/005039 and EP19930919916). Only HBc88–96 had previously been functionally identified as a natural HLA-A*1101-presented epitope derived from HBcAg (Tsai et al., 1996), but this epitope has not been well studied.

Next, we investigated whether these three peptides could induce a HLA-A*1101-restricted CD8+ CTL response. Fresh PBMCs were isolated from healthy HLA-A*1101 homozygous volunteer donors. Human PBMC-derived dendritic cells (DCs) were generated as described previously (Ho et al., 2006). PBMCs suspended at 1 x 10^6 cells per millilitre in complete RPMI 1640 medium were stimulated by incubation with each peptide (10 µg ml⁻¹) on day 1. Half the medium was replaced with complete medium supplemented with recombinant IL-2 (30 IU ml⁻¹; Roche) every 3–4 days. On day 7, lymphocytes were restimulated with 1 x 10^5 peptide-pulsed autologous DCs in medium containing 10 ng IL-7 ml⁻¹ and 20 IU IL-2 ml⁻¹. On day 10, autologous PBMCs pulsed with the corresponding peptides were used as stimulators in ELISPOT and intracellular cytokine-staining assays. The results of ELISPOT analysis, using a commercial ELISPOT kit (MabTech) according to the manufacturer’s instructions, demonstrated that significantly greater numbers of IFN-γ-producing cells were induced in PBMCs by stimulation with peptide HBs295–304, as compared with the identified epitope HBc88–96. Moreover, IFN-γ release induced by these two peptides was completely blocked by the anti-HLA-ABC antibody W6/32 (eBioscience) at a final concentration of 10 µg ml⁻¹, but not by an IgG2a isotype control (eBioscience). No specific IFN-γ-producing cell reactivity was detected in PBMCs stimulated with peptide HBs324–332, or in the no-peptide control cultures, or in cultures treated with anti-HLA-ABC antibody (Fig. 1a, b). For intracellular IFN-γ and granzyme B staining, Percp-cy5.5-conjugated CD3 mAb (BD Biosciences) and FITC-conjugated anti-CD8α mAb (BD Biosciences) were used to label cells for 30 min at room temperature, after a 5 h incubation with 0.65 µl GolgiStop ml⁻¹ (BD Biosciences) at 37 °C. After washing with flow cytometer buffer (PBS/1 % FBS), cells were fixed and labelled with PE-conjugated anti-human granulym B or allophycocyanin (APC)-conjugated anti-human IFN-γ at 4 °C in a permeabilization buffer. PE- or APC-conjugated isotype IgG1 was used as negative staining control (BD Biosciences). Flow cytometry data were acquired for each of the experiments using a BD Biosciences FACSCalibur. Data analysis was performed using FlowJo software. A representative result is shown in Fig. (2a). When compared with HBs324–332 or the no-peptide control, both HBs88–96 and HBs295–304 stimulated a significantly increased proportion of both IFN-γ-secreting and granulyme B-expressing CD8+ T-cells (Fig. 2b). It is noteworthy that the proportion of IFN-γ-secreting CD8+ T-cells in the HBs295–304-stimulated PBMCs was much higher than that obtained upon stimulation with HBs88–96 (Fig. 2b). The results show that peptide HBs295–304 can induce a much stronger peptide-specific CD8+ T-cell response than HBc88–96 in the context of HLA-A*1101, whereas peptide HBs324–332 cannot. We observed a high baseline expression of granulyme B in CD8+ T-cells in the absence of peptide (Fig. 2a), which may be due to the production of granulyme B by other antigen-encountering effector or effector-memory CD8+ T-cells pre-existing among healthy donor PBMCs. It had previously been demonstrated that effector and effector-memory CD8+ cells remain granulyme B-positive for 1 month or longer in the absence of ongoing antigen stimulation (Rock et al., 2005).
To visualize and enumerate peptide-specific CD8\(^+\) T-cells among PBMCs stimulated with peptides, HLA-A*1101/peptide pentamer staining was performed. HLA-A*1101-homozygous healthy PBMCs, with or without two rounds of peptide stimulation, were incubated with Percp-cy5.5-conjugated CD3 mAb (BD Biosciences), FITC-conjugated anti-CD8\(^a\) mAb (BD Biosciences) and a PE-labelled HLA-A\(^*\)1101 pentamer refolded around the HBs 295–304 peptide or the HBc 88–96 peptide (Proimmune). Cells were then analysed by flow cytometry on a FACSCalibur analyser. As shown in Fig. 3(a, b), no pentamer-positive cells could be detected in either HBs 295–304- or HBc 88–96-stimulated PBMCs at day 0. At day 10, the HLA-A*1101/HBs 295–304 pentamer-positive CD8\(^+\) T-cells arising in PBMCs induced with HBs 295–304 constituted 4.51\% of the total number of CD8\(^+\) T-cells; however, HLA-A*1101/HBc 88–96 pentamer-positive CD8\(^+\) T-cells were present at a much lower frequency (1.79\%). No pentamer-positive CD8\(^+\) T-cells were detected in PBMCs untreated with peptides at either day 0 or day 10. As displayed in Fig. S1, CD8\(^+\) cells were not stained with the pentamers, additionally the HBc 88–96-induced CD8\(^+\) T-cell line was not stained with the HBs 295–304 pentamer, and the HBs 295–304-induced CD8\(^+\) T-cell line was not stained with the HBc 88–96 pentamer, indicating the specificity of pentamer staining. These results revealed that the HBs 295–304-specific CTLs were present at a much higher frequency in HBs 295–304-treated healthy PBMCs compared with the HBc 88–96-specific CTLs generated under the same conditions, suggesting that the capacity of HBs 295–304 for inducing CD8\(^+\) T-cells response is markedly greater than that of HBc 88–96.

HBV-specific CD8\(^+\) T-cells have been reported to exist at low frequencies in chronic HBV infection in numerous studies that used a limited set of well-characterized HLA-A*0201-restricted epitopes or tetramers (Webster et al., 2004; Wu et al., 2004). To date, few studies have used HLA-A*1101 peptide tetramers or pentamers to visualize HLA-A*1101-restricted HBV-specific CD8\(^+\) T-cells in CHB patients. Here, we screened fresh PBMCs from five HLA-A*1101-positive CHB patients for the existence of the HBs 295–304- and HBc 88–96-specific CD8\(^+\) T-cells by pentamer staining. The representative result shown in Fig. 3(c) demonstrates that both HBc 88–96- and HBs 295–304-specific CD8\(^+\) T-cells were detectable at low levels in the

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Fig. 1. ELISPOT analysis for peptide-induced IFN-\(\gamma\) secretion. (a) Representative ELISPOT well of each peptide-cultured PBMC sample. Numbers represent spot counts for each well. (b) Spot counts are normalized by calculating the number of spots per 10\(^5\) cells. Each bar represents the mean ± SD of three independent experiments. Anti-HLA-ABC (+) or isotypic control (–) was added to each well to analyse the HLA class I restriction in peptide-induced IFN-\(\gamma\) secretion. ***Compared to the no-peptide group, \(P<0.001\). NS, Not statistically significant.
circulation of one CHB patient carrying the HLA-A*1101 allele, but those peptide-specific CD8\(^+\) T-cells were undetectable in PBMCs from a HLA-A*1101\(^+\) healthy donor. Moreover, the frequency of HBs\(_{295-304}\)-specific CD8\(^+\) T-cells was significantly higher than the frequency of HBc\(_{88-96}\)-specific cells (Fig. 3c; paired \(t\)-test, \(P<0.05\)). This finding is in contrast with previous results that showed that the HLA-A*0201-restricted HBV-specific T-cell responses in CHB patients are primarily those directed against peptides from HBcAg, with an almost complete lack of reactivity against HBsAg (Boni et al., 2007). This difference may be caused by the intrinsic features of the epitopes restricted by different HLA class I molecules (such as affinity for pentamer binding, efficiency of peptide presentation and availability of a T-cell repertoire). In addition, we found that both of the peptide-specific CD8\(^+\) T-cell types in CHB patients were dysfunctional, as these cells did not produce IFN-\(\gamma\) upon stimulation with the corresponding peptides, in agreement with previous findings on the exhaustion of CTLs in chronic virus infection (Boni et al., 2007).

Few HLA-*1101-restricted epitopes in HBV antigens have been identified so far. Bertoni et al. (1997) demonstrated that several HLA-A*1101-binding peptides from HBV polymerase (Pol) could be recognized by a low frequency of acutely infected or convalescent patients. Recently, one HBcAg-derived peptide, HBc\(_{141}\), and several peptides derived from Pol (Pol\(_{149}\), Pol\(_{388}\) and Pol\(_{531}\)) restricted by HLA-A*1101 were characterized by immunization of HLA transgenic mice with a DNA vaccine (Depla et al., 2008).

HBV encodes three envelope proteins that share a common 226 aa carboxy terminus containing HBsAg. HBsAg is expressed at high levels by infected hepatocytes, and has been demonstrated to be highly immunogenic for both

![FACS analysis for peptide-induced IFN-\(\gamma\) and granzyme B production](image)

**Fig. 2.** FACS analysis for peptide-induced IFN-\(\gamma\) and granzyme B production. (a) Representative FACS data of intracellular IFN-\(\gamma\) and granzyme B staining. Numbers in quadrants indicate the percentages of IFN-\(\gamma^+\) or granzyme B\(^+\) cells among total CD8\(^+\) T-cells. (b) Each bar represents the mean±SD of three independent experiments with PBMCs from one donor. **\(P<0.01\), ***\(P<0.001\) determined by Student’s \(t\)-test. NS, Not statistically significant.
humoral and cellular immune responses. To date, therapeutic vaccine candidates for HBV have partly focused on the use of HBsAg (Schirmbeck & Reimann, 2001). To our knowledge, very few HLA-A*1101-restricted epitopes from HBsAg have been identified until now. In 1988, an HLA-A11-restricted CTL response to PreS1 (PreS1 10–17, PLGFFPDH) was described in a single uninfected individual who had been vaccinated with a plasma-isolated HBV envelope protein (Jin et al., 1988). In a single patient with CHB, Barnaba et al. (1989) had previously isolated intrahepatic CTL clones specific for PreS2 120–134 restricted by HLA-A3, which is grouped together with HLA-A*1101 in the A3 supertype. In this study, we identified that HBs295–304 is an HLA-A*1101-restricted immunodominant epitope.

Fig. 3. Detection of pentamer-reactive CD8\(^+\) T-cells in PBMC from healthy individuals and CHB patients. (a) Representative data of FACS analyses for detecting HLA-A11/peptide pentamer-reactive CD8\(^+\) T-cells generated from HLA-A*1101-homozygous healthy PBMCs at day 0 or day 10 after in vitro stimulation with HBc\(_{88–96}\), HBs\(_{295–304}\) or no peptide. Numbers in quadrants indicate the percentage of pentamer\(^+\) cells gated on the CD3\(^+\)CD8\(^+\) population. (b) Representative data of HLA-A11/peptide pentamer staining of peripheral blood obtained from one healthy donor and one CHB patient carrying the HLA-A*1101 allele. Numbers in quadrants indicate the percentage of pentamer\(^+\) cells among the total CD8\(^+\) cells gated on CD3\(^+\) populations. (c) Data are presented as percentage of pentamer\(^+\) cells gated on the CD3\(^+\)CD8\(^+\) population for five CHB patients carrying HLA-A*1101. *P<0.05, as determined by paired Student’s t-test.
derived from HBsAg, which is capable of eliciting stronger CTL responses than HBC$_{88-96}$, a known epitope from HBC$_{A3}$, in individuals carrying the HLA-A*1101 allele. Considering the overlapping peptide–HLA-binding specificities in one HLA class I supertype, we presume that this HLA-A*1101-restricted epitope may also be presented by other HLA-A3 supertype alleles, such as HLA-A*0301 and HLA-A*3301. Indeed, two patents have previously indicated that this 10-mer peptide binds well to HLA-A11 and HLA-A3 (PCT/US1998/005309 and EP1993099196). Interestingly, HBs$_{295-304}$ was also described as a chimaerin MHC class I allele (Patr-A*0101)-restricted immunodominant epitope in HBV-infected chimpanzees (Asabe et al., 2009). These studies suggest that HBs$_{295-304}$ may be a common CTL epitope with broad population coverage, which may benefit the development of immunotherapeutic approaches for HBV infection. However, this needs to be verified by further studies.

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