Absence of viral escape within a frequently recognized HLA-A26-restricted CD8+ T-cell epitope targeting the functionally constrained hepatitis C virus NS5A/5B cleavage site

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CD8+ T-cell responses are central for the resolution of hepatitis C virus (HCV) infection, and viral escape from these CD8+ T-cell responses has been suggested to play a major role in HCV persistence. However, the factors determining the emergence of CD8 escape mutations are not well understood. Here, the first identification of four HLA-A26-restricted CD8+ T-cell epitopes is reported. Of note, two of these four epitopes are located in the NS3/4A and NS5A/5B cleavage sites. The latter epitope is targeted in all (three of three) patients with acute, resolving HCV infection and in a relatively high proportion (four of 14) of patients with chronic HCV infection. Importantly, the epitope corresponding to the NS5A/5B cleavage site is characterized by the complete absence of sequence variations, despite the presence of functional virus-specific CD8+ T cells in our cohort. These results support previous findings that showed defined functional constraints within this region. They also suggest that the absence of viral escape may be determined by viral fitness cost and highlight an attractive target for immunotherapies.

Growing evidence suggests an important role of the adaptive T-cell response for control of acute hepatitis C virus (HCV) infection. The mechanisms by which HCV establishes persistent infection are not fully understood. T-cell failure may be caused by selection of viral escape mutations within targeted major histocompatibility complex (MHC) class I-restricted HCV epitopes (Bowen & Walker, 2005a, b; Cox et al., 2005; Erickson et al., 2001; Ray et al., 2005). However, the determinants of viral escape, as well as its relative contribution to T-cell failure and HCV persistence, are not yet characterized to date.

Recently, an association between expression of particular MHC class I alleles and the natural course of HCV infection has been described. For example, HLA-B27 is associated with spontaneous viral clearance, whilst HLA-B8 is associated with HCV persistence (McKiernan et al., 2004). We have recently studied the virus-specific CD8+ T-cell response and virus evolution in the context of HLA-B27 and HLA-B8, and found that, for both HLA alleles, viral escape within dominant virus-specific CD8+ T-cell epitopes is a common feature in chronically infected patients (Neumann-Haefelin et al., 2006; Timm et al., 2004).

HLA-A2 has not been found to be associated with a specific outcome of infection, even though most studies about the role of viral escape have been biased towards this allele and have focused on previously described epitopes (Chang et al., 1997; Spangenberg et al., 2005; Urbani et al., 2005). The frequency of viral escape seems to be different for individual HLA-A2 epitopes. For example, selection of escape mutations within the epitope NS3 1406 (KLVALGINAV) has been described, whereas they are rare in the epitopes NS5B 2594 (ALYDVVTKL) and NS3 1073 (CINGVCWTV) (Chang et al., 1997; Kantzanou et al., 2003; Spangenberg et al., 2005). For the latter, substantial constraints on the ability to accommodate sequence variation due to fitness costs have been suggested (Soderholm et al., 2006).

The aim of this study was to characterize further the determinants of viral escape and its relative contribution to...
CD8\(^+\) T-cell failure and viral persistence. In order to exclude a methodological bias by using previously described (but not necessarily immunodominant) epitopes, we used a comprehensive approach to determine the full breadth and hierarchy of the CD8 response and its impact on virus evolution in acute and chronic HCV infection in HLA-A26\(^+\) patients. Importantly, the relatively rare HLA allele A26 has not been shown previously to restrict HCV-specific CD8\(^+\) T-cell responses.

First, we performed a comprehensive analysis of the CD8\(^+\) T-cell response in three HLA-A26\(^+\) patients with acute, self-limited HCV infection (patients A1–A3; see Supplementary Table S1, available in JGV Online, for patients’ characteristics), as well as six HLA-A26\(^+\) patients with chronic HCV infection (patients C1–C4, C7 and C13; see Supplementary Table S1). A screening ELispot assay was performed in a matrix set-up, using 441 overlapping peptides spanning the complete HCV polyprotein (18-mers overlapping by 11 aa, derived from HCV strain H77, kindly provided by the NIH Reference and Reagent Program). Positive peptides were confirmed by intracellular gamma interferon (IFN-\(\gamma\)) staining as described previously (Neumann-Haefelin et al., 2006). Positive overlapping peptides were screened by an epitope-prediction program (http://www.syfpeithi.com) and given a score, leading to the identification of four novel HLA-A26-restricted HCV-specific CD8\(^+\) T-cell epitopes (Table 1). The predicted optimal epitopes were confirmed by serial dilution in comparison with the corresponding overlapping peptide; HLA-A26 restriction was confirmed by functional assays using autologous and partially HLA-matched Epstein–Barr virus (EBV)-infected cell lines [representative data for epitope NS5 2416 are shown in Fig. 1(a)]. Binding assays (Ruppert et al., 1993) showed that all four epitopes had a good HLA-A26-binding capacity (IC\(_{50}\) < 500 nM), with the NS5 2416 epitope displaying the best binding (IC\(_{50}\) 25 nM; Table 1). Next, we tested the four novel HLA-A26-restricted epitopes in a larger cohort of chronically HCV genotype 1-infected HLA-A26\(^+\) patients (in patients C1–C3, infected with genotype 1a, peptides with the genotype 1a consensus sequence were used; whilst in patients C4–C8, infected with genotype 1b, peptides with the genotype 1b consensus sequence were used; see Table 1 for details). Importantly, one of the four epitopes, NS5 2416, was targeted in all (three of three) patients with acute, resolving HCV infection (genotype 1) and in two of eight patients with chronic HCV infection (genotype 1). The sequence-matched peptide with a D2416S substitution was also tested in six patients infected with genotype 3 (patients C9–C14); two of the six patients targeted the epitope. Of note, a similar frequency has been described recently for immunodominant HLA-A2- and HLA-B27-restricted epitopes in chronic HCV infection (Lauer et al., 2004; Neumann-Haefelin et al., 2006; Spangenberg et al., 2005),

**Table 1.** HLA-A26-restricted CD8\(^+\) T-cell responses in patients with acute, resolving or chronic HCV infection

Values are number of IFN-\(\gamma\)\(^+\) CD8\(^+\) cells in CD8\(^+\) PBMCs/number of IFN-\(\gamma\)\(^+\) CD8\(^+\) cells in a peptide-specific cell line (%). – indicates no response detectable (<0.02 %). ND, Not determined.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Epitope (IC(_{50}), nM)</th>
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<tbody>
<tr>
<td></td>
<td>NS3 1383 (437)</td>
</tr>
<tr>
<td><strong>(a) A26(^+) patients with acute, self-limited HCV infection</strong></td>
<td></td>
</tr>
<tr>
<td>Genotype 1a</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>−/−</td>
</tr>
<tr>
<td>A2</td>
<td>−/−</td>
</tr>
<tr>
<td>Genotype 1b*</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>−/−</td>
</tr>
<tr>
<td><strong>(b) A26(^+) patients with chronic HCV infection</strong></td>
<td></td>
</tr>
<tr>
<td>Genotype 1a</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>−/−</td>
</tr>
<tr>
<td>C2</td>
<td>0.10/−</td>
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<tr>
<td>C7</td>
<td>−/−</td>
</tr>
<tr>
<td>C8</td>
<td>−/−</td>
</tr>
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</table>

* Differences between the genotype 1a and genotype 1b consensus sequence are indicated in bold.
† Peptides corresponding to autologous sequences tested negative (C1, EAIKGGRHL, ENFPYLVAY; C3, EAIKGGRHL; C5, ETIRGGRHL; C6, ETIRGGRHL).
Fig. 1. Characterization of HLA-A26-restricted CD8+ T-cell epitope NS5 2416. (a) HLA restriction of the NS5 2416 epitope. Peripheral blood mononuclear cells (PBMCs) from patient A2 were stimulated for 2 weeks with peptide NS5 2416 and then tested for IFN-γ production after 5 h stimulation with autologous or allogeneic HLA-matched or -mismatched EBV cell lines that were loaded with the peptide (filled bars) or not (empty bars). (b) NS5 2416-specific CD8+ T-cell responses in three patients with acute, self-limited HCV infection. IFN-γ production after 5 h stimulation with peptide NS5 2416 was tested in CD8+ T cells without prior peptide stimulation (left), as well as after 14 days peptide stimulation (right). (c) Course of infection and NS5 2416-specific CD8+ T-cell response in patient A1. At the time points marked by +, the autologous sequence was shown to be identical to the consensus sequence. (d) NS5 2416-specific CD8+ T-cell response in patients with chronic HCV infection. IFN-γ production after 5 h stimulation with peptide NS5 2416 was tested in CD8+ T cells without prior peptide stimulation (left), as well as after 14 days peptide stimulation (right). Four of 14 patients (C1–C8, genotype 1; C9–C14, genotype 3) targeted the epitope, but responses were detectable only after 14 days peptide stimulation.
Fig. 2. Viral sequences corresponding to HLA-A26-restricted epitopes. (a) Genotype 1a-infected patients. (b) Genotype 1b-infected patients. Patients with a positive response to the respective epitope are marked by an asterisk.
indicating that the NS5 2416 epitope is recognized frequently in the background of HLA-A26 + patients. Interestingly, this response was detectable directly without previous peptide-specific stimulation in patients with acute, resolving infection, but only after peptide-specific stimulation in patients with chronic HCV infection; in addition, peptide-specific cell lines remained reproducibly negative in patient A1, despite the presence of a detectable response directly ex vivo. These results support previous findings that some HCV-specific T-cell responses display an impaired proliferative capacity (Urbani et al., 2002; Wedemeyer et al., 2002).

Next, we studied the autologous viral sequences corresponding to the four novel epitopes in HLA-A26 + patients with chronic HCV infection and compared these with the sequences of 66 HLA-A26 − patients (genotype 1a; Fig. 2a) and 103 sequences from the Los Alamos HCV sequence database (genotype 1b; Fig. 2b) (http://hcv.lanl.gov; Kuiken et al., 2005). Strikingly, no viral sequence variations were detected within two epitopes, NS3 1655 and the frequently recognized epitope NS5 2416, in the nine HLA-A26 + patients studied (Fig. 2), indicating that viral escape had not taken place, even in two patients with CD8 + T-cell responses against epitope NS5 2416 (marked with an asterisk in Fig. 2). With respect to the latter epitope, the genotype 1b consensus sequence varies from the genotype 1a consensus sequence by a T2425S variation in the C-terminal flanking region of the epitope (compare Fig. 2a, b). However, the same polymorphism is also observed in genotype 1a and tends to be more frequent in subjects expressing the HLA-A26 allele (HLA-A26 + patients, three of six; HLA-A26 − patients, 11 of 66; P = 0.083). An impairment of proteasomal processing of the epitope through this polymorphism cannot be excluded completely, as has been described previously for an HLA-A2-restricted HCV-specific epitope (Seifert et al., 2004). However, the fact that patients infected by HCV genotype 1b (e.g. patients A3 and C6) also target this epitope argues strongly against a significant impairment of antigen processing by this polymorphism. We also analysed the evolution of this epitope region during acute HCV infection in a patient with intravenous drug use who was HCV RNA PCR-positive before the onset of clinical signs and the alanine aminotransferase (ALT) peak. Importantly, no variation of this epitope was observed in the presence of a strong CD8 + T-cell response during the 7 weeks of documented viraemia (Fig. 1c).

Interestingly, both epitopes without sequence variations, NS3 1655 and the frequently recognized NS5 2416, are located at cleavage sites of the NS3/4A protease, between NS3 and NS4A and NS5A and NS5B, respectively. Of note, only one HCV-specific CD8 + T-cell epitope has previously been located directly within a cleavage site (Los Alamos HCV Immunology Database; http://hcv.lanl.gov), an HLA-B37-restricted T-cell epitope within the NS4B/5A cleavage site (Lechner et al., 2000). The viral NS3/4A serine protease recognizes a distinct pattern of amino acid residues at positions P6 (d/e), P1 (t/c) and P1′ (s/a) with respect to the cleavage site (Grakoui et al., 1993) (see Supplementary Fig. S1, available in JGV Online). Thus, a high degree of conservation at these sites is needed for correct polypeptide processing. Indeed, most mutations at these positions abolish cleavage completely, as has been shown elegantly by introducing experimental mutations (Bartenschlager et al., 1995; Kolykhhalov et al., 1994) (Supplementary Fig. S1). Importantly, the NS5A/5B cleavage site is most sensitive to mutagenesis compared with the other cleavage sites of the NS3/4A serine protease (Kolykhhalov et al., 1994). In addition, the viral sequence of this epitope is highly conserved in all HCV genotypes. For example, the genotype 3a consensus sequence is identical to that of genotype 1 except for a D2416S substitution. However, these two peptide variants were highly cross-reactive (data not shown).

Taken together, our findings suggest that strong functional constraints in the NS3/4A and especially in the NS5A/5B cleavage sites may prevent mutational escape in HLA-A26-restricted epitopes, and illustrate the limits of virus evolution in the presence of selection pressure by a strong and functionally intact immune response. This supports the hypothesis that selection towards a consensus sequence providing optimal viral fitness is a major player acting as a driving force against evolution of a highly mutable RNA virus (Altman & Feinberg, 2004). It is important to note that, in addition to the hypothesis suggested above, host factors, such as the genetic restriction of the immune response (Neumann-Haefelin et al., 2006), T-cell receptor diversity (Meyer-Olson et al., 2004) or strong CD4 + T-cell help (Grakoui et al., 2003), may also contribute to the observed lack of viral escape. However, in the presence of viral factors constraining evolution of viral mutations CD8 escape will not occur, despite a strong and functional virus-specific CD8 + T-cell response. These findings have important implications for vaccine design, as the identification of CD8 + T-cell epitopes in a virus is an important prerequisite for the development of an effective therapeutic vaccine. Targeting of conserved viral regions with strong functional constraints should result in stable control of virus replication.

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