It has been well established that immunological escape mutations within the hepatitis C virus genotype (gt) 1a non-structural (NS) 3/4A protease are partly prevented by a reduction in viral protease fitness. Surprisingly little is known about whether similar mutations affect proteases from other genotypes. In the present study, we assessed both the HLA-A2-restricted CTL response and gt3a NS3/4A protease fitness. Similar to gt1, the 1073–1081 epitope was immunodominant within the gt3a-specific HLA-A2-restricted CTL response, despite sequence similarity of only 56% between the gt1a and gt3a genes. However, unlike the gt1a NS3/4A protease, all residues within the gt3a 1073–1081 epitope could be replaced sequentially by alanine while retaining protease activity, at least in part.
et al., 2011) and HLA-B08- (Salloum et al., 2008) restricted epitopes. Interestingly, it has been observed that gt1a and gt3a exhibit different viral polymorphisms (Rauch et al., 2009), so we wanted to extend our previous study to investigate gt3a NS3 protease fitness.

Venous blood samples were obtained in EDTA tubes from two chronically gt3a HCV-infected patients with undetermined HLA type at University College Dublin, Dublin, Ireland. One patient subsequently responded to treatment (SR gt3a; GenBank accession no. JQ514562), whereas the other did not (NR gt3a; JQ514563). Six- to 12-week-old inbred HHD (HHD\(^+\) H-2D\(^b\)\(^/-\) β2m\(^-/-\)) mice transgenic for the HLA-A2.1 monochain histocompatibility class I molecule were kindly provided by Dr F. Lemonnier, Institut Pasteur, France. The local ethical committee approved all sampling and analysis of human samples, and the animal ethics committee approved all experimental protocols. Groups of nine mice were anaesthetized using Hypnorm (VetaPharma) and immunized with 100 μg plasmid DNA suspended in reverse-osmosis water by needle injection in the tibialis cranialis muscle (Söderholm et al., 2006). At week 4, a booster dose of 100 μg plasmid DNA was given. The mice were sacrificed and the spleens were harvested at week 6. RMA-S (H-2\(^b\)) and RMAS-HHD cells (HHD\(^+\) H-2D\(^b\); kindly provided by Dr F. Lemonnier) were maintained as described previously (Söderholm et al., 2006).

Full-length gt3a NS3/4A genes were isolated and cloned as described previously (Söderholm et al., 2006). Plasmids expressing the gt1a wild-type gene and 1073–1081 alanine mutants have been described elsewhere (Söderholm et al., 2006). Peptides corresponding to HLA-A2 epitopes of the cytomegalovirus (CMV) pp65 protein (sequence NLVPVMATV) or the HCV NS3 sequences were synthesized by using an automated synthesizer with 9-flourenylmethoxycarbonyl-protected amino acids as described previously (Sällberg et al., 1991). Mutant plasmids in which residues were sequentially replaced by alanine were generated by site-directed mutagenesis; Invitrogen) using primers containing the specific mutation (Söderholm et al., 2006). The ability of NS3/4A-derived HLA-A2 peptides to bind HHD molecules was determined in an RMAS-HHD stabilization assay (Ljunggren et al., 1990; Söderholm et al., 2006). Murine NS3/4A-specific CTLs were detected as described previously (Söderholm et al., 2006). In brief, 5-day restimulation cultures were set up with 2.5 × 10\(^7\) pooled splenocytes from immunized mice, an equal number of irradiated (20 Gy) syngeneic splenocytes and 0.05 μM specific peptide. CTLs were detected in a 4-h \(^{51}\)Cr-release assay using specific peptide-pulsed RMAS-HHD target cells. Specific lysis (%) was calculated as [(sample c.p.m.− spontaneous lysis control c.p.m.)/(maximum lysis control c.p.m.− spontaneous lysis control c.p.m.)]×100. Functionality of the NS3/4A protease was analysed by an in vitro transcription and translation assay (TNT; Promega) of gt3a or gt1a plasmids for 90 min at 30 °C in the presence of \(^35\)S-methionine and separated on a 10% SDS-PAGE gel, as described previously (Frelin et al., 2003, 2004; Söderholm et al., 2006). Functional NS3 protease generated two protein bands, a free NS3 protein and an uncleaved NS3–NS4A fusion protein, whereas a non-functional protease only generated the NS3–NS4A fusion protein band (Söderholm & Sallberg, 2006). The bands were quantified using ImageJ 1.45s software (National Institutes of Health) (Abramoff et al., 2004).

Most studies on HCV immune responses are based on gt1 reagents (Bowen & Walker, 2005). Less is known about the immune response towards epitopes from other HCV genotypes. Numerous studies have shown the NS3/4A protease from HCV gt1 strains to possess key properties when establishing and/or maintaining chronicity (Duong et al., 2004; Foy et al., 2003; Gale & Foy, 2005; Li et al., 2005). In addition, we have also shown hepatic expression of NS3/4A to prevent lethal TNF-α-mediated liver disease (Frelin et al., 2006) by interfering with intrahepatic IFN-γ production (Brenndörfer et al., 2012). Based on these findings, it would be important to determine whether the NS3/4A proteases of other genotypes display the same characteristics as the gt1a protease. The NS3/4A genes from two gt3a patients were cloned and sequenced. These sequences were used to map the gt3a NS3 gene for HLA-A2-restricted epitopes (Fig. 1) using 20-mer peptides with 15-residue overlaps spanning the complete SR gt3a NS3 sequence. HLA-A2-binding peptide sequences were identified by the surface stabilization of HLA-A2 molecules on RMAS-HHD cells. By this approach, we could identify nine HLA-A2-binding regions (Fig. 2a). In order to fine-map these regions for HLA-A2 binding, we synthesized 10-mer peptides, with nine-residue overlaps, spanning the 20-mer HLA-A2 binding sequences (two of nine regions shown in Fig. 2b, c). To elucidate HLA-A2 binding further, we generated 9-mer peptides of the positive 10-mer peptides (data not shown), thus identifying 29 9- or 10-mer peptides that tentatively constitute CTL epitopes (Figs 1 and 2d). Four of these sequences corresponded to the locations of previously identified gt1 NS3 HLA-A2-binding peptides (GLFGTIVTS\(^{1038-1047}\), TVGGVMVT\(^{1073-1081}\), YLIRTDADV\(^{1131-1139}\), and VMCPAGHAV\(^{1169-1177}\), Söderholm & Sallberg, 2006). Almost all of the NR gt3a peptide sequences corresponding to the SR gt3a HLA-A2-binding peptides showed similar HLA-A2-binding ability to the corresponding SR gt3a peptide, except the NR gt3a GLFGTIVTS\(^{1038-1047}\) epitope, which showed drastically better HLA-A2 binding compared with the SR gt3a GLFGTIVTS\(^{1038-1047}\) epitope (data not shown).

To test whether the identified CTL epitopes were recognized by a primed immune response, we evaluated the peptides using T-cells from immunized HLA-A2-Tg mice. The immunodominance of the identified CTL epitopes was determined using standard techniques in HHD mice immunized with a plasmid expressing the SR gt3a NS3/4A gene (Söderholm et al., 2006). In this way, we showed that the previously observed immunodominance of the CINGCVWT\(^{1073-1081}\) epitope seen in HHD mice immunized with a gt1a-based NS3/4A DNA vaccine (Söderholm et al., 2011) and HLA-B08- (Salloum et al., 2008) restricted epitopes. Interestingly, it has been observed that gt1a and gt3a exhibit different viral polymorphisms (Rauch et al., 2009), so we wanted to extend our previous study to investigate gt3a NS3 protease fitness.
et al., 2006) was maintained using the gt3a sequence (Fig. 2d), despite only 56% (five of nine amino acids) sequence identity (Fig. 1). Responses to other putative HLA-A2-restricted gt3a epitopes were absent (Fig. 2d), suggesting that the TVGGVMWTV1073–1081 epitope is also immunodominant within the HCV NS3/4A gt3a-specific HLA-A2-restricted immune response. The TVGGVMWTV1073–1081 epitope has been shown previously to be CD8+ T-cell reactive in HLA-A2 patients infected with gt3a (Giugliano et al., 2009) and, in agreement with previous studies (Fytili et al., 2008; Giugliano et al., 2009), we did not detect any cross-genotype CD8+ T-cell recognition between gt1a and gt3a in CTLs.

Fig. 1. Amino acid sequence alignment of cloned gt3a sequences from a patient responding to treatment (SR) and a patient not responding to treatment (NR) with an HCV gt1a amino acid sequence (GenBank accession no. ACD13390.1). Underlined sequences indicate HLA-A2-binding peptides. The dotted line indicates the immunodominant gt1a and gt3a HLA-A2-restricted epitope.
Fig. 2. (a) Mapping of HLA-A2-binding peptides using 20-mer peptides with 15-residue overlaps spanning the complete HCV NS3 SR gt3a sequence. Values are percentages of the positive control, calculated as [fluorescence intensity of peptide−fluorescence intensity of negative control]/[fluorescence intensity of positive control peptide (CMV)−fluorescence intensity of negative control]×100. Above 25 % binding compared with the positive control was considered HLA-A2 binding. (b, c) Fine mapping of four gt3a HLA-A2-binding 20-mers within two HLA-A2-binding regions. (d) Immunodominance of the TVGGVMWTV1073−1081 epitope in HHD mice immunized twice with the SR gt3a NS3/4A plasmid. Splenocytes were restimulated and CTL activity was measured using peptide-loaded RMAS-HHD target cells using different effector:target (E:T) ratios. Data are given as percentages of specific lysis.
Fig. 3. (a) In vitro transcription and translation analysis of gt1a and SR gt3a plasmids in which each of the residues of the 1073–1081 epitope was replaced individually by alanine. The upper band corresponds to the NS3–NS4A polyprotein, whereas the lower band is cleaved NS3 protein. Each band was quantified using ImageJ (Abramoff et al., 2004). (b) Binding of the 1073–1081 alanine-substituted gt3a peptides to the HLA-A2 molecule. Values are presented as percentage binding relative to the original TVGGVMWTV1073–1081 peptide. (c) Polymorphisms within the 1073–1081 epitope for gt3a and gt1a. GenBank accession numbers for gt3a are indicated; GenBank accession numbers for 148 gt1a isolates are given elsewhere (Söderholm & Sallberg, 2006).
from gt3a-vaccinated mice after stimulation using a peptide corresponding to the gt1a CINGVCWTV\textsuperscript{1073–1081} sequence (data not shown).

As the 1073–1081 sequence also seems to be immunodominant within the gt3a NS3/4A gene, we were interested to investigate whether the suggested immune-escape mutations seen for the gt1a protease (Söderholm et al., 2006) could possibly mediate corresponding immune escape within gt3a and whether those mutations also exhibited negative effects on gt3a NS3/4A protease fitness. Each residue of the 1073–1081 epitope in the full-length SR gt3a NS3/4A was sequentially replaced with alanine using \textit{in vitro} mutagenesis. The mutant plasmids were subsequently tested for protease activity as determined by proteolytic cleavage at the NS3–NS4A junction after \textit{in vitro} transcription and translation. Interestingly, by this approach, we could show that all epitope residues could be replaced with alanine without adversely affecting NS3 protease activity against the NS3–NS4A junction (Fig. 3a). This contrasts with the gt1a NS3 gene, where A\textsuperscript{1074} and A\textsuperscript{1079} abolished NS3 protease activity almost completely. Thus, it seems that the gt3a NS3/4A protease exhibits higher plasticity in comparison with the gt1a protease. This could be explained, at least in part, by the higher wild-type NS3 protease activity for gt3a in comparison with gt1a (Fig. 3a). However, as we have shown previously, both gt1a N\textsuperscript{1075} to A\textsuperscript{1075} and G\textsuperscript{1076} to A\textsuperscript{1076} substitutions affected NS3–NS4A proteolytic cleavage only moderately (Fig. 3a) but were lethal for replication when introduced into the subgenomic HCV reporter replicon (Söderholm et al., 2006). This suggests that these substitutions affect other aspects of the NS3 protease that are important for HCV replication and that are not detected by the NS3–NS4A proteolytic cleavage assay. The lack of a gt3a \textit{in vitro} replication system limits our ability to study the V\textsuperscript{1074} to A\textsuperscript{1074} substitution, for example, with regard to viral replication. When analysing substitutions in the 1073–1081 epitope with respect to HLA-A2 binding, the same residues (1074, 1081 and possibly 1079) as for gt1a were also identified as essential for TVGGVMWTV\textsuperscript{1073–1081} HLA-A2 binding (Fig. 3b). In agreement with the higher possible plasticity seen within the immunodominant HLA-A2-restricted epitope of gt3a NS3, greater polymorphism within the 1073–1081 protein sequence was observed for 36 gt3a 1073–1081 sequences (SR gt3a, NR gt3a and 34 sequences from GenBank; Fig. 3c) compared with 148 gt1a sequences (Söderholm & Sallberg, 2006). However, the V\textsuperscript{1074} to I\textsuperscript{1074} or L\textsuperscript{1074} substitutions are conserved and, at least for gt1, the I\textsuperscript{1074} to V\textsuperscript{1074} substitution does not affect the immune response seen in immunized HHD mice (Söderholm et al., 2006). Also, the gt1a A\textsuperscript{1081} substitution was only seen in one of ten isolates from an HLA-A2-negative patient (Söderholm et al., 2006). Interestingly, and in agreement with these findings, gt3a NS3 protease HLA-associated viral escape has been reported for T\textsuperscript{1073} (HLA-A24) and V\textsuperscript{1135} (HLA-A3), whereas it was not seen for gt1a (Rauch et al., 2009).

The observations described above suggest that mutations within one genotype do not accurately predict effects in another HCV genotype. Despite the higher polymorphism seen for gt3a, we can only speculate as to why the putative HLA-A2 escape mutations (A\textsuperscript{1074}, A\textsuperscript{1079} or A\textsuperscript{1081}) have not been reported so far. This might be explained by inherent differences in viral properties for the gt3a virus compared with the gt1a virus; for example, the slightly higher NS3 protease activity observed in gt3a (Fig. 3a) could possibly allow the establishment of chronic gt3a infections without the need for immune escape. If so, the lesser need for the virus to adapt to the host could help in part to explain the better treatment response seen for gt3a patients, if the gt3a virus is less likely to have an optimal IFN-α immune escape phenotype. Also, these substitutions could affect other functions of the NS3 protease not measured in the present study. The higher plasticity and polymorphism seen for the gt3a immunodominant HLA-A2-restricted epitope of NS3 could possibly lead to an increased problem with resistance mutations for both the development of gt3a NS3 protease inhibitors and gt3a NS3/4A-based therapeutic vaccines compared with gt1-specific treatments. However, this study only investigated the immunodominant HLA-A2-restricted epitope in NS3/4A plasmid-immunized HHD mice, and more studies investigating the behaviour of the gt3a virus during both the acute and treatment response phases are needed.

In conclusion, there seems to be greater possible variability for mutations within the NS3 protease in gt3a compared with gt1a, although the location and hierarchy of T-cell epitopes are maintained from an immunological standpoint. Thus, the relationship between gains and losses for the virus and its fitness in adapting to a new environment seems to be highly complex, suggesting that each genotype should be studied separately in detail.

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