Hepatitis C virus (HCV) infects 2–3% of the world population and is a leading cause of liver disease (Freeman et al., 2001; Zhou et al., 2014). Early in infection the host immune system responds by producing neutralizing antibodies (Terilli et al., 2012). Multiple linear epitopes within the 27 aa hypervariable region 1 (HVR1), in the N terminus of the E2 envelope protein, play between viral diversity and suboptimal immunity. Viruses with hypervariable genomic regions evade host humoral immune response by several mechanisms (Brown et al., 2005; Quaranta et al., 2012; Thimme et al., 2006). The best understood mechanism for viral immune escape is single-point mutation which results in non-synonymous changes within the immunodominant viral envelope glycoprotein and NS3 (Cox et al., 2005; Ray et al., 2005; Thimme et al., 2006, 2012). Multiple linear epitopes within the 27 aa hypervariable region 1 (HVR1), in the N terminus of the E2 envelope protein, have been identified as the principle target of neutralizing antibodies (Ball et al., 2014; Fafi-Kremer et al., 2012; Tarr et al., 2015). Antibodies specific for epitopes within HVR1 have been reported to inhibit the binding of the E2 glycoprotein to cells and to block HCV infectivity in vitro and in vivo (Farcı et al., 1996; Haberszter et al., 1998; Ossianaka et al., 2001). However, HCV pseudoparticle and cell-culture-derived HCV experiments have shown poor cross-neutralization potential of isolate-specific neutralizing antibody response to HVR1 (Brown et al., 2005; Cashman et al., 2014; Larrubia et al., 2014). Cytotoxic T-lymphocytes drive evolution of the HVR1, which can lead to the emergence of escape variants (Cox et al., 2005; Ray et al., 2005). However, there is an absence of direct in vivo evidence of humoral immune escape by host-derived antibodies and viral glycoproteins (Chung et al., 2013).

Previous research from our group has observed, over a near 10 year period, the emergence, dominance and disappearance of distinct but related lineages (L1 and L2) in a treatment-naive patient chronically infected with HCV genotype 4a (Palmer et al., 2014). L1 dominated the virome for the first 8 years of the sampling period prior to population collapse and this led to the concomitant rise to prominence of L2. During the initial dominance of L1, IgG targeting of L1 was detected in five of the first seven samples which, in part, contributed directly to the extinction of this group of variants (Palmer et al., 2012, 2014). In spite of the near total dominance of L2 sequences in later samples (96.9 and 99.9% at T9 and T10, respectively; see Fig. 1 for details of sampling times), no IgG targeting of L2 virions was detected in this previous study (Palmer et al., 2014). Furthermore, the HVR1 of L2 variants remained predominantly under purifying selection across the 10 year period with a single principle HVR1 amino acid variant persisting during this time. Follow-up clonal analysis 1 year later (T11) revealed that a HVR1 variant with a single-point mutation had superseded the principle variant. There was no antibody-associated virus (AAV) found at T11 (Palmer et al., 2014) (Fig. 1). Fig. 1 summarizes the AAV profile of all samples analysed.
In the current follow-up study to Palmer et al. (2014) we mapped a further four serum samples T12–T15 that extended the sampling period to 13 years (Fig. 2). Serum samples were obtained from a treatment-naive patient. This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospital and written consent from the patient was obtained. The clonal sequence analysis of these samples identify the continued dominance of L2 sequences. The constituent virions were partitioned into antibody-free virus (AFV) and AAV fractions, as described previously (Moreau et al., 2008; Palmer et al., 2014). Of these samples, only T13 contained detectable levels of AAV (GenBank accession numbers KT595222 and KT595223) (Fig. 1). The antibody–virus complex of this fraction was dissociated and disruption of the virion was achieved by treatment with proteinase K (5 mg ml⁻¹) for 2 h at 37 °C with end-over-end mixing followed by overnight incubation at room temperature. Confirmation of the virus-free status of this proteinase K-treated T13 antibody (T13Ab) preparation was determined by the absence of an E1E2-specific amplicon following reverse transcription PCR; the virus-free T13Fab fragment was designated VF-T13Fab.

T11 had three different HVR1 amino acid variants, i.e. H₃₉₅-X-X-X-F₃₉₉, N₃₉₅-X-X-X-F₃₉₉ and N₃₉₅-X-X-X-L₃₉₉ (subscript numbering identifies specific amino acid positions within the 27 aa HVR1 with reference to GenBank accession number NC_004102). The presence of HVR1 variant H₃₉₅-X-X-X-F₃₉₉ in the AFV fraction of T12 was confirmed by amplicon sequencing only. The subsequent AFV fraction of T13 also had the H₃₉₅-X-X-X-F₃₉₉ amino acid profile (frequency 0.40). However, the N₃₉₅-X-X-X-L₃₉₉ variant was now dominant in T13 (frequency 0.60). Interestingly, the predicted HVR1 sequence from AAV RNA detected at T13 indicated N₃₉₅-X-X-X-L₃₉₉ motif targeting (Fig. 2). This Leu-containing motif was isolated in the succeeding samples, i.e. T14 and T15 (Fig. 2). AAV was not detected in samples T11, T12, T14 and T15. However, in order to exclude the possibility that the P2 variant motif containing N₃₉₅-X-X-X-L₃₉₉ was not accessible to antibodies, we similarly tested VF-T11Fab, VF-T12Fab and VF-T15Fab for binding affinity to the HVR1 peptide variants. A recent study by Guan et al. (2012) showed that neutralization epitopes can be between amino acid positions 16 and 24 (i.e. 399–407) in HVR1. It is interesting to note that

![Fig. 1. Ultra-deep pyrosequencing (UDPS) and clonal analysis of serum samples over 13 years. All the samples were screened for the presence or absence of AAV. The y-axis depicts the percentage of lineage L2 in all the samples in the study. The x-axis demonstrates antibody response to L2 over the period of 13 years. The black columns indicate dominance of L1, grey columns indicate dominance of L2. The plus sign in the bottom row at 11.6 years indicates detectable levels of T13Ab to L2. Neither clonal nor UDPS data is available for T12.](image-url)
in our study, amino acid variation was observed only at amino acid positions 395 and 399 within the entire HVR1. Based on the Guan et al. (2012) data, we hypothesized that the HVR1 variants with N_{395}-X-X-F_{399} (T11-AFV) and H_{395}-X-X-F_{399} (T13-AFV) motifs were potential humoral immune escape mutants which have Phe at position 399. To confirm the escape phenotype hypothesis, three N-terminally His_{6}-tagged, 27 aa HVR1 peptides were synthesized, i.e. P1 (H-HHHHHHTHTGAVASSNAQKFLTSLFTFGP-QQN-OH), P2 (H-HHHHHHTHTGAVASSNAQKFLTSLFTFGPQQN-OH) and P3 (HHHHHHHTHTGAVASSHAKFSLFTFGPQQN-OH) (Pepscan Presto), where underlining indicates the His_{6}-tag and bold indicates the variant amino acid at position 395 or 399. The HVR1 sequence of P1 and P3 corresponded to the dominant L2 HVR1 variant for the initial 10 years of in vivo infection for which no AAV was detected (Palmer et al., 2014). The P2 sequence corresponded to the predicted HVR1 of AAV RNA isolated at T13. Peptides were reconstituted in 100 % DMSO at a concentration of 1 mg ml^{-1} and stored at −20 °C. Peptide (100 ng µl^{-1}) was used in an ELISA-based method. These peptides were incubated with VF-T11Fab, VF-T12Fab, VF-T13Fab and VF-T15Fab at 1:10 dilution for 1 h followed by incubation with anti-human IgG (H&L)–HRP conjugate secondary antibody (Promega) at 1:5000 dilution for 1 h (Fig. 3). The ELISA results confirmed that the peptide containing the N_{395}-X-X-L_{399} mutation was recognized by VF-T13Fab. The binding phenotype of VF-T13Fab had the strongest affinity to P2 (P2>>P1>>P3) with Leu at position 399, compared with the predicted escape variant Phe in vitro (P = 0.06, Kruskal–Wallis test using Prism 4; GraphPad) (Fig. 3). Our results prove that, in this case, a naturally occurring single amino acid change to Phe in the HVR1 alone at position 399 can drive humoral immune escape after >10 years of immune silence. Our results demonstrated that none of VF-T11Fab, VF-T12Fab and VF-T15Fab bound to the HVR1 peptide variants P1–P3.

In our current study, analysis of serum samples over a 13 year period showed two distinct periods when AAVs were present. A window period of 5 years existed between the two periods during which AAVs were detectable. The antibody specificity of the latter time point, i.e. T13, targeted a different HVR1 lineage from that found previously (Palmer et al., 2012, 2014). The HVR1 variant captured by the T13Ab was first observed in pyrosequencing data at T10 (1.1 %) (Palmer et al., 2014). A further 2 years elapsed before T13Ab to this latter variant was detected (Fig. 2).

Interestingly, the HVR1 genomic sequence associated with virus captured by T13Ab was found to be present in the subsequent samples, i.e. T14 and T15. Analysis of samples T14 and T15 revealed that the T13Ab response was not sustained to detectable levels. The loss of neutralization antibodies is a recognized feature of the natural history of HCV infection (Shimizu et al., 1994). Additionally, it is also recognized that a sustained antibody response is likely a prerequisite for complete removal of viral variant(s).
The absorbance obtained by subtracting the three independent experiments. The P2 and baseline activity towards P3. The data were obtained from ary antibodies. VF-T13Fab showed the strongest affinity towards tides with only primary antibodies and peptides with only second-

of T11, and the P2 variant was observed in the AAV fraction (see text). The P1 and P3 variants were observed in the AFV fraction.

Fig. 3. ELISA-based detection of the binding of the VF-T13Ab to the predicted HVR1 derived His<sup>6</sup>-tag peptide epitopes (P1–P3, see text). The P1 and P3 variants were observed in the AFV fraction of T11, and the P2 variant was observed in the AAV fraction of T13. The control reference points included only peptides, peptides with only primary antibodies and peptides with only secondary antibodies. VF-T13Fab showed the strongest affinity towards P2 and baseline activity towards P3. The data were obtained from three independent experiments. The x-axis indicates the peptide used in the ELISA, i.e. P1, P2 and P3. The y-axis indicates the absorbance obtained by subtracting the $A_{560}$ reading from the $A_{560}$. *P = 0.06.

notable absence of a sustained and neutralizing antibody response (in T14 and T15), and likely superiorit, explains why the motif (N<sub>395</sub>-X-X-X-L<sub>399</sub>) persisted in subsequent samples.

In conclusion, our proof-of-concept study has confirmed that antibodies were naturally generated against a discrete viral variant (Fig. 3). We additionally confirmed that naturally occurring amino acid variations in this epitope representa mechanism by which HCV escapes humoral immunity.

Acknowledgements

This work was funded by Molecular Medicine Ireland as a part of the Clinical & Translational Research programme. We would also like to thank John Levis and Dr Kevin Hegarty for initial characterization of patient sera.

References


