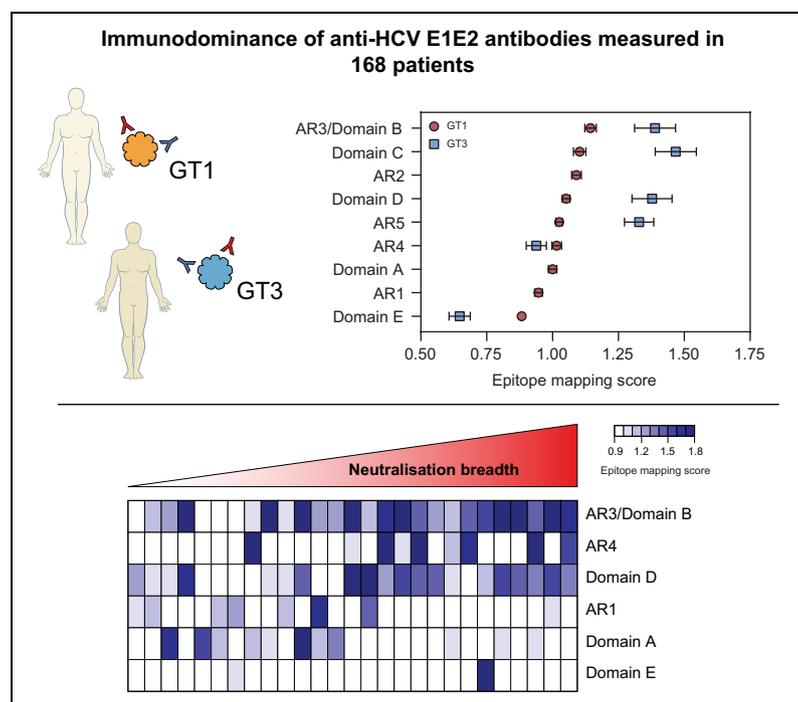# B cell immunodominance in primary hepatitis C virus infection

Graphical abstract

Authors

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Lay summary

Neutralising antibodies will likely form a key component of a protective hepatitis C virus vaccine. In this work we characterise the predominant neutralising and non-neutralising antibody (epitope) targets in acute hepatitis C virus infection. We have defined the natural hierarchy of epitope immunodominance, and demonstrated that viral genotype can impact on this hierarchy. Our findings highlight key epitopes that are associated with broadly neutralising antibodies, and the deleterious impact of mounting a response towards some of these domains on neutralising breadth. These findings should guide future efforts to design immunogens aimed at generating neutralising antibodies with a vaccine candidate.

Highlights

- Neutralising antibodies will likely form a key component of an HCV vaccine.

- We characterise the immunodominance of neutralising and non-neutralising epitopes in primary HCV infection.

- We identify that virus genotype might influence the epitopes targeted.

- We also identify that certain epitope target combinations are associated with greater breadth.

B cell immunodominance in primary hepatitis C virus infection

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See Editorial, pages 604–606

Background & Aims: Neutralising antibodies (NAbs) play a key role in clearance of HCV. NAbs have been isolated and mapped to several domains on the HCV envelope proteins. However, the immunodominance of these epitopes in HCV infection remains unknown, hindering efforts to elicit optimal epitope-specific responses. Furthermore, it remains unclear which epitope-specific responses are associated with broad NAb (bNAb) activity in primary HCV infection. The aim of this study was to define B cell immunodominance in primary HCV, and its implications on neutralisation breadth and clearance.

Methods: Using samples from 168 patients with primary HCV infection, the antibody responses targeted 2 immunodominant domains, termed domains B and C. Genotype 1 and 3 infections were associated with responses targeted towards different bNAb domains.

Results: No epitopes were uniquely targeted by clearers compared to those who developed chronic infection. Samples with bNAb activity were enriched for multi-specific responses directed towards the epitopes antigenic region 3, antigenic region 4, and domain D, and did not target non-neutralising domains.

Conclusions: This study outlines for the first time a clear NAb immunodominance profile in primary HCV infection, and indicates that it is influenced by the infecting virus. It also highlights the need for a vaccination strategy to induce multi-specific responses that do not target non-neutralising domains.

Lay summary: Neutralising antibodies will likely form a key component of a protective hepatitis C virus vaccine. In this work we characterise the predominant neutralising and non-neutralising antibody (epitope) targets in acute hepatitis C virus infection. We have defined the natural hierarchy of epitope immunodominance, and demonstrated that viral genotype can impact on this hierarchy. Our findings highlight key epitopes that are associated with broadly neutralising antibodies, and the deleterious impact of mounting a response towards some of these domains on neutralising breadth. These findings should

guide future efforts to design immunogens aimed at generating neutralising antibodies with a vaccine candidate.

Introduction

Induction of neutralising antibodies is a major goal in viral vaccine design. For highly mutable RNA viruses such as HIV, HCV and influenza, broadly neutralising antibodies (bNAb) are thought to be required if the vaccine is to protect against the diverse strains in circulation, as well as mutants generated within the host under immune pressure. Understanding the mechanisms underlying the development of bNAb in the context of these human viral infections is therefore essential for the rational design of bNAb-inducing vaccines.[1]

Neutralising antibodies generated against HCV are primarily directed towards the surface envelope glycoprotein E2,[2–8] but a few NAbs targeting E1, or the E1/E2 heterodimer have also been identified.[9,10] Extensive efforts to characterise the specificity of these NAbs have defined multiple epitopes within the surface proteins. The best characterised immunogenic epitopes are domains A-E, and antigenic regions (AR) 1-5. Residues that define these epitopes are usually determined by alanine scanning mutagenesis studies, which have revealed partial overlap between some sites such as domain B and AR3 (Table S1). Domains D and E (also termed epitope I) are defined by linear E2 peptides corresponding to sites 428-448 and 412-423, respectively, while the other domains are all defined by non-continuous sites. The neutralising activity of monoclonal Abs (mAbs) that bind these epitopes varies widely: domain A and AR1 specific antibodies have limited to no neutralising activity, while domain C and AR2 antibodies have neutralising activity restricted to certain genotypes/isolates.[5,11] AR3-, AR4-, domain D and domain E-binding mAbs have particularly broad neutralisation activities across multiple genotypes.[3,10]

A major challenge in directing antibody responses towards these epitopes is that little is known about their immunodominance hierarchy in natural HCV infections. The domains of some of the most potent bNAbs were found to be rarely targeted in natural HCV infections,[12] which suggests that such viral domains are naturally shielded from the immune system. This infers a difficult path towards the development of immunogens which elicit responses against these epitopes in a population-level

vaccine strategy, despite their appealing neutralisation potential. The hypervariable region 1 (HVR1) within E2 forms an immunodominant epitope in HCV infection, however antibodies that target this region are highly strain specific. Furthermore, HVR1 is prone to high levels of mutation, driving immune escape,[13] making it unsuitable for vaccine design. Characterising the immunodominance hierarchy of epitopes with favourable neutralising potential is therefore required to understand what constitutes protective immunity to HCV.

Understanding the functional consequences of variations in the immunodominance hierarchy is essential for the rational design of vaccine candidates that generate protective immunity. While early induction of NAb responses is associated with HCV clearance,[14,15] it remains unclear whether these responses are the result of unique antibody specificities. Indeed, until recently,[16,17] no antibodies had been isolated from patients that cleared HCV infection, hindering such analyses. In addition, while the neutralising activity and breadth of mAbs with unique specificities have been analysed *in vitro*, which responses are associated with potent and broad neutralisation of HCV at the population level remains to be resolved. Furthermore, it is unknown whether mounting an immune response towards a single epitope is sufficient, or whether multi-specific antibody responses explain the bNAb activity observed in some patients.

Here, the immunodominance of well-defined B cell epitopes was examined in a large cohort of patients with early primary HCV infection. The analysis revealed a clear immunodominance hierarchy across the cohort, but also responses unique to individual patients. While this hierarchy did not associate with the clearance or chronic infection outcomes, analysis of patients infected with different HCV genotypes indicated unique genotype-specific responses. Finally, the analysis revealed that mounting an immune response towards 2 or more neutralising domains was associated with increased breadth of the polyclonal response. In contrast, mounting an immune response of similar magnitude towards non-neutralising domains was associated with narrow neutralising activity. These findings highlight the need to engineer potential HCV vaccine(s) so that immune responses are generated towards select immunodominant epitopes.

Patients and methods
Study participants
Blood samples were available from the H̲epatitis C̲ I̲ncidence and T̲ransmission S̲tudies in prisons and in the community (HITS-p and HITS-c),[18,19] which were prospective cohorts of HCV seronegative and RNA negative people who inject drugs in New South Wales, Australia. Participants were recruited between 2005 and 2014, tested every 3 to 6 months for HCV seroconversion, and then followed regularly post infection until clearance or persistence, in which case antiviral treatment was offered to those chronically infected. An early infection case was defined by the availability of at least 1 viraemic sample prior to seroconversion. For this study, participants were included only if they met the following criteria: i) had samples collected during primary HCV infection, ii) were not treated prior to sample collection, and iii) were sampled within 9 months of the estimated date of infection. Ethical approvals were obtained from the research ethics committees of Justice Health (G304/11), the New South Wales Department of Corrective Services (05/0884), and UNSW Sydney (05094, 08081, 13237, 09075, 14170). The

study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Monoclonal antibodies
Representative mAbs were selected which bound antigenic regions AR1 to AR5 and domains A-E. Envelope-specific antibodies covering AR1-AR5 were sourced from Prof. Mansun Law (The Scripps Research Institute, La Jolla, California, USA). CBH-4G (domain A), CBH-5 (domain B) and CBH-7 (domain C) antibodies were purified from supernatants of hybridoma cell lines obtained from ATCC (PTA-4468, PTA-4469, and PTA-4470, respectively). Antibody HC84.26 was kindly provided by Prof. Steven Foung (Stanford University, California, USA). Antibodies HC84.27 (domain D), MAb24 and HCV1 (domain E), and mAb A8 were generated by transient transfection of FreeStyle™ 293-F cells with plasmids encoding heavy and light chains which were constructed as described previously.[20] Antibodies were biotinylated using EZ-Link® Sulfo-NHS-LC-Biotin kit (Thermo Fisher Scientific) according to the manufacturer's instructions. HC84.26 was used for all experiments with the patient plasma and as the biotinylated antibody for detection of domain D competition, but due to limited quantities of HC84.26, for some of the matrix competition assays, HC84.27, which is highly similar to HC84.26, was used (Fig. S3).

In order to minimise redundancy in the mAb panel, which would introduce a bias in the calculated epitope mapping score (EMS), mAbs were selected based on prior knowledge of unique epitope binding, but also based on cross-competition fingerprints for the mAbs. Competition between all mAbs revealed significant correlation between AR3, CBH-5 and A8, and between HCV1 and MAB24 (Fig. S8). Therefore, AR3 and HCV1 were chosen to represent AR3/domain B and domain E, respectively.

HCV neutralisation and E1E2 production
All UKN E1E2 expression plasmids were gifts from Prof. Jonathon Ball (The University of Nottingham, Nottingham, UK). A total of 11 E1/E2 expression plasmids were used to generate heterologous HCV pseudoparticles (HCVpp) from 6 HCVs,[21] as previously described.[22] These included H77.20, UKN1A20.8, UKN1B5.23, UKN2A1.2, UKN2B2.8, UKN3A1.28, UKN3A1.9, UKN3A13.6, UKN4.11.1, UKN5.14.4 and UKN6.5.340. For neutralisation assays, HCVpp was incubated for 1 h with heat-inactivated plasma at a 1:50 dilution before being added to Huh7.5 cells. Luciferase activity was measured 72 h later and compared to signals obtained from healthy control samples to determine neutralisation. Cells transiently transfected with the expression plasmids were also pelleted, and the lysate was used for E1E2 immunoassays.

To measure breadth and potency of individual plasma samples, a breadth score was calculated.[23] To that end, neutralisation activity against a specific HCVpp was given a score of 0 when neutralisation activity was below 20%, a score of 1 for 20% to 50% neutralisation, a score of 2 for 50% to 80%, and a score of 3 for >80% neutralisation. Breadth score was calculated as the sum of scores for all HCVpps assessed for that plasma sample.

Neutralisation against cell culture-derived HCV (HCVcc) was performed as previously described with GT2a Jc1Flag2 (p7-nsGluc2A) and GT3a (S52) JFH-1 chimeras.[24]

HCV E1E2 binding and competition
ELISAs were performed as described previously.[4] Briefly, microtiter plates were pre-coated with *Galanthus nivalis* lectin

(GNA), blocked with 5% non-fat dry milk before capturing GT1 (H77.20) or GT3 (UKN3A13.6) E1E2 for 1.5 h at saturating concentration. Heat-inactivated plasma was added at 1:10 to 1:1,000 dilutions and left for 1.5 h before anti-human horseradish peroxidase (HRP, Jackson Immunoresearch) was added at a 1:6,000 dilution. One hour later, bound IgG was detected using TMB Chromogen Solution (Thermofisher). The threshold for positive antibody detection was determined at 2xSD of the average signal detected using the healthy plasma. For competition, assays were performed in 5% w/v BSA (Sigma) instead of non-fat dry milk. Biotinylated mAbs were added at a concentration corresponding to 60–75% maximal binding and mAb binding was detected using Streptavidin-HRP (Dako) used at 1:6,000 dilution. The EMS was calculated as follows:

$$EMS = \frac{1}{\left(\frac{Resid_{B*Ab}}{Mean.Resid\ OD_{B*Ab}}\right)}$$

Where $Resid_{B*Ab}$ is the residual binding of a biotinylated antibody in the presence of the patient plasma, compared to no plasma. $Mean.Resid_{B*Ab}$ is the mean residual binding of all biotinylated antibodies in the presence of patient plasma, compared to no plasma.

Viral genome sequencing and analysis

Viral RNA was extracted from plasma samples and amplicons covering the E1E2-coding sequence were generated as described previously.[25,26] Next-generation sequencing of the amplicons was conducted using the Illumina MiSeq sequencing platform as previously reported.[25,27] Sequence alignments were generated against the corresponding genotype reference sequence: GT1a (GenBank accession numbers AF009606), GT1b (AJ238799), GT2b (AB030907) and GT3a (D17763). Consensus sequences were used for downstream analysis. N-linked glycosylation was predicted using the N-GlycoSite online tool, and amino acid enrichment analysis was performed using VESPA (https://hcv.lanl.gov/).

Results

Sample cohort

Data and samples were available from 2 prospective cohorts of acute HCV infection in New South Wales, Australia, recruited between 2005 and 2014 in prisons (HITS-p) and in the community (HITS-c).[18,19] Seronegative and aviraemic participants were enrolled and monitored for seroconversion at approximately 3–6 monthly intervals, and then followed intensively for at least 24 weeks after the onset of infection, when clearance or persistence was determined, and treatment was offered to those with chronic infection. The estimated date of infection was calculated as described previously.[27] For this study, only samples collected within 9 months of the estimated date of infection were included. The study participants included 168 individuals in total (Table 1), of whom 58 (35%) cleared the infection, 69 (41%) developed chronic infection, and 41 (24%) had insufficient follow-up to determine the infection outcome. The majority of infections were due to GT1 (32%) and GT3 (34%) viruses. In addition, samples collected from 14 HCV-negative volunteers were used as controls.

B cell immunodominance in primary HCV

To examine the prevalence of antibodies directed towards each HCV epitope, samples were initially screened for anti-envelope

Table 1. Summary of samples included in the study by infection outcome, genotype, and gender.

Variable	Number of participants n = 168 (%)
Infection outcome	
Chronicity	69 (41%)
Clearance	58 (35%)
Unknown	41 (24%)
Infection genotype	
1	54 (32%)
2	2 (1%)
3	57 (34%)
6	4 (2%)
Mixed	18 (10%)
Unknown	33 (21%)
Female gender	66 (39%)

antibodies. Plasma samples were tested for binding to lectin-captured HCV GT1 (strain H77) and GT3 (UKN3A13.6) E1E2 antigen. Of the 168 participants, 96 (57%) were found to have anti-GT1 E1E2 immunoreactive Abs, whereas 47 (28%) were found with GT3 immunoreactivity. Thirty-nine (23%) patients had anti-E1E2 reactive Abs to both GT1 and GT3. There were no significant differences in binding ($p = 0.09$ and $p = 0.22$ for GT1 and GT3, respectively) between participants that ultimately cleared (54% GT1, 30% GT3) or developed persistent infection (60% GT1, 35% GT3) (Fig. S1). The number of anti-GT1 or -GT3 E1E2 Ab positive participants was significantly higher in patients infected with GT1 HCV (78%), compared to those infected with GT3 (51%, $p = 0.005$) or other strains (58%, $p = 0.028$, Fisher's exact test).

To understand the immunodominance hierarchy of E1E2 epitope domains, we established a competitive GT1 immunoreactivity assay of patient plasma against a panel of 9 biotinylated monoclonal antibodies (a sub-panel of 6 mAbs was used for GT3 assays) that covered all known E1E2 mAb domains, excluding HVR1 (Table S1). These mAbs were carefully chosen from an expanded panel of antibodies to minimise redundancies and biases in downstream analysis of immunodominance (see methods). We found that plasma derived from HCV-infected participants variably blocked the binding of all mAbs in the panel (Fig. S2A). As expected, the observed binding of the mAbs was negatively correlated with the total E1E2 binding for the plasma samples (Spearman's correlation coefficient (r) values -0.38 to -0.88, Fig. S2B-D). To examine the relative abundance of epitope-specific responses, an EMS was devised, whereby antibody competition values were normalised by the geometric mean competition across all antibodies. In order to assess the usefulness of the EMS for the deconvolution of polyclonal responses, the score was calculated after competition immunoassays were performed on GT1 E1E2 using unlabelled mAbs that bound to the different domains, as well as mixtures of up to 5 other mAbs (to simulate a polyclonal response). When the competing antibodies were mixed at equimolar ratios, the GT1 EMS displayed good accuracy in prediction of the targeted epitopes (Table S2), even with mixtures of 5 mAbs. Misidentification was restricted to false negative detection of CBH-4G (domain A), false positive identification of AR3A when domain D/HC84 was present, and also false negative detection of AR4A when HC84.27 was added, although this could be overcome by adding a larger amount of AR4A (Fig. S3C).

This assay thus enabled investigation of immunodominance for each sample (Fig. 1A) and revealed a clear immunodominance
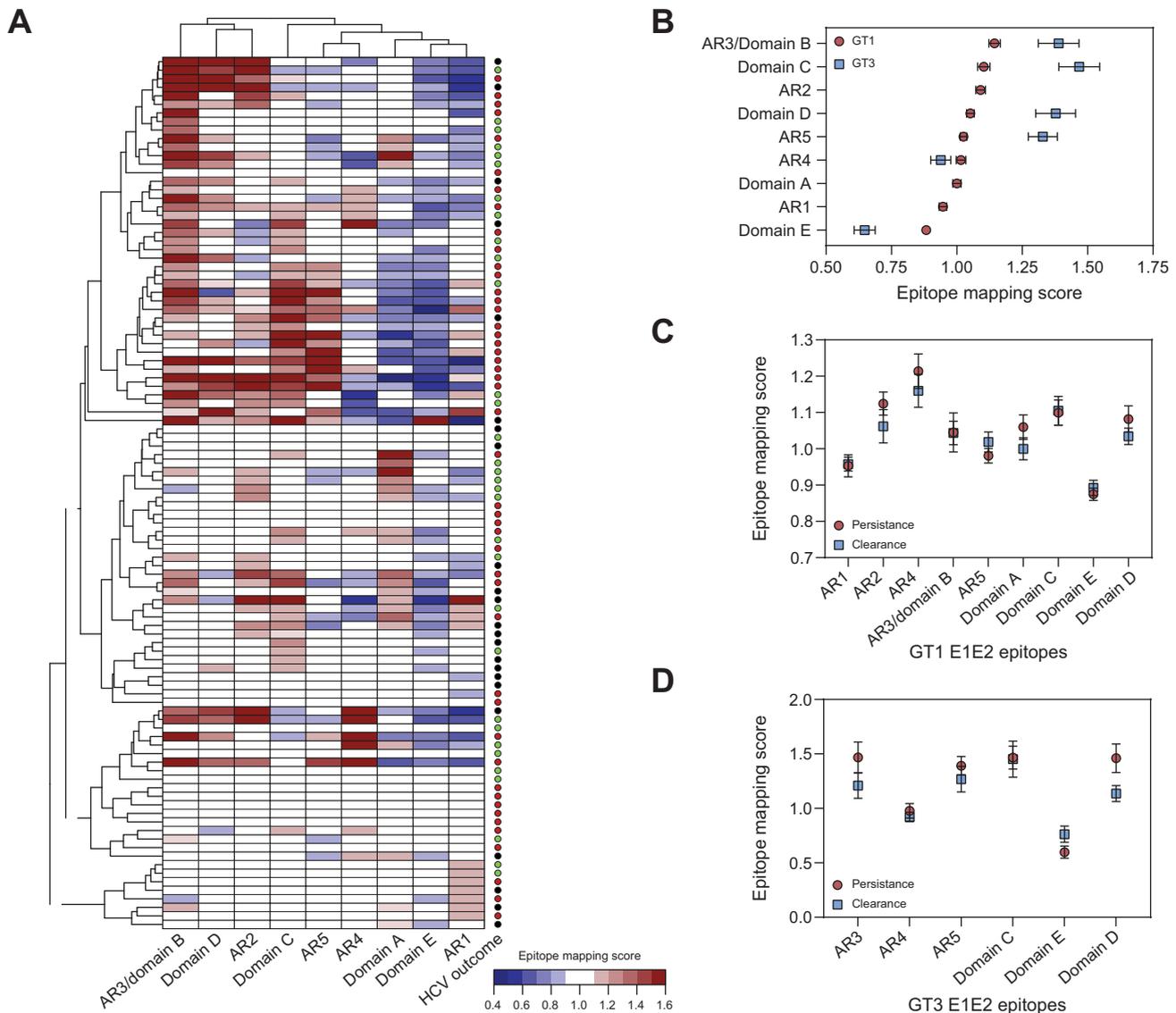
Fig. 1. Immunodominance hierarchy of antibody epitopes in primary HCV infection. Polyclonal responses of 102 participants that were positive for anti-envelope antibodies were mapped to the 9 major antibody domains using a panel of reference antibodies specific to each epitope. An EMS was calculated and presented as a heatmap, with dendograms representing hierarchical clustering of the EMS profiles (A). Every row represents scores from a single plasma sample and columns show the corresponding EMS for a specific envelope epitope. The outcome of HCV infection is shown next to each row as red (chronic), green (clearance) or black (unknown) circles. The figure shows the relative abundance of epitope-specific responses in each sample, highlighting unique immuno-dominance profiles detected for individual samples. (B) Average EMS values for each Envelope epitope are shown with the SEM, showing the relative abundance of antibodies directed towards epitopes like AR3 and domain C, and the low dominance of AR1 and domain E. Epitope rankings for GT1 and GT3 EMS values were determined to be equivalent, as assessed using the Wilcoxon test. The calculated EMS values for GT1 (C) and GT3 (D) E1E2 were compared between participants that ultimately cleared HCV infection (n = 32, 11 for GT1 and GT3, respectively) and those that developed chronic infection (n = 46, 15 for GT1 and GT3, respectively). No association was detected between the infection outcome and any of the examined epitopes. Differences in EMS values were assessed using the Mann-Whitney test. Data is shown as the average EMS with the SEM. AR, antigenic regions; EMS, epitope-mapping score.

hierarchy for the HCV epitopes examined from the 102 antibody-positive patients (Fig. 1B). This hierarchy was relatively conserved for both the GT1 and GT3 analyses (no significant difference, Wilcoxon matched-pairs signed rank test). Consistent with several studies that have reported the isolation of mAbs targeting AR3/domain B[5,17,28] and domain C, responses to these 2 domains dominated the antibody response in the study cohort reported here. These domains were then followed in prevalence by those targeting AR2 and domain D. AR4, domain A and AR1 exhibited relatively lower dominance (Fig. 1B). This analysis also

demonstrated the relatively low dominance of domain E antibodies in primary HCV infections (Fig. 1B), consistent with previous analyses in chronic HCV infections.[12] No competition against any mAbs was detected when using negative control plasma obtained from HCV-negative volunteers, confirming the specificity of the competition assay (Fig. S2B).

To investigate whether the earlier development of bNAb in clearers was a result of immune responses targeting unique epitopes on the viral envelope, the immunodominance hierarchy and the calculated EMS was compared between participants that

cleared infection (n = 32) and those that developed chronic infection (n = 46). Surprisingly, none of the examined epitopes were associated with infection outcome, and EMS scores were comparable between persistent and cleared infection for both the GT1 and GT3 samples (Fig. 1C,D).

Overall these findings demonstrate a clear B cell immunodominance hierarchy in primary HCV infection independent of genotype, but also suggest that antibodies can be generated towards non-immunodominant epitopes in individual infection cases. Clearance of primary HCV was not associated with unique responses towards the examined epitopes.

Specificity of E1E2 antibodies predicts HCV neutralisation

To understand whether the immunodominance hierarchy of the polyclonal E1E2 response influenced antibody function, neutralisation activity of the mapped patient plasma was measured against GT1 or GT3 E1E2 HCVpp. Neutralisation of both pseudoparticles correlated with the measured E1E2-specific antibody titres but was less pronounced for GT3 HCVpp than for GT1 HCVpp (Fig. S4).

Neutralisation of GT1 and GT3 HCVpp for the entire cohort was tested for correlation with EMS values for each epitope calculated from either GT1 or GT3 E1E2 mapping. This analysis separated known broadly neutralising domains from weak or non-neutralising ones. Neutralisation of GT1 and GT3 HCVpp displayed the strongest correlation with AR3 EMS (r = 0.7, Fig. 2A,C). Domain D was also associated with increased neutralisation potency of GT1 and GT3 HCVpp. bNAb epitope AR5 GT3 E1E2 EMS scores did correlate with HCV neutralisation activity but did not for GT1 E1E2 EMS scores. This may be due to the relatively low abundance of AR5 responses in the cohort.

Interestingly, the rare AR5 responses in the GT1 mapped samples were potent neutralisers of GT1 HCVpp (Fig. S5). Domain C was only positively correlated between genotype matched EMS and neutralisation. AR4 EMS values did not associate with neutralisation, but like AR5, AR4 had a limited number of responses with the rare AR4 responses being potent neutralisers (Fig. S5-8). Responses towards the weak and non-neutralising domains A and AR1 showed a negative correlation with HCVpp neutralisation (Fig. 2C). This was only examined for GT1 mapping as these Abs did not bind to GT3 E1E2. For the entire cohort, domain E EMS negatively correlated with HCVpp neutralisation. This was primarily due to increased EMS of other specificities, which lowered the EMS for domain E (see methods). The only sample with a dominant domain E response also displayed potent neutralisation of GT1 HCVpp (Fig. 3A).

These findings show that neutralisation of HCV by polyclonal plasma is predominantly due to targeting of known bNAb epitopes, particularly AR3 and domain D. Antibody responses against non-neutralising domains were not associated with HCV neutralisation.

Breadth of neutralisation in serum samples is explained by epitope specificity

Next, the specificity of the polyclonal response mounted during early HCV infection was examined in relation to the breadth of neutralising activity. Five samples that had the highest EMS for each epitope were selected, with the exception of domain E, for which only a single sample was available. A total of 27 samples were examined for bNAb activity using an established panel of 11 pseudoparticles covering 6 HCV genotypes.[21] Neutralisation potency and breadth were estimated by calculating a breadth
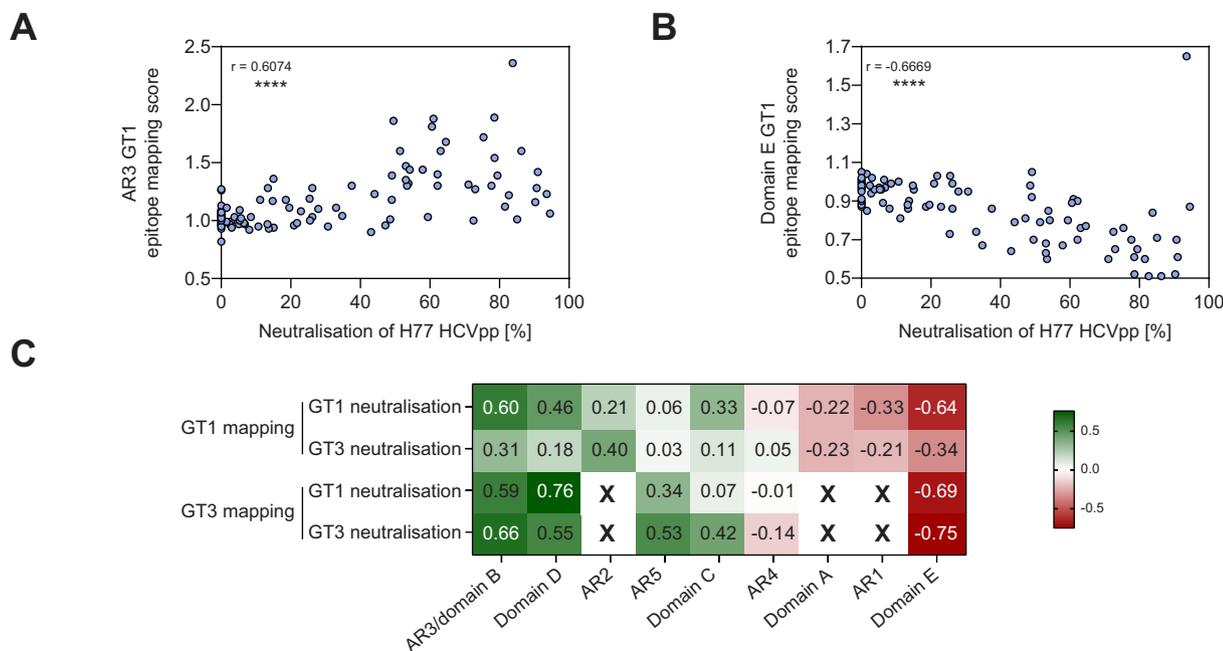
A

B

C

Fig. 2. Specificity of anti-envelope antibodies in primary infection is correlated with HCV neutralisation. The ability of participants' plasma to neutralise HCV was measured using GT1 and GT3 HCVpp. Correlation was then sought for HCVpp neutralisation and EMS values for each epitope. (A) Neutralisation of GT1 HCVpp strongly correlated with antibody responses towards the bNAb epitope AR3 on GT1 E1E2 (p <0.0001), while (B) a negative correlation was observed for domain E (p <0.0001). (C) Spearman's correlation coefficient was similarly calculated for each epitope on GT1 and GT3 E1E2 and neutralisation of GT1 and GT3 pseudoparticles, highlighting that responses towards known neutralising domains were associated with potent HCV neutralisation *in vitro*. Conversely, responses towards weakly neutralising, or non-neutralising domains were either not correlated, or negatively correlated with HCVpp neutralisation. AR, antigenic regions; bNAbs, broad neutralising antibodies; EMS, epitope mapping score; HCVpp, HCV pseudoparticles.
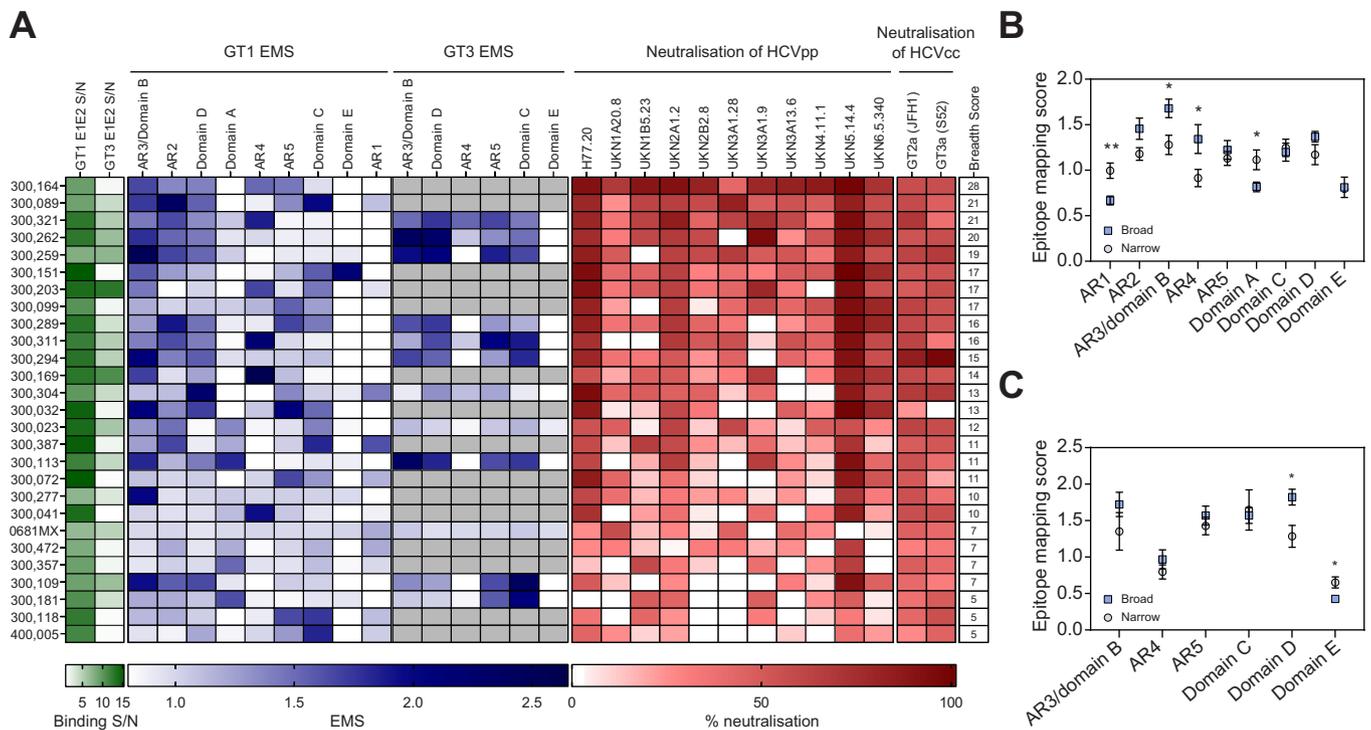
Fig. 3. **Breadth of the neutralising response is associated with multi-specific antibodies that do not target non-neutralising domains.** A panel of 11 HCV pseudoparticles representing all 6 genotypes was used to examine bNAb activity in participants' plasma samples. (A) Samples were ranked according to their breadth score[21] and shown in rows, together with the corresponding anti-envelope titre, GT1 and GT3 EMS values, and HCVpp and HCVcc neutralisation percentages shown as a heatmap. While all samples had comparable anti-envelope antibody titres, the specificity of some antibodies was associated with the breadth of HCVpp neutralisation. Samples with bNAb activity (upper half of the panel) were particularly enriched for multi-specific antibodies targeting AR3, AR2 and domain D. Narrowly neutralising samples were enriched for antibodies targeting the non-neutralising domains C and E. Samples which were not mapped to GT3 are marked as grey in corresponding GT3 EMS rows of the heatmap. (B) EMS values for each epitope were compared between broadly neutralising samples and those with narrow neutralisation of HCVpp. Breadth of neutralisation was significantly associated with elevated AR3 and AR4 EMS values, whereas narrowing of the neutralisation was associated with elevated AR1 and domain A scores. (C) When this comparison was analysed using GT3 EMS data, breadth was significantly associated with elevated domain D values, with narrowing of neutralisation being associated with elevated domain E scores. Means and standard errors are indicated (*$p < 0.05$, **$p < 0.01$, 2-tailed Mann-Whitney test). AR, antigenic regions; bNAbs, broad neutralising antibodies; EMS, epitope mapping score; HCVcc, cell culture-derived HCV; HCVpp, HCV pseudoparticles.

score,[23] which accounts for both the number of different pseudoparticles neutralised, as well as the observed percentage neutralisation for each.

For the 27 samples included in this set, the calculated breadth score ranged between 5 and 28 (Fig. 3A). This variability was observed despite having comparable anti-E1E2 antibody titres across the sample set, indicating that antibody specificity rather than titre was likely to account for this difference. Ranking the GT1 E1E2 mapped samples according to their breadth score revealed a clear association between antibody specificity and neutralising breadth. While the observed breadth could not be uniformly explained by the score of a single epitope, samples with bNAb responses, defined by a breadth score ≥14, had significantly increased EMS values for the neutralising epitopes, AR3 and AR4 ($p = 0.012$ and 0.029, respectively). Conversely, samples that exhibited narrow neutralisation had significantly higher scores for the non-neutralising domain AR1 ($p = 0.002$, Fig. 3A). Remarkably, samples that exhibited bNAb demonstrated increased EMS for multiple neutralising epitopes simultaneously, particularly for AR3, AR4 and domain D (Fig. 3B). In addition, the induction of AR1 and domain A antibodies appeared to correlate with narrow antibody responses despite high EMS for AR3 (participants 300023, 300387, 300181), AR4 (participant 300041) or the induction of a combination of AR3/domain D

antibodies (participant 300113, Fig. 3A). Similar trends were also observed for a subset of GT3 E1E2 EMS values with AR3/domain B, AR4 and domain D binding having higher bNAb responses, but only domain D was significantly associated with bNAb responses, likely due to the limited sample size (Fig. 3C). Domain E GT3 E1E2 EMS responses were significantly associated with narrow NAbs. Differences in neutralisation breadth were not associated with the genotype of the infection (Fig. S9).

For confirmation of the NAb activity in the 27 samples, neutralisation was also measured against GT2 and GT3 HCVcc (Fig. S10). The NAb activity of all 27 patients against GT2 and GT3 HCVcc were positively correlated with the breadth scores, and a mid-point breadth score of 14 was comparable to 50% neutralisation of the HCVcc. The association was only statistically significant for GT2 HCVcc and the breadth score.

These findings show that a multi-specific antibody response, particularly targeting bNAb domains, is associated with increased neutralisation breadth.

B cell immunodominance is associated with antigenic diversity

Examining the prevalence of certain specificities in a polyclonal response is complicated by the relationship antibodies have with each other, and upon binding to the antigen. Although there was

a clear immunodominance hierarchy between the antigenic domains in the group analysis, unique immunodominance patterns were observed within individuals, where the dominant epitopes were domain C, AR4 and AR5 (Fig. 1A). The rarely dominant domain E responses also exhibited the highest EMS score in a single individual. These observations raise the possibility that both viral and host factors play a role in B cell immunodominance. To address this, the influence of the infecting HCV genotype was analysed in relation to the quality of the NAb response elicited. This analysis was restricted to participants mono-infected with GT1 (n = 43) or GT3 (n = 27), which accounted for 66% of this cohort (Table 1) and was mapped onto GT1 E1E2 (Fig. 4A).

Remarkably, the bNAb domain AR3 responses exhibited a significantly higher EMS in GT3 samples compared to GT1 samples (p = 0.0002, Fig. 4A). Although rare in either group, domain D responses were also more dominant in GT3 infections (p = 0.027, Fig. 4A). GT1 samples displayed a significant increase in EMS for the narrowly neutralising domain C, and the weakly neutralising AR1 (p = 0.003 and 0.013, respectively). These results, however, could be confounded by the use of a GT1 E1E2 construct in this analysis, and the reduced reactivity of antibodies like AR1B to GT3 antigens.[10] Therefore, in order to ensure that the associations detected between the infecting genotype and epitope responses were not an artefact of this system,

mapping results of GT1-infected samples mapped onto GT1 E1E2 and GT3-infected samples mapped onto GT3 E1E2 were compared (Fig. 4B). For some epitopes, domain C, AR5 and domain E, the genotype of the E1E2 used for mapping did affect the mapping score, whereas for other epitopes the same trends were observed independently of which genotype the samples were mapped on, and indicated that domain D and AR3 were enriched in GT3 infections, although the latter was not significant for GT3 infections. Overall, these results suggest that different infecting genotypes may be associated with divergent responses towards particular bNAb domains.

To establish whether specific viral signatures were associated with unique epitope responses, 10 viraemic samples with the greatest observed EMS values for each epitope were selected, with the exception of domain E for which only 1 sample was available (Fig. 1A). For these samples, the E1E2-encoding regions were amplified from the same sampling time-point used for mapping or, if available, a sample taken prior to it. Sequencing was performed on the Illumina MiSeq platform.[29] In total 44 sequences were available for analysis, covering a range of HCV subtypes, including GT1a (n = 19), GT3a (n = 17), GT1b (n = 4), GT6a (n = 3) and GT2b (n = 1). E1E2 sequences were analysed for enrichment of amino acid residues in patients that mounted epitope-specific responses compared to those that did not, response was defined by the GT1 E1E2 mapped data. The
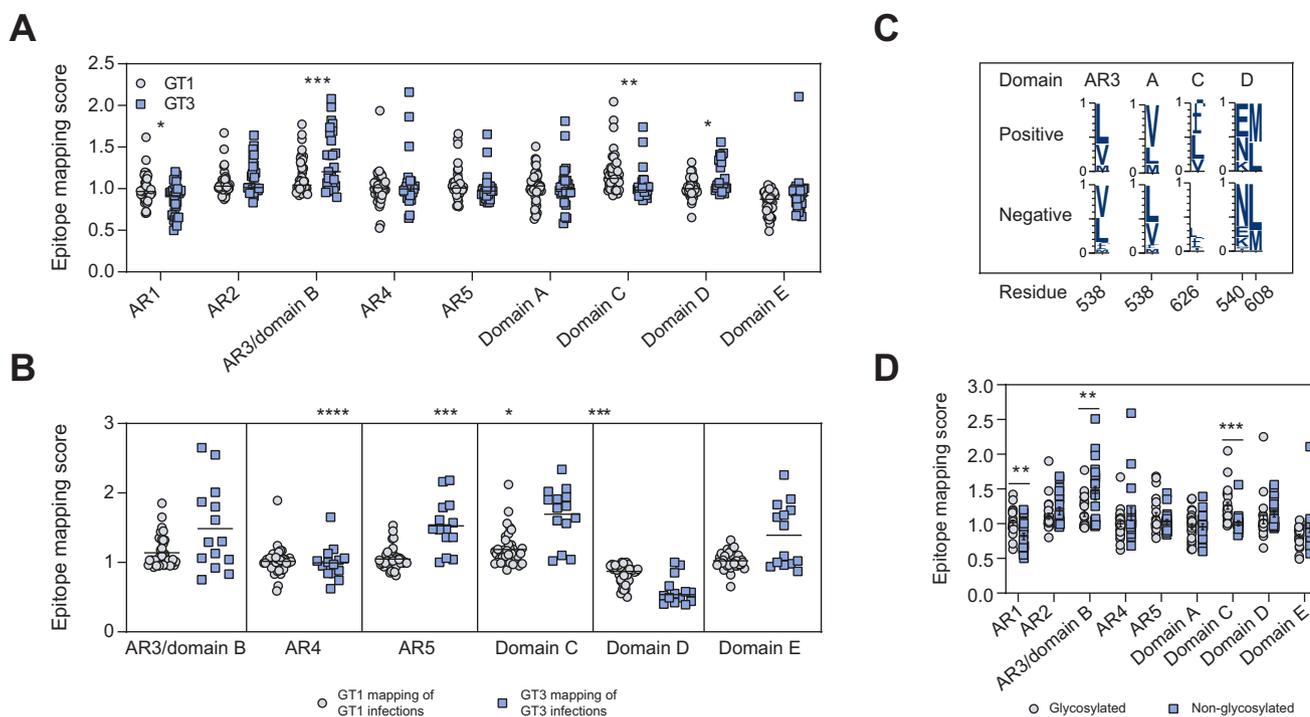

Fig. 4. Immunodominance hierarchy is associated with antigenic diversity. (A) The impact of HCV sequence on the observed GT1 epitope immunodominance was examined by comparing the specificities observed in patients infected with GT1 (n = 43) to those infected with GT3 (n = 27). Infection with GT3 was significantly associated with an increased response towards bNAb domains AR3 and domain D, while GT1 samples displayed a significant increase in EMS values for the narrowly neutralising domains C and AR1. (B) The EMSs of GT1-infected patients' plasma mapped to GT1 E1E2 were compared to GT3-infected patients' plasma mapped to GT3 E1E2. The results are shown for Abs against all 6 epitopes in the GT3 panel, indicating significant differences in EMS for all epitopes except those bound by AR3A and AR4A. (C) E1E2-encoding regions from 44 sequences were analysed for enrichment of amino acid residues that might be associated with epitope-specific responses. The frequency of amino acid residues that were significantly different between samples with (top) or without (bottom) epitope-specific antibodies is shown, highlighting differences in AR3 and domains A, C and D. (D) N-linked glycosylation of GT1 E2 site 540 is significantly associated with an increased dominance of domain E targeting antibodies, whereas the lack of a glycan at GT3 site 540 was associated with an increased AR3/domain B, AR5, domain C and domain D EMS (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001, 2-tailed Mann-Whitney test). Results are shown as mean ± SEM. AR, antigenic regions; bNAbs, broad neutralising antibodies; EMS, epitope mapping score.

analysis was restricted to known binding sites for the mAbs used in this study (Table S1). This revealed differences in sequences derived from patients with AR3/domain B and domain A responses, which were enriched for leucine and valine residues in position 538, respectively (Fig. 4C). Similarly, domain C responses were associated with an increased frequency of I626 within the E2 sequences. Enrichment for E540 and M608, positions known to be important for HC84.26 binding,[30] was also seen in patients with domain D antibody responses (Fig. 4C). Substitutions of the aforementioned residues have also been shown to affect the binding of a range of other antibodies.[30]

In order to examine whether differences in E1E2 glycosylation could modulate host immune responses, sequences were analysed for predicted N-linked glycosylation, and correlations were sought with the calculated GT1 E1E2 EMS values for each epitope. This analysis revealed a strong association between the presence of a glycan at position 540 with the epitope scores for AR1, AR3 and domain C ($p = 0.0002$, Fig. 4D). As this glycan has been shown to have a genotype-specific distribution,[31] this observation was not independent of the genotype-associated differences for these domains (Fig. 4D). Nevertheless, the associations appeared to be of higher significance, particularly for AR1 ($p = 0.006$), perhaps due to the inclusion of GT2 and GT6 samples in this analysis.

Overall the findings suggest that the responses against important bNAb epitopes vary significantly between HCV genotypes, and that these differences may be partly attributed to glycosylation of E2 at position 540, which might play a role in modulating the immune response towards AR1, AR3 and domain C.

Discussion

The antibody response to HCV is key to natural clearance of primary infection and is likely to be critical for effective protection using a prophylactic vaccine. In natural infection, the antibody response is complicated by the presence of multiple epitopes on the viral envelope, not all of which induce neutralising antibodies. In the present study, the immunodominance of these epitopes in a large cohort of patients with early infection with different HCV genotypes was defined, and the consequences of mounting epitope-specific responses on neutralisation breadth and infection outcome were determined.

By examining antibody responses in a primary HCV infection cohort of clearers and comparing them to those that progressed to chronic infection, the findings indicate that B cell immunodominance is preserved despite the varied outcomes. In 2 recent reports, mAbs were isolated from patients after successful clearance of HCV,[16,17] revealing mAbs which predominantly bound the AR3 domain and which did not feature sufficient somatic mutations to achieve breadth. This preliminary finding raises the suggestion that, at least in primary infection, bNAb control of HCV viraemia is not dependent on the generation of a unique specificity or extensive maturation of induced mAbs.

The aim of a B cell-based vaccine is to induce responses that protect from multiple virus genotypes and subtypes. In this cohort, plasma samples with bNAb activity were enriched for multi-epitope responses, particularly directed towards AR2, AR3, AR4 and domain D. Interestingly, these combinations were recently shown by 2 independent reports to have synergistic activity in vitro,[11,32] which was superior to individual mAb activities. This supports a recent paper by Kinchen and colleagues,[33] who reported that the induction of AR3 or domain D

targeting bNAbs may be important in developing an effective NAb response in combination with AR4-targeting bNAbs. This concordance of findings provides strong evidence for the importance of these epitopes in bNAb responses. The role of synergistic antibody responses in overall bNAb activity has also become apparent for other viruses.[34,35] These findings have implications for vaccine design, in that the inclusion of multiple epitopes may enhance bNAb induction. Indeed, failure to account for B cell immunodominance has been suggested to have contributed to the failure of HIV vaccine candidates, as non-neutralising antibodies were induced at the expense of bNAbs.[36] The data reported here and by Kinchen et al.[33] similarly suggest that epitopes for vaccine candidates need to be carefully selected, as direction of the natural immune response towards non-neutralising domains was associated with reduced NAb breadth. Subjects with high domain A EMS had significantly more narrow NAb responses compared to those with low domain A scores. The presence of non-neutralising antibodies to domain D has been shown to interfere with NAbs specific to the adjacent domain E.[37] However, domain A antibodies have also been shown to have minimal impact on the activity of domain B and C targeting antibodies.[38] The increased neutralisation breadth in samples with low domain A responses might therefore be due to "focusing" of the immune response on bNAb epitopes. Engineering of envelope constructs to remove domain A may be a strategy to improve bNAb responses with an immunogen, comparable to the strategy with a current HCV vaccine candidates, in which HVRs have been deleted from the E2 immunogen.[13]

The choice of an antigenic variant is an important consideration for the development of a protective vaccine, particularly for highly variable antigens, like HCV E1E2. Epitope presentation on different variants can have significant impacts on the quality of the host immune response. For instance, H1N1 influenza infections have been shown to induce a significantly broader antibody response compared to infections with other strains, including seasonal H3N2 viruses.[39] In HIV, bNAbs targeting the CD4-binding site are significantly more prevalent in infections with subtype B viruses, while bNAbs targeting the V2 glycan are associated with non–subtype B viruses.[23] Efforts to develop HCV vaccines have focused on GT1-based envelope formulations, primarily due to the worldwide prevalence of this genotype. The findings here suggest that the genotype from which an immunogen is derived has broad implications for the desired immune response, and that native GT3 E1E2 may induce a more favourable bNAb response compared to GT1 antigens, potentially by focusing the immune response on bNAb AR3/domains B and D. A potential confounder of this finding is that mAbs homologous to either GT1 and GT3 are not available and this may have unknowingly biased the findings. Biologically, however, this difference might, in part, be due to the glycosylation profile of the proteins, which are known to modulate antibody activity and escape.[31] Additionally, HVR1 has been shown to regulate HCV neutralisation in part by altering envelope conformation and therefore potentially shielding certain epitopes from NAb binding.[40] This is likely to cause variation between individuals, particularly between those with differing infecting genotypes. It is worth noting, however, that while general trends between GT1- and GT3-infected patients were observed, unique dominance profiles were observed in individual patients, which suggests that further determinants of immunodominance hierarchy

could be encoded by unique viral strains, or by host factors. A recent analysis of HIV transmission pairs revealed a significant effect of the infecting virus strain on the outcome of the bNAb response, suggesting an "imprinting" effect by the virus on the humoral response.[41] Future studies should examine whether certain viral signatures, or host genetic determinants impact HCV immunodominance, as demonstrated for the influenza B cell hierarchy.[42]

A potential confounder in this study is the cross-reactive nature of the mAbs used, as demonstrated in Fig. S3. Interestingly, many of the mAbs increased binding of the CBH4G detection antibody, so poor competition was observed with this antibody and clearly distinguishable competition results could not be observed. Likewise, HC84 cross-competed with AR3A, and could potentially have increased its false detection, while conversely HC84 increased AR4A binding and AR4A competition may only be detected in high concentrations, so samples with low AR4A titres might not be detected. Given that the presence of HC84/domain D was low, we believe these affects were minimal. This study was also limited by the lack of mAbs that target non-GT1 envelope proteins, particularly within non-neutralising domains. This has implications for the interpretation of the lack of AR1-like antibodies in GT3-infected patients, as AR1B does not bind GT3 antigens and therefore could not be assessed in the GT3 E1E2 ELISA.[10] For bNAb domains, however, the difference between GT1 and GT3 infections was confirmed using both GT1 and GT3 E1E2.

In conclusion, this study outlines a clear immunodominance profile for known HCV neutralising and non-neutralising domains. It also suggests that immunodominance may be preserved across genotypes and enrichment of certain epitopes might be influenced by variations in the protein sequence of the infecting virus. Given the clear relevance of bNAb induction for vaccine design, detailed understanding of B cell immunodominance is critical for the success of future protective immunogens.

Abbreviations

AR, antigenic regions; bNAbs, broad NAbs; EMS, epitope mapping score; HCVcc, cell culture-derived HCV; HCVpp, HCV pseudoparticles; HRP, horseradish peroxidase; mAbs, monoclonal antibodies; NAbs, neutralising antibodies.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Authors AAE, NAB, AU, IB and CR completed the laboratory work and data analysis. NT, FL ARL and RAB contributed to the study design. Authors ARL, HED and LM contributed samples and reagents. HED and IB conducted HCVcc neutralisation assays. The first manuscript was drafted by NAB, AAE, and RAB. All authors contributed to and have approved the final manuscript.

Supplementary data

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Author names in bold designate shared co-first authorship

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