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The broad assessment of HCV genotypes 1 and 3 antigenic targets reveals limited cross-reactivity with implications for vaccine design

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ABSTRACT

Objective Developing a vaccine that is cross-reactive between HCV genotypes requires data on T cell antigenic targets that extends beyond genotype-1. We characterised T cell immune responses against HCV genotype-3, the most common infecting genotype in the UK and Asia, and assessed within genotype and between genotype cross-reactivity.

Design T cell targets were identified in 140 subjects with either acute, chronic or spontaneously resolved HCV genotype-3 infection using (1) overlapping peptides and (2) putative human leucocyte antigens (HLA)-class-I wild type and variant epitopes through the prior assessment of polymorphic HCV genomic sites associated with host HLA, in IFNγ-ELISpot assays. CD4+/CD8+ T cell subsets were defined and viral variability at T cell targets was determined through population analysis and viral sequencing. T cell cross-reactivity between genotype-1 and genotype-3 variants was assessed.

Results In resolved genotype-3 infection, T cells preferentially targeted non-structural proteins at a high magnitude, whereas in chronic disease T cells were absent or skewed to target structural proteins. Additional responses to wild type but not variant HLA predicted peptides were defined. Major sequence viral variability was observed within genotype-3 and between genotypes 1 and 3 HCV at T cell targets in resolved infection and at dominant epitopes, with limited T cell cross-reactivity between viral variants. Overall 41 CD4/CD8+ genotype-3 T cell targets were identified with minimal overlap with those described for HCV genotype-1.

Conclusions HCV T cell specificity is distinct between genotypes with limited T cell cross-reactivity in resolved and chronic disease. Therefore, viral regions targeted in natural HCV infection may not serve as attractive targets for a vaccine that aims to protect against multiple HCV genotypes.

Significance of this study

What is already known on this subject?

- HCV genotype-3 is the most prevalent HCV strain in South Asia and the UK.
- HCV viral genotypes share approximately 80% sequence homology.
- Limited data on T cell specificity is available for HCV genotypes other than HCV genotype-1.
- Population studies assessing the association of HLA-class-I with viral genomic polymorphisms suggest that T cell specificity differs between genotypes 1 and 3.

What are the new findings?

- A comprehensive assessment of HCV genotype-3 T cell specificity identifies 41 CD4+ and CD8+ genotype-3 specific T cell targets across the viral genome.
- A novel sequence led approach can be used to identify HLA-class-I epitopes under T cell selection.
- T cell targets in HCV genotype-3 infection are distinct from those targeted in HCV genotype-1 in resolved and chronic disease.
- T cell cross-reactivity to genotype-1 and genotype-3 sequence variants in resolved infection and at dominant HCV genotype-3 epitopes is limited.

How might it impact on clinical practice in the future?

- Distinct T cell specificity and limited T cell cross-reactivity between HCV genotypes are important considerations for the development of vaccines aiming to induce T cell responses cross-reactive against multiple HCV genotypes.

INTRODUCTION

HCV infection is a major health risk, infecting approximately 170 million people worldwide.[1] The majority of infected patients develop persistent infection, which may lead to liver cirrhosis, hepatocellular cancer and death.[2] Even though major advances in HCV treatment with directly acting antivirals (DAAs) have been achieved over recent years,[3 4 5] costs are high and treatment may remain inaccessible for many, particularly in health resource poor countries. In addition, reinfection with DAA resistant variants can occur following successful therapy.[6] An effective prophylactic HCV vaccine remains an unmet clinical need.

HCV is a highly genetically diverse pathogen that is divided into 7 major genotypes and 67

subtypes[7] that are broadly distributed by geographical location. Even within a single host infected with one subtype HCV exists as multiple closely related but distinct viral quasi species.[8] HCV genotype-1 is the dominant genotype globally,[9] but HCV genotype-3 is now the major infecting genotype in the UK[10] and in large parts of Asia,[1] infecting approximately 53 million people globally[9] and commonly associated with injecting drug use and interventional medical practice.[11–13]

HCV genotype-1 vaccines based on viral vectored technology used in heterologous prime/boost regimens are currently in development and able to induce a high magnitude of T cells that target multiple HCV antigens.[14 15] However, the success of T cell vaccines in regions where multiple HCV genotypes coexist will depend on the generation of T cells that have the capacity to target multiple viral strains or viral regions that are conserved between genotypes. A better understanding of genotype-specific immune responses will therefore aid the development of vaccines active against multiple genotypes.

Based on significant viral genetic differences between HCV genotypes,[7] we hypothesised that T cell targets differ in HCV genotypes 1 and 3. Comparative studies on HCV genotype-1 and HCV genotype-3-specific T cell immunity to date include an analysis of genotype-specific sequence polymorphisms linked to HLA types, suggesting that T cell targets are distinct between HCV genotypes.[16] However T cell immune pressure was not confirmed by experimental T cell assays. Although patients infected with HCV genotype-3a have been included in numerous publications addressing HCV specific T cell immunity experimentally, studies using specific HCV genotype-3 cohorts and genotype-3 peptide sets are limited to a single study evaluating T cell responses to the NS3 protein only[17] and our previous study that primarily evaluated the impact of therapy on HCV genotype-3-specific T cell responses.[18]

To date, no specific cross-reactive T cell targets linked to spontaneous resolution of infection have been described, and no comprehensive assessment of T cell cross-reactivity between HCV genotypes 1 and 3 has been performed. Even if T cell targets are shared between genotypes, a single amino acid (AA) substitution may abort or substantially decrease recognition of the epitope by wild type primed T cells.[19–21] Although some T cell cross-reactivity between HCV genotypes has been described,[17 18] several small-scale studies in patients with evidence of multiple infections have shown lack of cross-reactivity of CD4+ T cell responses between genotypes.[22 23] Furthermore, systematic analysis assessing all possible sequence variants between genotypes is limited to a singe epitope, showing that CD8+ T cells primed against the dominant HCV genotype-1 epitope $NS3_{1073}$ do not recognise HCV genotype-2 and genotype-3 viral variants at that location.[21]

T cell cross-reactivity between heterologous viral strains can also be evaluated in the context of human reinfection observational studies and chimpanzee rechallenge experiments. Published studies suggest that chimpanzees[24 25] and humans[26] that spontaneously clear acute HCV infection are more likely to clear subsequent infections. However the role of cross-reactivity in preventing chronic disease upon reinfection is not clear and while clearance of heterologous HCV reinfection is reported,[24] persistent infection on rechallenge with heterologous strains is also common.[25 27] Furthermore, these studies have not evaluated T cell cross-reactivity at epitope level, and other factors may explain the phenomenon of repeated viral resolution such as a favourable innate immune response and host genetic make up.[24]

ELISpot assays have been established as a reliable method to define T cell epitopes in chronic viral infections[18 28] and T cell vaccine studies.[15 29] Overlapping peptides homologous with the pathogen genome are commonly used as a screening tool to identify T cell epitopes. However, it has been reported previously that the detection of T cell responses in IFNγ-ELISpot assays may be dependent on the position of the presented optimal epitope within an overlapping peptide[30] and T cell responses may be missed when screening with this approach. To address this, we assessed T cell responses using two complementary HCV genotype-3-specific peptide sets; one based on a novel, sequence-led approach using wild type and variant peptides corresponding to putative HLA class-I restricted epitopes under T cell selection identified in a large HCV genotype-3 sequence data set;[16] the other based on a consensus sequence derived from 15 chronically genotype-3 infected patients spanning the whole HCV genome.[18]

We aimed to comprehensively characterise T cell immune responses against HCV genotype-3 and to compare T cell specificity between HCV genotype-1 and genotype-3. Finally we assessed T cell cross-reactivity between common HCV genotype-1 and genotype-3 sequence variants, focusing particularly on dominant genotype-3 T cell epitopes, in addition to a cohort of patients with resolved infection where cross-reactive T cells associated with viral control may have the greatest implications for vaccine design.

METHODS

Patient cohort

One hundred and forty HCV genotype-3a infected individuals including 16 acutely infected, 108 chronically infected and 16 with spontaneously resolved infection were recruited (John Radcliffe Hospital Oxford, MGH Boston, and the BBAASH cohort, Baltimore[31]). Informed consent and local ethical approval was obtained for all patients. Patient details are summarised in online supplementary table S1. Acute patients were defined as those within the first 6 months of infection (n=16), of whom 12 were not treated (n=4 cleared infection spontaneously; n=6 proceeded to chronic infection; n=2 lost to follow-up), and 4 were treated during acute infection (n=3 sustained virological response; n=1 non-responder) (see online supplementary table S2). HCV genotype could not be determined in spontaneously resolved individuals by conventional genotyping; however, to define the infecting genotype, T cell responses to genotype-1 and genotype-3 peptides were assessed in this group.

Peptide sets and approaches used to identify HCV-specific T cell targets

(1) *Overlapping peptides* for HCV genotype-1 and genotype-3: A genotype-1b peptide set containing peptides 15 to 18 AA in length overlapping by 10 AA derived from HCV J4 sequence (AF054250); A genotype-3a peptide set based on 18 full-length genotype-3a sequences as previously described, spanning the whole viral genome (GQ356200-GQ356215, GQ356217 and JF509175-JF509177).[18]

(2) *HLA-predicted peptides* for genotype-3 were based on a novel sequence led peptide design approach aiming to identify HLA class-I restricted optimal epitopes: Associations between HLA-class-I alleles and HCV viral sequence polymorphisms within NS2-NS5B were identified in a cohort of 136 HCV genotype-3a infected patients.[16] Epitope computer prediction programmes were used to identify putative T cell epitopes (9–10AA) hosting HLA-associated polymorphic sites (BioInformatics and Molecular Analysis Section (BIMAS) score ≥50, Syfpeithi score ≥20; http://www-bimas.cit.nih.gov, http://

www.syfpeithi.de). Fifty-five epitopes were predicted to contain HLA-associated polymorphic sites within the peptide (see online supplementary table S3), whereas in 10 peptides the polymorphic site was flanking the epitope (see online supplementary table S4). Wild type (defined as the consensus AA at each position in an HCV genotype-3 sequence alignment)[16] and variant (defined as the second most common AA at each position linked to patient HLA) peptides were subsequently evaluated in T cell assays matched to the patients' HLA type. We have previously published T cell responses in 10 spontaneously resolved and 17 chronically HCV genotype-3 infected patients using overlapping peptides only[18]; these have been included in this manuscript for assessment using HLA-predicted peptides and comparative analysis of T cell specificity.

Detected T cell responses to overlapping peptide pools (HCV core, E1, E2, p7/NS2, NS3 protease (NS3p), NS3 helicase (NS3h), NS4, proximal NS5B (NS5BI), and distal NS5B (NS5BII)) were mapped to subpools and single peptides. T cell responses to both peptide sets were compared at pool level, and at single epitope level in patients with mapped responses. CD4+/CD8+ restriction was defined using CD8+ depletion assays and intracellular staining assays as previously described.[18] For further analyses, dominant responses were defined as those targeted in more than four patients within the Oxford cohort.

HLA typing

DNA was extracted using the DNeasy Blood And Tissue Kit (Qiagen) from peripheral blood mononuclear cells (PBMCs) or whole blood using the Gentra Puregene kit (Qiagen) as per manufacturer's instructions and then HLA typed (Transplant Immunology Lab, Oxford Radcliffe Hospitals).[32]

ELISpot assays

Human PBMCs were separated, frozen immediately and stored in liquid nitrogen as previously described.[18] T cell responses were assessed using thawed PBMCs in IFNγ-ELISpot assays as previously described.[33] In brief, precoated ELISpot plates (anti-IFNγ monoclonal antibody (0.5 µg/well, Mabtech)) were blocked with R10 (RPMI Sigma, 10% fetal calf serum (FCS), penicillin and streptomycin added). For 18 h, 200 000 PBMCs/well were stimulated with HCV genotype-3 peptide sets (3 µg/mL), cytomegalovirus (CMV) lysate (0.05 µg/mL, Chiron), influenza, Epstein Barr virus and CMV (FEC) CD8+ epitopes in a single pool (3 µg/mL BEI resources) in duplicates for each condition. Dimethyl sulfoxide (DMSO) and concanavalin A (10 µg, Sigma) served as negative and positive controls, respectively; all ELISpot assays were strongly positive for concanavalin A. Additionally, 101/140 patients were positive for CMV lysate and 68/140 patients were positive for FEC antigens (mean spot-forming units/10^6PBMCs 661.77 and 686.45). All patients were tested using overlapping peptide pools. HLA-predicted peptides were tested in HLA-typed patients with cells available. SFUs were counted on an automated ELISpot plate reader. A positive cut-off of 40 SFUs/10^6PBMCs for the HCV genotype-3 peptides and 43 SFUs/10^6PBMCs for genotype-1b peptides was defined previously in healthy volunteers using; (mean SFU/10^6PBMCs in test wells—negative control wells)+3×SD.[18]

Viral sequencing

HCV viral sequencing was performed as previously published.[18] In brief, patient plasma was concentrated by centrifugation (1 h, 23 000 rpm, 4°C) and viral RNA was extracted using a QIAmp Viral RNA Mini Kit (Qiagen). Reverse-transcription and first round PCR were performed in a single step (Superscript III OneStep RT-PCR system, Platinum Taq enzyme (Invitrogen)). In a second step single proteins were amplified in multiple nested PCR reactions (High Fidelity Taq DNA polymerase (Roche), for primers see refs. [18] and [34]). Amplified PCR fragments were gel purified and sequenced bidirectionally with Prism Big Dye (Applied Biosystems) on an ABI3100 DNA automated sequencer. Sequences were edited using the Sequencher 4.8 Software (Gene Codes), and aligned using Se-Al (http://tree.bio.ed.ac.uk). Sequence entropy was calculated using the Shannon entropy score (http://evolve.zoo.ox.ac.uk/Evolve/SHiAT.html) using HCV genotype-3 sequences derived from the Los Alamos sequence database (http://hcv.lanl.gov/content/index).35

Analysis of HCV genotype-1 and genotype-3 T cell targets

HCV genotype-1 and genotype-3 epitopes were obtained from the immune epitope database resource (IEDB, http://www.iedb.com). To ensure data quality, epitopes were crosschecked with primary publications; epitope duplications, sequence variants and epitopes described in non-human organisms were excluded. Dominant HCV genotype-1 targets were defined as those described in more than five publications, and were compared with all genotype-3 epitopes defined in this study. Experimentally identified HCV genotype-3 targets were compared with all HCV genotype-1 epitopes described on the IEDB. Targets previously described were defined as 'overlapping' with those detected experimentally if epitopes exhibited >80% AA sequence homology or 'not overlapping' if <80% AAs sequence homology, or if epitopes overlapped by less than 7 AA (left coloured bar, online supplementary tables S7 and S8).

Statistical analysis

Non-parametrical tests were used throughout, paired for within-individual comparisons (Wilcoxon) and unpaired for group comparisons (Mann-Whitney). A p value of <0.05 was considered statistically significant. Prism (V.4.0 for Mac) was used.

RESULTS

T cell specificity differs in patients with spontaneously resolved and chronic HCV genotype-3 infection

T cell responses to HCV genotype-3 were first assessed in 20 patients with spontaneously resolved infection since these responses are most likely to be causally related to viral resolution. We included four patients with acute infection, assessed at the earliest available time point after presentation, who subsequently resolved infection. Using HCV genotype-3 peptides, T cell responses were identified in 19/20 (95%) patients targeting a broad range of viral regions (figure 1A), as reported for spontaneously resolved HCV genotype-1 infection.[35 36] In patients with acute HCV genotype-3 infection who did not resolve infection, T cell responses were identified in 6/12 patients (50%), predominantly targeting non-structural proteins (figure 1B). In chronic HCV genotype-3 infection T cell responses were detected in 56/108 patients that mainly targeted the HCV core (39/56) and NS3 proteins (26/56). Similar to previous data in HCV genotype-1, no T cell responses were detected in 48% of HCV genotype-3 chronically infected individuals (figure 1C).[18 37] As previously described in HCV genotype-1, the total magnitude of T cell responses in spontaneously resolved infection was significantly stronger and targeted more viral peptide pools compared with chronic infection (p<0.0001, online supplementary figure S1A).[35] In defining T cell specificity, we observed that patients with resolved infection preferentially targeted HCV non-structural regions and at higher magnitude compared with patients with chronic

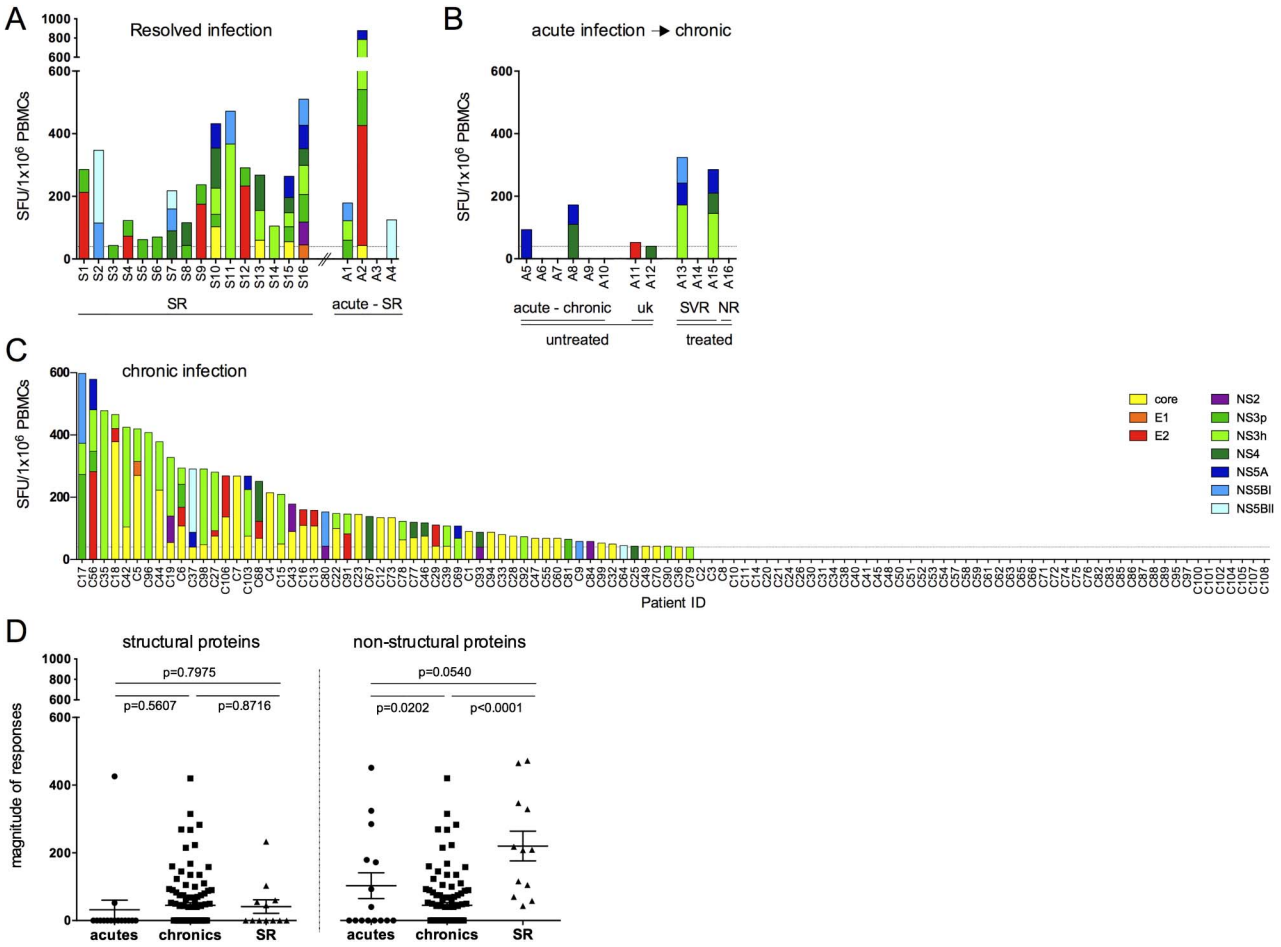

Figure 1 T cell responses against an HCV genotype-3 overlapping peptide set in HCV genotype-3 infected patients. HCV genotype-3 specific T cell responses were measured by IFNγ-ELISpot assays (SFU/10^6 PBMCs) using an HCV genotype-3-specific peptide set spanning the entire HCV genome. T cell responses over cut-off were detected in (A) 16/16 individuals with spontaneously resolved infection and 3/4 patients with acute infection that subsequently spontaneously resolved infection (acutely infected→SR); (B) 6/12 patients with acute HCV genotype-3 infection that did not clear infection; and (C) 56/108 individuals chronically infected with HCV genotype-3. (D) Comparison of total magnitude of T cell response in IFNγ-ELISpot assays to HCV structural and non-structural viral regions is depicted. SFU, spot forming units; NS3p NS3 protease; NS3h NS3 helicase; NS5BI proximal NS5B region; NS5BII distal NS5B region; ns, not significant; PBMC, peripheral blood mononuclear cell; SR, spontaneously resolved patients; C, chronic; uk, unknown; SVR, sustained virological response; NR, non-responder. P values are given between patient groups.

infection, whereas the magnitude of responses to HCV structural proteins did not differ between patient groups (see figure 1D and online supplementary figure S1B).

Additional HCV genotype-3 T cell targets are identified using putative HLA class-I peptides associated with viral genomic polymorphisms

Using overlapping peptides, T cell responses were mapped to individual peptides in 55 patients (see online supplementary tables S5 and S6); 35 HCV genotype-3-specific T cell targets were identified, 10 located in HCV structural and 25 in non-structural regions.

Recognising the fact that T cell responses may be missed using overlapping peptides,[30] wild type and variant peptides corresponding to putative HLA class-I restricted epitopes were assessed in 88 genotype-3 patients with matching HLA types in IFNγ-ELISpot assays. Using this approach, additional T cell targets were identified in 20 patients. Overall, nine T cell epitopes were identified in four different viral regions (NS2, NS3, NS4B, NS5B) in 6/16 (37.5%) patients with acute, 12/64 (18.75%) with chronic and 2/8 (25%) patients with spontaneously resolved infection (figure 2A). Epitopes ATDALMTGY ($NS3_{1442}$, A*01 restricted) and IPFYGKAIPI ($NS3_{1379}$, B*51 restricted) have been previously identified in HCV genotype-1 (at positions $NS3_{1436}$[15 17 20 35 37 39 40] and $NS3_{1373}$[17 41 42]). The seven remaining epitopes were novel HCV genotype-3-specific epitopes: (1) [L]LYPSLIFDI ($NS2_{886}$; restricted by A*02 and A*24); (2) LVRSVMGGKY ($NS2_{931}$; A*03 restricted) (3) FQMIILSIGR ($NS2_{941}$; B*27 restricted); (4) LVTRDADVI ($NS3_{1139}$; A*03 restricted) (5) RVLLDILAGY ($NS4b_{1853}$; A*26 restricted); (6) VLDDHYKTAL ($NS5b_{2490}$; A*02 restricted); and (7) RVKARMLTI ($NS5b_{2508}$; B*08 restricted). Although T cell responses to wild type peptides were readily detected, T cell responses to the variant peptide were only detected in a single epitope ($NS3_{1442}$) in two patients with chronic infection; these were at a lower magnitude than that made to wild type peptide (figure 2A). Viral sequence analysis in these two patients showed that the circulating HCV viral sequence was identical to the variant peptide sequence (see online supplementary table S6). T cell responses using HLA-predicted peptides were identified in the minority of patients with a matched HLA type, ranging from 2.3–40% (figure 2B), with the exception of $NS4b_{1853}$ that was identified in 5/8 (62.5%) HLA A*26 positive patients.

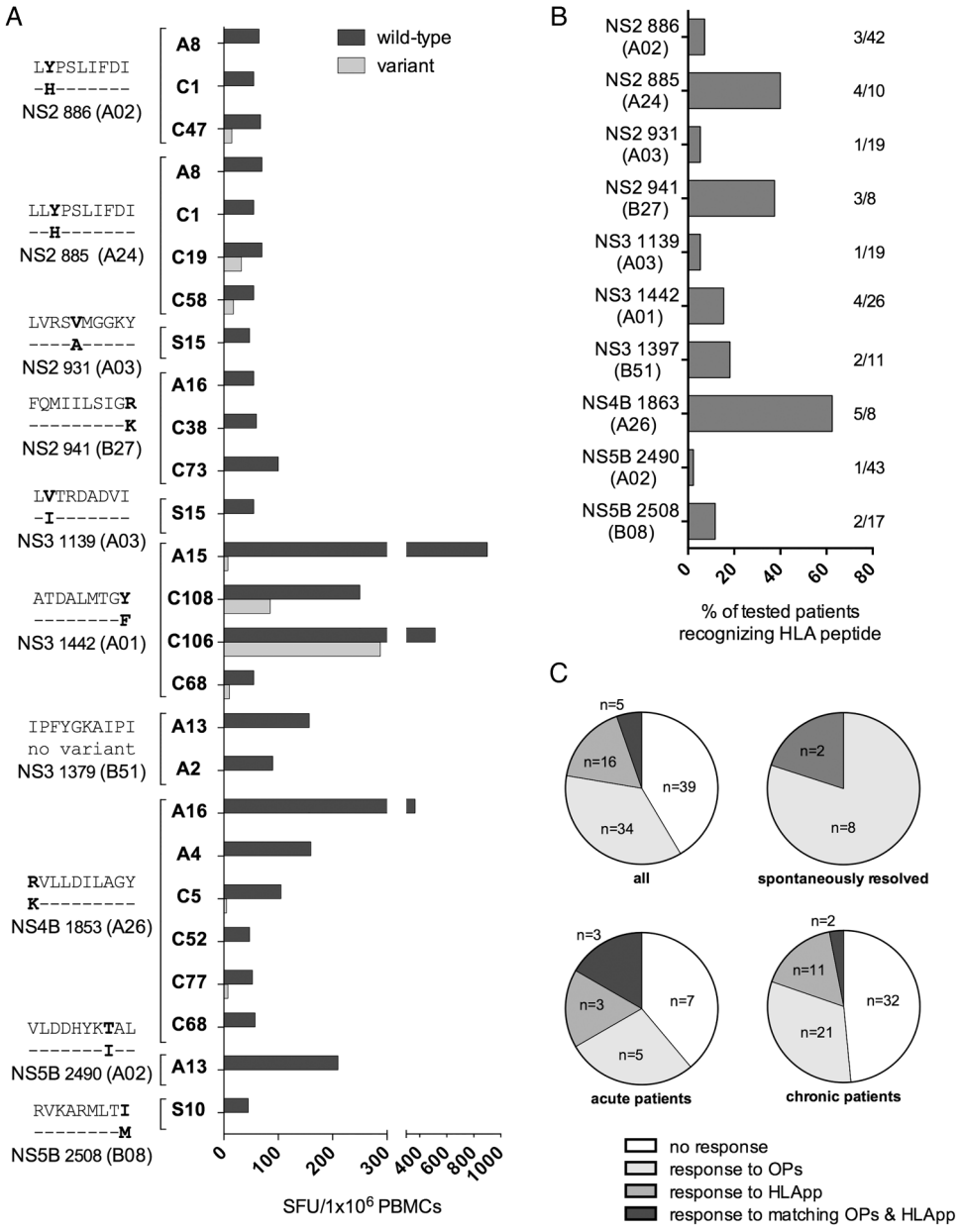

Figure 2 T cell responses detected using HLA-predicted peptides in HCV genotype-3 infected patients. (A) HCV genotype-3 specific T cell responses measured by IFNγ-ELISpot assays (spot-forming units (SFUs)/10^6 peripheral blood mononuclear cells (PBMCs)) using an HLA predicted peptide set. The magnitude of T cell responses to HLA predicted wild type and variant peptides are shown. For each T cell response the responding patient, epitope wild type and variant sequence, HLA restriction and the according HCV viral region are depicted. (B) The percentage of tested HLA-matched patients mounting a detectable T cell response to HLA predicted peptides in IFNγ-ELISpot assays is depicted; also shown as number of patients responding/total number of tested HLA-positive patients. (C) Comparison of T cell responses to HLA-predicted peptides and overlapping pools (only responses to non-structural proteins) for acute, chronic and spontaneously resolved patients, performed at the level of targeted peptide pools. Responses were classified as either: no response to both peptide sets, response to either overlapping pools (OPs) or HLA predicted peptides (HLApp), response to matching OPs and HLApp. * not done; A, acute; C, chronic; SR, spontaneous resolved; HLApp, HLA predicted peptides; OPs, overlapping peptide pools; WT, wild type; V, variant; ID, patient identity number.

Overall, using two distinct peptide-screening approaches we identified 41 distinct genotype-3 T cell targets. However, assessed at the level of peptide pools, only the minority of responses (5.7%) were detected by both approaches (see figure 2C and online supplementary figure S2) and mapped to peptide epitopes at three T cell targets ($NS3_{1379}$, $NS3_{1442}$ and $NS5b_{2490}$, online supplementary table S5). Detection by both methods was highest in acutely infected patients (18.6%), whereas no overlap was observed in spontaneously resolved patients.

T cell subset analysis and viral diversity at HCV genotype-3 T cell targets

The requirement for T cell cross-reactivity at a known target to protect against heterologous infection is dependent on the degree of viral variability at that target in the circulating viral populations. In HCV genotype-1 infection, sequence polymorphisms are more commonly observed at CD8+ compared with CD4+ epitopes.[43] For HCV genotype-3 T cell targets defined in this study, CD4+/CD8+ subset analysis was performed at 25 targets, with 18 CD8+ and 7 CD4+ targets

clearly defined. Viral sequence diversity at these targets was assessed by determining Shannon entropy scores,[44] using an alignment of HCV genotype-3 sequences obtained from the Los Alamos sequence repository and additional inhouse sequences. Although sequence variability was higher at CD8+ than CD4+ targets (mean Shannon entropy score 0.056 vs 0.031), this did not reach statistical significance (p=0.34, see online supplementary figure S4A–H). Analysis of sequence diversity at targeted epitopes within the Oxford cohort showed more polymorphic sites relative to consensus in CD8+ compared with CD4+ epitopes (p=0.0152, see online supplementary figure S4I).

Limited T cell cross-reactivity at HCV genotype-3 T cell targets detected in spontaneously resolved infection to viral variants found between genotypes

First, we assessed T cell cross-reactivity in patients with resolved infection using genotype-specific overlapping peptides across the whole genome. We observed that T cell responses that were almost universally present to the genotype-3 peptide pools (figure 1A) were largely absent using HCV genotype-1 peptides (see online supplementary figure S4 and figure 3A, p<0.0001).

We then determined whether HCV genotype-3-specific T cells were able to recognise common genotype-1 sequence variants at

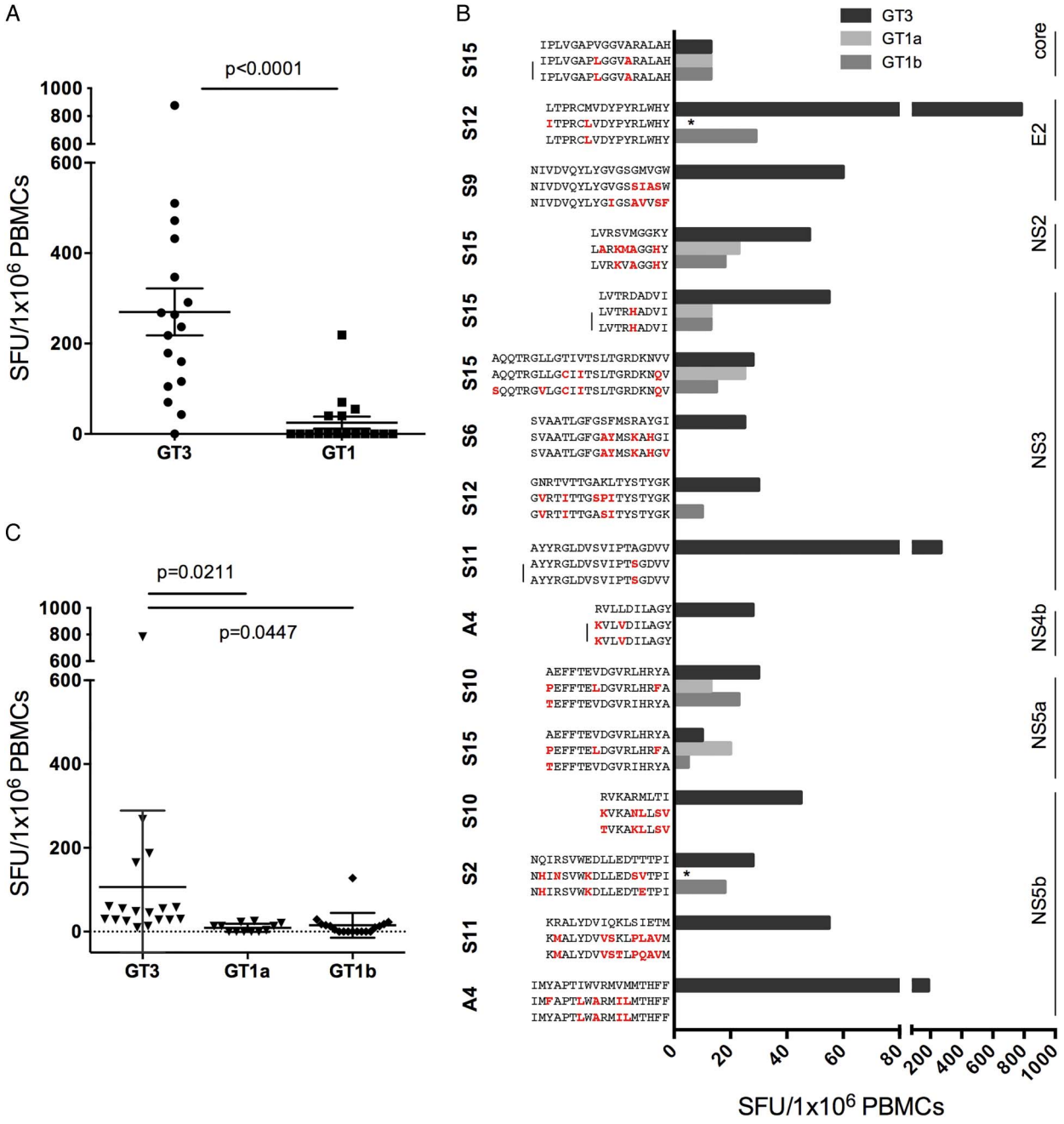

Figure 3 Limited cross-reactivity between HCV genotype-3 and genotype-1 in spontaneously resolved infection. HCV genotype-3 and genotype-1 specific T cell responses were measured by IFNγ-ELISpot assays (spot-forming units (SFUs)/10^6 peripheral blood mononuclear cells (PBMC)) using (A) an HCV genotype-3 and genotype-1 specific overlapping peptide set spanning the entire HCV genome; or (B) HCV genotype-3 and genotype-1 individual peptide variants at T cell targets detected in spontaneously resolved infection. Sequence variants identical between HCV genotype-1a and 1b are marked by a bar, those not assessed by IFNγ-ELISpot assays are marked with a star. (C) Significantly reduced T cell cross-reactivity at T cell targets identified in spontaneously resolved infection was detected against common HCV genotype-1a and genotype-1b sequence variants at individual peptide level.

Table 1 T cell responses detected in spontaneously resolved patients

HCV genotype-3-specific T cell response detected in spontaneous resolved patients						Number of patients responding to epitope			
Viral region	AA position	Sequence	HLA	CD4/CD8	Pept. set	S	C	A	Total
Core	27–51	GGQIVGGVYVLPRRGPRL VYVLPRRGPRLGVRATRK	ND	ND	OP	1	2	–	**3**
	143–158	IPLVGAPVGGVARALAH	ND	CD4	OPs	1	–	–	**1**
E2	610–625	LTPRCMVDYPYRLWHY	ND	ND	OPs	2	1	–	**3**
	702–719	NIVDVQYLYGVGSGMVGW	ND	CD8	OPs	2	–	1TxN	**3**
NS2	931–940	LVRSVMGGKY	A03	CD8	HLA	1	–	–	**1**
NS3	1040–1062	AQQTRGLLGTIVTSLTGR LGTIVTSLTGRDKNVV	ND	ND	OPs	1	–	–	**1**
	1139–1147	LVTRDADVI	A03	CD8	HLA	1	–	–	**1**
	1198–1213	KALQFIPVETLSTQAR	ND	ND	OPs	1	–	–	**1**
	1246–1261	KVPAAYVAQGYNVLVL	ND	ND	OPs	1	–	–	**1**
	1264–1281	SVAATLGFGSFMSRAYGI	ND	ND	OPs	1	–	1UK	**2**
	1282–1305	DPNIRTGNRTVTTGAKL GNRTVTTGAKLTYSTYGK	ND	ND	OPs	2	–	–	**2**
	1379–1387	IPFYGKAIPI	B51	CD8	HLA	–	–	1SR 1TxS	**2**
	1423–1440	AYYRGLDVSVIPTAGDVV	ND	CD4	OPs	1	1	–	**2**
	1520–1537	RPSGMFDSVVLCECYDA DSVVLCECYDAGCSWYDL	ND	CD8	OPs	2	12	–	**14**
NS4b	1805–1822	TSPLTTNQTMFFNILGGW	ND	ND	OPs	2	–	–	**2**
	1853–1862	RVLLDILAGY	A26	CD8	HLA	–	3	1TxS 1SR	**5**
NS5a	2126–2141	AEFFTEVDGVRLHRYA	ND	CD8	OPs	2	–	2TxS	**4**
NS5b	2508–2516	RVKARMLTI	B08	CD8	HLA	1	–	1TxS	**2**
	2548–2565	NQIRSVWEDLLEDTTTPI	ND	CD4	OPs	1	–	–	**1**
	2603–2618	KRALYDVIQKLSIETM	ND	CD4	OPs	1	–	–	**1**
	2844–2861	IMYAPTIWVRMVMMTHFF	ND	ND	OPs	–	–	1SR	**1**
	2893–2908	IIERLHGLSAFTLHSY	ND	CD4	OPs	1	–	–	**1**

T cell targets detected in spontaneously resolved patients and patients acutely infected who subsequently resolved infection spontaneously. For each targeted epitope, the amino acid (AA) position, peptide sequence, restricting HLA type, CD4/CD8 restriction and detecting peptide sets are specified. The total number of patients responding to the peptide and their status of infection (S, spontaneously resolved; C, chronic; A, acute; TxS, treated achieving SVR (sustained virological response); TxN, not responding to treatment; SR, spontaneously resolved) is detailed.
Underlining represents amino acids that are common between overlapping peptides.
ND, not determined; OPs, overlapping peptides.

the peptide level (table 1) in spontaneously resolved patients. We used an alignment of HCV sequences obtained from the Los Alamos sequence repository and additional inhouse sequences to identify common genotype-1 sequence variants (defined as >15% of sequences) at HCV genotype-3-specific T cell targets (see online supplementary figure S5). Sequence identity between genotype-3 and genotype-1 was observed at only 1/19 T cell targets detected in spontaneously resolved infection ($NS3_{1379}$, see online supplementary figure S5). At other T cell targets with distinct sequences between genotypes, limited cross-reactivity

Table 2 Dominant HCV genotype-3-specific T cell responses

Dominant HCV genotype-3-specific T cell response						Number of Patients responding to epitope			
Viral region	AA position	Sequence	HLA	CD4/CD8	Pept. set	S	C	A	Total
Core	66–83	PKARRSEGRSWAQPGYPW	ND	CD4	OP	–	5	–	**5**
	143–158	PVGGVARALAHGVRAL	ND	CD4	OPs	–	11	1TxN	**12**
NS2	886–896	LLYPSLIFDI	A02	CD8	HLA	–	2	1AC	**3**
		LYPSLIFDI	A24	CD8		–	3	1AC	**4**
NS3	1443–1451	ATDALMTGY	A01	CD8	HLA	–	3	1TxS	**4**
	1520–1537	RPSGMFDSVVLCECYDA DSVVLCECYDAGCSWYDL	ND	CD8	OPs	2	12	–	**14**
NS4b	1853–1862	RVLLDILAGY	A26	CD8	HLA	–	3	1TxS 1SR	**5**
NS5a	2126–2141	AEFFTEVDGVRLHRYA	ND	CD8	OPs	2	–	2TxS	**4**

Dominant T cell responses, defined as targeted in more than four patients within the Oxford HCV genotype-3 cohort, are depicted. For each targeted epitope, the amino acid (AA) position, peptide sequence, restricting HLA type, CD4/CD8 restriction and detecting peptide sets are specified. The total number of patients responding to the peptide and their status of infection (S, spontaneously resolved; C, chronic; A, acute; AC, acute proceeding to chronic; TxS, treated achieving SVR (sustained virological response); TxN, not responding to treatment; SR, spontaneously resolved) is detailed.
Underlining represents amino acids that are common between overlapping peptides.
ND, not determined; OPs, overlapping peptides.

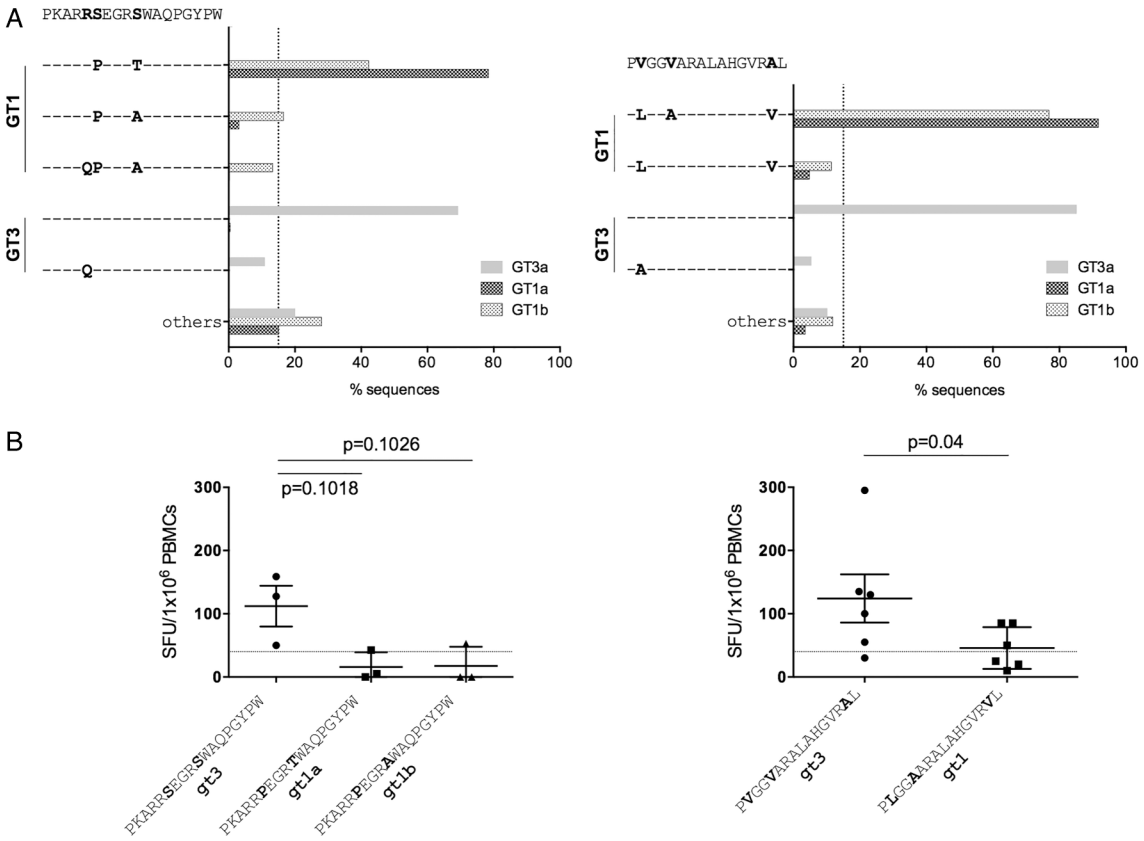

Figure 4 Dominant CD4+ T cell targets are variable between HCV genotypes, with limited T cell cross-reactivity against identified sequence variants. (A) HCV genotype-1 and genotype-3 sequences variants at dominant CD4+ T cell targets $core_{66}$ and $core_{143}$ are depicted. Sequences were obtained from the Los Alamos database, with additional HCV genotype-3 sequences generated inhouse. (B) T cell cross-reactivity as assessed by IFNγ-ELISpot assay (spot-forming units (SFUs)/10^6 peripheral blood mononuclear cells (PBMCs)) against identified sequence variants at dominant CD4+ T cell targets $core_{66}$ and $core_{143}$. GT, genotype; v, variant.

between genotype-1 and genotype-3 variants was observed at 15 T cell targets, tested in 16 patients with PBMC available; with reduced responses at 8 targets and no cross-reactivity at 7 targets (figure 3B, C).

Limited cross-reactivity within and between genotypes at dominant HCV genotype-3 T cell targets

We also assessed T cell cross-reactivity against common genotype-1 and genotype-3 sequence variants at seven dominant T cell targets identified across the entire HCV genotype-3 cohort; two were CD4+ targeting HCV core, and five were CD8+ targeting HCV non-structural proteins (table 2). At the two dominant genotype-3 CD4+ T cell targets ($core_{66}$ and $core_{143}$) no common genotype-3 variants were identified (figure 4A). In contrast, dominant CD4+ core T cell targets varied between HCV genotypes 1 and 3 by one to three AAs (figure 3A), with limited T cell cross-reactivity detected in IFNγ-ELISpot assays (figure 4B). For the majority of CD8+ epitopes (4/5), common HCV genotype-3 sequence variants were identified, with only epitope $NS3_{1520}$ showing a high level of conservation within genotype-3 (figure 5B, left panel). In addition, dominant CD8+ epitopes were highly divergent between HCV genotype-1 and genotype-3, with the exception of epitope $NS3_{1442}$, which has been previously reported to be highly conserved between genotypes (figure 5B, left).[20] Limited T cell cross-reactivity against identified HCV sequence variants was observed at all dominant CD8+ T cell targets, with reduced or abrogated recognition of common genotype-3 and genotype-1 sequence variants (figure 5, right panel).

T cell specificity is distinct between HCV genotypes 1 and 3 infection across the HCV genome

Finally, the overlap in T cell specificity between HCV genotypes 1 and 3 was evaluated across the viral genome. For HCV genotype-1, previously described T cell targets were obtained from the immune epitope database (http://www.iedb.org/) and these were aligned with HCV genotype-3 T cell targets detected in the Oxford cohort for comparison (see figure 6A and online supplementary tables S7 and S8). The majority (11/18) of HCV genotype-3-specific CD8+ T cell targets did not overlap with epitopes previously described in genotype-1. However, six out of seven HCV genotype-3-specific CD4+ epitopes overlapped with those previously described in HCV genotype-1 infection. Next, dominant published HCV genotype-1 epitopes were compared with the Oxford HCV genotype-3 T cell targets (see figure 6B and supplementary figure S6). Minimal overlap in T cell specificity was found at 18 CD8+ epitopes dominant in HCV genotype-1 infection: only one epitope overlapped with those detected in the Oxford HCV genotype-3 cohort. Similarly, of 20 HCV regions frequently targeted by CD4+ cells in HCV genotype-1 infection, overlapping T cell responses in HCV genotype-3 infection were only detected in 2 cases. Overall, T cell specificity was markedly different between HCV genotypes in patients with resolved infection (figure 6C).

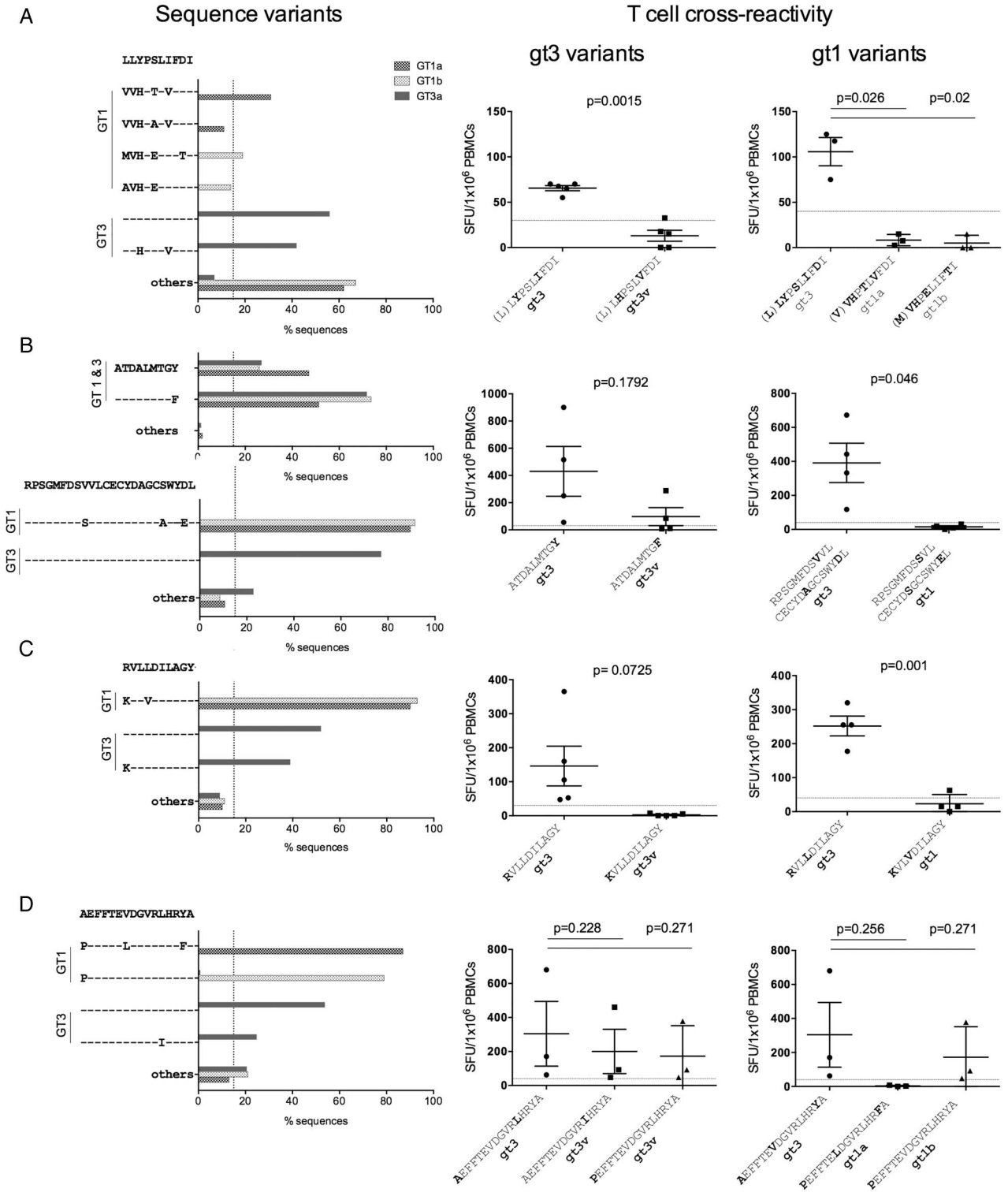

Figure 5 Dominant CD8+ T cell targets are variable within HCV genotype-3 and across HCV genotypes, with limited T cell cross-reactivity against identified sequence variants. (Left panel) HCV genotype-1 and genotype-3 sequence variants at dominant CD8+ T cell targets (A) $NS2_{886}$, (B) $NS3_{1443}$ and $NS3_{1520}$ (C) $NS4b_{1853}$, and (D) $NS5a_{2126}$ are depicted. Sequences were obtained from the Los Alamos database, with additional HCV genotype-3 sequences generated inhouse. (Right panel) T cell cross-reactivity of epitope-specific T cells against identified common sequence variants at dominant CD8+ T cell targets, as assessed by IFNγ-ELISpot assays (spot-forming units (SFUs)/10^6 peripheral blood mononuclear cells (PBMCs)) (A) $NS2_{886}$, (B) $NS3_{1443}$ and $NS3_{1520}$ (C) $NS4b_{1853}$, and (D) $NS5a_{2126}$ is shown. GT, genotype; v, variant.

DISCUSSION

To date, the assessment of T cell immunity in HCV has focused on HCV genotype-1 infection since this infection is dominant in wealthy countries, and was historically more difficult to treat. However, globally more than 53 million people are infected with genotype-3, and in the era of DAA therapy genotype-3 is more difficult to treat.[45 46] This means that the evaluation of T cell immunity in genotype-3 with a view to developing vaccines

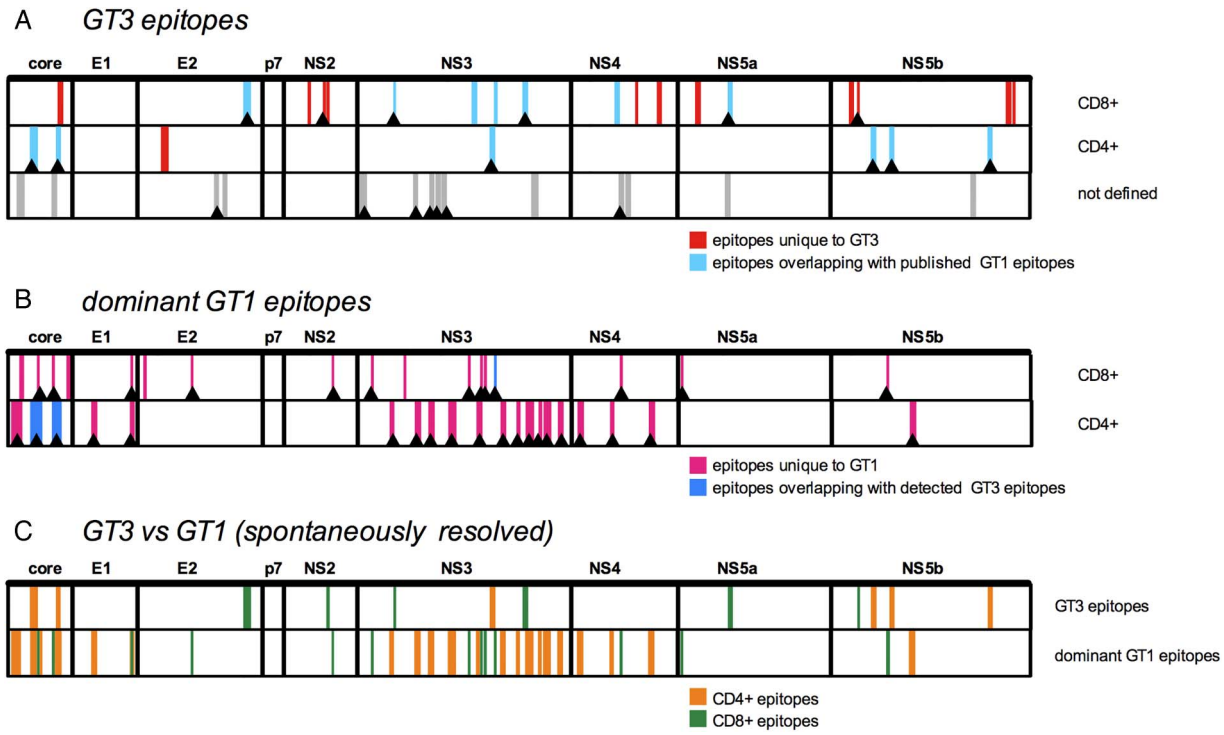

Figure 6 T cell targets are distinct in HCV genotypes 1 and 3. Comparison of T cell specificity in HCV genotypes 1 and 3: (A) HCV genotype-3 CD4+ and CD8+ T cell targets and those without defined CD4/CD8 restriction described in this study are depicted. Genotype-3 T cell targets previously described/not described in HCV genotype-1 infection (as deposited on the immune epitope database, IEDB) are colour coded in light blue/red, respectively. T cell targets detected in at least one patient with spontaneously resolved infection are marked with an arrow. (B) Dominant HCV genotype-1 epitopes as derived from the IEDB are depicted; those detected/not detected in HCV genotype-3 in this study are colour coded in blue/pink, respectively. Genotype-1 T cell targets identified in patients with spontaneously resolved infection in the literature are marked with an arrow. (C) Comparison of HCV immunogenic regions in HCV genotype-3 infection (identified in this study) and HCV genotype-1 infection (from IEDB) that were targeted in patients with spontaneously resolved infection. CD4+ (orange) and CD8+ (green) T cell targets are colour coded. GT, genotype.

capable of targeting multiple genotypes is increasingly relevant. In this study, we set out to perform a comprehensive assessment of T cell specificity in a large cohort of HCV genotype-3 infected patients with acute, resolved and chronic HCV infection. In addition we assessed T cell cross-reactivity with common genotype-3 and genotype-1 viral variants focusing particularly on people with resolved infection, where T cell induction has been shown to play a critical role in viral control. Overall, we show that only the minority of T cell targets is recognised by both genotypes, and that cross-reactivity between common circulating genotype-1 and genotype-3 viral sequence variants is limited.

Similar to published data for HCV genotype-1, we show that T cell responses are readily detectable in resolved infection using genotype-3 specific peptides, target multiple HCV antigenic regions and are of a higher magnitude compared with people with chronic disease, where responses are undetectable in approximately 50% of people[35 47–49] We also observed that overall, patients with resolved genotype-3 infection preferentially targeted HCV non-structural proteins. Together, this data suggests that T cells, particularly to the non-structural regions play an important role in viral clearance irrespective of the viral genotype and that early maintenance of this response is important in viral control.

The detailed assessment of T cell specificity revealed notable differences with limited cross-reactivity between HCV genotypes 1 and 3; to assess T cell specificity we used overlapping peptides in pools derived from a genotype-3 sequence spanning the entire HCV genome. In addition we used a sequence-based screening approach to identify putative HLA-class-I epitopes through the prior assessment of polymorphic HCV genomic sites associated with host HLA in a large cohort of patients with HCV genotype-3 infection.[16] The advantage of the latter approach is that the optimal epitope length, HLA restriction, and functionally relevant 'escape' peptide variants linked to HLA associated T cell escape are predefined. However, this approach is dependent on bioinformatic analysis with a reduced capacity to identify epitopes restricted by rare HLA alleles where information of HLA/peptide binding may be lacking, and by necessity will only identify epitopes where viral variation as a result of T cell pressure occurs. In contrast, overlapping peptides allow for the detection of T cell epitopes across the entire genome in regions where viral escape does not or cannot occur, but that nevertheless may play an important role in viral control. These two approaches were complementary and together identified 41 distinct CD4+ and CD8+ T cell targets in HCV genotype-3.

The requirement for T cell cross-reactivity at a known target to protect against heterologous infection either in natural infection, or following vaccination is dependent on the degree of viral variability at that target in the circulating viral population. At a population level, the majority of T cell targets were not conserved within genotype-3, or between genotype-1 and genotype-3. An analysis of the viral diversity at targeted epitopes within our cohort showed more variability within CD8+ compared with CD4+ targets, consistent with published longitudinal data showing that viral escape to CD4+ epitopes is relatively unusual.[43]

We assessed T cell cross-reactivity in patients with resolved infection first using genotype-specific overlapping peptides and showed that cross-reactivity was minimal. However, we also

found that T cell cross-reactivity was absent or reduced when assessed at a peptide level using common circulating genotype-1 peptide variants. Similarly, there was minimal evidence of T cell cross-reactivity when we assessed dominant genotype-3 responses among the whole cohort. This is in line with previously published cross-reactivity data at dominant HCV genotype-1 epitopes.[21] We cannot exclude the possibility that some of the patients in our cohort were infected with multiple HCV genotypes. New next generation sequencing technologies currently in development may improve the resolution in detecting mixed genotype infection. Nevertheless this is not expected to impact on our measurement of T cell cross-reactivity ex vivo.

Finally, we show that T cell specificity across the HCV genome differs between HCV genotypes 1 and 3, including people with resolved genotype-1 and genotype-3 infection consistent with previous results reporting substantial differences in the patterns of viral adaptation to HLA-restricted immune pressure[16] and differences in T cell responses to the NS3 region between HCV genotypes 1 and 3.[17] In contrast, a recent study analysing responses in HCV genotype-1 and genotype-4 infection suggested that similar HCV regions are targeted in these genotypes, however, responses were not mapped to epitope level.[50]

HCV sequence diversity is thought to be one of the major obstacles in the development of an effective vaccine. Currently HCV T cell vaccines have completed phase-I assessment and are now in phase-IIb efficacy testing.[15 51] In these studies we have shown that HCV vaccines based on simian adenoviral vectors encoding an HCV genotype-1b strain shown some cross-reactivity (approximately 30%) to non-genotype-1 HCV. Parallel efforts in the development of B cell vaccines that aim to induce cross-protective neutralising antibodies against the HCV envelope in distinct viral genotypes are also underway.[52] To date, HCV sequence diversity has been rarely taken into account in the design of HCV immunogens for prophylactic vaccines; a single study specifically aiming to induce cross-reactive T cell responses has assessed the ability of HCV genotype-1 ancestral and consensus sequences to prime T cell immune responses,[53] and we have recently published an in vivo priming model that seeks to identify T cell variants that are maximally cross-reactive for inclusion into a HCV immunogen.[54]

Future HCV immunogens that aim to target multiple genotypes may need to focus on new approaches to target multiple HCV genotypes to generate vaccines that are applicable in settings where mixed genotypes circulate in the population. This may be possible using viral vectored strategies that can encode large immunogens.[15] Some approaches that are currently in development for vaccines against immunodeficiency virus may be readily also applied to HCV. This may include vaccines encoding viral regions that are conserved between genotypes,[29] excluding variable epitopes dominant in natural infection, with the hope of inducing T cells to subdominant epitopes. Alternative approaches include the use of multivalent mosaic immunogens that encode antigens derived from multiple genotypes.[55]

In conclusion, we show that HCV T cell specificity is distinct between two highly prevalent global genotypes with limited T cell cross-reactivity between common viral variants at dominant epitopes. Since this also holds true for people with resolved infection, our data suggests that regions frequently targeted in natural HCV infection may not serve as attractive targets for a vaccine that aims to protect against multiple HCV genotypes.

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Data sharing statement We will share our raw anonymised data after publication with interested parties.

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		spontaneous resolvers		acutely infected		chronic patients	
number of patients		16	%	16	%	108	%
sex	female	5	**31.3**	7	**43.8**	31	**28.7**
	male	11	**68.8**	9	**56.3**	77	**71.3**
Age	>40	7	**43.8**	5	**31.3**	35	**32.4**
	<40	9	**56.3**	11	**68.8**	73	**67.6**
Ethnicity	Caucasian	16	**100.0**	15	**93.8**	87	**80.6**
	Asian					16	**14.8**
	Hispanic			1	**6.3**	1	**0.9**
	not known					4	**3.7**
Risk factor	IVDU	11	**68.8**	9	**56.3**	70	**64.8**
	unkown	1	**6.3**			14	**13.0**
	BP			1	**6.3**	12	**11.1**
	cocaine					6	**5.6**
	tattoo	2	**12.5**	1	**6.3**	3	**2.8**
	needlestick	1	**6.3**			2	**1.9**
	sexual	1	**6.3**	5	**31.3**	0	**0.0**
	vertical					1	**0.9**
HCV subtype	Genotype 3		N/A	8	**50.0**	14	**13.0**
	Genotype 3a		N/A	8	**50.0**	94	**87.0**
Viral load	IU/ml (mean)		N/A		**1827490**		**872828**
	IU/ml (range)		N/A		6290-8930000		3614-17880410
Outcome	cleared spontaneously	16	**100.0**	4	**25.0**	0	**0.0**
	chronic	0	**0.0**	10	**62.5**	108	**100.0**
	not treated	N/A		6	60.0	30	27.8
	treated	N/A		4	40.0	78	72.2
	unkown			2	**12.5**		
Treatment	SVR			2	**50.0**	42	**53.8**
	REL			1	**25.0**	25	**32.1**
	NR			1	**25.0**	6	**7.7**
	incomplete					3	**3.8**
	not known					2	**2.6**

Table S1: Patient details of patients with spontaneously resolved, acute and chronic HCV genotype-3 infection.
ID: patient identifier; IVDU: intravenous drug use; BP: blood products; SVR: sustained virological response; REL: viral relapse; NR: treatment non-response.

ID	Age	Sex	Race	Risk factor	GT	HCV VL	ALT	Treatment	outcome
						Iu/ml			
A1	23	F	W	Sexual	3	>700000	68	naïve	cleared spontaneously
A2	31	M	W	IVDU	3a	6290	not known	naïve	cleared spontaneously
A3	23	M	W	IVDU	3a	8930000	46	naïve	cleared spontaneously
A4	21	M	W	IVDU	3	15900	72	naïve	cleared spontaneously
A5	19	M	W	IVDU	3	168000	229	naïve	chronic
A6	20	F	W	IVDU	3	16100	38	naïve	chronic
A7	46	M	W	blood exposure	3	204000	592	naïve	chronic
A8	28	M	W	Sexual	3	136000	698	naïve	chronic
A9	45	F	W	Tattoo	3a	>700000	201	naïve	chronic
A10	21	F	W	IVDU	3a	600000	not known	naïve	chronic
A11	18	F	W	IVDU	3	102000	88	naïve	lost in <6 months
A12	25	F	W	IVDU	3a	not known	21	naïve	lost in <6 months
A13	40	F	W	Sexual	3a	>700000	464	treated	cleared on Tx
A14	41	M	H	Sexual	3a	67535	250	treated	cleared on Tx
A15	49	M	W	Sexual	3a	46603	1441	treated	cleared on Tx
A16	20	M	W	IVDU/Tattoo	3	>700000	383	treated	chronic (non-responder)

Table S2: Patient details acute HCV genotype-3 cohort.
ID: patient identifier; M: male; F: female; W: white; H: Hispanic; IVDU: intravenous drug use; GT: genotype; ALT: alanine aminotransferase; Tx: treatment.

Protein	HLA	Wildtype	Variant	Amino Acids	Peptide ID	Predicted Epitope
NS2	A0101	V	A	879-887	001-A0101	**V**ILLTSLLY
	A02	L	P	862-871	049-A02	ALQVWVPPL**L**
		L	P	870-879	048-A02	L**L**ARGSRDGV
		Y	H	881-890	052-A02	LLTSLL**Y**PSL
		Y	H	885-894	047-A02	LL**Y**PSLIFDI
	A0301	V	A	926-935	006-A0301	RLCMLVRS**V**M
		V	A	929-938	004-A0301	MLVRS**V**MGGK
		V	A	930-939	005-A0301	LVRS**V**MGGKY
	A2402	Y	H	886-894	007-A2402	L**Y**PSLIFDI
	B1501	T	A	878-887	008-B1501	GVILL**T**SLLY
	B15	I	V	946-954	055-B15	S**I**GRWFNTY
	B2705	R	K	940-949	010-B2705	FQMIILSIG**R**
		R	K	948-956	009-B2705	G**R**WFNTYLY
		H	Y	962-971	011-B2705	MQ**H**WAAAGLK
	B4402	I	V	822-831	012-B4402	ATLGAG**I**LVL
	B44	I	V	826-835	057-B44	AG**I**LVLFGFF
	B5101	S	G	871-880	013-B5101	LARG**S**RDGVI
	C03	L	M	829-838	050-C03	I**L**VLFGFFTL
	C04	V	I	981-990	051-C04	IFSPMEIK**V**I
NS3	A0101	Y	F	1442-1450	014-A0101	ATDALMTG**Y**
	A0201	A	D	1389-1398	015-A0201	**A**LLKGGRHLI
	A0301	V	I	1138-1146	016-A0301	L**V**TRDADVI
	B1501	K	R	1296-1305	017-B1501	**K**LTYSTYGKF
	B2705	L	I	1379-1388	018-B2705	IPFYGKAIP**L**
		V	I	1632-1641	019-B2705	YRLGP**V**QNEI
	B4402	G	S	1407-1416	020-B4402	DEIASKLR**G**M
	B4403	L	S	1639-1647	021-B4403	NEIC**L**THPI
	B5101	A	D	1388-1397	023-B5101	I**A**LLKGGRHL
NS4B	A02	A	T	1873-1882	062-A02	KIMGGELPT**A**
		A	T	1880-1889	065-A02	PT**A**EDMVNLL
	A0301	I	V	1901-1910	026-A0301	GV**I**CAAILRR
	A2601	R	K	1852-1861	027-A2601	**R**VLLDILAGY
	A68	A	I	1736-1744	067-A68	EK**A**LGLLQR
	B27	R	K	1948-1957	061-B27	A**R**VTALLSSL
	B4001	I	V	1847-1855	028-B4001	G**I**GLGRVLL
	B51	A	T	1879-1888	066-B51	LPT**A**EDMVNL
		A	T	1881-1889	064-B51	T**A**EDMVNLL
	C0401	V	I	1733-1742	025-C0401	QFKEK**V**LGLL
NS5A	A0201	G	S	2321-2330	029-A0201	ALPPR**G**APPV
	A0301,	V, P	A, S	2382-2391	030-A0301	K**V**PPS**P**GGES
	A2601	D	G	2268-2276	033-A2601	ET**D**AELSVA
	A68	T	S	1989-1998	070-A68	WVC**T**VLSDFK
	B0702	V	E	2309-2317	036-B0702	APDY**V**PPTV
		V	E	2313-2322	035-B0702	**V**PPTVHGCAL
		T	A	2332-2341	034-B0702	PPRRKR**T**IQL
	B0801	I	V	2251-2259	037-B0801	ESETKVV**I**L
	B44	R	Q	2097-2105	071-B44	VEVR**R**VGDF
NS5B	A0201	T	I	2489-2498	038-A0201	VLDDHYK**T**AL
		N	D	2540-2549	040-A0201	SLSSKAI**N**QI
		N	D	2544-2552	039-A0201	KAI**N**QIRSV
	A1101	R	K	2500-2509	041-A1101	EVKERAS**R**VK
	A2601	K	R	2537-2545	042-A2601	DVRSLSS**K**A
	B0801	I	M	2507-2515	043-A2601	RVKARMLT**I**
	B1501	Q	L	2476-2484	044-B1501	S**Q**RQKKVTF
	B5101	K	R	2474-2482	045-B5101	SASQRQ**K**KV

Table S3: HLA-associated polymorphisms – sequence polymorphisms located *within* the predicted epitope sites.

HCV viral region, associated HLA type, epitope position, peptide identifier and sequence, as well as wild-type and variant sequences are depicted. Polymorphic sites associated with patient HLA are marked in in bold.

Protein	HLA	Wildtype	Variant Residue	Amino Acids	Polymor-phic Site	Peptide ID	Predicted Epitope and Wild-type Residue ()
NS2	A0201	I	V	934-942	943	002-A0201	VMGGKYFQM(**I**)
		I	V	944-953	943	003-A0201	(**I**)ILSIGRWFNT
	A02	L	P	861-870	871	054-A02	AALQVWVPPL(**L**)
		Y	H	878-886	887	053-A02	GVILLTSLL(**Y**)
NS3	A68	L	F	1047-1056	1046	058-A68	(**L**)GTIVTSLTGR
	B5101	A	D	1379-1388	1389	022-B5101	IPFYGKAIPI(**A**)
		A	D	1390-1398	1389	024-B5101	(**A**)QLKGGRHLI
NS5A	A1101	V	A	2374-2382	2383	031-A1101	DTQSSTTSK(**V**)
	A2402	D	E	2138-2147	2148	032-A2402	RYAPPCKPLL(**D**)
NS5B	B5101	M	L	2846-2854	2855	046-B5101	APTIWVRMV(**M**)

Table S4: HLA-associated polymorphisms – sequence polymorphisms located *flanking* the predicted epitope sites.
HCV viral region, associated HLA type, epitope position, peptide identifier and sequence as well as wild-type and variant sequences are depicted. Polymorphic sites flanking epitope are indicated in bold within brackets ().

Protein	Position	3a peptide sequence	Patient		Patient viral sequence	CD8/CD4
core	27-51	GGQIVGGVYVLPRRGPRL	C58	*	GGQIVGGVYVLPRRGPRL	N/A
		VYVLPRRGPRLGVRATRK	C106		VYVLPRRGPRLGVRATRK	
			S11		SR	
	66-90	PKARRSEGRSWAQPGYPW	C5		ND	CD4
		GRSWAQPGYPWPLYGNEG	C12		PKARRSEGRSWAQPGYPW	
			C37		PKARRSEGRSWAQPGYPW	
			450		PKARRSEGRSWAQPGYPW	
			C68	*	GRSWAQPGYPWPLYGNEG	
	130-147	FADLMGYIPLVGAPVGGV	C15		FADLMGYIPLVGAPVGGV	N/A
			C5		ND	
	137-154	IPLVGAPVGGVARALAH	S15		SR	N/A
	143-158	PVGGVARALAHGVRAL	A16		PVGGVARALAHGVRAL	CD4
			C6	*	P**A**GGVARALAHGVRAL	
			C12		PVGGVARALAHGVRAL	
			C13		PVGGVARALAHGVRAL	
			C18		PVGGVARALAHGVRAL	
			C19		PVGGVARALAHGVRAL	
			C22		PVGGVARALAHGVRAL	
			C23		PVGGVARALAHGVRAL	
			C27		PVGGVARALAHGVRAL	
			331	*	PVGGVARALAHGVRAL	
			C70		PVGGVARALAHGVRAL	
			C77	*	PVGGVARALAHGVRAL	
	148-165	ARALAHGVRALEDGINFA	C103		ARALAHGVRALEDGINFA	CD8
E2	460-476	CKPITFFRQGWGSLTDA	A15		CKPITFFRQGWGSLTDA	CD4
		FRQGWGSLTDANITGPSD	C106		F**N**QGWGSLTDANI**S**GPSD	
	610-625	LTPRCMVDYPYRLWHY	C106		LTPRC**L**VDYPYRLWHY	N/A
			S1	*	SR	
			S12		SR	
	635-650	KVRMFVGGFEHRFTAA	A11		KVRMFVGGFEHRFTAA	N/A
			C13		KVRMFVAGFEHRFTAA	
	696-719	LIHLHQNIVDVQYLYGV	S4	*	SR	CD8
		NIVDVQYLYGVGSGMVGW	A16		NIVDVQYLYGVGSGMVGW	
			S9	*	SR	

Table S5: HCV genotype-3-specific T-cell targets in HCV structural regions.
HCV genotype-3-specific T-cell targets identified using overlapping peptide pools in HCV structural regions are depicted. All patients targeting specific individual peptides are specified (colour coding: orange-SR, blue-acute, yellow chronic). Circulating viral sequence is depicted when obtained, as well as CD4+ /CD8+ subset analysis. Sequence polymorphisms differing from overlapping peptide set sequence are marked in red. * Epitopes previously described in (Humphreys et al. 2012). N/A not available.

Protein	Position	3a peptide sequence		Patient		Patient viral sequence	CD8/CD4
NS2	886-896	(L)LYPSLIFDI	#	A8		LLYPSLIFDI	CD8
			#	A8		LYPSLIFDI	
			#	C1		LYPSLIFDI	
			#	C19		LYPSLIFDI	
			#	C47		LYPSLIFDI	
			#	C58		LY**S**SLIFDI	
	931-940	LVRSVMGGKY	#	S15		SR	CD8
	941-951	FQMIILSIGR	#	A16		FQM**A**ILSIGR	CD8
			#	C38		FQMIIL**GV**GR	
			#	C73		FQM**V**ILSIG**K**	
NS3	1040-1062	AQQTRGLLGTIVTSLTGR LGTIVTSLTGRDKNVV		S15		SR	N/A
	1139-1147	LVTRDADVI	#	S15		SR	CD8
	1198-1213	KALQFIPVETLSTQAR		S5	*	SR	N/A
	1246-1261	KVPAAYVAQGYNVLVL		S8	*	SR	N/A
	1264-1281	SVAATLGFGSFMSRAYGI		A11		SVAATLGFGSFMS**H**AYGI	N/A
				S6		SR	
	1282-1305	DPNIRTGNRTVTTGAKL GNRTVTTGAKLTYSTYGK		S1	*	SR	N/A
				S12		SR	
	1370-1387	EEVALGSEGEIPFYGKAI		A13		ND	CD8
	1379-1387	IPFYGKAIPI	#	A13		ND	
			#	A2		ND	
	1423-1440	AYYRGLDVSVIPTAGDVV		C22		AYYRGLDVSVIPTAGDVV	N/A
				S11	*	SR	CD4
	1436-1447	GDVVVCATDALMTGF		A15		GDVVVCATDALMTG**Y**	CD8
	1442-1447	ATDALMTGY	#	A15		ATDALMTG**Y**	
			#	C68		ATDALMTG**F**	
			#	C106		ATDALMTG**F**	
			#	C108		ATDALMTG**F**	
	1520-1537	RPSGMFDSVVLCECYDA DSVVLCECYDAGCSWYDL		C5		ND	CD8
				C7		RPSGMFDSVVLCECYDAGCSWYDL	
				C15	*	RPSGMFDSVVLCECYDAGCSWYDL	
				C17		RPSGMFDSVVLCECYDAGCSWYDL	
				C19		RPSGMFDSVVLCECYDAGCSWYDL	
				C27	*	RPSGMFDSVVLCECYDAGC**A**WYDL	
				C35		ND	
				C42		ND	
				C44		RPSGMFDSVVLCECYDAGCSWYDL	
				C58	*	RPSGMFDSVVLCECYDAGCSWYDL	
				C96		ND	
				C98		ND	
				S13		SR	
				S14		SR	
	1547-1569	RAYLSTPGLPVCQDHLDF GLPVCQDHLDFWESVF		C19		RAYLSTPGLPVCQDHLDFWESVF	N/A
NS4B	1792-1808	PAVASLMAFTASVTSPL		A8		ND	CD8
				C68	*	PAVASLMAFTASVTSPL	
				C77	*	ND	
	1805-1822	TSPLTTNQTMFFNILGGW		S8	*	SR	N/A
				S13		SR	
	1825-1842	THLAGPQSSSAFVVSGLA		C77	*	ND	N/A
	1853-1862	RVLLDILAGY	#	A16		**K**VLLDILAGY	CD8
			#	A4		ND	
			#	C5		**K**VLLDILAGY	
			#	C52		ND	
			#	C77		ND	
	1917-1932	EGAVQWMNRLIAFASR		C67	*	EGAVQWMNRLIAFASR	CD8
NS5A	2030-2047	GVMSTRCPCGASIAGHVK		C13	*	GVMSTRCPCGASI**T**GHVK	CD8
	2119-2136	CPCQVPAAEFFTEVDGVR		A8		CPCQVPA**P**EFFTEVDGVR	
	2126-2141	AEFFTEVDGVRLHRYA		A13		ND	CD8
				A15		ND	
				S10		SR	
				S15		SR	
	2145-2162	KPLLRDEITFMVGLNSYA		A5		ND	N/A
NS5B	2484-2499	TFDRLQVLDDHYKTAL		A13		TFDRLQVLDDHYKTAL	CD8
	2490-2499	VLDDHYKTAL	#	C68		ND	
	2508-2516	RVKARMLTI	#	A13		RVKARMLTI	CD8
			#	S10		SR	
	2548-2565	NQIRSVWEDLLEDTTTPI		S2	*	SR	CD4
	2603-2618	KRALYDVIQKLSIETM		S11	*	SR	CD4
	2844-2861	IMYAPTIWVRMVMMTHFF		A4		ND	N/A
	2893-2908	IIERLHGLSAFTLHSY		S2	*	SR	CD4
	2947-2964	GKAKICGLYLFNWAVRTK		C37	*	GKAKI**T**GLYLFNWAVRTK	CD8
	2967-2976	KLTPLPAAGQL		450	*	KLTPLPAAG**LL**	CD8

Table S6: HCV genotype-3-specific T-cell targets in HCV non-structural regions. HCV genotype-3-specific T-cell targets identified using overlapping peptide pools and HLA predicted peptides (#) in HCV structural regions are depicted. All patients targeting specific individual peptides are specified (colour coding: orange-SR, blue-acute, yellow chronic). Circulating viral sequence is depicted when obtained, as well as CD4+ /CD8+ subset analysis. Sequence polymorphisms differing from overlapping peptide set sequence are marked in red. *Epitopes previously described in (Humphreys et al. 2012). N/A not available.

GT3 CD8+ epitopes (Oxford Study Cohort)						GT1 CD8+ epitopes in corresponding regions		
Position	3a peptide sequence	Viral protein	HLA	Pt ID	HLA type	Peptide (Literature)	HLA	First author
931-940	LVRSVMGGKY	NS3	A03	S15	A*3201 A*0301 B*1401 B*0702 C*0802 C*0702 #	no CD8 epitopes described		
941-951	FQMIILSIGR	NS2	B27	A16	A*0201 A*2601 B*2702 B*3801 C*1203 #	no CD8 epitopes described		
				C38	A*0201 A*1101 B*1801 B*2705 C*0102 C*1203 #			
				C73	A*0201 B*5701 B*2702 C*0701 C*1501 #			
1917-1932	EGAVQWMNRLIAFASR	NS4B		C67	A*0101 A*3001 B*1302 B*4402 C*0602 C*0501 # *	no CD8 epitopes described		
2030-2047	GVMSTRCPCGASIAGHVK	NS5A		C13	A*1101 A*7401 B*4403 B*38 C*04 C*0702 # *	no CD8 epitopes described		
2484-2499	TFDRLQVLDDHYKTAL	NS5B		A13	A*0101 B*0801 B*5101 C*0102 C*0701 #	no CD8 epitopes described		
2490-2499	VLDDHYKTAL		A02	C68	A*0101 A*0201 B*0801 B*5701 C*0602 C*0701 #			
2508-2516	RVKARMLTI	NS5B	B08	A13	A*0101 B*0801 B*5101 C*0102 C*0701 #	no CD8 epitopes described		
				S10	A*3201 A*0101 B*0801 B*4402 C*0501 C*0701			
2967-2976	KLTPLPAAGQL	NS5B		450	# *	no CD8 epitopes described		
1853-1862	RVLLDILAGY	NS4B	A26	A16	A*0201 A*2601 B*2702 B*3801 C*120301 #	ILAGYGAGV	A2	M Battegay
				A4	A*0201 A*2601 B*3801 B*4402 C*0501 C*1203 #	ILAGYGAGV	A2	N H Gruener
				C6	A*2301 A*2601 B*3801 B*4901 C*0701 C*1203 #	ILAGYGAGV	A2	T Kuntzen
				C52	A*0101 A*2601 B*4501 B*0801 C*0602 C*0701 #			
				C77	A*0201 A*2601 B*3801 B*2702 C*1203 C*0102 #			
886-896	LLYPSLIFDI/LYPSLIFDI	NS2	A02/A24	A8	A*2402 A*0201 B*3502 B*4403 C*0401 C*1601 #	**H**P**T**L**V**FDITK	Class I	A L Cox
				C1	A*1101 A*2404 B*18 B*3501 C*0401 C*1203 #	**H**P**T**L**V**FDITKL	Class I	T Kuntzen
				C19	A*24 B*3501 B*4403 C*0401 C*0409 #			
				C47	A*0205 A*2402 B*1302 B*4901 C*0602 C*0701 #			
				C1	A*0205 A*2402 B*1302 B*4901 C*0602 C*0701 #			
				C58	A*0101 A*0201 B*0702 B*4001 C*0304 C*0702 #			
2947-2964	GKAKICGLYLFNWAVRTK	NS5B		C37	A*1101 B*0702 B*4402 C*0501 C*0702 # *	RGG**R**A**A**ICG**K**YLFNWAVR	Class I	C Neumann-Haefelin
						G**R**A**A**ICG**K**Y	B27	C Neumann-Haefelin
						G**R**A**A**ICG**K**YLFNWAV	Class I	P T F Kennedy
						KYLFNWAV**K**	A2	Z Guo
148-165	ARALAHGVRALEDGINFA	core		C103	#	GVR**V**LEDG**V**	A2	H F Löhr
						R**V**LEDGVN**Y**	Class I	D D Anthony
						VLEDGVN**Y**ATGNLPG	Class I	K Sugimoto
1139-1147	LVTRDADVI	NS3	A03	975	A*3201 A*0301 B*1401 B*0702 C*0802 C*0702 #	VTR**H**ADVIPV	Class I	T Kuntzen
1520-1537	RPSGMFDSVVLCECYDAGCSWYDL	NS3	B35/C04	C5	A*1101 A*1101 B*3501 B*5101 C*0401 C*1402	MFDS**S**VLCECYDAGC	Class I	D Ciuffreda
				C7	A*0101 A*0201 B*35 B*14 C*0401 C*0802			
				C15	A*2402 A*3002 B*0702 B*3501 # *			
				C17	A*1101 A*1101 B*1517 B*3501 C*0401 C*0701			
				C19	A*1101 A*2402 B*18 B*3501 C*0401 C*1203			
				C27	A*0301 A*3002 B*0702 B*3501 C*0401 C*0702 # *			
				C35				
				C42	A*0101 A*2901 B*3501 B*4403 C*0401 C*1601			
				C44	A*0301 A*2402 B*3501 B*5501 C*0301 C*0401			
				C58	A*2402 B*3501 B*4403 C*0401 C*0409 # *			
				C96				
				C98	A*0201 A*1101 B*3501 B*4001 C*0401 C*0301			
				S13	A*0301 A*2601 B*3501 B*2701 C*0401 C*0202			
				S14	A*0201 A*1101 B*4402 B*3501 C*0401 C*0501			
2126-2141	AEFFTEVDGVRLHRYA	NS5A		A13	A*0101 B*0801 B*5101 C*0102 C*0701 #	FFTE**L**DGVRLHRFAP	Class I	D Ciuffreda
				A15	A*0101 B*5201 B*5701 C*0602 C*1202			
				S10	A*3201 A*0101 B*0801 B*4402 C*0501 C*0701			
				S15	A*3201 A*0301 B*1401 B*0702 C*0802 C*0702			
1792-1808	PAVASLMAFTASVTSPL	NS4B		6-23	A*2402 A*0201 B*3502 B*4403 C*0401 C*1601	SLMAFTA**A**V	A2	B Rehermann
				C68	A*0101 A*0201 B*0801 B*5701 C*0602 C*0701 # *	SLMAFTA**A**V	A2	K M Chang
				C77	A*0201 A*2601 B*3801 B*2702 C*1203 C*0102 # *	SLMAFTA**A**V	A2	N H Gruener
696-719	LIHLHQNIVDVQYLYGVGSGMVGW	E2		S4	A*02 A*3201 B*2707 B*1501 C*0602 C*0304 # *	ALSTGLIHLHQNIVD	Class I	D Ciuffreda
				A16	A*0201 A*2601 B*2702 B*3801 C*1203	LHQNIVDVQYLYGVG	Class I	D Ciuffreda
				S9	# *			
1379-1387	IPFYGKAIPI	NS3	B51	A13	A*0101 B*0801 B*5101 C*0102 C*0701 #	IPFYGKAI &	B51	S Giugliano
				A2	B*4403 B*5101 C*1402 C*1601 #	IPFYGKAIP**L**	B51	D Yerly
1436-1447	GDVVVCATDALMTGF	NS3		A15	A*0101 B*5201 B*5701 C*0602 C*1202	ATDALMTGY	A1	G M Lauer, 2002
1442-1447	ATDALMTGY		A01	A15	A*0101 B*5201 B*5701 C*0602 C*1202 #	**V**ATDALMTGYTGDFD	Class I	A M Werthheimer
				C108	A*0101 A*2402 B*0801 B*3906 C*0701 C*0702 #	ATDALMTGF &	A1	S Giugliano
				C106	A*0101 A*0301 B*0702 B*0702 C*0702 C*0702 #	ATDALMTGF	A1	T Kuntzen
				C68	A*0101 A*0201 B*0801 B*5701 C*0602 C*0701 #	ATDALMTGY	A1	A L Cox
						ATDALMTGY	A1	E Barnes
						ATDALMTGY	A1	G M Lauer, 2004

Table S7: Overlap between genotype-3-specific CD8+ T-cell targets defined in this study and previously described HCV genotype-1-specific CD8+ targets.
HCV genotype-3-specific CD8+ epitopes and described genotype-1-specific CD8+ epitopes were classified by overlap (left coloured bar): 'overlapping' (blue), 'likely overlapping' (light blue, <20% sequence differences within targeted area), 'unlikely overlapping' (light red, >20% differences within targeted area, or less than 7 amino acids overlap), and 'not overlapping' (red). ***Left:*** For each epitope, viral region, position and genotype-3 sequence is given, as well as patients targeting the epitopes (colour coding: orange-SR, blue-acute, yellow chronic), and patient's HLA class-I types. ***Right:*** Published overlapping genotype-1 CD8+ epitopes are specified, including sequence, restricting HLA type and first author of the publication; overlap between epitopes is underlined and genotype-3/genotype-1 sequence differences marked bold. # CD8 restriction for marked patient experimentally defined; * Epitopes

previously described in (Humphreys et al. 2012); & Epitope previously described for genotype-1 and 3.
(M Battegay et al. 1995; Rehermann et al. 1996; K. M. Chang et al. 1997; Löhr et al. 1999; Grüner et al. 2000; Wertheimer et al. 2003; Sugimoto et al. 2003; Lauer et al. 2004; Cox et al. 2005; Kennedy et al. 2006; Kuntzen et al. 2007; Yerly et al. 2008; Ciuffreda et al. 2008; Giugliano et al. 2009; Neumann-Haefelin et al. 2008; Barnes et al. 2012; Guo et al. 2012)

GT3 CD4+ epitopes (Oxford Cohort)							GT1 epitopes described in corresponding regions		
Position	3a peptide sequence	Viral protein	Patient ID				Peptide (Literature)	HLA	First author
453-476	CPQRLSSCKPITFFRQGWGSLTDANITGPSD	E2	A15	DRB1*0701	DRB1*1502	#	no CD4 epitopes described		
			C106						
2603-2618	KRALYDVIQKLSIETM	NS5B	S11	DRB1*0404	DRB1*0701	# *	KL**PLAV**MGSSYGFQYSPGQR	Class-II	J Schulze zur Wiesch, 2007
							KL**PLAV**MGSSYGFQYSPGQR	Class-II	C L Day
66-90	PKARRSEGRSWAQPGYPWPLYGNEG	core	C5	DRB1*0101	DRB1*0701		RRQPIPKARR**P**EGR**T**WAQPG	Class-II	J Schulze zur Wiesch, 2012
			C12	DRB1*0301	DRB1*0401		RQPIPKVRR**P**EGR**T**	HLA-DR	J J Lasarte
			C37	DRB1*0401	DRB1*0407		K**V**RR**P**EGR**T**WAQPG	HLA-DR	J J Lasarte
			450			#	PEGR**T**WAQPGYPWPLYGNEG	Class-II	J Schulze zur Wiesch, 2007
			C68	DRB1*0101	DRB1*0701	# *	PEGR**T**WAQPGYPWPLYGNEGCGW	Class-II	H F Löhr
							PEGR**T**WAQPGYPWP	HLA-DR	J J Lasarte
							PEGR**T**WAQPGYPWPL	Class-II	P T F Kennedy
143-158	PVGGVARALAHGVRAL	core	A16				ADLMGYIPLVGAP**L**GG**A**ARA	Class-II	J Schulze zur Wiesch, 2007
			C6	DRB1*0801	DRB1*1101	# *	ADLMGYIPLVGAP**L**GG**A**AR	HLA-DR	F A Castelli
			C12	DRB1*0301	DRB1*0401	#	ADLMGYIPLVGAP**L**GG**A**ARA	Class-II	J Schulze zur Wiesch, 2012
			C13	DRB1*1504	DRB1*0701		LMGYIPLVGAP**L**GG**A**	Class-II	D Ciuffreda
			C18	DRB1*0301	DRB1*1602	#	LVGAP**L**GG**A**ARAL	Class-II	H F Löhr
			C19	DRB1*0403	DRB1*0701		LVGAP**L**GG**A**ARALAH		K Sugimoto
			C22	DRB1*0404	DRB1*1104	#	GAP**L**GG**A**ARALAHGVR**V**LED	Class-II	J Schulze zur Wiesch, 2007
			C23	DRB1*0701	DRB1*0801	#	GAP**L**GG**A**ARALAHGVR**V**LED	Class-II	A J MacDonald
			C27	DRB1*0101	DRB1*1501		GAP**L**GG**A**ARALAHGVR**V**LED	Class-II	C L Day
			331			# *	GAP**L**GG**A**ARALAHGVR**V**LED	Class-II	J Schulze zur Wiesch, 2012
			C70	DRB1*0101	DRB1*1501		GG**A**ARALAHGVR**V**LE	Class-II	D Ciuffreda
			C77	DRB1*0101		# *	AL**A**HGVR**V**L	Class-II	H F Löhr
							LAHGVR**V**LEDGVNYATGNLP	Class-II	J Schulze zur Wiesch, 2012
1423-1440	AYYRGLDVSVIPTAGDVV	NS3	C22	DRB1*0404	DRB1*1104		GINAVAYYRGLDVSVIPT**S**G	Class-II	J Schulze zur Wiesch, 2007
			S11	DRB1*0404	DRB1*0701	# *	GINAVAYYRGLDVSV	Class-II	A M Wertheimer
							GINAVAYYRGLDVSVIPT**S**G	Class-II	C L Day
							VAYYRGLDVSVIPT**S**	Class-II	A M Wertheimer
							LDVSVIPT**S**GDVVVVATDAL	Class-II	J Schulze zur Wiesch, 2007
							IPT**S**GDVVVVSTDALMTG	Class-II	N M Tabatabai
2548-2565	NQIRSVWEDLLEDTTTPI	NS5B	S2	DRB1*0701	DRB1*1454	# *	HARKAV**TH**I**N**SVWKDLLED**N**	Class-II	J Schulze zur Wiesch, 2012
							SVWKDLLED**NV**TPIDTTIMA	Class-II	C L Day
2893-2908	IIERLHGLSAFTLHSY	NS5B	S2	DRB1*0701	DRB1*1454	# *	PII**Q**RLHGLSAF**S**LHSYSPG	Class-II	J Schulze zur Wiesch, 2007
							PII**Q**RLHGLSAF**S**LHSYSPG	Class-II	J Schulze zur Wiesch, 2012

Table S8: Overlap between genotype-3-specific CD4+ T-cell targets defined in this study and previously described HCV genotype-1-specific CD4+ targets.
HCV genotype-3-specific CD4+ T-cell targets and described genotype-1-specific CD4+ targets and those without defined CD4/CD8 restricted were classified by overlap (left coloured bar): 'likely overlapping' (light blue, <20% sequence differences within targeted area), 'unlikely overlapping' (light pink, >20% differences within targeted area, or less than 7 amino acids overlap), and 'not overlapping' (pink). ***Left:*** For each epitope, viral region, position and genotype-3 sequence is given, as well as patients targeting the epitopes (colour coding: orange-SR, blue-acute, yellow chronic), and patient's HLA class-II types. ***Right:*** Published overlapping genotype-1 CD8+ epitopes are specified, including sequence, restricting HLA type and first author of the publication; overlap between epitopes is underlined and genotype-3/genotype-1 sequence differences marked bold. # CD4 restriction for marked patient experimentally defined; * Targets previously described in (Humphreys et al. 2012).
(Lasarte et al. 1998; Lamonaca et al. 1999; Löhr et al. 1999; Tabatabai et al. 1999; Day et al. 2002; MacDonald et al. 2002; Sugimoto et al. 2003; Wertheimer et al. 2003; Kennedy et al. 2006; Castelli et al. 2007; Schulze zur Wiesch et al. 2007; Ciuffreda et al. 2008; Schulze Zur Wiesch et al. 2012).

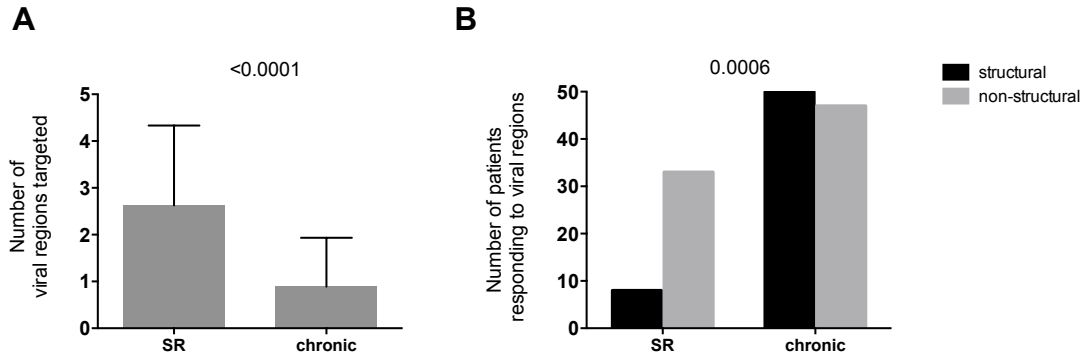


Figure S1: Comparison of breadth and targeted viral regions in spontaneously resolved individuals and patients with chronic HCV genotype-3 infection.
(A) Numbers of viral regions targeted in spontaneously resolved HCV infection and chronic HCV genotype-3 infection (unpaired t-test). (B) Comparison of numbers of patients targeting structural and non-structural regions in patient with chronic HCV genotype-3 infection and patients with spontaneously resolved HCV infection (Fisher's exact).

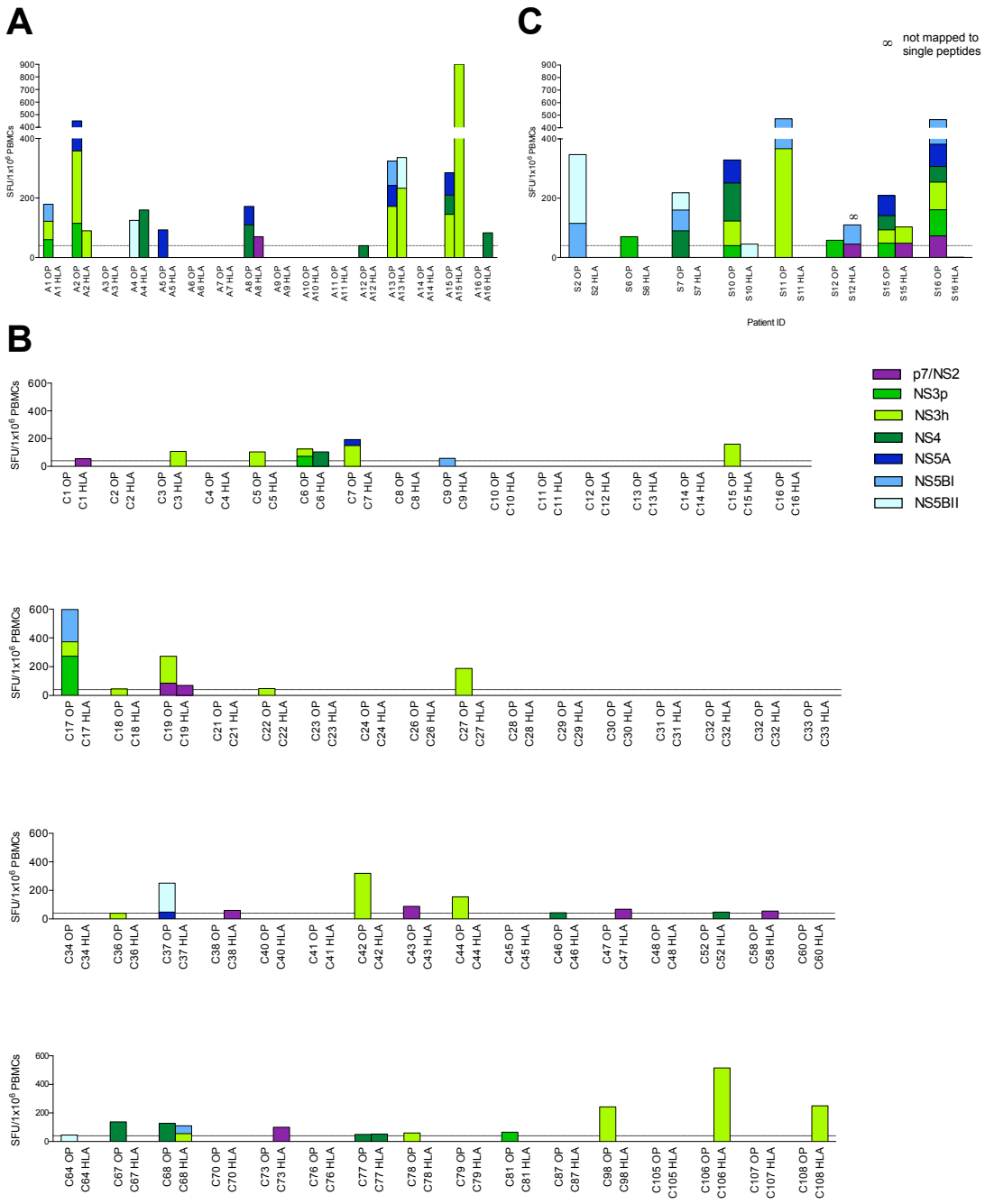
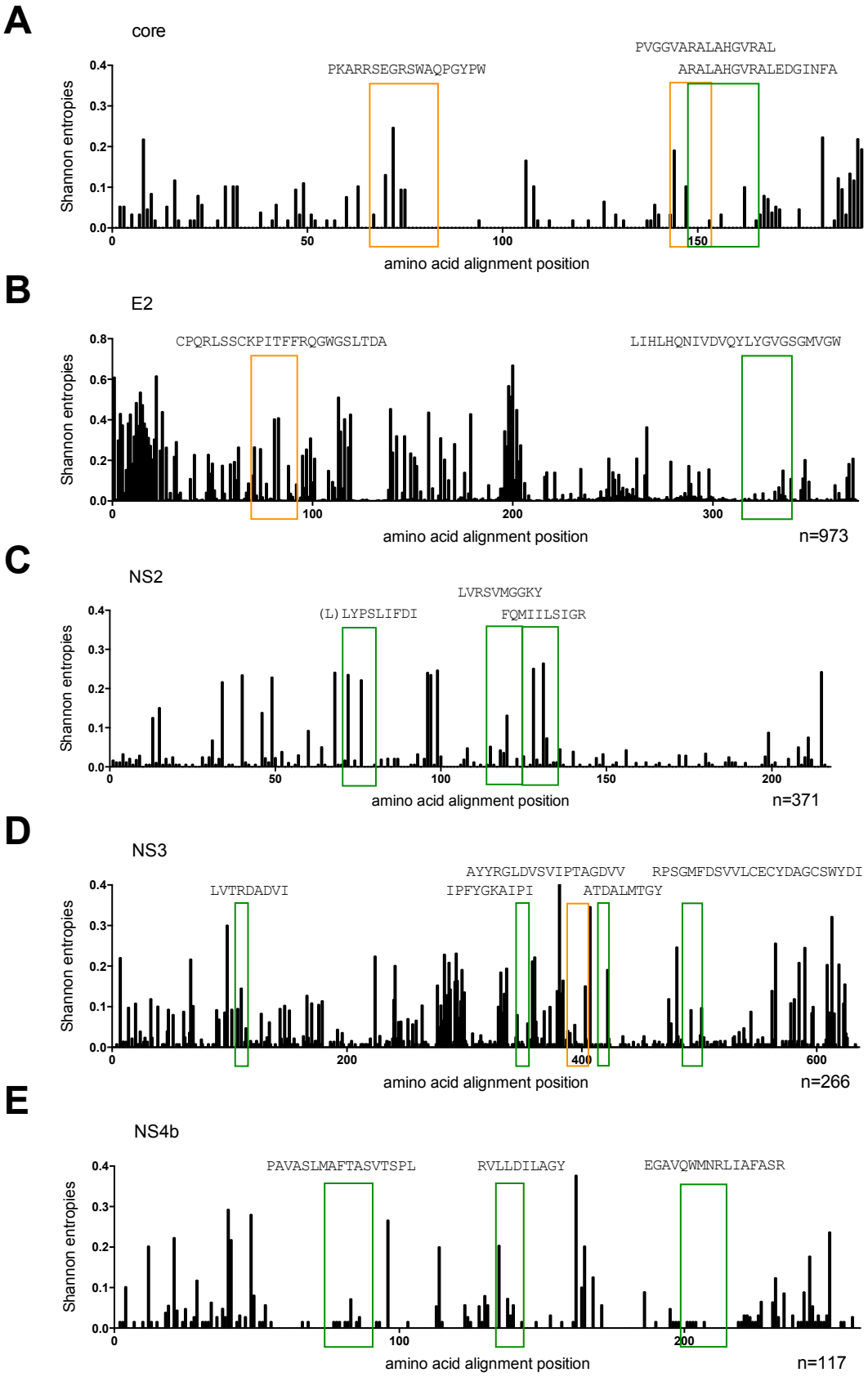


Figure S2: Comparison of responses to overlapping pools and HLA predicted peptides in acutely and chronically HCV genotype-3 infected patients, and patients with spontaneously resolved infection.

T-cell responses detected by IFNγ ELISpot assay to HLA predicted peptides and overlapping peptide pools (OP) were measured in patients with (A) acutely (n=16) and (B) chronically HCV genotype-3 infected (n=64) patients, and (C) spontaneously resolved infection (n=8). Responses to overlapping pools are compared to the HCV viral region in which the HLA predicted peptide falls (NS3p, NS3h, NS4, NS5a, NS5b1, NS5b2). SFU: Spot forming units.


A
core
PKARRSEGRSWAQPGYPW
PVGGVARALAHGVRAL
ARALAHGVRALEDGINFA
Shannon entropies
amino acid alignment position
B
E2
CPQRLSSCKPITFFRQGWGSLTDA
LIHLHQNIVDVQYLYGVGSGMVGW
Shannon entropies
amino acid alignment position
n=973
C
NS2
LVRSVMGGKY
(L)LYPSLIFDI
FQMIILSIGR
Shannon entropies
amino acid alignment position
n=371
D
NS3
AYYRGLDVSVIPTAGDVV
RPSGMFDSVVLCECYDAGCSWYDI
LVTRDADVI
IPFYGKAIPI
ATDALMTGY
Shannon entropies
amino acid alignment position
n=266
E
NS4b
PAVASLMAFTASVTSPL
RVLLDILAGY
EGAVQWMNRLIAFASR
Shannon entropies
amino acid alignment position
n=117

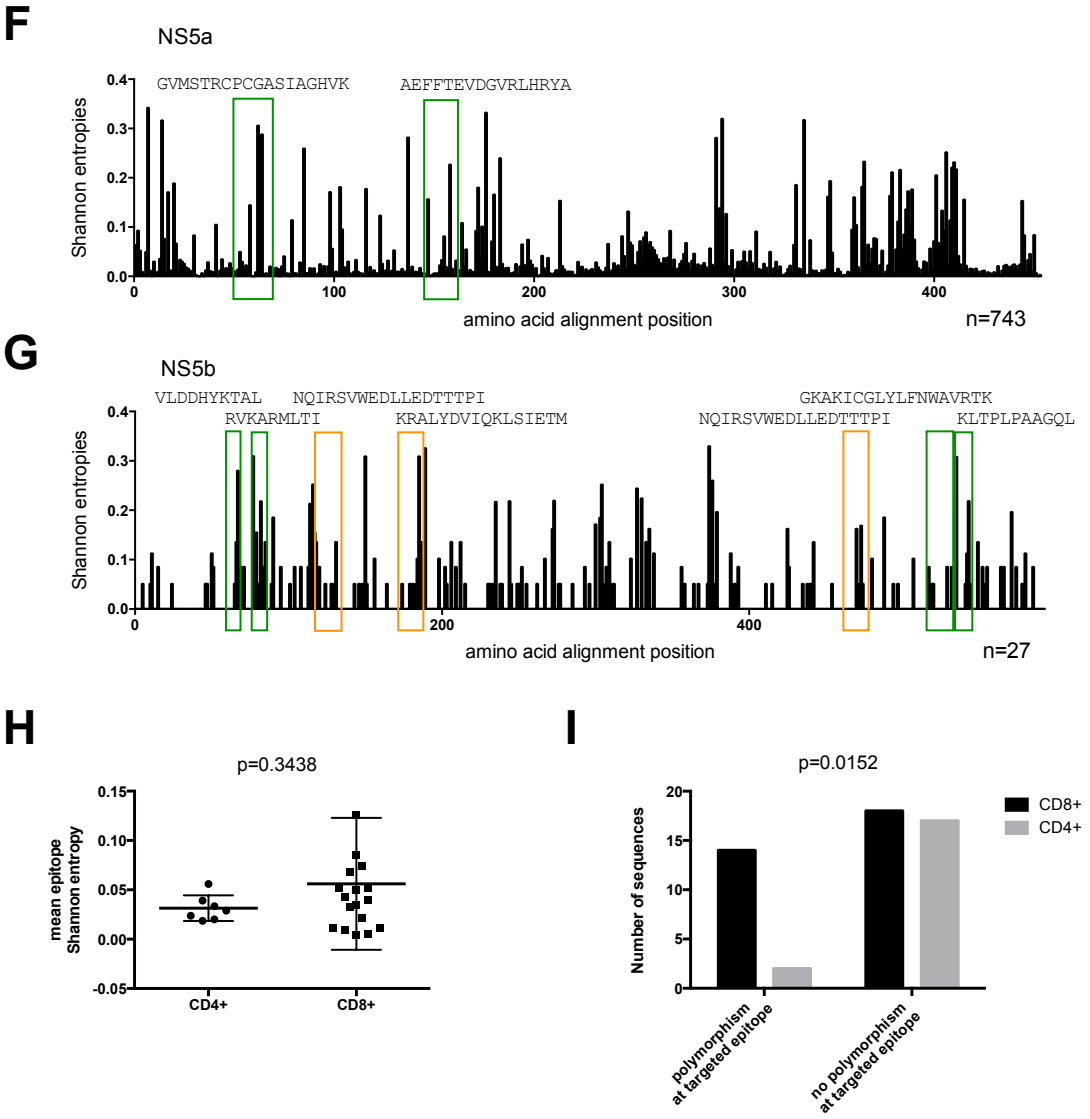


Figure S3: Evaluation of sequence heterogeneity at targeted HCV genotype-3-specific epitopes.

Sequence heterogeneity was evaluated by Shannon entropy scores in HCV viral regions (A) core, (B) E2, (C) NS2, (D) NS3, (E) NS4b, (F) NS5a and (G) NS5b on population level using Los Alamos genotype-3 sequences. The number of sequences used for each calculation is given. CD4+ (orange) and CD8+ (green) T-cell targets detected within the Oxford genotype-3 cohort are marked. (H) Mean Shannon entropy scores for CD4+ and CD8+ epitopes were calculated (p=0.3438, unpaired t-test). (I) Sequence polymorphisms within the Oxford cohort were evaluated at 16 CD8+ and 4 CD4+ T-cell targets in patients with detected T-cell responses. Sequence polymorphisms at CD4+ epitopes were detected in 2/19 sequenced patients, whereas polymorphisms at CD8+ epitopes were detected in 14/32 sequenced patients, respectively (p=0.0152, Fisher's exact).

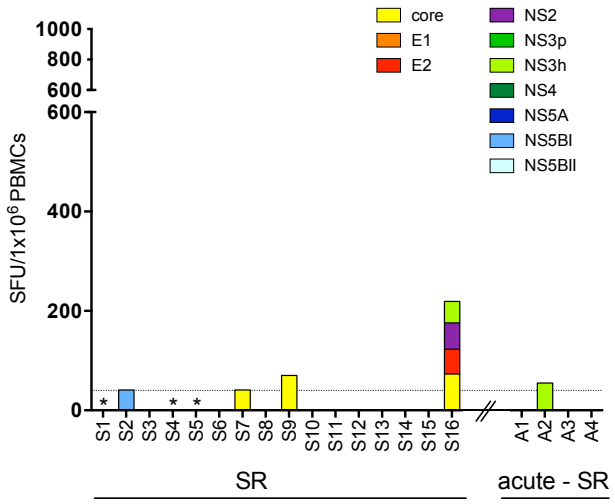


Figure S4: T-cell responses detected against HCV genotype-1 peptide sets in spontaneously resolved patients and acutely infected patients with subsequent resolution of infection.

Hepatitis C virus (HCV) genotype-1 specific T-cell responses were measured by IFNγ-ELISpot assays (spot-forming units (SFU)/10^6 peripheral blood mononuclear cells (PBMC)) using an HCV genotype-1b-specific peptide set spanning the entire HCV genome in 16 patients with spontaneously resolved infection (SR), and 4 patients acutely infected with HCV genotype-3 who subsequently spontaneously resolved infection (A-SR).

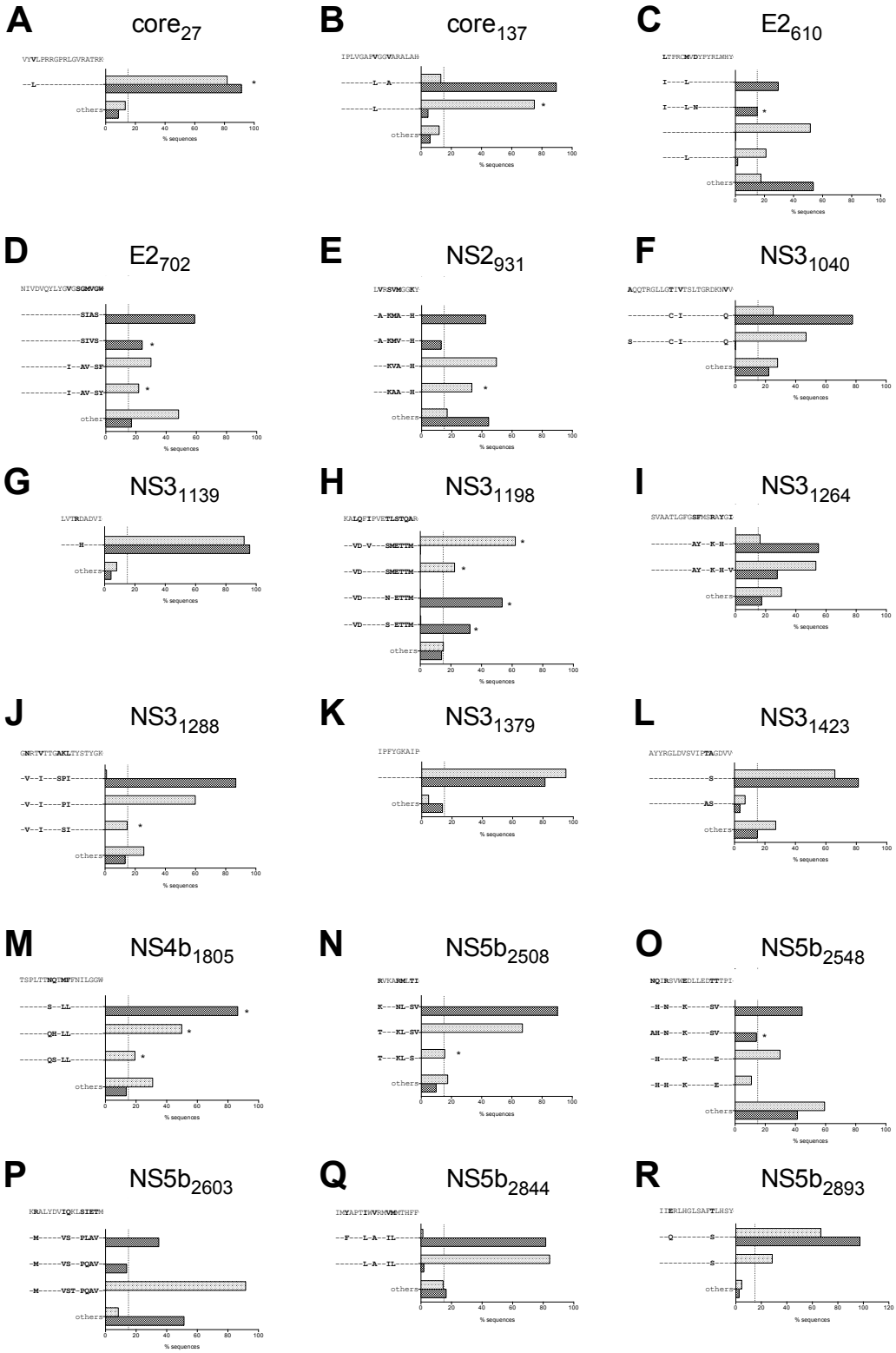


Figure S5: Hepatitis C virus (HCV) genotype-1 sequences variants at T-cell targets identified in spontaneously resolved patients.

HCV genotype-1 sequences were obtained from the Los Alamos database at T-cell targets identified in spontaneously resolved patients, to define common HCV genotype-1 sequence variants (frequencies >15%). For each T-cell target (A-R), HCV genotype-3 consensus sequence (top of each graph) and common HCV genotype-1 variants are specified. Where T-cell cross-reactivity to common variants has not been assessed experimentally due to lack of PBMC, sequences are marked with a star.

A

	Epitope sequence	Viral region	start AA	end AA	HLA type	number of publications	T cell targets in Oxford gt3 cohort	Restriction of HCV gt3 epitopes
CD8	YLLPRRGPRL	core	35	44	A2	10		
	GPRLGFRAT	core	41	49	B7	5		
	NEGLGWTGW	core	87	95	B44	5		
	ADLMGYIPLV	core	131	140	A2	11		
	LLALLSCLTV	core	178	187	A2	5		
	SMVGNWAKV	E1	363	371	A2	5		
	SLLAPGAKQNV	E2	401	411	A2	5		
	NTRPPLGNW	E2	541	549	B57	5		
	RDWAHNGL	NS2	957	964	B37	7		
	CINGVCWTV	NS3	1073	1081	A2	31		
	LLCPAGHAV	NS3	1169	1177	A2	5		
	HPNIEEVAL	NS3	1359	1367	B35	7		
	HSKKKCDEL	NS3	1395	1403	B8	8		
	KLVALGINAV	NS3	1406	1415	A2	32		
	ATDALMTGY	NS3	1436	1444	A1	7	+	CD8+
	LLFNILGGWV	NS4b	1807	1816	A2	7		
	VLSDFKTWL	NS4a	1987	1995	A2	7		
	ALYDVVTKL	NS5b	2594	2602	A2	11		
CD4+	NKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRL	core	11	44	ND	9		
	KVRRPEGRTWAQPGYPWPLYGNEGLGWAGWLLSPRGS	core	67	103	ND	10	+	CD4+
	ADLMGYIPLVGAPLGGAARALAHGVRVLED	core	131	160	ND	12	+	CD4+
	ATRDGKLPATQLRRHIDLL	E1	247	265	ND	7		
	YFSMVGNWAKVLVVL	E1	361	375	ND	6		
	SSDLYLVTRHADVIP	NS3	1127	1141	ND	6		
	LETTMRSPVFTDNSSPPVVP	NS3	1201	1220	ND	6		
	PAAYAAQGYKVLVLNPSVAA	NS3	1241	1260	ND	8		
	LADAGCSGGAYDIIICDECHSTDAT	NS3	1300	1324	ND	8		
	EVIKGGRHLIFCHSKKKCD	NS3	1383	1401	ND	9		
	CNTCVTQTVDFSLDPTFT	NS3	1454	1471	ND	7		
	FVAPGERPSGMFD	NS3	1501	1513	ND	6		
	YELTPAETTVRLRAYMNTPGLPVAQD	NS3	1528	1553	ND	11		
	THIDAHFLSQTK	NS3	1566	1577	ND	6		
	ENLPYLVAYQATVCARAQAPPPSW	NS3	1581	1604	ND	10		
	PLLYRLGAVQNEITLTHP	NS3	1623	1640	ND	8		
	VIVGRVVLSGKPAIIPDREV	NS4a	1681	1700	ND	6		
	GLSTLPGNPAIASL	NS4a	1777	1790	ND	6		
	ALVVGVVCAAILRRHVGPGE	NS4a	1891	1910	ND	6		
	QCCDLDPQARVAIKSLTERL	NS5b	2661	2680	ND	6		

B

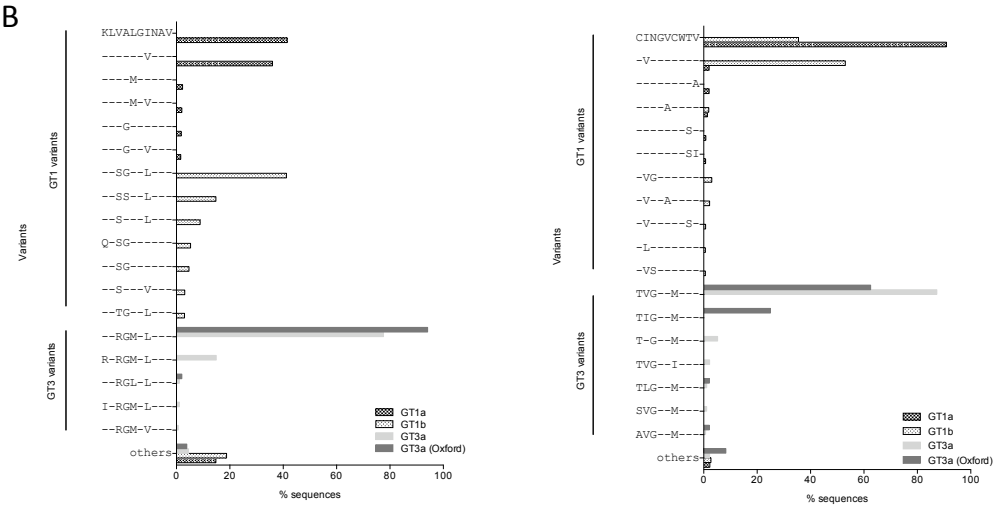


Figure S6: T-cell targets frequently detected in HCV genotype-1 infection are not targeted in HCV genotype-3 infection, likely due to sequence differences between genotypes.

(A) Comparison of CD8+ restricted epitopes dominant in HCV genotype-1 infection (defined as described in 5 or more publications on the IEDB). If the epitope was targeted in the Oxford HCV genotype-3 cohort, it is marked with +. Epitopes described as CD4+ restricted epitopes in the Oxford genotype-3 cohort falling into regions of CD8+ restricted epitopes described as HCV genotype-1 epitopes in the literature are marked in grey. (B) Comparison of circulating viral genotype-1 and genotype-3 sequences at dominant HCV genotype-1 epitopes. Sequences were obtained from the Los Alamos database, with additional in-house genotype-3 sequences.

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