

# Do antiviral CD8+ T cells select hepatitis C virus escape mutants? Analysis in diverse epitopes targeted by human intrahepatic CD8+ T lymphocytes

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**SUMMARY.** Hepatitis C virus (HCV) is a variable RNA virus that can readily establish persistent infection. Cellular immune responses are important in the early control of the virus. Evidence from animal models suggests that mutation in epitopes recognized by CD8+ T lymphocytes may play an important role in the establishment of persistence but in human persistent infection, equivalent evidence is lacking. We investigated this by analysing a unique resource: viruses from a set of chronically HCV-infected individuals in whom the CD8+ T-cell responses in liver had previously been accurately mapped. Virus was sequenced in seven individuals at 10 epitopes restricted by 10 human leucocyte antigen (HLA) molecules. Two main patterns emerged: in the majority of epitopes sequenced, no variation was seen.

In three epitopes, mutations were identified which were compatible with immune escape as assessed using phylogenetic and/or functional studies. These data suggest that – even where specific intrahepatic T cells are detectable – many epitopes do not undergo mutation in chronic human infection. On the contrary, virus may escape from intrahepatic CD8+ T-cell responses in a ‘patchy’ manner in certain specific epitopes. Furthermore, longitudinal studies to identify the differences between ‘selecting’ and ‘nonselecting’ intrahepatic CD8+ T-cell responses are needed in HCV infection.

**Keywords:** CD8+ T cell, evolution, HCV, immune escape, liver.

## INTRODUCTION

Hepatitis C virus (HCV) infects approximately 170 million people worldwide. Unlike the hepatitis B virus, persistence is readily established after infection in adults, although both viruses, once established, can cause progressive liver fibrosis, liver failure and hepatocellular carcinoma. In some individuals the virus is controlled after acute infection and they remain viral RNA-negative in blood thereafter. Cellular immune responses are assumed to be very important in this process. Evidence for this comes from studies of acutely infected chimpanzees and humans [1,2]. Both CD4+ and CD8+ T cells are involved in this process. The differences between cellular immune responses that are successful in controlling virus and those which fail to do so are not fully

understood, although it is assumed that a broad and vigorous response is most likely to lead to clearance [1]. Once persistence is established, *ex vivo* CD8+ and CD4+ T-cell responses often become difficult to detect by a variety of methods. However, it is still possible to find CD8+ T cells at low levels *ex vivo*, by *in vitro* expansion and within liver tissue [2–7].

The mechanisms behind the establishment and/or maintenance of persistence are not understood, yet are of major importance when considering vaccines and immunotherapy for HCV. Two major hypotheses exist, similar to those proposed for human immunodeficiency virus (HIV). In the first, immune responses are effective but variability within the virus leads to emergence of mutants which escape recognition by T cells, in particular, CD8+ T cells [8]. There is substantial evidence for this in HIV infection, at the level of individual patients, at the population level and also especially clearly in the macaque/simian immunodeficiency virus (SIV) model. In the case of HCV, there is compelling evidence from the chimpanzee model that viral escape can occur after acute HCV infection [9], although the evidence from humans is more limited [10]. There is a great deal of natural

Abbreviations: HCV, hepatitis C virus; HLA, human leucocyte antigen; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; PCR, polymerase chain reaction.

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variability in the virus, but it is not yet clear as to what extent evolution of viral mutants within an infected individual contributes to escape from CD8+ T cells and the establishment of persistence.

An alternative set of hypotheses suggests that CD8+ T cells lack the capability to effectively control virus *in vivo*. In the case of HIV, there is some evidence that CD8+ T-cell function may be impaired [11], and there are a range of viral mechanisms which might interfere with the recognition or destruction of a virally infected cell. For HCV, there are suggestions that the function of specific CD8+ T cells is impaired both acutely, and potentially in the long term [12]. More importantly, the frequencies of virus-specific CD8+ T cells are very low compared with HIV, as measured in blood [2,3]. In the liver, there is some enrichment of CD8+ T cells (although this is also seen in nonhepatotropic infections) [7,13]. As this is the primary site of viral production, it is these T cells which might be expected to exert maximal selective pressure and, if effective, their presence would then be associated with escape mutants.

We addressed this directly by analysing the viral sequence associated with a range of CD8+ T-cell responses which have already been extensively mapped in the livers of chronically infected patients [14]. These responses are particularly informative from the point of view of escape because they have been mapped using an initial screen which was comprehensive and not epitope-specific. The responses obtained were highly focused and thus we can pinpoint the precise sites of immunological selective pressure within the viral genome. Evidence for the corresponding adaptive evolution of the virus at these sites was investigated by phylogenetic analysis of the genetic diversity of the virus population in patients. As the study included a wide range of epitopes and HLA restriction molecules, we could investigate the relative importance of immunological escape in human chronic HCV infection. We provide some evidence for sequence variation which is compatible with immune escape in a proportion of epitopes studied, although this phenomenon is by no means universal and other explanations for persistence must also be sought.

## MATERIALS AND METHODS

### Study subjects

The complete data on the original cohort are available in the paper by Wong *et al.* [14], but also Table 1 and the Results section.

### RNA extraction and amplification by PCR

Viral RNA was extracted from 70 µL of plasma or serum using a kit (QIAamp viral RNA mini kit; Qiagen, Crawley, UK). RNA was eluted in 60 µL of RNase-free water. After incubation at 70 °C for 5 min, the extracted RNA (10 µL) was reverse transcribed at 37 °C for 60 min in a 25-µL reaction mixture with 100 ng of random hexamers (Gibco-BRL, Gaithersburg, MD, USA) and 125 U of Moloney murine leukaemia virus reverse transcriptase (ABgene, Epsom, UK) and then at 94 °C for 5 min. Nested primer sets were synthesized to amplify each region of the HCV genome that contained class major histocompatibility complex (MHC)-restricted epitopes. The sequences of the primers were as follows: (a) core region (subjects 92H, 93I): external sense (ES): 5'-TGCCCCGCGAGATCACTAGC-3', external antisense (EA): 5'-CCTTGTGGTACTGCCTGATAG-3', internal sense (IS): 5'-TTCATCATCATATCCCAAGCC-3', and internal antisense (IA): 5'-GTCMKCKGCTCATAACAAT-3'; (b) E1 region (91E): (ES): 5'-GCGTCCGGGTTCTGGAAGACGGCGTGAAGTATGCAACAGG-3', (EA): 5'-AGGCTTTCATTGCAGTTCAAGCCGTGCTATTGATGTGCC-3', (IS): 5'-AAGACGGCGTGAAGTATGCAACAGGGAACCTTCCTGGTTG-3', (IA): 5'-GACCAGTTCATCATCATATCCCATGCC-3'; (c) NS2 region (91E, 93I): (ES): 5'-CTGTTCTTCTGCTTGAGACGC-3', (EA): 5'-CCGTTGATGATGTCACCGCA-3', (IS): 5'-TGCTCCTGCTTGATGATG-3', (IA): 5'-GCCACGGC-CAGATCTCGAAGCC-3'; (d) NS3 region (93K): (ES): 5'-AT-CACGGCGTACGCCAGCAGA-3', (EA): 5'-ATCGCGTGCC-TCGTGACCA-3', (IS): 5'-AGCCTAACTGGCCGGGACAA-3', (IA): 5'-TCCGAGGAGCCGCAAGTGCA-3'; (e) NS5B (93I,

Subject	Epitope	HLA restriction	Sequence length (bp)	Subject sequences studied	Total sequences aligned
93I	Core 41-49	B7	414	20	65
	NS2 838-46	A23	420	18	39
	NS5B 2588-96	A3	525	23	53
92H	Core 1-9	A11	414	23	65
91E	NS2 827-34	A29	420	16	39
	E1 232-42	B35	303	18	39
93K	NS3 1073-81	A2	213	16	33
94K	NS5B 2629-37	B57	525	19	53
95I	NS5B 2794-804	B38	288	18	35
92G	E2 568-78	B50	410	20	20

**Table 1** Study subjects, epitopes studied in each and other details as shown

94K): (ES): 5'-TCTGCGTTCAGCCTGAGAA-3', (EA): 5'-CAAGTCGTATTCTGGTTGTGG-3', (IS): 5'-AGCTCGTCTCATC-GTGTTC-3', (IA): 5'-CTGGTCATAGCCTCCGTGAA-3'; (f) NS5B (95I): (ES): 5'-GGCGACGACTTAGTCGTTATCTGTG-3', (EA): 5'-TTCAAGCTGGTCCCTGGCTATAAGG-3', (IS): 5'-GT-TATCTGTGAAAGTGGGGGTCC-3', (IA): 5'-GGCTATA-AGGACGCTAAAGAAATGG-3'.

Two microlitres of the cDNA was used as the template for amplification. PCR was performed in a total volume of 50  $\mu$ L, in a reaction mixture containing 50 pmol of each of the respective primers and 2.5 U of *Taq* DNA polymerase (Bioline, London, UK). The first round of PCR amplification was performed for 35 cycles as follows: denaturation at 94 °C for 30 s, annealing at 45–55 °C for 60 s, and extension at 72 °C for 60 s. Then, 2  $\mu$ L of the first PCR reaction product was reamplified with inner primers for 35 cycles under the same reaction conditions as in the first-round PCR. A product of the predicted size was observed after electrophoresis on a 1% agarose gel when visualized under ultraviolet light after ethidium bromide staining. This DNA band was excised from the gel and the DNA was purified using a DNA purification kit (QIAquick gel extraction kit; Qiagen). PCR products were cloned and sequenced using a commercial kit (TOPO TA-Cloning Kit; Invitrogen, Carlsbad, CA, USA). The extracted PCR products were ligated into the plasmid pCR 2.1 (Invitrogen), according to the manufacturer's instructions. The ligated vector was introduced into competent Top10F' cells (Invitrogen) by transformation and the cells were plated on Luria-Bertani (LB) agar plates containing ampicillin and X-Gal. Single colonies were picked up and then cultured in LB medium containing ampicillin. Plasmid DNA was isolated by the miniprep method. DNA sequences were determined using the ABI Prism 377 DNA Sequencing System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems) and M13 sequencing primers.

#### Analysis of sequence diversity

A phylogenetic approach was used to look for evidence of adaptive evolution in the virus. To be consistent with basic evolutionary principles, the analysis took the following factors into account: (1) not all amino acid variability in the viral population is indicative of adaptive evolution. Many observed amino acid changes represent selectively neutral (or slightly deleterious) mutations that exist only transiently in the viral population. (2) Amino acid changes that are at a high frequency in the population, or that have been maintained in the population for some time, are consistent with adaptive evolution. (3) By comparing the viral genetic diversity within a patient with that observed in closely related database sequences, it is possible to investigate when an amino acid change arose, and thereby judge if it was present in the virus transmitted to that

patient, or alternatively, if that change appeared in the patient *de novo*.

For each viral genomic region studied, the sequences obtained from the infected patients were aligned with a set of reference sequences obtained from the HCV Sequence Database (<http://s2as02.genes.nig.ac.jp>). Reference strains that were most closely related to the virus present in the infected patients. Details of the alignments are provided in Table 1. A phylogeny was estimated from each alignment using the maximum likelihood approach, as implemented in PAUP\* 4.0b10 [15]. The HKY85 substitution model was used with a gamma distribution model of rate heterogeneity among sites. The estimated phylogenies were rooted using outgroup sequences belonging to subtype 1b.

Each alignment was subsequently translated into amino acids and the evolutionary history of each amino acid change was inferred by mapping the change onto its appropriate phylogeny. Changes that occurred only among reference strains were ignored. The mapping was calculated using the parsimony algorithm implemented in MacClade 3.07 (Sinaver Associates, Sunderland, MA, USA). Changes that were located on internal branches in the phylogeny have, by definition, been maintained in the viral population for at least some period of time, and were therefore considered to be strongly suggestive of adaptive evolution. Amino acid changes that occurred on external branches in the phylogeny could represent either transient neutral/deleterious mutations, or advantageous mutations that did not have the chance to spread before the population was sampled. Such changes were therefore considered to be only weakly suggestive of viral adaptation (see Fig. 3).

#### Functional analysis of variants

Cytotoxic T lymphocyte (CTL) clones were thawed and interferon-gamma ELISpot assays (Mabtech, Stockholm, Sweden) performed as previously described [16]. Clones were derived from subjects 94K and 93I as previously reported [14]. Peptides were titrated out at 10-fold dilutions starting at 10  $\mu$ M. Plates were incubated overnight. The spots were developed and counted on an ELISpot plate reader as previously described [16].

## RESULTS

#### Variation and conservation in distinct epitopes

The samples analysed were derived from study subjects in the cohort originally described by Wong *et al.* [14]. Briefly in this original study, 22 subjects with chronic stable HCV infection were initially screened for CD8+ T-cell responses in liver biopsy samples by non-specific expansion of CD8+ T cells which were then tested using vectors expressing HCV structural and nonstructural proteins (derived from genotype 1a). Forty-five per cent of subjects showed T-cell

responses in these assays. The responses detected were heterogeneous: only one peptide was targeted more than once between individuals. In the original study, 19 epitopes were fully mapped in 13 patients. Of these, HCV-RNA-positive serum samples from seven patients were available for the study of 10 epitopes. In the other cases, concurrent samples were either no longer available or RNA was no longer detectable after an 8–10-year interval. The relevant immunological data of the patients studied are summarized in Table 1 – the clinical data are summarized in the original paper by Wong *et al.* [14].

Figure 1 displays the sequence results from epitope-specific regions obtained from individual patients. In each case, the consensus sequence from genotype 1a used in the detection of CD8+ T-cell responses is indicated, and the number of clones containing different predicted amino acid sequences indicated beneath. These can be divided into two major groups simply on sequence changes. (A) The biggest group includes six responses, which are either entirely conserved [e.g. 93K responding to NS3 (1073–1081)], or show only minor variation [e.g. 93I responding to A3 NS5B (2588–2596)]. (B) In four cases – 93I responding to B7 core (41–49), 95I responding to B38 NS5B (2794–2804) and 94K responding to B57 NS5B (2629–2637) – we find a single amino acid change which dominates the viral population [92H responding to A11 core (1–9) is also strictly included in this category, but the K to R change at position 9 is the common polymorphism at this site and the R in fact dominates all genotype 1a sequences (see below)]. A sub-category includes only 92G responding to B50 E2 (569–578). This response falls within a highly variable region and the genotype of the individual's virus is in fact genotype 3a, while the clone was raised against a quite distinct genotype 1a epitope. This interesting and unusual result will be discussed separately.

We performed control analyses by looking at sequences in a set of these epitopes in individuals who cannot respond to them because of HLA mismatch (HLA–, CTL–); five epitopes were studied (Fig. 2a). In none of these cases was a variant sequence dominant, apart from the K9R change in A11 core [1–9], which, as described above is the dominant polymorphism in genotype 1a. Thus, in the absence of any potential selective pressure from CD8+ T cells, the relevant epitopes retained wild-type sequences.

Similarly, we analysed sequences in previously described epitopes which were appropriate for the HLA type of the patient, but where no CTL responses had been identified (HLA+, CTL–; Fig. 2b). Eight epitopes were studied and in five of these, the dominant species or uniform sequence was equivalent to that of the prototype sequence. In one case where the epitope resides in a variable region of E1, a significant change from wild type was seen. These results are relevant in that in the majority of cases, lack of response to these epitopes is not because of mutation which may have either been already established in the infecting strain or

emerged within the individual under previous CTL selection. In the case of the E1 epitope, this has only been reported in a single case as an epitope and lies within an area under antibody selection, so the significance of this change as a reflection of T-cell immunological pressure is doubtful. In two cases, significant mutation within 'HLA+CTL–' epitopes was seen (the HLA A3-core epitope in individual 93I, and in the HLA A3-NS5b epitope in 94K). Escape within CTL epitopes was observed in both these individuals; this is discussed further below.

Thus the sequence analysis alone suggests that, in certain cases, escape mutation may have occurred, but that other epitopes remain unmutated in the presence of CD8+ T-cell responses within the liver. As viral sequences were available only from one time point in each patient, additional phylogenetic data were obtained to address whether the amino acid changes observed were likely to have evolved within the patients themselves and therefore represent adaptation to T-cell pressure.

#### *Phylogenetic analysis of regions showing epitope mutation*

The phylogenetic analysis detected several mutations that were considered as strongly suggestive of viral adaptation at potential T-cell epitopes (i.e. changes that occurred on internal branches of the phylogeny). Three of these changes (93I B7 position 48, 94K B57 position 2633, and 95I B38 position 2796) occurred on the most ancestral branch of their respective infections, i.e. they were found in all, or most of the sequences from these infections but almost never found in the closely related reference strains (Fig 3a,b,c). Thus it appears that these mutations arose during their respective infections and have subsequently spread to fixation within each patient. In the absence of sequences at the start of infection, it is impossible to rule out the possibility that these changes were present at transmission. However, the amino acid composition of the reference strains at these positions suggests that this is highly unlikely. In 95I B38 there appears to have been two more recent mutations after the initial selective sweep at position 2796. As B57 and B38 are uncommon HLA alleles, this reduces the probability that the escape mutation arose in a previous infection and strengthens the probability that such escape occurred in the patient studied.

There was also strong evidence for adaptive evolution at two HLA-matched epitopes for which no CD8+ T-cell response was detected, namely 93I A3 position 48 and 94K A3 position 2595 (HLA+, CTL–; Fig. 2b). Overall, this analysis suggests the appearance and spread in three patients of distinct viral strains carrying apparent epitope mutations. However, by itself, the genetic data do not provide direct evidence that these mutations were immunologically selected adaptations leading to functional escape. We therefore analysed the functional effects of these mutants on recognition by the T-cell clones derived from the same patients.

92H A11 Core (1-9)	93K A2 NS3 (1073-1081)
MSTNPKPQK	CINGVCWTV
-----R 21/23	----- 16/16
-I-----R 1/23	
-G-----R 1/23	
<div style="border: 1px solid black; padding: 5px; display: inline-block;">93I B7 Core (41-49)</div>	93I A3 NSSB (2588-2596)
GPRLGVRAT 20/2	RVCEKMALY
-----T- 0	----- 22/23
	-----D-- 1/23
91E B35 E1 (234-242)	<div style="border: 1px solid black; padding: 5px; display: inline-block;">94K B57 NSSB (2629-2637)</div>
NASRCWVAM	KSKKTPMGF
----- 17/18	-----A----- 19/19
-----I 1/18	
91E A29 NS2 (827-834)	<div style="border: 1px solid black; padding: 5px; display: inline-block;">95I B38 NSSB (2794-2804)</div>
MALTLSPY	HDGAGKRVYYL
----- 16/16	--A----- 15/18
	--V----- 2/18
	--T----- 1/18
93I A23 NS2 (838-846)	92G B50 E2 (569-578) (Genotype 3)
YISWCLWWL	CVIGGAGNNT
----- 18/18	-N-Y-DKREP 10/20
	-N-Y-DRRNR 06/20
	-N-Y-DRRNG 01/20
	-N-Y-DRRNP 01/20
	-N-Y-DRRSP 01/20
	-D-Y-GEGNR 01/20

**Fig. 1** Sequence variation in epitopes targeted by intrahepatic T cells. The sequence data derived from each individual's serum is shown. The predicted genotype 1A sequence is shown at the top. The figures refer to the number of clones analysed and the number representative of that sequence. Only coding changes are indicated, and unchanged codons are indicated by a dash. The sequences in which evidence for escape was obtained are shown in a box.

#### *Analysis of the effects of variation on T-cell recognition*

Patient-derived CTL clones were available for the study of two of the potential escape mutations. In the first case (94K NS5b), no recognition of the variant was observed across a range of peptide concentrations. In the second case (93I core), recognition of the autologous variant was observed, but with weaker recognition at lower peptide concentrations (a difference in about 1 log of peptide; Fig. 4). These functional data are consistent therefore with the evolutionary data suggesting selection for T-cell escape mutants in these cases. In the case of the 92H core response, where the genetic evidence does not suggest escape, but rather the intra-patient sequence reflects that of the typical strain, strong recognition of the autologous sequence was observed (data not shown).

#### DISCUSSION

Immune escape from CD8<sup>+</sup> T cells has been well demonstrated in many viral systems [8,17-19]. Direct evidence for immune escape in HCV has come from the chimpanzee model, where cloned virus can be tracked over time in relationship to fully mapped T-cell responses [1]. Early escape can be seen in multiple epitopes [9]. Late escape has also been noted [20]. The CTL in these studies have largely been from intrahepatic samples, as in our study.

In human infection, the data have been much more patchy. A study of a set of A2-restricted responses did show rare mutation in persistently infected individuals [10], although such mutations do not accumulate in HLA-A2+ individuals [21]. A study of immune escape in persistently

(a) Mismatched HLA, response(-)		(b) HLA matched, response (-)	
<u>Core</u>	<u>N5B</u>	<u>93I</u>	<u>91E</u>
93I Core (1-9)	93I NS5B (2629-2637)	A3 Core(43-51)	A2 E1 (220-227)
MSTNPKPQK	KSKKTPMGF	RLGVRATRK	ILHTPGCV
-----R 16/18	----- 20/21	----T--- 19/20	----- 18/18
----- 1/18	--E----- 1/21	----T-G- 1/20	
-----E--R 1/18			A2 E1 (257-266)
		A3 Core(51-59)	QLRRHIDLLV
92H Core (41-49)		KTSERSQPR	----- 15/18
GPRLGVRAT		----- 20/20	--P----- 1/18
----- 23/24			-----G 1/18
-----A 1/24			-----A 1/18
		B7 Core(111-119)	A2 E1 (285-293)
<u>NS2</u>		DPRRRSRNL	FLVSQLFTF
93I NS2 (827-834)		----- 20/20	---G-V--- 14/14
MALTLSPY			
----- 19/19		B7 Core(169-177)	<u>94K</u>
		LPGCSFSIF	A3 NS5B (2588-2596)
91E NS2 (838-846)		----- 18/20	RVCEKMALY
YISWCLWWL		----S--- 1/20	----- 12/17
----- 13/14		-----L 1/20	-----F- 5/17
--N----- 1/13			

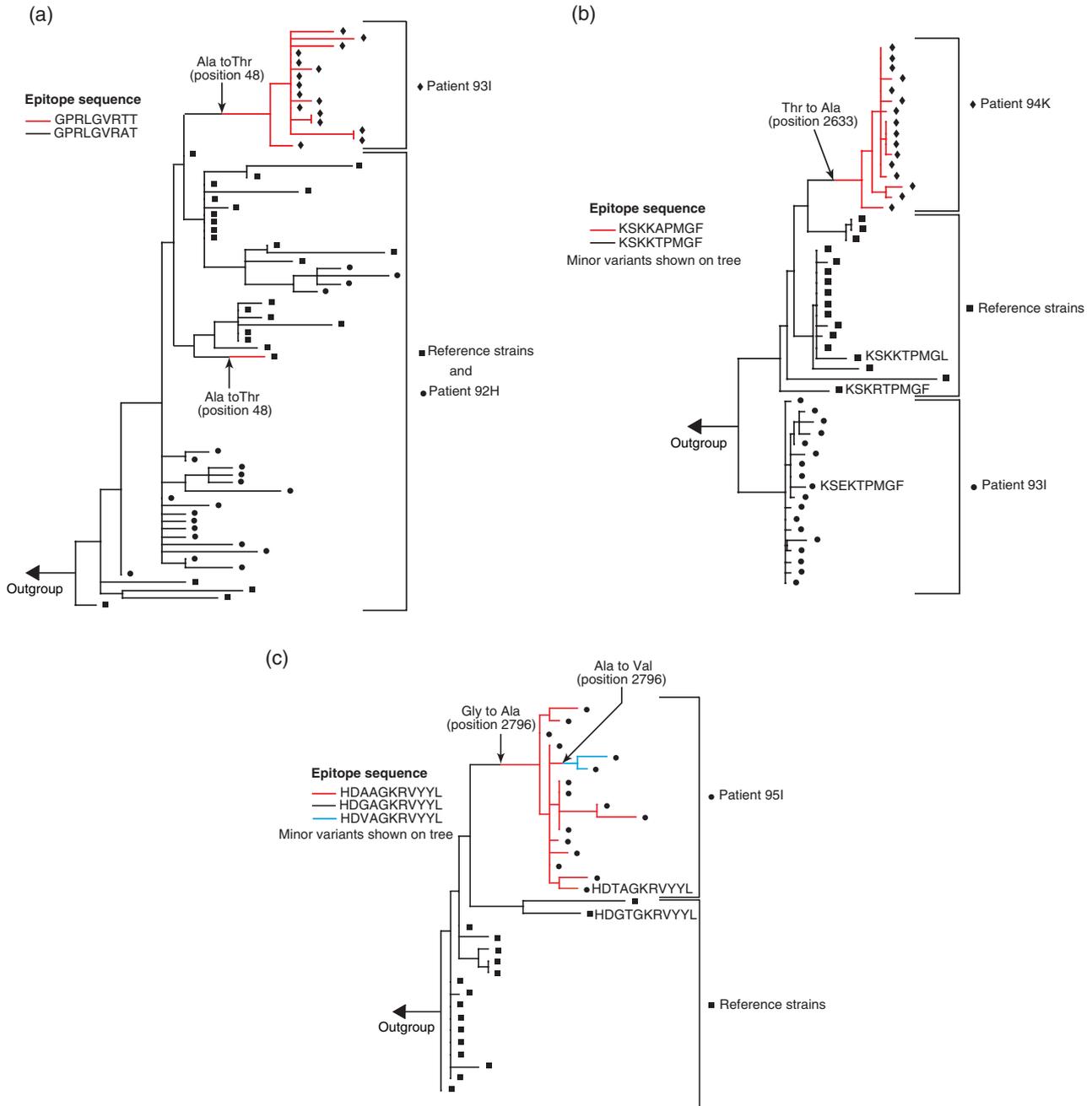
**Fig. 2** Sequence variation in control epitopes not targeted by intrahepatic T cells. (a) Control study of epitopes which are not presented in the individual due to HLA mismatch (HLA-, CTL-). Data presentation is similar to that in Fig. 1. (b) Control study of epitopes where no T cell response was identified, although epitopes were potentially presented due to HLA match (HLA+, CTL-).

infected antibody-deficient patients, where *ex vivo* T-cell responses were detectable did not reveal escape mutation [22]. A single study analysed the relationship between intrahepatic CTL and the virus within the patients. This concluded that the responses cloned were in some cases unable to recognize the virus replicated *in vivo*. In that study, the clones were grown by stimulation with index peptide and it is not clear whether there were variant-specific clones [23].

An early study of acute infection did show evolution of mutations in an HLA-A2-restricted epitope within the E2 hypervariable region. In this case, although escape was evident, it is difficult to disentangle the potential role of antibody-induced mutation [24]. A recent study of escape in early infection did demonstrate clear evolution in a B8-restricted epitope [25].

This study has exploited a unique resource of diverse intrahepatic clones, the targets of which have been accurately mapped. The patients studied had established persistent infection – it would be ideal to analyse such data early in disease but biopsies are not performed in such circumstances, and therefore a longitudinal component to such studies is not readily created. To deal to

some extent with this issue we have attempted to use phylogenetic data in order to gain as much 'historical' data on the origins of the variants which were identified in these patients. In this study, we did see some variation within epitopes that was suggestive of escape from CD8+ T cells. These occurred in three individuals, in epitopes from different genes (core and NS5b) and restricted by diverse HLA molecules (HLA B57, 38 and B7). Two of these were shown to be associated with functional escape using patient-derived clones and thus the combination of genetic and immunological evidence indicates immune escape in these cases. In two further cases where CD8+ responses were not found, genetic evidence for selection was obtained. Both of these responses were HLA-A3 restricted (again core and NS5b, from subjects 93I and 94K). The core-derived, HLA-A3 restricted epitope concerned (RLGVRATRK) was under selection pressure from the B7-restricted response in the same individual, since the epitope overlaps (GPRLGVRAT). Thus, it is theoretically possible that a single mutation (A48T) led to escape from both responses, with maintenance of the B7-restricted response and loss of the A3-restricted response. Overall, we therefore identified potential escape within five of 18



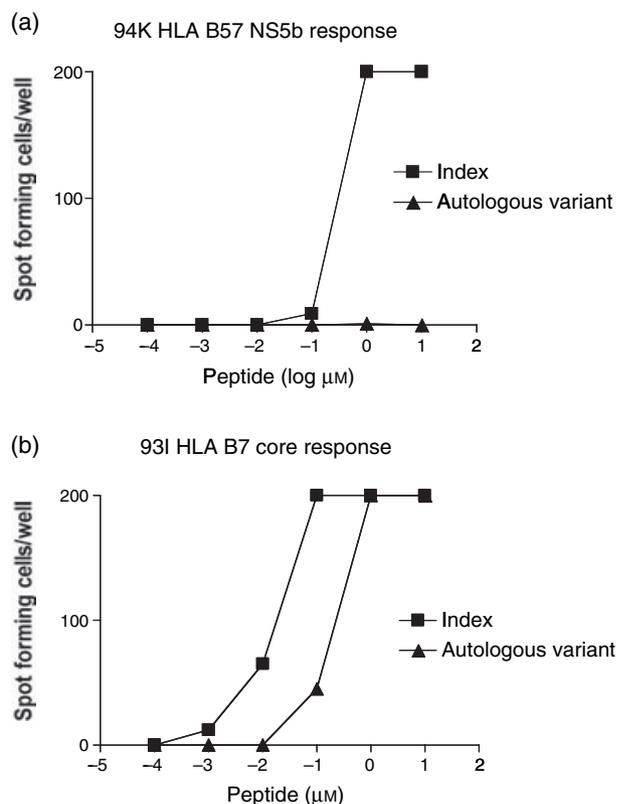
**Fig. 3** Genetic evidence for novel mutations occurring within patient strains. The relationship between inpatient sequences and those derived from nearest neighbours is described. See Materials and Methods for details of tree generation and analysis: (a) 93I B7; (b) 94K B57; (c) 95I B38.

studied epitopes (3/10 where responses were identified and 2/8 where they were not).

Patient-derived clones were no longer available for all specificities to define further the functional consequences of all the selected putative escape mutants. It is also possible that in such assays, minor mutants which were not positively selected could also act as viral T-cell receptor antagonists [26,27], an issue which could be examined in a future prospective study, although the *in vivo* relevance of such activity is

not clear. In such a study it would also be important to specifically analyse – as in HIV [28] and lymphocytic choriomeningitis virus (LCMV) [29] – whether variant-reactive clones could be generated, in order to assess whether a novel cross-reactive or variant-specific response can arise.

A further probable selected site which may have relevance for T-cell escape was identified in an additional epitope recognized by patient 93I. In this case, the mutation lies in a region flanking the NS2-derived epitope.



**Fig. 4** Recognition of autologous variants by liver-derived clones. (a) Titration of autologous variant derived from subject 94K (NS5b, HLA B57 restricted response). Dilutions of the index peptide KSKKTPMGF and the variant KSKKAPMGF were performed in parallel and the spot forming cells per well indicated; >200 cells per well were not countable and such numbers are indicated as 200 on the *y*-axis. (b) Similar titration using index and variant derived from subject 93I (core, HLA B7-restricted response). Results are representative of two independent experiments.

Similar such 'flanking' mutations have been observed in large-scale studies of immune selection in HIV [17] and it has been suggested also in HCV that interference in antigen processing might influence the presentation of such peptides from the protein [25]. In this case, this possibility was not tested further, but analysis of such peri-epitope mutations should form an important part of future studies.

In a single case, the epitope present *in vivo* was derived from a completely distinct sequence and indeed the genotype of the virus concerned was 3a. It is possible that this represents superinfection of a genotype 3a virus in an individual who had previously been infected with or cleared genotype 1 HCV. This exact occurrence has been seen previously in an individual who mounted a CD4+ T-cell response to two novel peptides in NS3 which were genotype 1 specific [30]. These T cells failed to recognize the significantly mutated

genotype 3 peptide, derived from the superinfecting virus. The continued presence of these 'memory' T-cell responses in the face of a different circulating virus is reminiscent of the phenomenon of original antigenic sin [29]. Similar CD8+ T-cell responses to genotype 1 peptides in those infected with genotypes 2 and 3 have been noted elsewhere, including cases where the epitopes concerned are highly divergent [16].

One limitation of clones is that it may only give a partial representation of the responses *in vivo* – important responses may be missed through failure to proliferate *in vivo*. It is possible that such responses include significant populations which could lead to strong selection pressure and hence immune escape. An alternative approach for this issue is to link sequence polymorphisms to specific HLA alleles – independent of T-cell data – as has been used in HIV [17, 31], and more recently in HCV [25]. We only sequenced limited numbers of clones from HLA-mismatched patients within the study cohort – the data from this study suggests that a larger-scale analysis of responses restricted by HLA B57, B7/A3 and B38 would be of interest in defining the importance of the mutations observed.

The data in this study, although limited by the above constraints, suggest, nevertheless, that in many cases escape through mutation is not the explanation for the persistence of virus in the face of an ongoing T-cell response. A variety of explanations for such persistence without escape might be suggested. In one scenario, the responses are not able to exert significant immunological pressure because the epitope is weakly bound to MHC class I and therefore present at low levels on MHC class I molecules. This may be the case for NS3 1073, which in one patient was found to be the weakest of all the binding peptides in the eight responses detected [2]. Whether this is true for all the unmutated epitopes is not yet known. A second potential explanation is that the low level of MHC class I on the hepatocyte does not allow effective presentation of the endogenously presented peptides. It may be that presentation or cross-presentation of the same peptides on professional antigen presenting cells is efficient and allows priming of these responses, but their effectiveness in the liver is lowered by the significant deficiency of MHC class I on the hepatocyte surface [32]. A third explanation is that the effector functions of the CD8+ T cell are impaired, potentially because of the lack of interferon gamma secretion, or intracellular perforin. Such cells have been noted in HCV infection, but mainly in the blood [12] – the clones which had been restimulated *in vitro* had intact killing functions, although their function *ex vivo* was not addressed [14]. A similar effect might be due to a viral gene product which interferes with the ability of the cell to be killed by CTL or respond to secreted viral cytokines. A variety of these have been suggested, notably core [33]. Finally, and most simply, although these CD8+ T cells might be detectable, they may be too rare to generate a selective pressure that is sufficiently strong to promote viral escape.

In summary, this study not only provides evidence that immunological escape from CD8+ T cells may occur in the course of human HCV infection, but also that it is not uniform. About half the individuals studied showed some evidence of such escape, but half did not, even in the presence of intrahepatic T-cell responses, which may have been continuous for years. If escape through mutation occurs it may occur early, as is clear from both the HCV chimpanzee model, HIV and SIV [1,9,34,35]. As the study was performed only on individuals with established infection, it is not possible to define whether the mutants contributed to the initial escape, or whether they simply contribute to maintaining persistence. Perhaps only key epitopes require escape through mutation for persistence to be established – once this has occurred, failure to eradicate virus with perhaps less efficient responses may give rise to the picture seen here. For HIV, we have proposed that type A ‘driver’ epitopes may drive down viral load efficiently and be associated with escape, whereas less-effective type B ‘passenger’ epitopes might simply follow virus without immune selection [8]. Genetic evidence in HIV also suggests that evolution is HLA class I associated but is not uniform [17]. Further studies to identify both the mechanisms which lead to persistence without the requirement for mutation in T-cell epitopes, and which elucidate the differences between ‘driver’ and ‘passenger’ CD8+ T-cell responses, if these exist in HCV, are required.

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#### REFERENCES

- Cooper S, Erickson AL, Adams EJ *et al.* Analysis of a successful immune response against hepatitis C virus. *Immunity* 1999; 10(4): 439–449.
- Lechner F, Wong DK, Dunbar PR *et al.* Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; 191(9): 1499–1512.
- Lechner F, Gruener NH, Urbani S *et al.* CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur J Immunol* 2000; 30(9): 2479–2487.
- Lechner F, Sullivan J, Spiegel H *et al.* Why do cytotoxic T lymphocytes fail to eliminate hepatitis C virus? *Phil Trans R Soc Lond B Biol Sci* 2000; 355(1400): 1085–1092.
- Gerlach J, Diepolder H, Jung M-C *et al.* Recurrence of HCV after loss of virus specific CD4+ T cell response in acute hepatitis C. *Gastroenterology* 1999; 117: 933–941.
- He X-S, Rehermann B, Lopez-Labrador F *et al.* Quantitative analysis of HCV-specific CD8+ T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci (USA)* 1999; 96: 5692–5697.
- Grabowska AM, Lechner F, Klenerman P *et al.* Direct ex vivo comparison of the breadth and specificity of the T cells in the liver and peripheral blood of patients with chronic HCV infection. *Eur J Immunol* 2001; 31(8): 2388–2394.
- Klenerman P, Wu Y, Phillips R. HIV: current opinion in escapology. *Curr Opin Microbiol* 2002; 5: 408–413.
- Erickson AL, Kimura Y, Igarashi S *et al.* The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. *Immunity* 2001; 15(6): 883–895.
- Chang K, Rehermann B, McHutchinson J *et al.* Immunological significance of CTL epitope variants in patients chronically infected by HCV. *J Clin Invest* 1997; 100: 2376–2385.
- Appay V, Nixon DF, Donahoe SM *et al.* HIV-specific CD8 (+) T cells produce antiviral cytokines but are impaired. *J Exp Med* 2000; 192(1): 63–75.
- Gruener NH, Lechner F, Jung MC *et al.* Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol* 2001; 75(12): 5550–5558.
- Masopust D, Vezys V, Marzo A, Iefrancois L. Preferential localisation of effector memory cells in nonlymphoid tissue. *Science* 2001; 291: 2413–2417.
- Wong D, Dudley D, Afdhal N *et al.* Liver derived CTL in HCV infection; breadth and specificity of responses in a cohort of patients with chronic infection. *J Immunol* 1998; 160: 1479–1488.
- Swofford DL, Waddell PJ, Huelsenbeck JP, Foster PG, Lewis PO, Rogers JS. Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. *Syst Biol* 2001; 50(4): 525–539.
- Lauer GM, Barnes E, Lucas M *et al.* High resolution analysis cellular immune responses in resolved and persistent HCV infection. *Gastroenterology* 2004; 127: 924–936.
- Moore C, John M, James I, Christiansen F, Witt C, Mallal S. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 2002; 296: 1439–1443.
- Klenerman P, Zinkernagel R. What can we learn about HIV infection from a study of LCMV? *Immunol Rev* 1997; 159: 5–16.
- Pircher H, Moskphidis A, Rohrer U, Burki K, Hengartner H, Zinkernagel RM. Viral escape by selection of cytotoxic T cell-resistant variants in vivo. *Nature* 1990; 346: 629–633.
- Weiner AJ, Erickson AL, Kansopon J *et al.* Association of cytotoxic T lymphocyte (CTL) escape mutations with persistent hepatitis C virus (HCV) infection. *Princess Takamatsu Symp* 1995; 25: 227–235.
- Kantzanou M, Lucas M, Barnes E *et al.* Viral escape and T cell exhaustion in hepatitis C virus infection analysed using class I peptide tetramers. *Immunol Lett* 2003; 85(2): 165–171.
- Christie JM, Chapel H, Chapman RW, Rosenberg WM. Immune selection and genetic sequence variation in core and envelope regions of hepatitis C virus. *Hepatology* 1999; 30(4): 1037–1044.
- Giuggio V, Bonkovsky H, Smith J, Rothman A. Inefficient recognition of autologous viral sequences by intrahepatic HCV-specific CTL in chronically infected subjects. *Virology* 1998; 251: 132–140.

- 24 Tsai S, Chen Y, Chen M *et al.* HCV variants circumventing CTL activity as a mechanism of chronicity. *Gastroenterology* 1998; 115: 954–966.
- 25 Timm J, Lauer G, Kavanagh D *et al.* CTL epitope escape and reversion in acute HCV infection. *J Exp Med* 2004; 200: 1593–1604.
- 26 Klenerman P, Rowland-Jones S, McAdam S *et al.* Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. *Nature* 1994; 369(6479): 403–407.
- 27 Bertoletti A, Sette A, Chisari FV *et al.* Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 1994; 369(6479): 407–410.
- 28 Klenerman P, Meier UC, Phillips RE, McMichael AJ. The effects of natural altered peptide ligands on the whole blood cytotoxic T lymphocyte response to human immunodeficiency virus. *Eur J Immunol* 1995; 25(7): 1927–1931.
- 29 Klenerman P, Zinkernagel RM. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. *Nature* 1998; 394(6692): 482–485.
- 30 Harcourt GC, Lucas M, Godkin AJ, Kantzanou M, Phillips RE, Klenerman P. Evidence for lack of cross-genotype protection of CD4+ T cell responses during chronic hepatitis C virus infection. *Clin Exp Immunol* 2003; 131(1): 122–129.
- 31 McMichael A, Klenerman P. HIV/AIDS HLA leaves its footprints on HIV. *Science* 2002; 296(5572): 1410–1411.
- 32 Willberg C, Barnes E, Klenerman P. HCV immunology – death and the maiden T cell. *Cell Death Differ* 2003; 10: S39–S47.
- 33 Large MK, Kittlesen DJ, Hahn YS. Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. *J Immunol* 1999; 162(2): 931–938.
- 34 O'Connor D, Friedrich T, Hughes A, Allen T, Watkins D. Understanding CTL escape during SIV infection. *Immunol Rev* 2001; 183: 115–126.
- 35 Borrow P, Lewicki H, Wei X *et al.* Antiviral pressure exerted by HIV-1 specific CTL during primary infection demonstrated by rapid selection of CTL escape virus. *Nature Med* 1997; 3: 205–211.