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Broad Repertoire of the CD4$^+$ Th Cell Response in Spontaneously Controlled Hepatitis C Virus Infection Includes Dominant and Highly Promiscuous Epitopes[1]

Julian Schulze zur Wiesch,*‡ Georg M. Lauer,* Cheryl L. Day,* Arthur Y. Kim,* Kei Ouchi,* Jared E. Duncan,* Alysse G. Wurcel,*¶ Joerg Timm,* Andrea M. Jones,* Bianca Mothe,§ Todd M. Allen,* Barbara McGovern,¶ Lia Lewis-Ximenez,‖ John Sidney,** Alessandro Sette,** Raymond T. Chung,† and Bruce D. Walker[2]*‡

A vigorous hepatitis C virus (HCV)-specific Th cell response is regarded as essential to the immunological control of HCV viremia. The aim of this study was to comprehensively define the breadth and specificity of dominant HCV-specific CD4$^+$ T cell epitopes in large cohorts of subjects with chronic and spontaneously resolved HCV viremia. Following in vitro stimulation of PBMC, HCV-specific cell cultures from each subject were screened with an overlapping panel of synthetic 20-mer peptides spanning the entire HCV polyprotein. Of 22 subjects who spontaneously controlled HCV viremia, all recognized at least one of a group of six epitopes situated within the nonstructural (NS) proteins NS3, NS4, and NS5, each of which was detected by >30% of subjects, but most subjects recognized additional, more heterogeneous specificities. In contrast, none of the most frequently targeted epitopes was detected by >5% of persons with chronic infection. The most frequently recognized peptides showed promiscuous binding to multiple HLA-DR molecules in in vitro binding assays and were restricted by different HLA-DR molecules in functional assays in different persons. These data demonstrate that predominant CD4$^+$ T cell epitopes in persons with resolved HCV infection are preferentially located in the nonstructural proteins and are immunogenic in the context of multiple class II molecules. This comprehensive characterization of CD4$^+$ T cell epitopes in resolved HCV infection provides important information to facilitate studies of immunopathogenesis and HCV vaccine design and evaluation. *The Journal of Immunology,* **2005, 175: 3603–3613.**

Worldwide, over 170 million people are infected with the hepatitis C virus (HCV).[3] Chronic HCV infection often results in cirrhosis of the liver and increases the probability of developing hepatocellular carcinoma (1, 2). There is no HCV vaccine available at this time (3) despite the fact that a significant subset of persons with acute HCV infection spontaneously clear the virus, suggesting the possibility of immunological control (4–7).

A growing body of evidence indicates that spontaneous clearance of HCV is associated with a strong HCV-specific proliferative CD4$^+$ Th cell response (8–12). A number of studies on persistent murine and human viral infections indicate that virus-specific CD4$^+$ T cells play a critical role in the outcome of viral infections (13–18), and seem to be required to maintain effective cytotoxic T cell responses (19) and neutralizing Abs (20). Experiments in the chimpanzee model of HCV demonstrate that depletion of CD4$^+$ T cells before reinfection of two previously resolved animals resulted in persistent, low-level viremia despite the presence of intrahepatic memory CD8$^+$ T cell responses (21). Incomplete control of HCV replication in the absence of adequate CD4$^+$ T cell help was associated with the emergence of viral escape mutations in class I MHC-restricted epitopes and failure to resolve HCV infection.

To understand the differences associated with control or failure of the HCV-specific Th response, a necessary first step is to define the breadth and specificity of the immune responses associated with viral control vs persistence (12, 22).

This study was designed to comprehensively determine the breadth and fine specificities of HCV-specific CD4$^+$ T cell responses for each individual in a large cohort of subjects with diverse HLA backgrounds who spontaneously resolved HCV, and to compare these to an equally large group of persons with persistent viremia. The results show that the CD4$^+$ T cell response in spontaneously resolved HCV infection is broadly directed, targeting up to 28 specificities within a single subject. We demonstrate that all subjects with resolved viremia recognize one or more of a group of immunodominant HCV CD4$^+$ T cell epitopes that are highly promiscuous in that they are recognized in the context of multiple HLA class II molecules. However, these frequently targeted epitopes rarely account for the majority of responses, but instead

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[2] Address correspondence and reprint requests to Dr. Bruce D. Walker, Partners AIDS Research Center, Howard Hughes Medical Institute, Massachusetts General Hospital, 149 13th Street, Room 5212D, Charlestown, MA 02129. E-mail address: bwalker@partners.org

[3] Abbreviations used in this paper: HCV, hepatitis C virus; ALT, alanine transaminase level; ICS, intracellular cytokine assay; BCL, B-lymphoblastoid cell line; LCL, L cell line; LPA, lymphoproliferative assay; SI, stimulation index.

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the response in persons with resolved viremia almost always includes additional more heterogeneous and less frequently detected $CD4^+$ T cell epitopes. These findings are important for the understanding of HCV pathogenesis (12), and will facilitate further studies on the function of HCV-specific $CD4^+$ Th cells on the single epitope level (23).

Materials and Methods

Subjects

Twenty-two subjects with spontaneously controlled HCV infection and 23 subjects with chronic HCV infection gave written informed consent and were enrolled in this study which was approved by the Institutional Review Boards of the Massachusetts General Hospital and the Lemuel Shattuck Hospital. All subjects were anti-HCV positive as measured by third-generation enzyme immunoassay, and no subject had been treated with HCV antiviral therapy. HCV RNA was measured by the Roche Amplicor Monitor assay (detection limit of 600 HCV RNA IU/ml plasma). All individuals with spontaneously controlled HCV viremia had documented undetectable plasma viral loads (<600 IU/ml plasma) at multiple time points for >7 mo (range, >7 mo to >3 years). The HCV viral loads of the individuals with chronic HCV infection ranged from 2240 to >850000 IU/ml plasma (HCV RNA positive >1 year). Serum alanine transaminase levels (ALT) were normal (<28 U/L) in individuals with resolved infection, with the exception of subject R1, who had intermittent mild elevation in ALT levels (range, 23–72 U/L) attributed to chronic hepatitis B virus infection. Subjects R16, R17, R18 were HIV positive, with HIV viral loads <50 copies/ml for >3 mo and $CD4^+$ counts >350/μl (patients R17 and R18 were being treated with highly active antiretroviral therapy; R16 had never been on HIV antiretroviral therapy). In subjects with spontaneously resolved infection, serotyping for the HCV genotype was performed using the Murex HCV Serotyping 1–6 assay (Abbott) according to the manufacturer's instructions except in cases where the genotype had already been documented during acute infection. There was no serum or plasma available for subject R9. Data from subjects R1–3 have been described (22). Twenty-three subjects with persistent HCV viremia served as a control group.

Synthesis of HCV-derived peptides

Peptides corresponding to the amino acid sequences of the HCV-1a strain HCV-1 (GenBank accession number: M62321) were synthesized as free acids using the 9-fluorenylmethoxy carbonyl method. A total of 301 peptides spanning the entire polyprotein were used for the screening. These peptides were 20 aa in length, overlapping adjacent peptides by 10 aa (22).

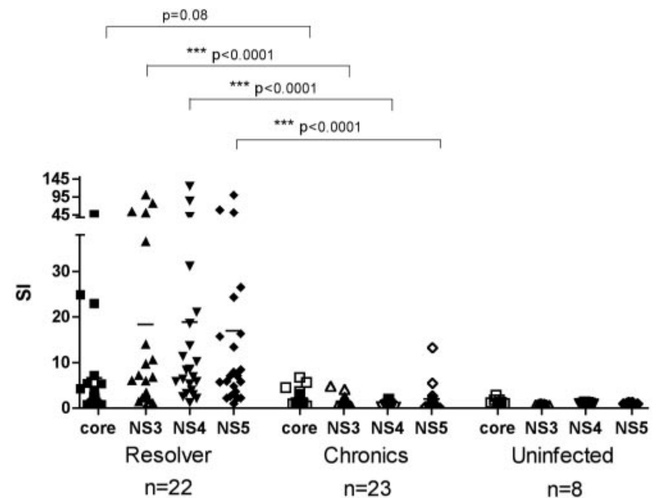

FIGURE 1. Comparison of lymphoproliferative responses to recombinant HCV Ags in subjects with spontaneously resolved and chronic infection. LPAs with recombinant HCV Ags expressing the HCV proteins: core (c22.3), NS3 (c33c), NS4 (c100.3), and NS5 were performed on PBMC of 22 spontaneously resolved and 23 chronically HCV infected subjects as described in *Materials and Methods*. Eight uninfected seronegative subjects served as controls. In contrast to the weak proliferative responses displayed by subjects with chronic HCV infection, multiple regions of HCV were targeted by subjects with spontaneously resolved HCV, with recombinant NS3, NS4, and NS5 proteins each inducing similar strong proliferative responses (NS3 mean stimulation index (SI) = 18.3, range 0.5–101; NS4 mean SI = 18.9, range 1.2–124; NS5 mean SI = 17, range 1–100.3). The recombinant core protein induced the weakest average proliferative response in subjects with resolved HCV (mean SI = 6.6, range 0.7–46).

Table I. *Clinical, virological, and immunological data*[a]

Subjects with Chronic HCV								Subjects with Resolved HCV					
ID no.	Age/sex	HCV VL IU/ml	Genotype	ALT	$CD4^+$ response in short-term culture	DR	DQ	ID no.	Age/sex	Serotype	$CD4^+$ responses in short-term culture	DR	DQ
C1	47/M	>100,000	1	88	0	3, 14	2, 5	R1	44/M	1	16	11	3
C2	45/M	233,000	4a/b	57	7	1, 11	3, 5	R2	42/M	1	13	4, 10	3, 5
C3	44/M	>500,000	1a	43	0	3, 7	2, 3	R3	50/F	1	14	8, 4	3
C4	47/F	>500,000	4a/b	83	1	4, 15	3, 6	R4	40/F	4	3	11, 4	3
C5	49/M	>500,000	2	111	0	12, 13	3, 5	R5	40/F	1	7	4, 15	6, 3
C6	41/M	>500,000	1b	57	0	3, 7	2, 3	R6	44/F	4	5	14, 11	3, 5
C7	48/F	308,000	1b	216	1	13, 14	3, 5	R7	52/M	1	16	1, 7	3, 5
C8	31/F	>850,000	1a	126	3	8, 13	4, 6	R8	43/M	3	14	7, 13	6, 3
C9	43/F	810,000	1b	11	0	7, 15	2, 6	R9	54/M	ND	8	7, 3	2
C10	22/F	2,240	1a	67	0	1,	5,	R10	45/M	1	4	15, 3	2, 6
C11	29/F	>500,000	1a	67	0	4, 15	3, 6	R11	44/M	1a[b]	8	11, 3	2, 3
C12	37/F	87,200	3a	89	0	1, 3	2, 5	R12	41/F	1a/1b[b]	6	4, 7	3
C13	39/M	>850,000	3a	207	0	1, 11	5, 6	R13	73/F	4	5	15, 13	2, 6
C14	38/M	120,000	4	55	0	3, 11	2, 5	R14	50/M	1	12	8, 10	4, 5
C15	59/F	39,600	1a	27	0	ND	ND	R15	23/M	3	5	1, 3	2, 5
C16	64/F	>500,000	1a	88	0	4, 14	3, 6	R16	55/M	1	28	1, 8	4, 5
C17	38/M	452,000	1a	76	0	9, 15	3, 6	R17	49/F	1	12	4, 15	3, 6
C18	45/F	750	2	45	8	1, 3	5, 6	R18	48/M	1b[b]	9	12, 4	3
C19	27/M	102,000	3a	45	0	4, 7	3, 4	R19	38/M	3	6	10, 15	5, 6
C20	41/F	38,600	2a/c	21	2	4, 15	ND	R20	25/F	1	6	7, 15	2, 6
C21	20/F	164,000	1b	69	0	3, 7	3, 5	R21	43/F	1	9	16, 12	5
C22	37/M	13,582	ND	29	0	1, 15	5, 6	R22	46/M	3	8	4, 10	3
C23	39/M	2,940	4	123	4	1, 15	5, 6						

[a] Clinical, virological, and immunological data of subjects with chronic HCV viremia (left) and spontaneously resolved HCV viremia (right) included in this study.
[b] Genotype.

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Recombinant HCV proteins

The recombinant HCV proteins used in this study were expressed as C-terminal fusion proteins with human superoxide dismutase in *Saccharomyces cerevisiae* or *Escherichia coli* and were kindly provided by M. Houghton (Chiron, Emeryville, CA). These proteins were derived from the HCV-1 sequence and encoded core (C22-3 aa 2–120), NS3 (C33C aa 1192–1457), NS4 (C100.3 aa 1569–1931), NS3/NS4 (C200 aa 1192–1931), and NS5 (NS5 aa 2054–2995).

HLA typing

DNA for HLA typing was extracted using the Puregene DNA Isolation kit for blood (Gentra Systems) according to the manufacturer's instructions. HLA class II typing was performed at the MGH Tissue Typing Laboratory and by Dynal Biotech HLA Diagnostics using sequence-specific primer PCR (24, 25).

Proliferation assays

Lymphocyte proliferation assays were performed as described previously (22, 26), using c22.3, c33c, c100.3, C200, and NS5 HCV proteins (Chiron) at concentrations of 10 μg/ml. PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque-density gradient centrifugation and plated at 100,000 cells/well in 96-well U-bottom plates (Costar) in 200 μl of R10/HAB medium (RPMI 1640 (Sigma-Aldrich), 10% human AB serum (Sigma-Aldrich), and 10 mM HEPES buffer (Sigma-Aldrich) with 2 mM glutamine and antibiotics (penicillin-streptomycin, 50 U/ml)) and the designated proteins in quadruplicate wells. After a 6-day incubation at 37°C and 5% CO_2, wells were pulsed for 6 h with 1 μCi of [^{3}H]thymidine (PerkinElmer). Cells were then collected on filters, and the amount of incorporated radiolabel was measured with a beta counter (22).

In vitro HLA-DR peptide binding assays

Frequently targeted peptides were tested for in vitro binding to up to 15 common HLA-DR molecules. HLA-DR molecules were purified, and binding assays were performed as previously described (27). Purified human HLA-DR molecules were incubated with unlabeled HCV peptides and 1–10 nM ^{125}I-radiolabeled probe peptides for 48 h. MHC binding of the radiolabeled peptide was determined by capturing MHC-peptide complexes on LB3.1 (anti-HLA-DRA) Ab-coated Lumitrac 600 plates (Greiner

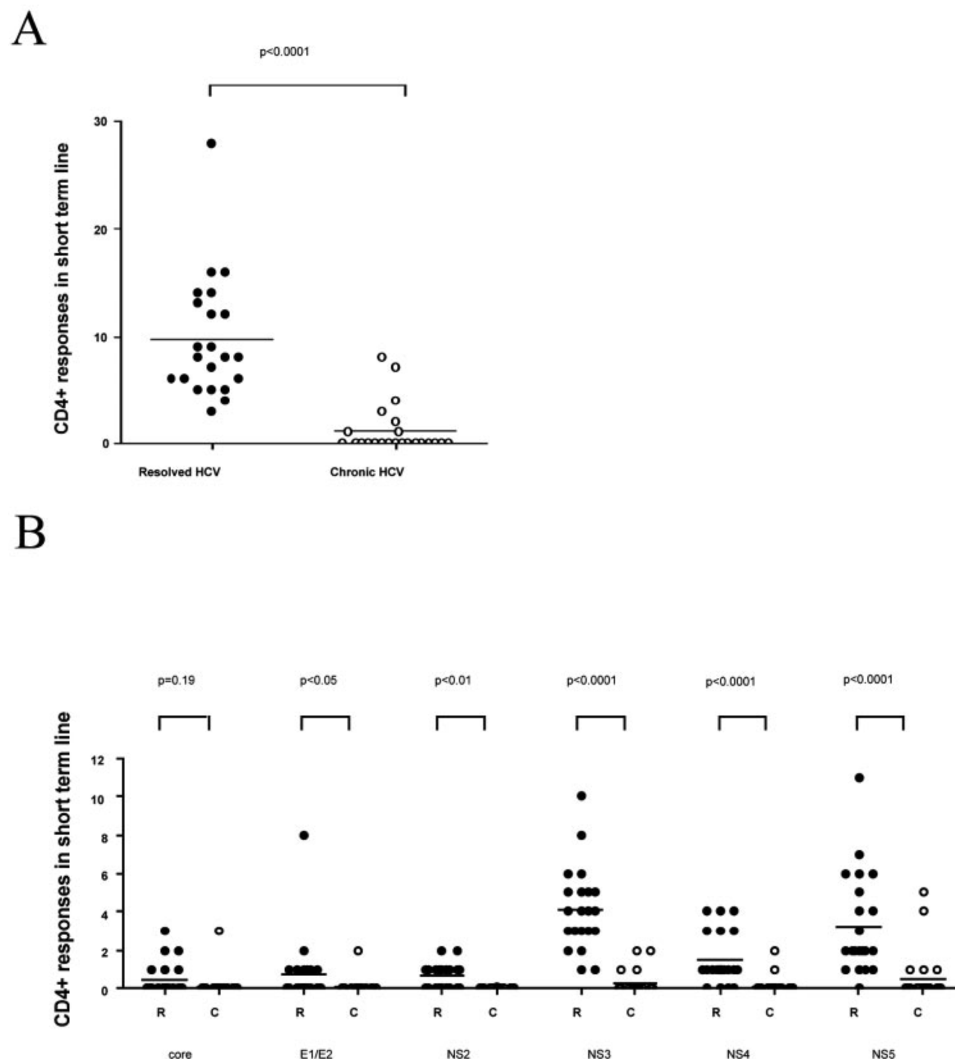

FIGURE 2. Spontaneous resolution of HCV viremia is associated with recognition of multiple $CD4^+$ T cell epitopes. PBMC from 22 subjects with resolved HCV infection and 23 subjects with chronic HCV infection were $CD8^+$-depleted and stimulated with recombinant Ags (or overlapping peptides) covering the entire polyprotein in the presence of recombinant IL-2. Expanded cell lines were tested for IFN-γ production upon stimulation with pools of 10–20 overlapping peptides by ELISPOT. All peptide pools eliciting a positive response by IFN-γ production were further deconvoluted to identify specific 20-mer peptides targeted by the $CD4^+$ T cell response. Each single response detected in the ELISPOT assay was confirmed by ICS for IFN-γ production as well. Horizontal bars indicate the median numbers of targeted HCV $CD4^+$ T cell responses against single peptides for each subject as tested by ICS. The number of responses is significantly higher ($p < 0.0001$) in persons with resolved HCV than in chronically infected persons when comparing the number of $CD4^+$ T cell responses detected in short-term lines across the entire polyprotein (resolvers: mean 10, range 3–29; chronic infection: mean 1, range 0–8) (*A*), and when comparing the number of responses in HCV resolvers (R) or chronically infected (*C*) analyzed and plotted for each of the proteins E1/E2, NS2, NS3, NS4, and NS5 individually (*B*). Although recombinant core protein induced more responses in persons with resolved HCV than in chronically infected persons this trend did not reach significance.

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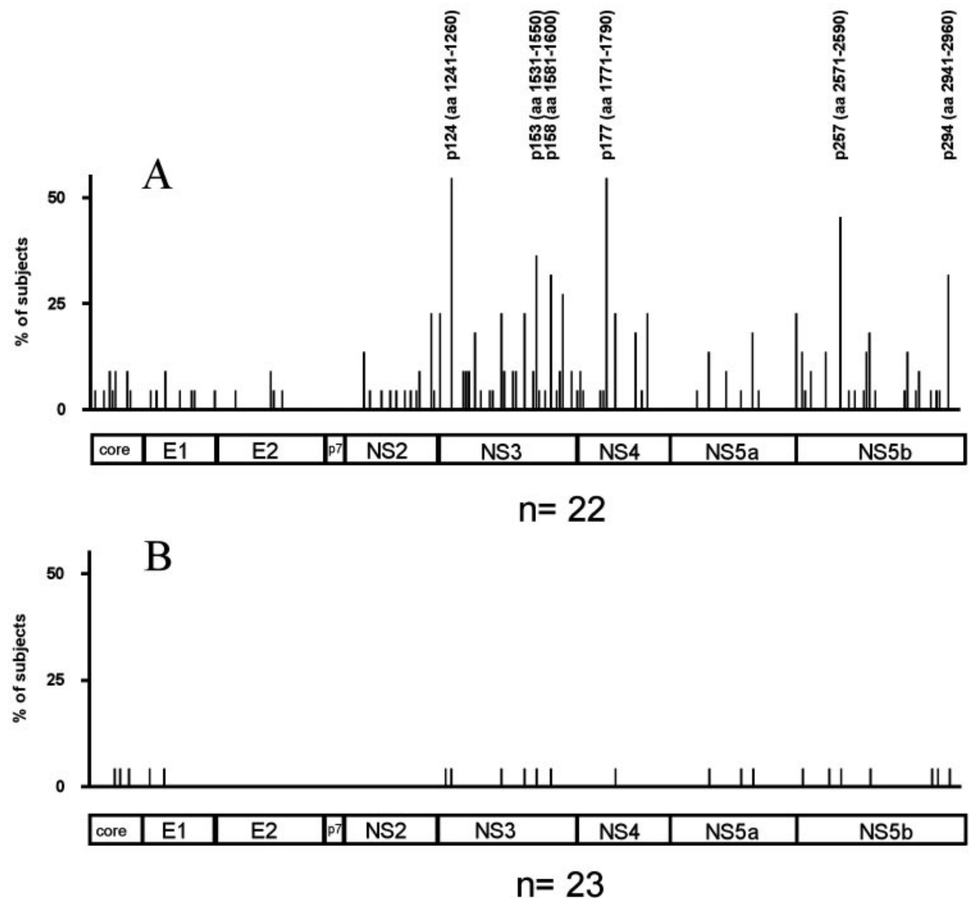

FIGURE 3. Frequency of detection of HCV peptides in HCV-specific CD4^{+} T cell cultures and localization of these specificities within the HCV polyprotein of 22 subjects with spontaneously resolved HCV infection (*A*) and 23 chronically HCV-infected subjects (*B*).

Bioone) and measuring bound cpm using the TopCount (Packard Instrument) microscintillation counter. The binding data were analyzed and IC_{50} (nanomolar) determined as previously described (27, 28).

Bulk stimulation of PBMC

Twenty million fresh or thawed PBMC were depleted of CD8^{+} cells with magnetic beads according to the manufacturer's instructions (M-450 CD8 beads; Dynal Biotech) and the CD8^{+}-depleted cells were stimulated with 1 μg/ml recombinant HCV Ags (either C22–3, C33C, C100.3, C200, or NS5) in 2 ml of R10/50 medium (RPMI 1640 medium, 10 mM HEPES with 2 mM glutamine and antibiotics (penicillin-streptomycin, 50 U/ml)) and 10% FCS (Sigma Aldrich) supplemented with recombinant IL-2 (rIL-2) (50 U/ml)) (22). Alternatively, for the regions of the HCV genome for which recombinant Ags were not available (E1, E2, p7, and NS2) PBMC were stimulated with overlapping 20-mer peptides (1 μg/ml each) in pools of 10 peptides (22). After 11–13 days cells were assayed for IFN-γ production by ELISPOT and intracellular cytokine staining in response to stimulation with HCV peptides. In fine-mapping and restriction experiments, HCV-specific CD4^{+} T cell lines were directly used if the specificity of the cell line was $\geq$3%. If the specificity was $<$3%, peptide-specific cells were selected by an IFN-γ capture assay (Miltenyi Biotec), as previously described (29). Briefly, the cells were stimulated with 10 μg of peptide/ml and costimulatory CD28 and CD49d mAbs (1 μg/ml; BD Biosciences). After incubation for 5 h at 37°C, IFN-γ was processed according to the manufacturer's protocol and isolated on a magnetic cell sorting column. Selected cells were expanded with IL-2 and irradiated allogenetic feeder cells for an additional 10–14 days and then used in HLA restriction experiments.

ELISPOT assay

ELISPOT assays were performed as previously described (30). Polyvinylidene plates (96-well; Millipore) were coated with 2.5 μg/ml recombinant anti-human IFN-γ Ab (Endogen) in PBS at 4°C overnight. Peptides were directly added to the wells at a final concentration of 10 μg/ml–0.01 ng/ml. Cells from polyclonal HCV-specific CD4^{+} T cell cultures were taken out of R10/50 by washing three times with media and incubating for 6 h at 37°C in R10 and were then added at 50,000 cells/well in 140 μl of R10 medium. The plates were incubated for 18 h at 37°C in 5% CO_2. Plates were then washed, labeled with 0.25 μg/ml biotin-labeled anti-IFN-γ (Endogen), and developed by incubation with streptavidin-alkaline phosphatase (Bio-Rad), followed by incubation with 5-bromo-4-chloro-indolylphosphate-NBT (Bio-Rad) in Tris buffer (pH 9.5). The reaction was stopped by washing with tap water, and the plates were dried overnight, before counting on an ELISPOT reader (AID). Responses were considered positive if the number of spots was three times greater than the background; PHA served as a positive control. All positive responses were reconfirmed by intracellular cytokine assay (ICS) following stimulation with the specific peptide.

Intracellular IFN-γ staining by flow cytometry

Intracellular cytokine staining was performed as previously described (22, 31). Briefly, 5×10^5 bulk expanded PBMC were added to 10^5 autologous B cells and incubated with 4 μM peptide and anti-CD28 and anti-CD49d mAb at 37°C and 5% CO_2 for 1 h before the addition of GolgiPlug (1 μl/ml; BD Biosciences). The cells were incubated for an additional 5 h at 37°C and 5% CO_2. PBMC were then washed and stained with surface Abs, allophycocyanin-conjugated anti-CD3, and peridin-chlorophyll protein-conjugated anti-CD4 (BD Biosciences) at room temperature for 20 min. Following washing, the PBMC were fixed and permeabilized (Caltag Laboratories), and the FITC-conjugated anti-IFN-γ mAb (BD Biosciences) was added. Cells were then washed and analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Single peptides that elicited distinct responses of more than three times background IFN-γ production were considered positive (22).

HLA restriction experiments

HLA restriction experiments were performed as described before (22, 29). Autologous and partially HLA-matched EBV-transformed B-lymphoblastoid cell lines (BCL) and L cell lines (LCL) stably transfected with HLA class II molecules were pulsed with 0.4 μg of peptide/ml for 90 min at 37°C and 5% CO_2. HLA-DR-transfected cell lines were kindly donated by Epimmune. The peptide-pulsed BCL or LCL were then washed four times,

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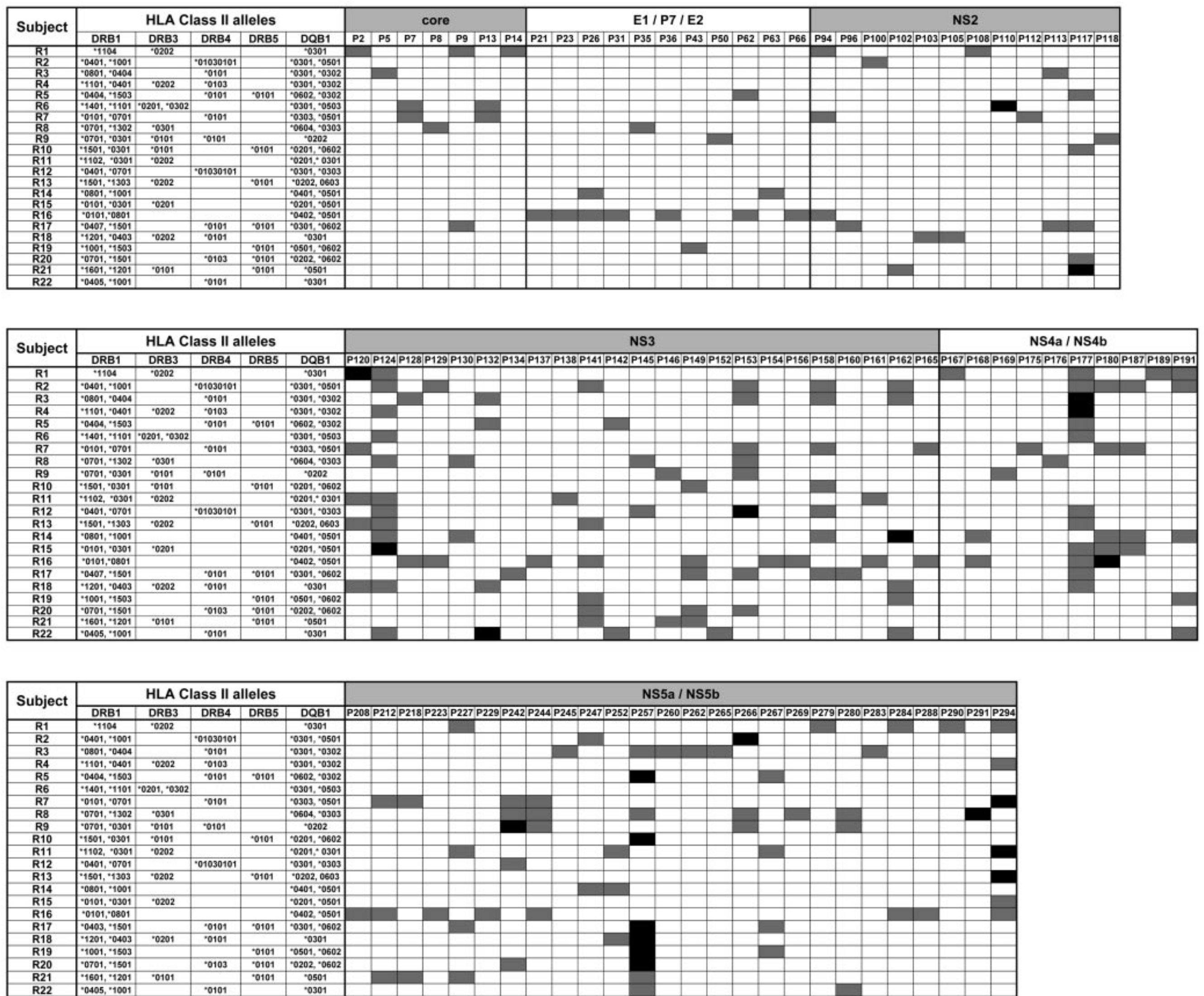

FIGURE 4. Summary of all the specificities recognized by each individual with resolved HCV infection. Shaded boxes indicate a positive CD4$^+$ response. Black boxes represent the strongest CD4$^+$ response in a single subject. Peptides: p2 = aa 21–40; p3 = aa 31–50; p294 = aa 2941–2960.

incubated together with peptide-specific cells at a ratio of 1:10, and stained as described above. The peptide-specific CD4$^+$ T cell response was analyzed by intracellular cytokine staining for IFN-γ production as described above.

Statistical analysis

Nonparametric Mann-Whitney tests were performed using GraphPad Prism 4.0 software (GraphPad Software). To avoid overestimation of the total breadth of antiviral responses, responses to adjacent overlapping peptides were counted as responses to 1 epitopic region, with only the stronger response shown.

Results

Immunodominance of selected peptides located within NS3, NS4, and NS5 in spontaneously resolved HCV viremia

Spontaneous resolution of HCV infection has been shown to be associated with a vigorous lymphocyte proliferative response (8, 10, 32). Therefore, we stimulated PBMC with recombinant HCV core (c22.3), NS3 (c33c), NS4 (c100.3), and NS5 proteins in standard lymphoproliferative assays (LPA) in all 22 subjects with resolved HCV infection and 23 subjects with chronic HCV infection (for clinical and virological data for the cohort see Table I). There was a significant difference in the magnitude of the lymphoproliferative responses to all three nonstructural proteins when compared with persons with unresolved infection (Fig. 1). The lowest magnitude of responses in persons with resolved infection was to the relatively small core protein, and these responses were not significantly different from persons with chronic infection, although there was a trend toward significance ($p = 0.08$). Proliferative responses were infrequently detected in persons with chronic infection (Fig. 1).

We next performed a more detailed analysis of each subject to define the fine specificities of the HCV CD4$^+$ T cell epitopes targeted within each of the HCV proteins by testing HCV-specific polyclonal CD4$^+$ T cell cultures that were generated with slight modifications of the protocol described previously (Ref. 22, see also *Materials and Methods*). Cells were initially tested with separate pools of 10 overlapping peptides covering the whole HCV polyprotein after 11–13 days. Positive pools were deconvoluted the next day by single peptide ELISPOT. Single-positive responses in ELISPOT were then confirmed in a single peptide intracellular cytokine staining assay for specific IFN-γ production.

The 22 subjects with resolved HCV infection demonstrated broadly distributed responses (mean 10; range 3–28) against different 20-mer peptides (Fig. 2*A*) and most subjects (21 of 22) each showed one or more responses in at least three of four of the

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Table II. *Summary of frequently targeted HCV specificities*[a]

Position of Peptide	HCV Protein	Sequence	% of Resolvers that Recognize Peptide
p94 (aa 941–960)	NS2	LGALTGTYVYNHLTPLRDWA	13
P117 (aa 1171–1190)	NS3	CPAGHAVGIFRAAVCTRGVA	22
P120 (aa 1201–1220)		LETTMRSPVFTDNSSPPVVP	22
P124 (aa 1241–1260)[b]		**PAAYAAQGYKVLVLNPSVAA**	**54**
P132 (aa 1321–1340)		TDATSILGIGTVLDQAETAG	18
P141 (aa 1411–1430)		GINAVAYYRGLDVSVIPTSG	27
P149 (aa 1491–1510)		TGRGKPGIYRFVAPGERPSG	22
P153 (aa 1531–1550)[b]		**TPAETTVRLRAYMNTPGLPV**	**36**
P158 (aa 1581–1600)[b]		**ENLPYLVAYQATVCARAQAP**	**31**
P162 (aa 1621–1640)		PTPLLYRLGAVQNEITLTHP	27
P177 (aa 1771–1790)[b]	NS4b	**GIQYLAGLSTLPGNPAIASL**	**54**
P180 (aa 1801–1820)		LTTSQTLLFNILGGWVAAQL	22
P187 (aa 1871–1890)		GEVPSTEDLVNLLPAILSPG	18
P191 (aa 1911–1930)		GAVQWMNRLIAFASRGNHVS	22
P212 (aa 2121–2140)	NS5a	FFTELDGVRLHRFAPPCKPL	13
P227 (aa 2271–2290)		PAEILRKSRRFAQALPVWAR	18
P242 (aa 2421–2440)	NS5b	SMSYSWTGALVTPCAAEEQK	22
P244 (aa 2441–2460)		LPINALSNSLLRHHNLVYST	18
P252 (aa 2521–2540)		FGYGAKDVRCHARKAVTHIN	10
P257 (aa 2571–2590)[b]		**KGGRKPARLIVFPDLGVRVC**	**45**
P266 (aa 2661–2680)		QCCDLDPQARVAIKSLTERL	13
P267 (aa 2671–2690)		VAIKSLTERLYVGGPLTNSR	18
P280 (aa 2801–2820)		VYYLTRDPTTPLARAAWETA	13
P294 (aa 2941–2960)[b]		**CGKYLFNWAVRTKLKLTPIA**	**31**

[a] Summary of frequently targeted HCV specificities by the CD4$^+$ T cell response of subjects with resolved HCV (detected in at least 3 of 22 subjects).
[b] Six most frequently recognized peptides are shown in bold.

nonstructural proteins (NS2, NS3, NS4a/b, or NS5a/b). In all cases (22 of 22) of resolved viremia one or more epitopes in NS3 were targeted (Fig. 2*B*) whereas responses were less frequently detected in the highly conserved, but small, core protein (consistent with the data generated in LPA assays), and in the variable regions of the HCV envelope proteins (Figs. 2*B* and 3*A*) (33–36).

Altogether, 89 of the 301 overlapping peptides tested were targeted by at least one person (Fig. 4). Whereas some peptides were only recognized by 1–2 subjects, each of the 24 peptides shown in Table II was recognized by at least 3 of the 22 subjects with resolved infection, even though in some cases these subjects did not share class II molecules (see below).

The two most frequently detected specificities in persons with resolved infection, p124 (aa 1241–1260) and p177 (aa 1771–1790) (situated in NS3 and NS4, respectively) were each recognized by >50% of the subjects with resolved HCV infection. These two peptides contain CD4$^+$ T cell epitopes that have previously been described in smaller and less comprehensive studies as frequently recognized epitopes (37, 38). An additional four peptides were targeted in >30% of persons with resolved infection (p153 (aa 1531–1550), p158 (aa 1581–1600), p257 (aa 2571–2590) and p294 (aa 2941–2960)), all of which are located within one of the different HCV nonstructural proteins. Each of the 22 subjects with spontaneously resolved infection targeted at least one (mean 2.5; range 1–4) of these six most frequently targeted peptides.

In addition to defining the breadth of the targeted epitopes, we also examined the dominant response in each subject with resolved viremia, defined as the peptide that induced the strongest IFN-γ response as measured by single peptide ICS in expanded cells (Fig. 4). Even though peptide p124 (aa 1241–1260) is one of the most frequently recognized peptides in persons with resolved HCV, this peptide was found to be the dominant response in only 1 of 22 subjects. In contrast, p257 (aa 2571–2590) was the dominant response in HCV-specific short-term lines of 6 of the 10 subjects that recognize this peptide.

In the comprehensive high-resolution screening of HCV-specific polyclonal cell lines, only 7 of the 23 subjects with chronic infection showed one or more positive HCV-specific CD4$^+$ T cell response, and these responses were narrowly targeted against few peptides (average 1, range 0–8) (Fig. 3*B*). Of those epitopes targeted in chronic infection, all were also detected in resolved infection.

Frequently detected HCV specificities show degenerate binding in in vitro assays

The finding that certain peptides were recognized by a large proportion of subjects with resolved HCV infection, despite the fact that they expressed different HLA class II molecules, raised the possibility of promiscuous HCV CD4$^+$ T cell epitopes that are recognized in the context of multiple HLA class II molecules. We first tested this hypothesis by analyzing the capacity of frequently recognized HCV peptides to bind to a panel of up to 15 of the most prevalent human HLA-DR molecules in an in vitro assay (29, 39, 40). Peptides with an IC_{50} of 1000 nM or lower were considered to bind to the prospective DR molecule since this has been shown in previous studies to represent an affinity threshold associated with immunogenicity in the context of HLA-DR molecules (28, 29). These most frequently recognized peptides were highly promiscuous in binding to multiple HLA-DR molecules with each of these six peptides binding between 7 and 12 different molecules (Table III). Furthermore, some of these peptides were able to bind to several HLA-DR molecules with extremely high affinities (IC_{50} <10 nM). These results indicate that HCV peptides capable of

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Table III. *Most frequently recognized HCV CD4$^+$ T cell epitopes*[a]

Position	Length	Sequence	Binding Capacity (IC_{50} nM)															Molecules bound
			DRB 1*0101	DRB 1*0301	DRB 1*0401	DRB 1*0404	DRB 1*0405	DRB 1*0701	DRB 1*0802	DRB 1*0901	DRB 1*1101	DRB 1*1201	DRB 1*1302	DRB 1*1501	DRB 3*0101	DRB 4*0101	DRB 5*0101	
p124C (AA 1243–1260)	18 aa	AYAAQGYKVLVLNPSVAA	**8**	**594**	**4**	**48**	**51**	**192**	18,665	3,436	**37**	**278**	**9**	**873**	1,541	**59**	5455	**10/15**
p153 (AA 1531–1550)	20 aa	TPAETTVRLRAYMNTPGLPV	**168**	10,193	**43**	**17**	**16**	**20**	**818**	1,448	**277**	1,565	**562**	**9**	10,289	**16**	6815	**10/15**
p158 (AA 1581–1600)	20 aa	ENLPYLVAYQATVCARAQAP	**4**	**471**	**4**	**25**	**76**	**267**	2,502	2,608	**111**	**636**	**457**	**30**	36,805	**8**	**135**	**10/15**
P177 (AA 1771–90)	20 aa	GIQYLAGLSTLPGNPAIASL	**1**	>69,767	**13**	ND	**6**	2,176	ND	ND	**5**	ND	**214**	**378**	>100,000	ND	**357**	**7/9**
p257 (AA 2571–2590)	20 aa	KGGRKPARLIVFPDLGVRVC	**283**	**152**	**900**	**127**	**45**	**458**	3,515	9,058	3,311	2,593	**33**	**50**	3,336	**6**	**535**	**10/15**
p294 (AA 2941–2960)	20 aa	CGKYLFNWAVRTKLKLTPIA	**48**	**546**	**187**	1,003	**91**	**131**	**457**	4,909	**138**	**876**	**118**	**122**	8,776	**455**	**125**	**12/15**

[a] Most frequently recognized HCV CD4$^+$ T cell epitopes show a "degenerate binding" pattern and bind to most *HLA-DR* alleles in in vitro binding assay with high affinity. Peptides were tested in binding assays to prevalent HLA-DR molecules. The IC_{50} for each *DR* allele is shown. A total of 1000 nM was defined as an affinity threshold associated with immunogenicity (shown in bold).

binding multiple HLA-DR molecules with high affinity are likely to be frequently recognized by subjects with resolved infection.

Frequently targeted peptides are restricted by multiple HLA class II molecules and share largely overlapping binding repertoires

The above binding data as well as the diverse HLA class II molecules expressed in persons targeting the same peptides (Fig. 4) suggest that these peptides may be promiscuously presented by multiple class II molecules. To address this experimentally, cells were stimulated with peptide-pulsed partially HLA-matched BCL and/or the corresponding HLA-DR-transfected fibroblasts in an assay described previously (22, 29). Representative data demonstrating HLA class II restriction of a p153 (aa 1531–1550)-specific cell line in subject R8 are shown in Fig. 5, demonstrating recognition of this epitope in the context of HLA DRB1*0701, an allele to which p153 (aa 1531–1550) showed strong binding in the in vitro assay (Table III).

Of the six most frequently targeted peptides in persons with resolved HCV infection, restricting HLA class II molecules were demonstrated for each epitope, and for three of these multiple restricting class II molecules were readily defined. As an example, peptide p124 (aa 1241–1260) overlaps with a previously described and well-defined highly promiscuous CD4$^+$ T cell epitope. This epitope has been previously described to be restricted by at least five different HLA-DR or DQ molecules (37, 38, 41), and additionally was found to be restricted by HLA-DR1*1001 in subject R2 and HLA-DRB3 0301 in subject R8. Thus together with previous data, this peptide can be presented in the context of at least seven different class II molecules, further emphasizing the promiscuity of this peptide (Table IV).

Data from all restriction experiments that we performed on individual subjects are summarized in Table IV. Of note, using this experimental approach, which incorporates functional FACS analysis with partially HLA-matched cell lines, all HCV HLA class II epitopes for which HLA restrictions could be defined were presented by HLA-DR molecules and not by DQ molecules.

The above data indicate that the 20-mer peptides can be presented in the context of multiple class II molecules, suggesting that either the epitopes within the longer peptides are identical, or that the peptides contain multiple overlapping epitopes. The generation of CD4$^+$ T cell lines provided a means to define the minimal (and optimal) length of some frequently recognized HCV 20-mer peptides in individual subjects with different HLA class II molecules. For the fine-mapping analysis of the six most frequently recognized peptides, we defined the "minimal-epitope" as the smallest peptide triggering a response at least 50% as strong as that observed with the full-length peptide in the ICS assay. This definition is consistent with criteria that have been used in studies of HIV and CMV-specific CD4$^+$ T cell responses (29, 42). We confirmed the results from the ICS experiments by conducting ELISPOT dilution assays (data not shown).

The effects of serial truncations on the magnitude of the IFN-γ responses are illustrated in Fig. 6 which shows representative ICS results from subjects R1, R3, R12, and R14, all of whom targeted peptide p177 (aa 1771–1790) in the context of different HLA class II molecules. Serial N-terminal and C-terminal truncations showed an almost identical pattern in all four subjects, suggesting that the minimal epitope was the 11-mer ^{1775}LAGLSTLPGNP.

Optimal responses for this and most of the other epitopes we analyzed were seen with peptide truncations flanking (43) the minimal epitope that induced IFN-γ production by two to four N- and C-terminal residues (Table IV). For some peptides (p227 (aa 2271–2290), p242 (aa 2421–2440)) the minimal epitope was only

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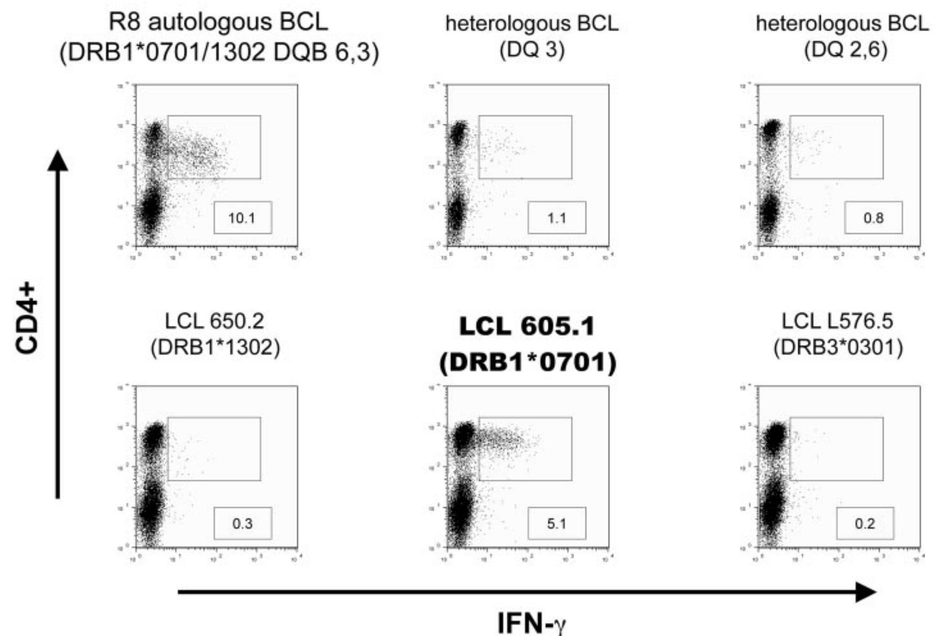

FIGURE 5. HCV-specific CD4$^+$ T cell response against p153 (aa 1531–1550) is restricted by HLA-DRB1*0701 in subject R8. Peptide-pulsed (0.4 μg/ml autologous and partially HLA-matched B cells, as well as L cells L650.2 (DRB 1*1302), L605.1 (DRB1*0701), and L576.5 (DRB3*0301)) were incubated with CD4$^+$ T cell lines from subject 1 for 6 h and analyzed by flow cytometry for IFN-γ production as described in *Materials and Methods*. Non-peptide-pulsed B cells and L cells were used as a control (data not shown).

narrowed down to 14 aa. For many applications like the construction of new class II tetramers (23), the knowledge of the peptide which triggers the optimal response rather than the minimal epitope may have the greatest relevance.

The data from the functional truncation experiments performed in this study are supported in part by commonly used prediction algorithms (44–46), but there are multiple peptides for which the functional data indicate the need for a longer peptide than would be predicted (Table IV).

Discussion

HCV-specific proliferative CD4$^+$ T cell responses to recombinant HCV proteins are associated with self-limited HCV infection as demonstrated in this and previous studies (8–12). Beyond this finding, our current understanding of the role of HCV-specific CD4$^+$ T cells in acute infection and the mechanism(s) by which the HCV virus successfully impairs this response in chronically evolving acute HCV is limited. This in part due to the fact that memory CD4$^+$ T cells appear to be present at significantly lower frequencies than their CD8$^+$ counterparts among circulating cells (23, 47, 48) requiring sophisticated in vitro assays to characterize CD4$^+$ T cell responses against certain specificities.

Among the questions that have yet to be addressed is whether it is the specific CD4$^+$ T cell response against certain epitopes (37), the breadth of the response (22), the vigor and kinetics (41) or the functional properties of the response that is essential to a successful resolution of the virus.

This study advances these efforts by providing a comprehensive and detailed picture of the breadth and specificity of the HCV CD4$^+$ T cell specificities targeted in a large cohort of subjects with varying HLA class II molecules that spontaneously resolved HCV, and comparing these to responses to persons with chronic infection. Subjects with spontaneously resolved HCV in this study universally targeted a broad range of epitopes that were located in several (>3 or more) of the HCV proteins (Figs. 2*B* and 4). Six peptides, all situated within the NS3, NS4 and NS5 protein, are each recognized by >30% of persons with resolved HCV infection, regardless of their HCV serotype or HLA background.

In fact, all 22 subjects with resolved infection showed positive responses against one or more of these six most frequently recognized specificities. However, and of significance for vaccine considerations, almost all of these subjects targeted additional peptides. Indeed, in terms of the contribution of these six epitopes to the total number targeted, it ranged from as high as 100% in one subject to <8% of the total response in another. This common feature of multiple targeted CD4$^+$ T cell responses in persons with resolved HCV infection may have important implications for future HCV vaccine strategies, since it would argue against vaccines that prime a narrow range of CD4$^+$ T cell responses that only target a small region of the entire HCV polyprotein.

The most frequently targeted specificities were characterized by degenerate binding to multiple HLA-DR molecules in in vitro assays, demonstrating that they are restricted by different HLA class II molecules in different subjects. Additionally, some of these epitopes have been described as well conserved throughout all six genotypes (35, 37, 38, 49), and are located in functional regions of the HCV polyprotein (Refs. 37, 49, and 50), and reviewed in Ref. 51). Peptide p257 (aa 2571–2590) for example, one of the six most frequently recognized peptides in our cohort, is situated within the conserved motif F of the HCV NS5B polymerase. Engineered mutations in this region have shown to be detrimental to replication of HCV replicons due to the disruption of the polymerase activity (52).

Although promiscuously binding epitopes in highly conserved regions of HCV are attractive targets for HCV vaccines and immunotherapies (53, 54), the exact prerequisites and number of HCV CD4$^+$ T cell specificities that are essential for a sufficient response are still not clear. It is important to note that resolution of infection is nearly always associated with a broad range of CD4$^+$ T cell responses in addition to the responses against these promiscuous epitopes mentioned above.

Promising in this respect is the fact that we characterize altogether 24 specificities that are recognized by >10% of our cohort of subjects with resolved infection (Table II). These peptides account for more than half of the total number of responses in subjects with resolved HCV infection (mean 6, range 2–10). These epitopes are mostly located in relatively conserved regions of the HCV polyprotein.

This might explain why, despite the use of recombinant HCV proteins and peptides that are based on a genotype 1a sequence,

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Table IV. *Summary of HLA-restriction and fine specificities of frequently recognized CD4^{+} T cell epitopes tested in this study*

	20-mer Sequence	Optimal Length According to Experimental Results	Minimal Length According to Experimental Results	Minimal Length as Suggested by Prediction Programs[a] (43–45)	Restriction Element of Original 20-mer Peptide as Tested in Subject(s)
Six most frequently recognized peptides:					
p124 (aa 1241–1260)[b]	PAAYAAQGYKVLVLNPSVAA	^{1247}QGYKVLVLNPSVAAT^{1261} (16 aa)	^{1251}VLVLNPSVAA^{1260} (10 aa)	YKVLVLNPSVAA	DRB1*1104 (R1), DRB1*0401 (R12) DRB1*1001 (R2), DRB3*0101 (R8)
p153 (aa 1531–1550)	TPAETTVRLRAYMNTPGLPV	^{1535}TTVRLRAYMNTPGLPV^{1550} (16 aa)	^{1542}YMNTPGLPV^{1550} (9 aa)	YMNTPGLPV	DRB1*0701 (R7, R8)
p158 (aa 1581–1600)	ENLPYLVAYQATVCARAQAP	^{1585}YLVAYQATVCARAQAP^{1600} (16 aa)	^{1587}VAYQATVCARAQ^{1598} (12 aa)	VAYQATVCARA	DRB1*1001 (R2)
p177 (aa 1771–1790)	GIQYLAGLSTLPGNPAIASL	^{1773}QYLAGLSTLPGNPAIASL^{1790} (18 aa)	^{1775}LAGLSTLPGNP^{1785} (11 aa)	LAGLSTLPG	DRB1*0404 (R3, R5), DRB1*0407 (R14), DRB1*0401 (R12), DRB1*1104 (R1)
p257 (aa 2571–2590)	KGGRKPARLIVFPDLGVRVC	^{2575}KPARLIVFPDLGVRVC^{2590} (16 aa)	^{2577}ARLIVFPDLGVR^{2588} (12 aa)	LIVFPDLGV	DRB1*0404 (R5), DRB1*0407 (R14)
p294 (aa 2941–2960)	CGKYLFNWAVRTKLKLTPIA	^{2941}CGKYLFNWAVRTKL^{2954} (14 aa)	^{2944}YLFNWAVRTKL^{2954} (11 aa)	VRTKLKLTP	DRB1*1104 (R1)
Other peptides restricted in this study:					
p120 (aa 1201–1220)	LETTMRSPVFTDNSSPPVVP	^{1203}TTMRSPVFTDNSSPPVVP^{1220} (16 aa)	^{1209}VFTDNSSPPVV^{1219} (11 aa)	FTDNSSPPV	DRB3*0201 (R1)
p162 (aa 1661–1680)	PTPLLYRLGAVQNEITLTHP	ND	ND	NA	DRB1*1001 (R14)
p191 (aa 1911–1930)	GAVQWMNRLIAFASRGNHVS	DRB1*1104: ^{1911}GAVQWMNRLIAFAS^{1924} (14 aa)	^{1913}VQWMNRLIAF^{1922} (10 aa)	VQWMNRLIA	DRB1*1104 (R1)
		DRB1*1001: ^{1913}VQWMNRLIAFASRGNHVS^{1930} (18 aa)	^{1915}WMNRLIAFAS^{1924} (10 aa)	NA	DRB1*1001 (R2)
p227 (aa 2271–2290)	PAEILRKSRRFAQALPVWAR	^{2271}PAEILRKSRRFAQALPVWAR^{2290} (20 aa)	^{2273}EILRKSRRFAQALP^{2286} (14 aa)	LRKSRRFAQ	DRB1*1104 (R1)
p242 (aa 2421–2440)	SMSYSWTGALVTPCAAEEQK	^{2421}SMSYSWTGALVTPCAA^{2436} (16 aa)	^{2423}SYSWTGALVTPCAA^{2436} (14 aa)	MSYSWTGAL	DRB1*0701 (R9)

[a] www.imtech.res.in/raghava/propred/; http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/EpitopePrediction.htm
[b] Compare Diepolder et al. (36).

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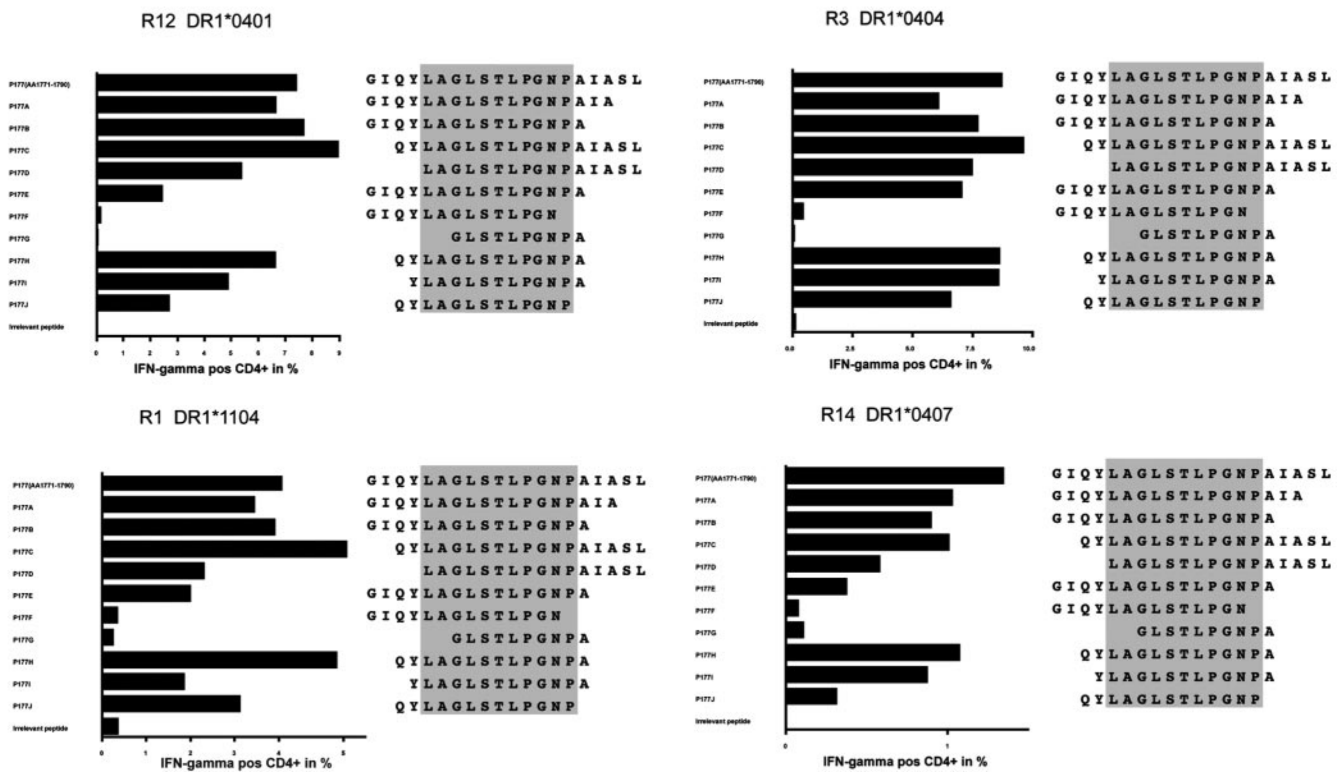

FIGURE 6. Defining the fine specificity of the response against peptide p177 (aa 1771–1790) in cell lines from multiple subjects with resolved HCV. Representative ICS data from CD4^{+} T cell lines of subjects R12, R3, R1, and R14 (the restricting HLA DR molecule is shown for each subject). Cells were stimulated with 0.4 μg/ml peptide p177 (aa 1771–1790) or its truncations 177A-J. The minimal region essential for recognition of this epitope is highlighted in gray.

only a slight decrease in the number of HCV-specific CD4^{+} T cell responses was detected in 7 of 22 subjects with serologically resolved non-genotype 1 infection in this study.

This study also raises additional important questions. Despite the addition of rIL-2 to our cell cultures (in contrast to the standard proliferation assay), we could detect only a limited number of HCV-specific CD4^{+} T cells in subjects with chronic infection. Recent data analyzing PBMC of persons with HCV infection with class II tetramer data suggest that these cells are not completely absent in subjects during early, acute disease, irrespective of outcome (55). Only by conducting comprehensive and more detailed studies during the early stages of acute infection will we be able to understand the evolution of differences in breadth, specificity, and functional properties of the HCV-specific CD4^{+} T cell response that lead to a favorable outcome. A better understanding of the mechanisms of establishment of immunodominance of certain CD4^{+} T cell epitopes during acute infection, as well as the fate of these cells during the transition to chronic infection, will be of importance for the understanding of disease pathogenesis and for the development of vaccines (12, 41). The identification of discrete epitopes targeted should assist greatly in such analyses.

In summary, we provide evidence that the HCV-specific CD4^{+} T cell response in subjects with resolved infection consists of multiple specificities predominantly located within the nonstructural HCV proteins. Additionally, we find that despite the high degree of polymorphism of the MHC-class II molecules expressed in the human population and the high sequence variability of the different HCV genotypes, many of the predominant HCV CD4^{+} T cell epitopes in persons with resolved HCV infection are widely recognized. The results of this study have important implications for future vaccine development and include data that will be important for a more sophisticated analysis of the HCV-specific CD4^{+} T cell response on the single epitope level, and are especially crucial to further the development of new MHC class II tetramers (56–58).

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Disclosures

The authors have no financial conflict of interest.

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