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*J Immunol* 2004; 172:483-492; doi: 10.4049/jimmunol.172.1.483
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Conserved Hierarchy of Helper T Cell Responses in a Chimpanzee during Primary and Secondary Hepatitis C Virus Infections

Naglaa H. Shoukry,* John Sidney,† Alessandro Sette,‡ and Christopher M. Walker*‡†

Control of hepatitis C virus (HCV) infection could be influenced by the timing and magnitude of CD4+ T cell responses against individual epitopes. We characterized CD4+ T cells targeting seven Pan troglodytes (Patr) class II-restricted epitopes during primary and secondary HCV infections of a chimpanzee. All Patr-DR-restricted HCV epitopes bound multiple human HLA-DR molecules, indicating the potential for overlap in epitopes targeted by both species. Some human MHC class II molecules efficiently stimulated IL-2 production by chimpanzee virus-specific T cell clones. Moreover, one conserved epitope designated NS3_{1248} (GYKVLVKNPSV) overlapped a helper epitope that is presented by multiple HLA-DR molecules in humans who spontaneously resolved HCV infection. Resolution of primary infection in the chimpanzee was associated with an initial wave of CD4+ T cells targeting a limited set of dominant epitopes including NS3_{1248}. A second wave of low-frequency CD4+ T cells targeting other subdominant epitopes appeared in blood several weeks later after virus replication was mostly contained. During a second infection 7 years later, CD4+ T cells against all epitopes appeared in blood sooner and at higher frequencies but the pattern of dominance was conserved. In summary, primary HCV infection in this individual was characterized by T cell populations targeting two groups of MHC class II-restricted epitopes that differed in frequency and kinetics of appearance in blood. The hierarchial nature of the CD4+ T cell response, if broadly applicable to other HCV-infected chimpanzees and humans, could be a factor governing the outcome of HCV infection. The Journal of Immunology, 2004, 172: 483–492.

The CD4+ helper T cell plays an essential role in control of virus replication (1–5) and their failure in human infections with the hepatitis C virus (HCV) is associated with persistent viremia (6–18). Helper responses are required to promote the generation of neutralizing Abs (19), induce maturation of APCs (20), and sustain effector functions of virus-specific CD8+ T cells (1–5). In addition, CD4+ T cells can directly inhibit virus replication through production of antiviral cytokines (21) and were shown to select for escape mutants in CD4+ T cell epitopes in vivo during infection with lymphocytic choriomeningitis virus (LCMV) (22). Breadth of the CD8+ T cell response and relative dominance of the epitopes they target has been recognized as a factor determining the outcome of infection with highly mutable RNA viruses like HCV (14, 23). There is comparatively limited information on the hierarchy of epitope recognition by CD4+ T cells and how this might influence the course of acute viral infection.

Loss of HCV-specific CD4 helper T cell responses was associated with recurrence of plasma viremia in humans (13) and Ab-mediated depletion of HCV-specific CD4+ T cells in chimpanzees facilitated the generation of virus escape mutations in MHC class I-restricted epitopes (24). In contrast, generation of a strong CD4+ and CD8+ T cell-mediated immune response in blood (6–18, 23, 25) and their accumulation in the liver (16, 23, 25) were required for successful control of HCV infection. Individuals with resolved HCV infections can target as many as 14 distinct MHC class II-restricted epitopes, while the number targeted by persistently infected individuals is significantly more limited (9). The magnitude and longevity of the CD4+ T cell response directed against individual MHC class II-restricted epitopes has not been studied and whether patterns of dominance are maintained upon reinfection with HCV is unknown. A helper T cell response that is narrowly focused on a few MHC class II epitopes could conceivably facilitate emergence of virus variants with the potential to evade or alter effector function. Indeed, the pattern of cytokine production by helper T cell lines can be skewed by stimulation with naturally occurring variants of MHC class II-restricted epitopes found in other HCV-infected individuals (27, 28).

The chimpanzee model of HCV infection is suitable for addressing these questions but the identity and number of epitopes targeted by CD4+ T cells from animals with resolved infections has not yet been described. In addition, although chimpanzees (Pan troglodytes) have MHC (Patr) class II loci orthologous to human HLA-DR, DP, and DQ, their involvement in presentation of HCV peptides for CD4+ T cell recognition has not been formally demonstrated. In this study, we observed that epitopes identified in a chimpanzee bound multiple human HLA-DR alleles and one overlapped a dominant helper epitope identified in human subjects with resolved HCV infections. Two distinct groups of epitopes targeted by CD4+ T cells were evident during primary infection with HCV. Some epitopes were considered dominant during the early phase of acute infection and the appearance of cognate CD4+ T cells in blood corresponded kinetically with a significant reduction in plasma virus load. CD4+ T cells targeting the remainder of the epitopes were present in blood at much lower frequencies and only

*Address correspondence and reprint requests to Dr. Christopher M. Walker, Center for Vaccines and Immunity, Columbus Children’s Research Institute, Columbus, OH, 43205; †La Jolla Institute for Allergy and Immunology, San Diego, CA 92121; and ‡College of Medicine and Public Health, Ohio State University, Columbus, OH 43205.

Received for publication June 26, 2003. Accepted for publication October 17, 2003.

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1 This work was supported by Public Health Service Grants AI-R01-A147367 and U19AI48231 (to C.M.W.) and N01-AI-95362 (to A.S.). N.H.S. is supported by post-doctoral fellowships from the Canadian Institute for Health Research and the American Liver Foundation.

2 Address correspondence and reprint requests to Dr. Christopher M. Walker, Center for Vaccines and Immunity, Columbus Children’s Research Institute, 700 Childrens Drive, Room W503, Columbus, OH 43205. E-mail address: walker@pediatrics.osu.edu

3 Abbreviations used in this paper: HCV, hepatitis C virus; BLCL, EBV-transformed B lymphoblastoid cell line; GE, genome equivalent; IBL, intrabiliary lymphocyte; LCMV, lymphocytic choriomeningitis virus; SFC, spot-forming cell; HS, human serum; p.i., postinfection.
after virus replication was controlled. Responses against all epitopes were accelerated and T cells targeting dominant epitopes were present at significantly higher frequencies during a second infection 7 years later. There was no delay in appearance of CD4+ T cells against the subdominant epitopes upon reinfection, although their frequencies in blood were still substantially lower than those targeting the dominant epitopes.

Materials and Methods

Animals and infection

Chimpanzee (Pan troglodytes) CB0572 was maintained under standard conditions for humane care and in compliance with National Institutes of Health guidelines at the New Iberia Research Center (New Iberia, LA). It was infected i.v. with 100 chimpanzee infectious doses of HCV-1/910 inoculum for the first time in 1994, then rechallenged with the same dose and strain 7 years later in 2001. Virus replication and immune responses after the two infections were described previously (26).

MHC class II typing of chimpanzees

Chimpanzee (Pan troglodytes) Patr MHC class II alleles were identified by cloning and sequencing the highly polymorphic exon 2. Briefly, RNA was purified from autologous EBV-transformed B lymphoblastoid cell lines (BLCLs) using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using a Reverse Transcription System (Promega, Madison, WI). Exon 2 of the different Patr molecules was PCR amplified using the primer pairs listed in Table I. PCR conditions were: an initial denaturation step at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step for 15 min at 72°C. PCR products were cloned in pCR2.1-Topo (Invitrogen) using the Topo TA cloning kit (Invitrogen), then sequenced. A minimum of 10 separate molecular clones were sequenced for each locus. Due to the multiple gene segments of the Patr DR-β locus, a minimum of 30 different molecular clones for each chimpanzee were sequenced.

HCV recombinant Ags and peptides

HCV-1 recombinant Ags C22–3 (Core aa: 2–120), C33c (NS3 aa: 1192–1205), C100 (NS3 aa: 1569–1931), C200 (NS3-NS4 a/b: aa 1192–1931), and NS5 (aa: 2054–2995) were expressed as C-terminal fusion proteins with human superoxide dismutase in yeast (Saccharomyces cerevisiae) or Escherichia coli and were kindly provided by Dr. M. Houghton (Chiron, Emeryville, CA). Peptides were synthesized by either Mimotopes Pty (San Diego, CA) or Invitrogen.

ELISPOT assays

Human IFN-γ ELISPOT kits from U-cytech (Utrecht, The Netherlands) were used and the assays were performed as previously described (26) using 1 μg/ml of each individual epitope. Cryopreserved PBMCs were used to assay the immune response during primary infection. Freshly isolated PBMCs were used in the secondary infection. The OVA-derived peptide SIINFEKL was used as a negative control. CD4+ intrahepatic lymphocytes (IHL) were isolated and expanded from liver biopsies as described below. For the ELISPOT assay, 1 × 10^5 irradiated autologous PBMCs were used as APCs with 0.5–1 × 10^5 expanded intrahepatic CD4+ T cells for 40–48 h and then processed for spot formation as described previously (25).

Generation of HCV-specific CD4+ T cell clones from liver

To recover CD4+ IHL, liver biopsies were gently homogenized in PBS containing 1% FCS. CD4+ T cells were enriched using anti-human CD4+ dynabeads (Dynal, Oslo, Norway) and cloned at a limiting dilution of 10 or 50 cells/well in 96-well plates. Briefly, CD4+ T cells were seeded in T cell clone medium (RPMI 1640 medium; Invitrogen), 10% heat-inactivated FCS, and 40 U/ml recombinant human IL-2 (a kind gift from Chiron). Cells were expanded using anti-human CD3 Ab (X35) (Immunotech, Marseille, France) at 0.05 μg/ml in the presence of 5 × 10^4 irradiated (3000 rad) human PBMCs per well as feeder cells. Cultures were fed every 3–4 days by replacing half of the culture medium. After 2 wk, growing clones were transferred to 24-well plates and subjected to another round of anti-CD3 Ab stimulation in the presence of 2 × 10^6 irradiated human PBMCs. Cultures were fed every 3–4 days as described above. Expanded cells were tested in a proliferation assay against autologous irradiated (10,000 rad) BLCLs pulsed with the different HCV-1 recombinant Ags described above. Positive clones were then picked and propagated using anti-CD3 stimulation. Epitope fine mapping was performed by proliferation assays as described below using peptide matrices spanning the Ag of interest. Peptides were 20 aa long, overlapping by 10 residues. Truncations of the specific 20-aa peptide were synthesized to determine the region crucial for recognition.

Generation of HCV-specific CD4+ T cell clones from peripheral blood

PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation. Briefly, 4 × 10^6 freshly isolated PBMCs were stimulated with 10 μg/ml recombinant HCV Ags (C22–3, C33c, C100, C200, or NS5) in one well of a 24-well plate in 2 ml of RPMI 1640-human serum (HS) medium ((RPMI 1640 medium; Invitrogen) containing 10% human AB serum (Gemini Biosciences, Woodland, CA)). Recombinant human IL-2 was added on day 3 at a final concentration of 40 U/ml. Cells were fed every 3–4 days by replacing half of the culture medium and incubated for a total of 10–14 days. Cells were then counted and restimulated in a 24-well plate at 1 × 10^5 T cells, 2 × 10^5 autologous irradiated (3000 rad) PBMCs, and 10 μg/ml recombinant HCV Ag in RPMI 1640-HS with addition of rIL-2 on day 3 as described above. Following the second stimulation, cells were subcloned at 1 and 10 cells/well of 96-well flat-bottom plates in the presence of 5 × 10^4 irradiated human PBMCs and anti-CD3 Abs at 0.05 μg/ml. Each clone was tested for specificity in a proliferation assay using autologous irradiated (10,000 rad) BLCLs pulsed with the recombinant HCV Ag as APCs. Positive clones were then propagated and epitope specificity was determined as described above for liver clones.

T cell proliferation assay

CD4+ T cell clone (5 × 10^5/well) were cocultured with irradiated (10,000 rad) autologous BLCLs (5 × 10^5/well) in the presence of the antigenic peptide at the indicated concentration in 200 μl of AIM-HS medium ((AIM-V lymphocyte medium; Invitrogen) containing 2% human AB serum (Gemini Biosciences)) in 96-well flat-bottom plates. Control wells containing no peptide, T cells alone, or BLCLs alone were included in all assays. Cultures were set in duplicate or triplicate and incubated at 37°C in 5% CO2 for 2 days and then labeled by incubation for another 18 h with 1 μCi/well of [3H]thymidine (Amersham Biosciences, Piscataway, NJ). Cells were collected and washed on filters using an automated cell harvester (Tomtec, Hamden, CT) and the amount of thymidine incorporated was measured using an automated beta counter (Wallac, Turku, Finland). Proliferation was determined as mean cpm of duplicate or triplicate wells.

MHC restriction assay

MHC restriction was first determined by inhibition of proliferation using the anti-HLA class II Abs: L243 (anti-HLA-DR), IVA-12 (anti-HLA-DR, DP, and DQ), IVD-12 (anti-HLA-DQ), and the anti-HLA class I Ab W6/32 (anti-HLA-A,B,C). Abs were purified from hybridoma supernatants using the HitTrap Protein G HP columns (Amersham Biosciences). All hybridomas were obtained from American Type Culture Collection (Manassas, VA). Cross-reactivity with the chimpanzee MHC class II molecules was used to determine the specificity of the proliferation assay using autologous irradiated (10,000 rad) BLCLs pulsed with the recombinant HCV Ag as APCs. Positive clones were then propagated and epitope specificity was determined as described above for liver clones.

Table I. Primers used for typing chimpanzee MHC class II via sequencing of exon 2

<table>
<thead>
<tr>
<th>Region/Primer</th>
<th>Sequence 5′ → 3′</th>
<th>Fragment size (bp)</th>
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<tbody>
<tr>
<td>DR-α</td>
<td>Invariant, no sequencing performed</td>
<td></td>
</tr>
<tr>
<td>DR-β Forward</td>
<td>GGGGACACCCCGAACACGTTTC</td>
<td>285</td>
</tr>
<tr>
<td>DR-β Reverse</td>
<td>ACTCGCGCGTCTAGTGAAGGC</td>
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</tr>
<tr>
<td>DQ-α Forward</td>
<td>GAAGACATGTTGCTCGACACGG</td>
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<tr>
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<td>CTAGACGAACTCATTGGAGAAGCC</td>
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</tr>
<tr>
<td>DP-α Forward</td>
<td>AGGACCTCTCCGAGAATTTC</td>
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</tr>
<tr>
<td>DP-β Forward</td>
<td>GCGGACATGTTGCTACATATTGC</td>
<td>297</td>
</tr>
<tr>
<td>DP-β Reverse</td>
<td>GCCCTCAGGCTCCTGTGGAA</td>
<td>297</td>
</tr>
<tr>
<td>Reverse</td>
<td>TAGGCTGGACAGGCGGCTGC</td>
<td>284</td>
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verified by flow cytometry. Briefly, proliferation assays were performed as described above using the optimal peptide epitope at 0.1 μg/ml in the presence or absence of 10–100 μg/ml of the inhibitory Ab. Following identification of the MHC class II-restricting molecule, fine allele restriction was determined by flow cytometry using the following semimatched chimpanzee BLCLs as APCs: autologous 572 (DRB1*1001, DRB1*0701, DRB5*0310); 264 (DRB1*0204, DRB1*0307, DRB3*0102, DRB5*0310), 297 (DRB1*1001, DRB1*0201, DRB5*0310), and 1558 (DRB1*0303, DRB1*0701, DRB5*0310). Briefly, 1 × 10^5 CD4^+ clonal T cells and 1 × 10^5 BLCLs were cocultured overnight in round-bottom 96-well plates in the presence or absence of 1 μg/ml of the optimal peptide in AIM-HS medium. Brefeldin A was added after the first hour at a concentration of 10 μg/ml. Following overnight incubation, cells were washed in FACs buffer (PBS containing 1% FCS and 0.01% sodium azide) and permeabilized using Cytofix-Cytoperm reagent (BD Pharmingen, San Diego, CA) for 15 min at 4°C. Cells were stained with anti-human CD4 (Leu-3a) and IL-2 Abs (both from BD Pharmingen) and analyzed on a FACS Calibur instrument using CellQuest software (BD Pharmingen). A total of 10,000 events was acquired in the CD4^+ T cell gate and analyzed for IL-2 expression.

**HLA-DR peptide binding**

HLA class II molecules were purified, as previously described (29), from EBV-transformed homozygous BLCLs or transfected fibroblasts by affinity chromatography. Peptide-binding assays were performed by incubating purified human MHC class II molecules (5–500 nM) with various concentrations of unlabeled test peptides as inhibitors and 1–10 nM ^3^H-labeled probe peptides for 48 h in PBS containing 0.05–0.15% Nonidet P-40 in the presence of a protease inhibitor mixture (29, 30). MHC class II peptide complexes were separated from free peptide by gel filtration on TSK200 columns (part no. 16215; Tosoh Biosciences LLC, Montgomeryville, PA) and the fraction of bound peptide was calculated. Alternatively, the percentage of MHC-bound radioactivity was determined by capturing MHC-peptide complexes on LB3.1 Ab-coated Optiplates (Packard Instrument, Meriden, CT) and determining bound cpm using the TopCount (Packard Instrument) microscintillation counter.

**Results**

**Mapping and MHC restriction of HCV-specific CD4^+ T cell clones from a chimpanzee with resolved infection**

Chimpanzee CB0572 was infected with 100 chimpanzee infectious doses of the HCV-1/910 virus stock in 1994. The infection resolved spontaneously within 4 mo and HCV RNA was not detectable in plasma through 7 years of follow-up. Virus clearance was verified by ELISA. The infection resolved based on their frequency and kinetics of appearance and was associated with a multispecific CD4^+ and CD8^+ cellular immune response (25). At 6 years after primary infection, high frequencies of HCV-specific CD4^+ T cells were detected in peripheral blood by proliferative and IFN-γ ELISPOT assays against multiple HCV recombinant Ags (data not shown). To dissect this immune response against individual helper epitopes, we cloned HCV-specific CD4^+ T cells from the blood and liver of this animal.

Intrahepatic CD4^+ T cell clones were derived by limited dilution cloning using non-Ag-specific stimulation with anti-CD3 Ab as described in Materials and Methods and then screened for their reactivity to the various HCV Ags. HCV-specific T cell lines were derived from blood by repeated stimulation with the recombinant HCV Ags C22-c (Core), c200 (NS3/4 a/b), and NS5. Six different clones were isolated (Table II). In a first step to map the targeted epitopes, we measured proliferative responses to peptide matrices spanning the entire polyprotein as described in Materials and Methods (data not shown). Following mapping of the response to the specific 20-aa peptide(s), further truncations were designed to map the region crucial for recognition (Figs. 1 and 2). MHC restriction was determined using a panel of anti-MHC class II Abs to block proliferative responses as described in Materials and Methods. Four clones were restricted by the chimpanzee MHC (Patr) DR molecule, one by DP, and one by DQ (Figs. 1 and 2 and Table II). Allelic restriction for the DP- and DQ-restricted clones was not determined due to the polymorphism of the Patr DP-α and DQ-α chains and lack of matched APCs. Patr-DR restriction of the clones was determined using a set of semimatched chimpanzee BLCLs (Fig. 2). Fine mapping and MHC restriction assays for each clone are shown in Figs. 1 and 2 and a summary of all six identified epitopes and their MHC restriction are shown in Table II. Interestingly, clones 5A and 1C isolated from liver and blood, respectively, targeted the same minimal epitope in NS5A (NS5_2081–2090) and were both restricted by the Patr DRB1*0701 allele (Fig. 2, C and D). However, clone 5A demonstrated an optimal response to a version of the peptide that was extended by 2 aa at its amino terminus (NS5_2079–2090). This difference in epitope recognition probably reflects a difference in the TCR α- and/or β-chains expressed by each of these clones.

**Tracking the development of helper responses during primary infection**

Our previous studies using HCV peptide pools demonstrated that CD4^+ T cells appeared in blood just before the infection resolved (26). However, this approach could not reveal whether CD4^+ T cells targeting certain epitopes are more crucial than others for infection resolution based on their frequency and kinetics of appearance during acute hepatitis C. We therefore sought to understand the evolution of the helper response to individual epitopes during primary infection. Immune responses to the six mapped epitopes (Table II) were monitored by IFN-γ ELISPOT analysis on cryopreserved PBMCs collected at different time points during primary infection. In addition, we monitored the immune response to a Patr-DRB5*0310-restricted epitope identified in another animal and designated NS3_1380 (1380-YGKAIPLEVI-1389) (31). Similar to what we and others have reported previously, there was a marked delay in the generation of HCV-specific immune responses despite robust virus replication (16, 17, 23, 26). An immune response was first detected against the NS3_1248 and NS3_1380 epitopes on day 56 postinfection (p.i.) when virus replication peaked at 4 × 10^6 genome equivalents (GE)/ml plasma (Fig. 3A). Importantly, HCV RNA in plasma was undetectable shortly afterward (Fig. 3A). Response to these two epitopes disappeared transiently following virus clearance but was re-detected on day 121 p.i. (Fig. 3A). Interestingly, no response was detected to the other five helper epitopes (Table II) until day 121 p.i. and it was very transient (Fig. 3A). We cannot exclude the possibility that the

<table>
<thead>
<tr>
<th>Table II. HCV helper epitopes targeted in CB0572 at 6 years following resolution of primary HCV infection</th>
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<tr>
<td>Clone</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>35D</td>
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<tr>
<td>8D</td>
</tr>
<tr>
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</tr>
<tr>
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biphasic response peaking on days 70 and 121 p.i. was due to the use of PBMCs cryopreserved at different time points 7 years earlier. Nevertheless, it is noteworthy that a slight rebound in plasma viremia on day 99 p.i. preceded the second increase in T cell frequencies. Breakthrough virus replication may have triggered a limited anamnestic response in an infection that was not completely controlled. The overall response then declined (NS31248 and NS31380) or became undetectable (Fig. 3A). At 7 years p.i. (day 2541), frequencies of CD4+/H11001 T cells targeting all seven epitopes were equivalent at around 100 spot-forming cells (SFC)/10^6 PBMCs (Fig. 3A).

Dominance of HCV helper epitopes upon homologous rechallenge

To evaluate the dominance of the various helper epitopes upon reinfection, the animal was challenged again with the same dose of the HCV-1/910 virus stock at 7 years after primary infection. Secondary infection was characterized by a shorter period (14 vs 86 days) and reduced peak (1 × 10^5 vs 4 × 10^6 GE/ml) of plasma viremia (26). Virus clearance was associated with an accelerated CD4+ and CD8+ T cell-mediated response (26). In this study, the immune response to the individual MHC class II-restricted helper epitopes in blood was monitored by an IFN-γ ELISPOT. Responses to all seven epitopes were accelerated by several weeks as compared with the primary infection and responses to dominant and subdominant epitopes were all clustered in a single early wave that appeared in blood on days 14–21 p.i. (Fig. 3B). Nevertheless, the hierarchy of the response targeting individual epitopes was conserved as the NS31248 and NS31380 epitopes again dominated the response. Specifically, frequencies of CD4+ T cells targeting all seven epitopes were stable around 100 SFC/10^6 PBMCs through day 10 p.i. (Fig. 3B). A 28-fold boost to 2800 SFC/10^6 in the frequency of NS31248-specific IFN-γ-producing cells occurred on days 14 and 21 p.i. coincident with virus clearance (Fig. 3B). This represents a dramatic expansion when compared with the primary infection where the response peaked at 350 SFC/10^6 PBMCs. The response declined gradually to baseline levels by day 94 p.i. The frequencies of IFN-γ-producing T cells targeting the other helper epitopes (Table II) did not show any significant increase (Fig. 3B). The response to the NS31380 epitope was measured retrospectively in a proliferation assay on frozen PBMCs. Comparison of proliferative responses to all seven defined epitopes near the peak of the response again revealed dominance of the NS31248 and NS31380 epitopes with stimulation indices 10- to 20-fold higher than those of the subdominant epitopes (data not shown).
FIGURE 2. Fine mapping and MHC restriction of HCV-specific Patr-DR-restricted CD4⁺ T cell clones generated from the blood and liver of CB0572 at 6 years after primary infection. Peptide truncations were used to fine map each of the individual epitopes in a 2-day proliferation assay as outlined in Materials and Methods. MHC restriction was determined by monitoring IL-2 production by flow cytometry in response to presentation of the optimal epitope (1 μg/ml) using a panel of semimatched chimpanzee BLCLs. Only MHC class II alleles shared between the autologous and the partially matched BLCLs are shown.
Helper responses in the liver during secondary infection

We next investigated whether all T cell populations found in blood were also present in the liver after reinfection. CD4-H11001 T cells recovered from the liver at the peak of the immune response on day 21 p.i. were expanded nonspecifically using anti-CD3 Ab stimulation. An IFN-γ ELISPOT assay on the expanded CD4-H11001 IHL demonstrated that T cells against all seven epitopes were present in the liver (Fig. 4). The requirement to expand CD4-H11001 IHL before the ELISPOT assay makes it impossible to draw conclusions about whether the pattern of immune dominance observed in blood is conserved in the liver. Nevertheless, this observation suggests that most HCV-specific CD4-H11001 T cell populations do home to the liver regardless of their frequency in blood.

Homology between helper epitopes identified in humans and chimpanzees

Human and chimpanzee MHC class II molecules share a high degree of homology particularly in the peptide-binding groove and one chimpanzee allele was capable of cross-species presentation of MHC class II-restricted peptides to human T cell clones (32). Furthermore, MHC class II supertypes characterized by overlapping peptide-binding repertoires have been described in humans (30) and were found to extend to a more distant nonhuman primate, the rhesus macaques (33) but little is known about the overlap between the human and chimpanzee repertoires. This suggested to us that HCV helper epitopes identified in the chimpanzee model are likely to bind and be presented by the corresponding human alleles with highest homology and might even be potential epitopes in humans infected with HCV. Interestingly, all Patr-DR-restricted epitopes identified in this study (Table II) fit the HLA-DR supertype peptide-binding motif. This motif is characterized by a large aromatic or hydrophobic residue in position 1 (Y,F,W,L,I,V,M) and a small noncharged residue in position 6 (S,T,C,A,P,V,I,M) (30). To investigate whether these were potential epitopes in humans, we tested their ability to bind different HLA-DR alleles. Each of the Patr-DR-restricted epitopes bound at least two different HLA-DR molecules with high affinity (Table III). Most importantly, the NS3_1248 epitope (GYKVLVLNPSV) targeted by clone 35D (Fig. 2A) bound eight different HLA-DR molecules (Table III). This epitope overlapped a number of helper epitopes identified in earlier studies of humans with resolved HCV infection (Table IV) and is highly conserved among different HCV genotypes (12). Given its ability to bind multiple HLA-DR molecules, it was considered a universal helper epitope and a target for vaccine development (12).

Interestingly, the two overlapping epitopes NS5_2079 and NS5_2081 differed in their binding pattern to HLA-DR molecules. Although NS5_2079 bound six different alleles, NS5_2081 bound only two (Table III), suggesting that the two extra residues at the amino terminus stabilized peptide binding. Indeed, the NS5_2079 epitope has two potential DR-binding motifs with either phenylalanine (FxxxxV) or leucine in the P1 position (LxxxxA).
To determine whether the high homology and binding affinity reflected a functional capacity for the chimpanzee epitopes in the human population, we investigated the ability of the HLA-DR molecules of highest amino acid homology to present the identified HCV epitopes to their cognate chimpanzee T cell clones. The newly identified epitopes NS41790, NS52079, and NS52081 were restricted by the Patr DRB1*0701 molecule (designated Patr-DR7). The human MHC molecule of closest homology to the Patr-DR7 is HLA-DRB1*0701 (designated HLA-DR7). The Patr-DR7/H9252-chain differs from HLA-DR7 at only three-amino acid residues (H925211 G→S, H925267 I→L and H925286 G→L). A chimpanzee CD4+ T cell clone recognized the NS41790 epitope efficiently regardless of whether it was presented by the HLA-DR7/H11001 B cell line (Pitout) or the autologous chimpanzee BLCL (Patr-DR7) (Fig. 5A). Interestingly, the NS52081 peptide could not bind HLA-DR7 (IC50 = 927; Table III). This difference was reflected in presentation of the two epitopes to their cognate chimpanzee CD4+ T cell clone (clone 1C) by HLA-DR7. Although both epitopes were recognized equally when presented by the autologous chimpanzee BLCL (Patr-DR7), only the high-affinity binder NS52079 epitope was recognized in the context of HLA-DR7 (Fig. 5B). These results indicate that despite the high homology between the human and chimpanzee model for HCV infection, they are not identical.

**Discussion**

A correlation between generation of a strong helper response and successful control of HCV infection is well established (6–18). In this study, we investigated the CD4+ T cell response to the individual MHC class II epitopes in a chimpanzee who spontaneously resolved two HCV infections. During primary infection of this individual, CD4+ T cells targeting a subset of dominant epitopes appeared earlier and were detected at higher frequencies than those targeting subdominant epitopes. Importantly, the initial wave of dominant T cell populations appeared in blood at the peak of viremia and was associated with control of infection. NS31248 and NS31380 were the prototype dominant epitopes, as cognate T cells

<table>
<thead>
<tr>
<th>Table III. Affinity of binding of HCV chimpanzee helper epitopes to the most common HLA-DR alleles</th>
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<tbody>
<tr>
<td>Sequence</td>
</tr>
<tr>
<td>NS31248</td>
</tr>
<tr>
<td>NS31380</td>
</tr>
<tr>
<td>NS41790</td>
</tr>
<tr>
<td>NS52079</td>
</tr>
<tr>
<td>NS52081</td>
</tr>
<tr>
<td>NS52426</td>
</tr>
</tbody>
</table>

A binder for a specific molecule is defined as a peptide with a binding capacity < 1000 nM. Binding capacities > 1000 nM are highlighted by bold face.
Table IV. Sharing of a universal helper epitope of HCV between humans and chimpanzees

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Peptide sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS3&lt;sub&gt;1248&lt;/sub&gt;-1267</td>
<td>GYKVVLNPSVAAATLGFGAY</td>
<td>12</td>
</tr>
<tr>
<td>NS3&lt;sub&gt;1253&lt;/sub&gt;-1272</td>
<td>GYKVVLNPSVAAATLGFGAY</td>
<td>18</td>
</tr>
<tr>
<td>NS3&lt;sub&gt;1241&lt;/sub&gt;-1250</td>
<td>PAAYAGQGYKVLNLNSVAA</td>
<td>9</td>
</tr>
<tr>
<td>NS3&lt;sub&gt;1248&lt;/sub&gt;-1258</td>
<td>GYKVVLNPSVSV</td>
<td>This study</td>
</tr>
</tbody>
</table>

appeared in blood early on day 56 p.i., while responses to the other mapped epitopes did not appear until day 121 p.i. following virus clearance from plasma. In contrast, during secondary infection the response to all seven epitopes was accelerated and clustered in one early wave that appeared in blood on days 14–21 p.i. as the virus was cleared from plasma. Nevertheless, the pattern of dominance of individual epitopes was conserved as T cells targeting the NS3<sub>1248</sub> and NS3<sub>1380</sub> were present at substantially higher frequencies. Further studies are needed to confirm whether such hierarchical and biphasic CD<sup>4</sup> T cell responses are common during acute HCV infection of humans and other chimpanzees and whether this phenomenon might influence the outcome of the infection.

The number of epitopes targeted by the first wave of T cells during primary infection and their susceptibility to immune selection pressure could influence the outcome of the infection. The dominant NS3<sub>1248</sub> epitope spans a region that is highly conserved among various HCV genotypes, suggesting that it might be important for virus replication and would be less likely to mutate in response to selection pressure. An early helper response directed only against a less-conserved region of the virus like NS3<sub>1380</sub> might be more likely to generate virus escape mutations in MHC class II-restricted epitopes and facilitate chronic infection. In addition, failure to generate the late wave of helper immune responses targeting a larger number of epitopes might lead to rebound in plasma viremia and virus persistence. Further comparison of the breadth and kinetics of the helper response during acute HCV in individuals with different infection outcomes is needed to fully understand the mechanisms responsible for failure of the helper response in chronic HCV infection.

Little is known about the development of helper T cell responses targeting individual MHC class II-restricted epitopes of viruses. Studies in the LCMV infection model suggested that the initial peak frequency of T cells targeting different epitopes (designated clonal burst size) determines the frequency and longevity of the memory CD4<sup>+</sup> T cell pool (34, 35). A recent study using MHC class II tetramers demonstrated that LCMV-specific memory CD4<sup>+</sup> T cell numbers decline slowly over a period of 900 days while memory CD8<sup>+</sup> T cells remain stable (36). However, these studies in mice focused on CD4<sup>+</sup> T cells targeting two dominant epitopes only. In HCV infection, virus-specific T cells were long-lived for two decades in human subjects with resolved infection (8) but their stability during that period was not examined. We have demonstrated that frequency of both CD8<sup>+</sup> (26) and CD4<sup>+</sup> memory T cells targeting different epitopes were stable in blood over a period of 7 years. We cannot exclude the possibility of low level or periodic virus replication that contributed to the stability of the memory T cells. It is also possible that the decline in frequency of memory T cells occurs in the liver, the primary site of infection, and not in blood. Furthermore, there was no obvious relationship between the peak frequency of different T cell populations in blood during acute HCV and their contribution to the memory T cell pool, but the pattern of dominance was nonetheless conserved during secondary infection. This suggests that the initial clonal burst size is likely to determine dominance upon rechallenge but does not influence the size of the memory pool, at least in the blood of an HCV-infected chimpanzee.

The liver is considered a site of long-lived CD4<sup>+</sup> memory T cells (37, 38). Indeed, we isolated HCV-specific CD4 helper T cells from the liver of CB0572 at 6 years following resolution of primary infection when they were difficult to detect in blood. Earlier observations suggested compartmentalization of some HCV-specific CD4 helper T cell subsets to the liver of a chronically infected human (39). Recent observations demonstrated that resolution of HCV infection is associated not only with generation of a sustained (16) and broad (9) helper response in blood but also accumulation of HCV-specific CD4 helper T cells in the liver (16). Although it is difficult to compare the relative frequency or dominance of the different T cell populations in blood vs liver because of methodological differences, our data demonstrate that all populations are present in both compartments at least in this individual during a second HCV infection.

MHC class I supertypes with a broadly degenerate peptide-binding motif are common to humans and chimpanzees (40–42). MHC class II supertypes have also been described in humans (30) and rhesus macaques (33) and here we extended them to chimpanzees as well. Interestingly, the dominant NS3<sub>1248</sub> epitope identified in this study overlaps a number of dominant epitopes reported in human subjects with acute resolving HCV infection (9, 12, 18). This highly conserved region bound multiple HLA-DR alleles. It was considered a universal helper epitope and a target for vaccine development. Similarly, most epitopes identified in this study fit

![FIGURE 5. Cross-species presentation of Patr-DR7-restricted HCV helper epitopes by HLA-DR7<sup>+</sup> BLCLs. Patr DRB1<sup>*0701</sup>-restricted HCV epitopes NS4<sub>1790</sub> (A), NS5<sub>2079</sub> and NS5<sub>2081</sub> (B) were presented by either chimpanzee CB0572 BLCL (Patr-DR<sup>+</sup>) or the human BLCL Pitout (HLA-DR<sup>+</sup>), respectively. Presentation efficiency was determined by monitoring IL-2 production by flow cytometry as described in Materials and Methods.](http://www.jimmunol.org/ Downloaded from)
the HLA-DR supertype motif (30) and bound at least two different HLA-DR alleles with high affinities. In addition, Patr-DR7-restricted epitopes were presented efficiently by HLA-DR7 (the human allele with the highest homology). Such epitopes are likely to be targeted in humans with the HLA-DR7 allotype and could be potential targets for vaccination in this population. Epitope prediction algorithm strategies might be helpful in identifying broadly degenerate binders as potential targets for vaccination in populations with a variable MHC background. Cross-reactivity between MHC class I-restricted CD8 epitopes of HCV identified in humans and chimpanzees was recently reported (42). Furthermore, these epitopes were used to monitor immune responses in chimpanzees rechallenged with HCV (43). However, our data suggest that this strategy should be used with caution and that epitopes identified by prediction algorithms or sequence similarity, although useful for vaccine design, should not serve as surrogates to monitor the overall immune response for two reasons. First, individuals that share the same MHC background might target different epitopes revealing unanticipated specificities (7, 44). Second, sequence similarity of the MHC molecules alone does not provide an efficient prediction of peptide-binding pattern. Indeed, the two overlapping epitopes NS5b2079 and NS5b2081 were both presented with equal efficiency by the Patr-DR7 molecule but with only the longer epitope (NS5b2079) bound the HLA-DR7 molecule that differs by only three-amino acid residues.

Although this analysis involved only one chimpanzee, it clearly demonstrates the presence of two waves of helper T cell responses during primary infection with HCV. Most importantly, it included an early response targeting a limited set of dominant epitopes that coincided with virus clearance and a late response targeting both dominant and subdominant epitopes. Whether the striking hierarchy of epitope recognition during primary infection that is maintained through multiple exposures to the virus is a factor in the outcome of infection merits further study. Helper epitopes identified in the chimpanzee model could be predictive of their reactivity in humans with related MHC background. Potential therapeutic and preventative vaccines aimed at restoring the helper response in chronically infected individuals could focus on broadly degenerate MHC class II-binding epitopes such as those identified in this study and on strategies that would target CD4+ T cells to the liver.

Acknowledgments

We thank Dr. Dana Hasselschwert and Neal Smith of the New Iberia Research Center (New Iberia, LA) for outstanding veterinary and technical support. The efforts of Devon Conway and Elizabeth Seidler in MHC class II typing of chimpanzees are greatly appreciated.

References


