THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Identification of Hepatitis C Virus-Specific CD8+ T Cells Restricted by Donor HLA Alleles following Liver Transplantation[1]

Hugo R. Rosen,[2]†‡ David J. Hinrichs,†‡ Rachel L. Leistikow,‡ Glenda Callender,¶ Anne M. Wertheimer,†‡ Michael I. Nishimura,¶ and David M. Lewinsohn†‡§*

***By necessity, human liver transplantation is performed across HLA barriers. As a result, intracellular infection of the allograft presents a unique immunologic challenge for the recipient's immune system. In this study, we describe the presence of HLA-A2-restricted, hepatitis C virus (HCV)-specific CD8+ T cells in liver transplant recipients in whom the allograft is HLA-A2 positive and the recipient is HLA-A2 negative. These memory-effector T cells are recipient derived and recognize HCV peptide uniquely in the context of HLA-A2. Furthermore, these cells were absent before the transplant, suggesting that the allograft is capable of selectively expanding naive CD8+ T cells. The in vitro specificity to donor HLA allele-restricted CD8+ T cells suggests that these cells may function to control HCV spread in the allograft. The Journal of Immunology,* 2004, 173: 5355–5359.**

Hepatitis C virus (HCV)[3] causes chronic infection and liver injury in the majority of exposed individuals through pathogenic mechanisms that remain incompletely understood, although considerable evidence shows that the vigor and breadth of HCV-specific T cell responses correlate with viral clearance, recovery, and self-limited disease (1–3). Liver disease related to HCV infection is the single leading indication for liver transplantation worldwide, and its significance as a clinical problem cannot be overstated. HCV infection significantly diminishes patient and allograft survival following liver transplantation (4). Liver transplantation for HCV-related liver failure is invariably followed by acute infection of the allograft. The rapid decrease in HCV viral load noted immediately after graft perfusion, followed by a marked increase (~20-fold) in circulating viral titers by the first postoperative month suggest the massive uptake of HCV virions and establishment of competent replication within the allograft (5, 6). A recent immunohistochemical analysis (7) demonstrated frequent contact between CD8+ T cells and HCV-positive hepatocytes following liver transplantation. However, the characteristics of the virus-specific T cell response following liver transplantation have not been defined; in particular, it is unknown how the HCV-specific repertoire is shaped by donor allele(s).

Moreover, liver transplantation is performed with no regard to specific matching of donor-recipient MHC alleles, and this may serve as a barrier to the development of protective (i.e., antiviral) cell-mediated immunity directed against infected cells within the allograft. Although incompletely understood, the immune recognition of the HCV-infected allograft may be essential in the containment of infection. CD8+ T cells are the primary effector lymphocytes for provision of protective immunity against intracellular pathogen infection of parenchymal cells and are effective because of their ability to recognize infected cells as the combination of pathogen-derived peptides in the peptide-binding grooves of MHC class I molecules on the surface of cells. While incompletely understood, the immune recognition of the HCV-infected allograft may be essential in the containment of infection; however, the HLA incompatibility between donor and recipient may serve as a barrier to the development of protective immunity. Recognition of the allograft could occur either via recipient-derived T cells or via those derived from the donor. For the recipient-derived T cells, recognition could occur either through use of shared HLA molecules, or could occur through the expansion of recipient-derived T cells that are uniquely restricted by the HLA molecules of the donor liver. Little is known about the relative contribution of those cells uniquely restricted by the donor liver. In a mouse model of tumor immunity, Sadovnikova and Stauss (8) have demonstrated that CTLs uniquely restricted by the MHC of the allotumor can mediate antitumor activity against melanoma and lymphoma. In the current report, we test the hypothesis that receipt of an allograft results in the development of a

*Hepatology and Liver Transplantation Program, Portland Veterans Affairs Medical Center/Oregon Health and Sciences University, †Molecular Microbiology and Immunology, Oregon Health and Sciences University, ‡Portland Veterans Affairs Medical Center Research Services, §Division of Pulmonary and Critical Care, Portland Veterans Affairs Medical Center, Portland, OR 97207; and ¶Department of Surgery, University of Chicago, Chicago, IL 60637

Received for publication May 20, 2004. Accepted for publication August 19, 2004.

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[1] This research was supported by RO1 DK060590 and a Veterans Affairs Merit Review Grant (to H.R.R.) and CA102280 and CA90873 (to M.I.N.).

[2] Address correspondence and reprint requests to Dr. Hugo R. Rosen, Hepatology and Liver Transplantation Program, Portland Veterans Affairs Medical Center, 3710 SW U.S. Veterans Hospital Road, P3-GI, Portland, OR 97207. E-mail address: rosenhu@ohsu.edu

[3] Abbreviations used in this paper: HCV, hepatitis C virus; HS, human serum; LCL, lymphoblastoid cell line.

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0022-1767/04/$02.00

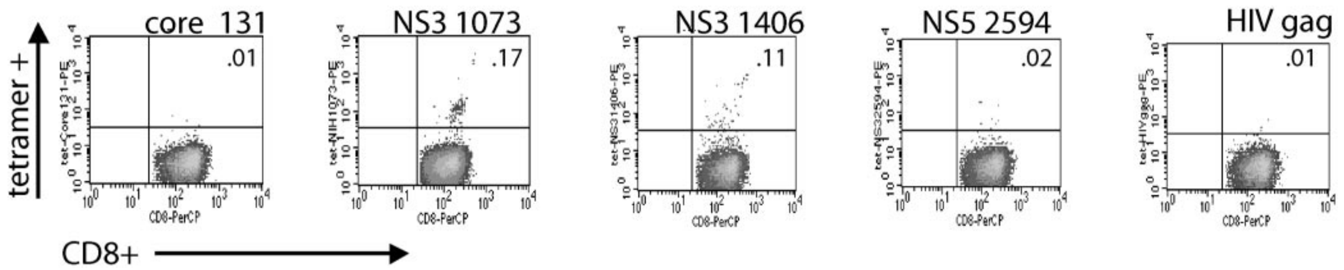

FIGURE 1. Enumeration of HCV-specific and HIV gag-specific tetramer-positive $CD8^+$ T cells 4 years after liver transplantation in patient 1.

unique population of recipient-derived cells capable of recognizing intracellular infection of HCV in the context of the donor HLA molecule. Using HCV-specific, HLA-A2 tetramers, we demonstrate that receipt of the allograft results in expansion of HLA-A2-specific $CD8^+$ T cells in HLA-A2-negative recipients. By cloning these cells, we demonstrate that they are incapable of recognizing HCV peptide in the context of any of the recipient HLA molecules. Consequently, developing a comprehensive understanding of protective immunity to HCV will require an assessment of both recipient and allograft-restricted CTL.

Materials and Methods

Patients

All patients received orthotopic liver transplantation for HCV genotype 1-related liver failure, and were immunosuppressed with tacrolimus and prednisone; three of the five patients additionally received azathioprine, and one received mycophenalate mofetil. Antiviral therapy with IFN-α and ribavirin was given to patient 1 (3 mo to 5 years posttransplant) and patient 2 (13–17 mo posttransplant).

Cell separation and culture

PBMC were isolated from whole blood using cellular preparation tubes (BD Biosciences, Franklin Lakes, NJ).

Tetramers

PE-labeled HLA-A*02 tetramers that had been folded around known HCV-immunodominant peptides ($core_{131–140}$ (ADLMGYIPLV), $NS3_{1073–1081}$ (CINGVWCTV), $NS3_{1406–1415}$ (KLVALGINAV), and $NS5_{2594–2602}$ (ALYDVVTKL)) were used. $CD8^+$ cells were separated from PBMCs using a positive-selection strategy and MACS superparamagnetic beads as per manufacturer's instructions. As previously described (9), the limit of detection was determined to be 0.04% by using the HIV gag tetramer, as well as cells from HLA-A2-negative and HCV-negative patients who had not undergone transplantation.

Cloning of tetramer-positive cells

Cells were separated and stained as described above, with the exception that cells were not fixed before being sorted by flow cytometry on a FACSVantage (BD Biosciences). One hundred fifty to 5000 $CD8^+$tetramer$^+$ cells were collected and allowed to rest in RPMI 1640 plus 10% human serum (HS) plus IL-2 (0.75 μg/ml). After a minimum of 2 h at 37°C, tetramer-positive cells were plated at limiting dilution (5, 10, and 100 cells/well) and cultured with 8×10^4 and 1.6×10^4 irradiated allogeneic PBMC and lymphoblastoid cell line (LCL), respectively, in a total volume of 250 μl per well of RPMI 1640 plus 10% HS with purified anti-CD3 (0.03 μg/ml) in 96-well plates. The next day, cultures were supplemented with IL-2 (1.25 ng/ml). Plates were incubated for 2 wk at 37°C and 5% CO_2. Wells showing growth after 14–21 days were transferred to T-25 flasks and restimulated with 25×10^6 and 5×10^6 irradiated allogeneic PBMC and LCL, respectively, in a total volume of 30 ml of RPMI 1640 plus 10% HS with anti-CD3 (0.03 μg/ml). After 2 days, cultures were supplemented with IL-2. Cultures were rinsed of anti-CD3 Ab after 5 days and fed with medium exchange and supplemented with IL-2 every other day thereafter until day 14 when cells were analyzed by FACS.

ELISPOT assay

IFN-γ ELISPOT assay as previously described by our group (9) was performed with cloned T cells and LCLs expressing different class I alleles to demonstrate HLA restriction.

Retroviral vectors, cell lines, and supernatant production

A SAMEN retroviral vector was used (10). cos (monkey kidney tumor, HLA-A2 negative), cosA2 (HLA-A2 positive), $Mel_{624–28}$ (human melanoma, HLA-A2 negative), Mel_{624} (HLA-A2 positive), RCC 1764 (human renal cell carcinoma, HLA-A2 negative), and RCC UOK 131 (HLA-A2 positive) were transduced with either empty retroviral vector or with retroviral vector containing the *HCV* minigene that encodes $NS3_{1406–1415}$ peptide. IFN-γ secretion (mean + SD) was assessed by ELISA (Pierce, Rockford, IL). Luminex assay was used to measure additional cytokines and chemokines.

HLA typing

HLA typing was performed using PCR amplification with sequence-specific primers (11). HLA haplotypes ($A2^+$ or $A2^-$) were further confirmed by staining PBMCs with mAbs MA2.1 (BD Biosciences).

mAbs and staining

Abs used in these experiments included the following: anti-CD3-allophycocyanin, anti-CD3-FITC, anti-CD8-PerCP, anti-CD8-allophycocyanin, anti-CD25-FITC, anti-CD28-FITC, anti-CD38-FITC, anti-CD45RO-FITC, anti-CD45RA-FITC, anti-CD69-FITC, anti-HLADR-PerCP, anti-CCR5-FITC, anti-CCR7-PE anti-IFN-γ-FITC, anti-IL-4-PE, anti-BCL2-FITC (BD Biosciences), anti-IL4-allophycocyanin, anti-IL10-allophycocyanin, and anti-TNF-α-FITC (BD Pharmingen, San Diego, CA). All flow cytometry data were analyzed with CellQuest program (BD Biosciences).

Cytotoxicity assay

HCV-specific cytotoxicity was determined by the recently described fluorometric assessment of T lymphocyte Ag-specific lysis assay using dual staining (PKH-26 and CFSE), which is at least as sensitive as the standard ^{51}Cr release assay (12).

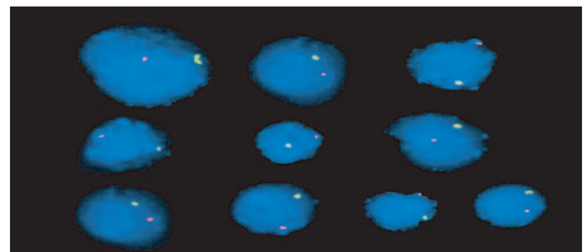

FIGURE 2. Fluorescence in situ hybridization analysis of tetramer-positive cells. Cells were treated in hypotonic solution and fixed in 3:1 methanol/acetic acid. Slide-mounted target DNA was then co-denatured with direct-labeled probes for X and Y α satellite DNA (CEP X and Y probe; Vysis, Downer Grove, IL), at 72°C for 2 min and allowed to renature for 4 h at 37°C. A Zeiss (Oberkochen, Germany) Axiophot fitted with appropriate filters for visualization of red and green probe signals was used for viewing specimens, and images were captured using a CytoVision system (Applied Imaging, Santa Clara, CA). A total of 100 interphase cells from each flow-sorted population was scored for X (green) and Y (red) signals, confirming that tetramer-positive and -negative cells were recipient derived.

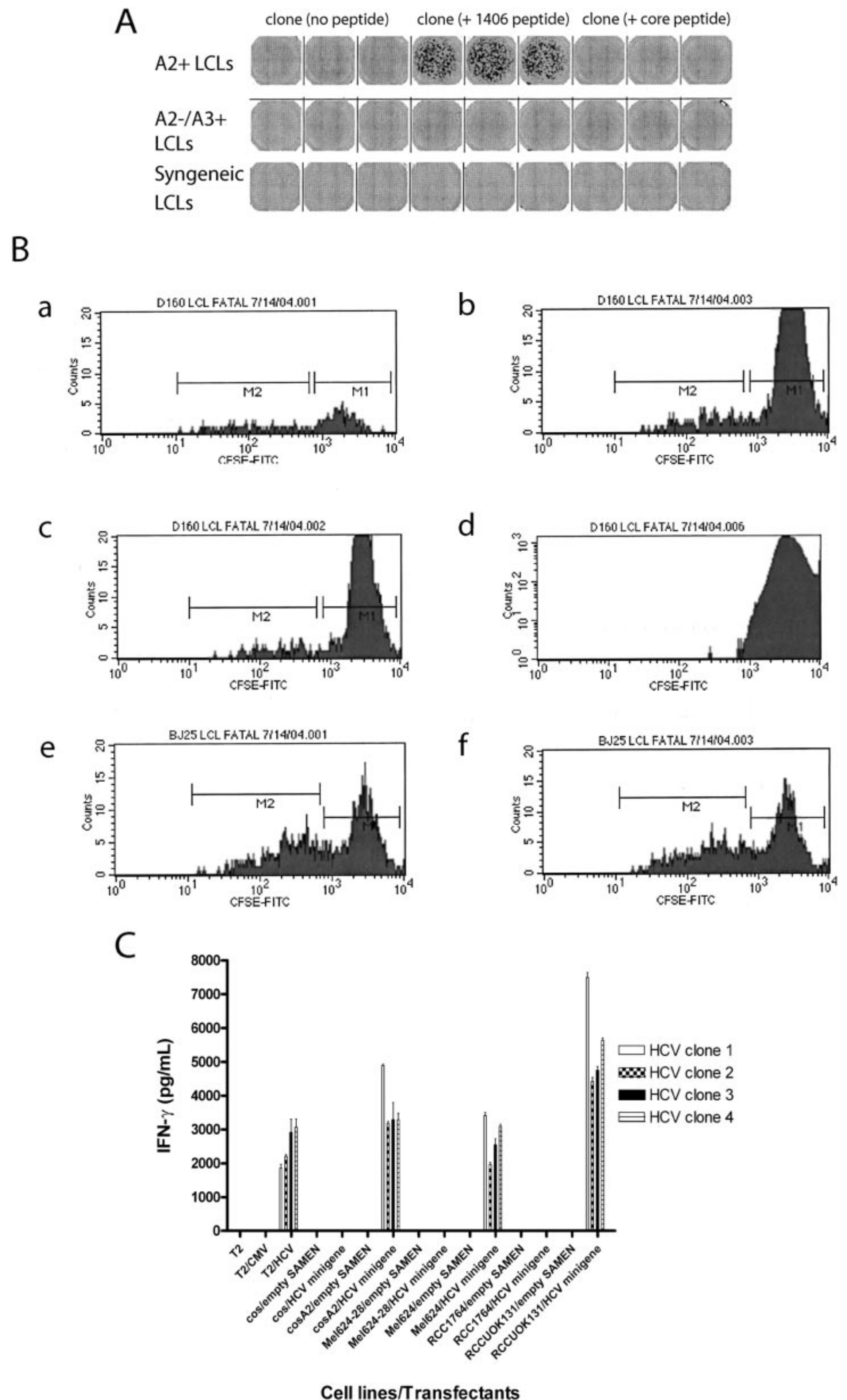

FIGURE 3. HLA restriction of HCV-specific clones. *A*, ELISPOT assay was performed with 1,000 T cells, 20,000 LCLs expressing A2 allele (*top row*), A3 allele (*middle row*), or syngeneic (recipient-derived) LCLs (*bottom row*) cocultured with no peptide, cognate peptide ($NS3_{1406-1415}$, KLVALGINAV) or irrelevant HCV core peptide ($core_{35-44}$, YLLPRRGPRL). *B*, Cytotoxic activity of HCV-specific tetramer^{+}CD8^{+} T cells. T cell clones were assayed for peptide-specific cytotoxicity using the fluorometric assessment of T lymphocyte Ag-specific lysis (FATAL) assay, which determines the percentage of labeled target cells surviving after 5-h incubation with effector cells; E:T ratio is 50:1 with 1×10^6 target cells. The target cells are selected by gating on the PKH-26^{high} population and demonstrate the reduction in CFSE fluorescence following incubation with $NS3_{1406}$-specific CTL, cognate peptide (KLVALGINAV), and HLA-A2 LCLs (*a*), compared with no peptide (*b*), irrelevant peptide $NS5_{2594-2602}$ (ALYDVVTKL) (*c*), and no effector cells (*d*) (200,000 total events were gated). Specific lysis was calculated as 30%. In contrast, the percentage killing of syngeneic LCLs was not different following incubation in the presence (*e*) or absence (*f*) of cognate peptide. *C*, $NS3_{1406}$-specific clones (HCV1–HCV4, represented by bars) recognize endogenously processed Ag. T2 cells were pulsed with $HCV_{1406-1415}$ peptide for 2 h. Cell lines (cos, cosA2, Mel_{624-28} (HLA-A2 negative), Mel_{624} (HLA-A2 positive), RCC 1764 (HLA-A2 negative), RCC UOK 131 (HLA-A2 positive)) were transduced with either empty retroviral vector or with retroviral vector containing the *HCV* minigene that encodes $NS3_{1406-1415}$ peptide. A total of 10,000 stimulator cells was incubated with 10,000 HCV-specific T cells per well for 18 h in triplicate; CMV-specific T cells were used as a control. IFN-γ secretion (mean + SD) was assessed by ELISA.

Results and Discussion

To determine whether or not HCV-specific CD8^{+} T cells restricted by donor HLA alleles were generated after liver transplantation, we studied patients in whom the donor was HLA-A2 positive, and the recipient was HLA-A2 negative. HLA-A2 was selected as the restricting allele, because a large number of HCV HLA-A2 binding peptides have been described as targets of HCV-specific CTLs (13). Fig. 1 shows class I tetramer results from patient 1 (donor A2, A24; recipient A3, A30) 4 years after liver transplantation when he was HCV RNA negative in the serum because of antiviral therapy following development of severe histologic recurrence (14). Both $NS3_{1073}$- and $NS3_{1406}$-specific CD8^{+} T cells were detectable, whereas core, NS5B-specific, and HIV gag-specific T cells were not

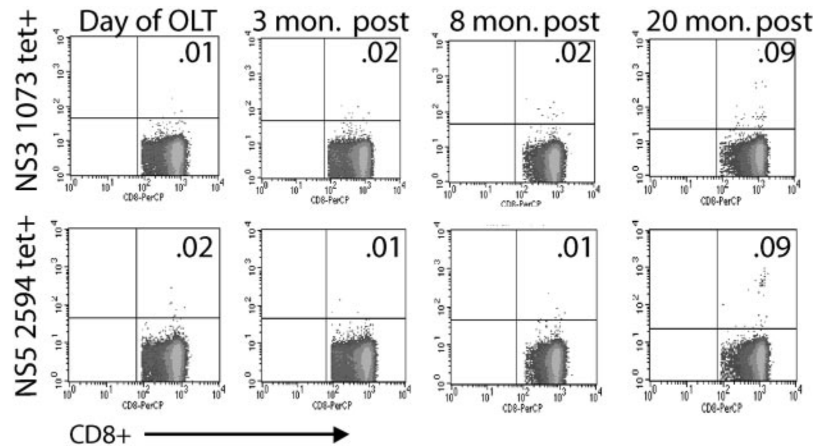

FIGURE 4. Prospective enumeration of HCV $NS3_{1073}$- and $NS5_{2594}$-specific tetramer responses in the blood (there were no tetramer responses to HCV $core_{131}$ or $NS3_{1406}$ epitopes; data not shown) in patient 2, who was an HLA-A2-negative recipient of an HLA-A2-positive liver transplant.

detectable by tetramer analysis. To generate sufficient numbers of cells for phenotypic and functional analysis, tetramer-positive cells were sorted and cloned using limiting dilution and a rapid expansion protocol as described in *Materials and Methods*. Although $NS3_{1073}$-specific $CD8^+$ T cells were most frequent, this epitope (CVNGVCWTV) recently (15) has been shown to have cross-reactivity with the influenza A IV neuraminidase epitope; therefore, we cloned $NS3_{1406}$-specific $CD8^+$ T cells. Phenotyping characterized these cells as $CD45RO^+RA^-$ memory cells with typically high expression level of activation markers CD38 and CD69. The absence of CD62L (L-selectin) and CCR7, essential for lymphocyte migration to lymph nodes, indicates these CTLs are highly differentiated long-lived effector populations that can enter peripheral tissues to mediate inflammatory reactions or cytotoxicity (16). Stimulation with the mitogen PMA-ionomycin revealed that these cells produced intracellular IFN-γ and TNF-α, but not IL-4 or IL-10.

To determine whether the T cells were of host or recipient origin, fluorescence in situ hybridization was used. Because donor (female) and recipient (male) were gender mismatched, it was possible to resolve this question by sorting on the tetramer-positive cells and demonstrating that all of the cells expressed the Y chromosome and were therefore recipient derived (Fig. 2). Moreover, analysis of genomic DNA from these clones by PCR amplification with sequence-specific primers revealed that the clone was positive for A3 and A30, but not A2 (data not shown), providing independent confirmation that they were of recipient origin.

Having demonstrated that these tetramer-binding $CD8^+$ T cells were of recipient origin, we sought to determine whether or not the cells were uniquely HLA-A2 restricted, or had cross-reactivity to other HLA molecules. To investigate the HLA restriction of these tetramer-specific T cells, assays were performed with cloned T cells and allogeneic LCLs expressing the A2 allele or the A3 allele, LCLs expressing all of the other donor HLA I alleles, or LCLs derived from the recipient cocultured with cognate or irrelevant HCV peptide. These analyses confirmed that these $CD8^+$ T cells are peptide specific and uniquely restricted by the HLA-A2 allele (Fig. 3*A* and data not shown). Moreover, as shown in Fig. 3*B*, these CTLs had cytotoxic activity in the presence of HLA-A2 LCLs but not syngeneic (recipient-derived) LCLs.

In order for these CTLs to be relevant in vivo, i.e., mediate anti-HCV-specific function within the allograft, they would need to recognize processed Ag presented by dendritic cells, hepatocytes, or liver sinusoidal endothelial cells (17). To this end, we constructed retroviral vectors containing a minigene encoding the $NS3_{1406}$ peptide, and then transduced a variety of cell lines with the vector plus *HCV* minigene, as well as an empty retroviral vector (Fig. 3*C*). Multiplex analysis of these supernatants demonstrated production of IFN-γ, TNF-α, IL-2, GM-CSF, and MCP-1α, but not IL-4, IL-5, IL-7, IL-10, or IL-13 in the presence of the HCV peptide and cell line expressing HLA-A2. Taken together, our results demonstrate for the first time the presence of recipient-derived, HCV-specific $CD8^+$ T cells that are selected and restricted by donor alleles following liver transplantation; these cells are memory, long-term effector cells that recognize endogenously processed Ag.

We next sought to determine the time course over which these $CD8^+$ T cells emerged in four HLA-disparate recipients with PBMC serially collected before and at multiple time points after transplantation. As shown in Fig. 4, patient 2 demonstrated novel HLA-A2-specific responses at 20 mo post-liver transplantation. Similar to the results derived from patient 1, analysis of cloned $NS3_{1073}$-specific CTLs from patient 2 confirmed that they were of recipient origin (A31, B40, B51) and restricted by donor HLA-A2 when tested in an ELISPOT assay.

In summary, our study shows the de novo acquisition of recipient-derived, HCV-specific $CD8^+$ T cells that are restricted by donor HLA alleles. Functionally, these CTL meet all traditional criteria for an adaptive HLA-Ia-restricted immune response in that T cell recognition occurs uniquely in the presence of HLA-A2 and the HCV peptide, and the response is clonally expanded following liver transplantation. Thus, our data are consistent with the hypothesis that the HCV-infected allograft is capable of stimulating and expanding naive $CD8^+$ T cells. Presuming normal thymic selection of T cells based on their restriction to self-HLA molecules, the mechanisms by which the allograft can shape the T cell repertoire remain incompletely understood; nonetheless, we acknowledge a cross-reactive memory response as a formal possibility (18).

Considering the inflammatory milieu of the HCV-infected allograft (19) coupled with the high viral load (and presumably robust display of viral peptides on hepatocytes), it is possible that direct, extralymphoid presentation drives the differentiation and maturation of naive recipient-derived CD8 T cells. Alternatively, we would hypothesize that the microenvironment

(e.g., lymphoid aggregates in the portal tracts or secondary lymphoid tissues) in which this immune response was initiated would contain dendritic cells that take up HCV-infected hepatocytes. Irrespective of the precise ontogeny of these $CD8^+$ T cells, their presence reveals the intrinsic plasticity (20) of the TCR that allows binding and recognition of HCV peptides on HLA-disparate APCs. Importantly, these CTLs were not simply alloreactive, because they did not bind irrelevant HLA-A2 tetramers that contained HIV gag peptide and did not respond when cocultured in an ELISPOT assay with HLA-A2-expressing LCLs alone (without cognate peptide) or with LCLs expressing the other donor alleles. The in vitro specificity of these donor HLA allele-restricted CTLs suggests that these CTL clones could be exploited for adoptive immunotherapy in liver transplant patients who develop severe recurrence of HCV infection within their allografts by specifically targeting infected donor organ tissues without triggering generalized alloimmunity against recipient tissues.

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